

Research Article

Use of carbon dioxide to prevent zebra mussel (*Dreissena polymorpha*) settlement and effects on native mussels (Order Unionoida) and benthic communities

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Abstract

We determined the efficacy of carbon dioxide (CO₂) for preventing larval (veliger) settlement of the invasive zebra mussel (*Dreissena polymorpha*) and compared the response of native juvenile mussels and macroinvertebrate and periphyton communities to hypercapnia. A microcosm system of control (ambient C_{amb}) and CO₂ treatments [low partial pressure of carbon dioxide, PCO₂ (C_{low}) = 35,094 μatm, and high PCO₂ (C_{high}) = 66,685 μatm] was supplied with river water for 11 weeks. We measured zebra mussel settlement, juvenile native mussel (*Lampsilis cardium* and *L. siliquoidea*) growth and condition, and macroinvertebrate and periphyton community composition. Zebra mussels settled in all C_{amb} tanks (*n* = 4) from early July through August compared to one mussel in a CO₂ treatment tank over the same period. Native mussel growth, tissue condition, and shell condition were reduced in CO₂, particularly in the highest treatment. Benthic biomass (excluding zebra mussels) was similar in C_{amb} and C_{low} but was reduced in C_{high}. Macroinvertebrate community composition differed among treatments due to greater abundance of Chironomidae in C_{low} and Oligochaeta in C_{high}. Periphyton abundance and richness increased in both CO₂ treatments and was driven by increases in Cyanobacteria, Bacillariophyta, and Chlorophyta. Our results indicate that efficacious levels of CO₂ (~ 35,000 μatm PCO₂) for reducing biofouling by dreissenids are tolerable to most freshwater benthic taxa. Chronic elevated CO₂ infusion (> 35,000 μatm PCO₂) may reduce native mussel growth and condition and alter benthic invertebrate and periphyton community composition. Further refinement of CO₂ application strategies is needed to determine minimum effective dose and duration to prevent dreissenid settlement and minimize effects on nontarget organisms.

Key words: biofouling, dreissenid, hypercapnia, macroinvertebrate, periphyton, unionid mussel, veliger

Introduction

Zebra mussels, *Dreissena polymorpha* (Pallas, 1771) and quagga mussels, *D. bugensis*, (Andrusov, 1897) are invasive bivalves to North America that were introduced in the 1980s presumably in ballast water of intercontinental ships entering the Great Lakes (Griffiths et al. 1991). Unlike native unionid mussels, dreissenids are epifaunal and secrete adhesive byssal threads for attachment to substrates. Their prolific

reproduction, microscopic larvae (herein referred to as veligers), and rapid growth rate enabled dreissenid mussels to quickly spread and establish in waterbodies across North America (Mackie 1991; Benson et al. 2020). As highly efficient filter feeders, dreissenids have substantially altered trophic structures and nutrient cycles in freshwater systems (Strayer et al. 1998; Madenjian et al. 2015) and caused the decline and extirpation of some native species, notably unionid mussels (Vanderploeg et al. 2002; Higgins and Van der Zanden 2010; Bootsma and Liao 2014; Mayer et al. 2014). Users of freshwater for industry, agriculture, municipal drinking water, and hydroelectric generation are especially affected by dreissenid fouling. Mussels cause reduced water flow through water intake and cooling systems, deterioration of surfaces, and contamination of potable water when mussels die. As evidence, water managers in North America have spent an estimated \$100,000 to millions of dollars per year to mitigate biofouling by dreissenids (Rosaen et al. 2016; Chakraborti et al. 2016).

Prevention and mitigation of macrofouling by mussels have focused primarily on chemical tools, especially oxidizing chemicals (e.g., chlorine), potassium compounds (permanganate, potassium hydroxide, potassium chloride; Mackie and Claudi 2010; Chakraborti et al. 2014; Davis et al. 2018), copper-based compounds (Claudi et al. 2014; Watters et al. 2013), and the biopesticide Zequanox® (Whitledge et al. 2015; Rackl and Link 2015). These tools are efficacious, but not without ecological and/or economic costs. Chlorine is commonly used in closed water systems for dreissenid control and has advantages of proven efficacy, cost-effectiveness, and simple application systems. However, chlorine can produce toxic byproducts, such as trihalomethane, poses a human safety concern in some forms, and must be neutralized before discharge into the environment (Mackie and Claudi 2010). Potassium and copper-based compounds have been used in both closed systems (Watters et al. 2013; Chakraborti et al. 2014) and open water (Offutt Air Force Base 2009; DFO 2014; Fernald and Watson 2014; Lund et al. 2017; Hammond and Ferris 2019). When used in open water, potassium and copper-based compounds can persist in the environment and may be toxic to other aquatic life at levels used in dreissenid control treatments (Offutt Air Force Base 2009; Fieldseth and Sweet 2016; Densmore et al. 2018).

Carbon dioxide has demonstrated effectiveness as a control for a variety of aquatic invasive species (AIS) including dreissenid mussels (McMahon et al. 1995; Waller and Bartsch 2018; Waller et al. 2020), invasive Asian carp (*Hypophthalmichthys* spp.) (Kates et al. 2012; Cupp et al. 2017b, c, 2018), bullfrogs (*Lithobates catesbeianus*) (Abbey-Lambertz et al. 2014), round goby (*Neogobius melanostomus*) (Cupp et al. 2017a), New Zealand mud snails (*Potamopyrgus antipodarum*) (Nielson et al. 2012), and invasive crayfishes (red swamp crayfish, *Procambarus clarkii* and rusty crayfish, *Faxonius rusticus*) (Fredricks et al. 2020). Carbon dioxide is easily off-gassed,

has low risk to human health, is widely available, can be repurposed from industrial sources and is inexpensive (Treanor et al. 2017). Carbon dioxide-carp was issued a Section 3 Federal Insecticide, Fungicide, and Rodenticide (FIFRA) registration in 2019 by the U.S. Environmental Protection Agency (USEPA 2019), and efforts to amend the label to include dreissenids are underway (Kim Fredricks, U.S. Geological Survey, *written communication*, 2021).

Carbon dioxide (CO₂) was first tested in the 1990s as a molluscicide and found to effectively reduce attachment and kill zebra mussels (Elzinga and Butzlaff 1994; McMahon et al. 1995; Payne et al. 1998). More recently, CO₂ treatment regimens were determined for detachment and lethality of adult zebra mussels at different water temperatures (Waller and Bartsch 2018; Waller et al. 2020). The lethal time to 99% mortality (LT99) of adult zebra mussels in 110,000 to 120,000 µatm partial pressure of carbon dioxide (PCO₂) ranged from 100 h at 20 °C to 300 h at 5 °C, and short-term infusion (48 h) of CO₂ at this level reduced attachment by 50% at both temperatures (Waller et al. 2020). A next step in an evaluation of CO₂ for dreissenid control is to determine treatments that are efficacious for the more sensitive veliger stage. Metamorphosis and settlement of veligers to the juvenile stage is reportedly prevented in water with pH < 7.1 (Claudi et al. 2012). Carbon dioxide reacts in water to form carbonic acid, causing mild acidification. Therefore, we expect that CO₂ levels that decrease pH to < 7.1 will prevent veliger settlement and effectively prevent biofouling of water infrastructures.

Our study site was located at a harbor that is used by the U.S. Fish and Wildlife Service (FWS) Genoa National Fish Hatchery (GNFH) for seasonal propagation of federally- and state-listed endangered native mussel species. Propagation cages and juvenile native mussels become infested with zebra mussels which increases maintenance costs and reduces juvenile production. As several studies have reported greater selective toxicity of CO₂ to zebra mussels compared to native mussels, we considered whether CO₂ could be used to prevent biofouling of native mussels by dreissenids. For example, Waller et al. (2020) found short-term PCO₂ treatments (maximum 300 h; 100,000 to 120,000 µatm) at 5, 12 and 20 °C that were lethal to adult zebra mussels and produced minimal mortality of juvenile *Lampsilis cardium* and *Leptodea fragilis*. However, other studies reported lethal and sublethal effects on juveniles of several species (Hannan et al. 2016a, b; Jeffrey et al. 2017, 2018a, b; Waller et al. 2017, 2019; Waller and Bartsch 2018). Survival of *Lampsilis* spp. was significantly reduced after 14-d exposure to 58,000 µatm PCO₂ (Jeffrey et al. 2018b) or 28-d exposure to 30,000 to 58,000 µatm PCO₂ (Waller et al. 2019). Physiological indicators of stress and reduced shell growth and condition from elevated CO₂ have also been reported (Waller et al. 2017, 2019; Jeffrey et al. 2018b). More investigation is needed to establish safe levels of CO₂ for juvenile native mussels and to determine

whether CO₂ could be a feasible option to reduce settlement of dreissenids in the vicinity of native mussels.

In addition to native mussels, other aquatic organisms in a natural water supply may be affected by elevated CO₂. Several studies have evaluated the effects of elevated CO₂ on nontarget fish (Tix et al. 2017; Schneider et al. 2019; Cupp et al. 2020), but few have monitored the response of nontarget freshwater communities to CO₂ at levels required for AIS control actions (~ 50,000 to 100,000 µatm PCO₂) (Treanor et al. 2017; Hasler et al. 2018). Levels of PCO₂ that prevent dreissenid settlement may also reduce colonization of pH-sensitive native species, especially calcifying organisms, and shift macroinvertebrate and periphyton community composition to more tolerant taxa.

In the present study, we developed a flow-through microcosm test system to assess the effectiveness of CO₂ for preventing the settlement of zebra mussel veligers in a water intake or confined open water system (e.g., marina, culture pond). We used a raw river water supply that continually seeded the system with phytoplankton, various life stages of zooplankton and benthic invertebrates, and zebra mussel veligers. We assessed changes in nontarget benthic taxa to levels of CO₂ that were predicted to reduce zebra mussel settlement. Our objectives were to: (1) determine the effectiveness of CO₂ to prevent veliger settlement, (2) evaluate lethal and sublethal responses of native juvenile mussels to chronic CO₂ exposure, and (3) describe shifts in resident benthic macroinvertebrate and periphyton communities to chronic CO₂ exposure at levels used in AIS control actions.

Materials and methods

Our test system was housed within a mobile laboratory and withdrew water from a harbor of the Mississippi River, Dubuque, IA, to supply a natural source of veligers and plankton to colonize the tanks, similar to that described in Claudi et al. (2012). Water was pumped from the adjacent harbor into a headbox (132 cm, length × 79 cm, width × 36 cm, height). A gravity-flow headbox outflowed to three infusion chambers (101.6 mm, diameter × 38 cm, length; Supplementary material Figures S1 and S2) where gas was mixed and dissolved in the water. Two chambers were infused with CO₂ and one chamber was infused with atmospheric air. We created a calibration curve of pH versus CO₂ concentration in the source water and then regulated CO₂ flow into each chamber based on pH probe measurements in the infusion chambers (Figure S2). Alkalinity was measured in the source water every week, and we recalibrated the pH-CO₂ curve when source water alkalinity changed by ± 50 mg/L. Target CO₂ concentrations for the low (C_{low}) and high (C_{high}) treatments were 30,000 µatm PCO₂ (~ 50 mg/L CO₂) and 55,000 µatm PCO₂ (~ 90 mg/L CO₂), respectively. The low CO₂ level corresponded to pH 6.9, just below the pH reported to prevent veliger settlement (Claudi et al. 2012). The high CO₂

was a maximum level expected to be used in long-term CO₂ AIS application for dreissenid control.

Each infusion chamber (ambient, low, and high CO₂) delivered water to four replicate tanks (66 cm, length × 30.5 cm, width × 30.5 cm, height; 51 L volume) per treatment (3 treatments × 4 replicates = 12 tank total). Flowrate to each tank produced about 1.5 tank exchanges per hour. Ancillary measurements (i.e., water quality, flowrate, temperature) were taken regularly to identify potential noncausal influences on tank position.

Settlement plate assessment

Four settlement samplers were placed into each test tank about 2 weeks before the onset of CO₂ infusion to establish invertebrate and periphyton communities on the plates. Sampler design was modified from Pucherelli and Claudi (2017) and consisted of six PVC plates (12 cm, length × 12 cm, width × 6 mm, thickness) mounted vertically into a slotted base (24 cm, length × 12 cm, width × 1.3 cm, thickness) and spaced ~ 3.0 cm apart (Figure S2). We collected settlement plates from two replicate tanks per treatment on alternate weeks beginning at week 4 (July 3) and extending to week 11 (August 19). During each sampling period, we removed one plate from each of 4 samplers within a given tank, gently scraped both sides of the plate with a razor blade, and returned the plate to the tank to maintain the geometry of the sampler. Each plate was sampled only once during the experiment. We composited the scrapings from the four plates per tank making a single sample per tank. Samples were preserved in ethanol for enumeration of zebra mussels and macroinvertebrates. On the last sampling date, we scraped four plates from all tanks. Macroinvertebrate identification and biomass measurements were conducted by Rhithron Associates, Inc., Missoula, Montana, USA.

Preserved macroinvertebrates were counted and identified to genus level, except Chironomidae which were identified to family level. If more than 300 organisms were present in the sample, macroinvertebrates were subsampled using a grid tray. If less than 300 organisms were present, the entire sample was identified. The subsample that was removed for identification was returned to the composite sample for determination of dry weight. Zebra mussels were separated from all other organisms for enumeration and determination of dry weight. Samples were divided into two mass groups (zebra mussel and “non-zebra mussel”), dried at 60 °C to a constant mass, and dried biomass (0.1 g) was measured. In C_{low} and C_{high} tanks, total biomass was equal to non-zebra mussel biomass. In C_{amb} tanks, we determined total biomass from the sum of zebra mussel and non-zebra mussel biomass.

Periphyton was collected on the last sampling day by scraping one plate per sampler in each test tank ($n = 4$ plates; $n = 12$ tanks). Scrapings from the four plates were combined, as described for benthic invertebrate

sampling, and preserved with 2% Lugol's solution. Periphyton analysis was conducted by BSA Environmental Services, Inc., Beachwood, Ohio, USA. Briefly, cell numbers of all identified algal and cyanobacteria taxa were quantified and converted to cell density (cells per cm²) and biovolume (µm³/L) using either the Utermohl method (Lund et al. 1958) or the membrane filtration technique (McNabb 1960). The abundance of common taxa was estimated by random field counts of 300 to 400 natural units (colonies, filaments, unicells) or a minimum of 50 random fields enumerated to the lowest possible taxonomic level.

Native mussels

Juvenile (1-yr-old) *L. cardium* (Rafinesque, 1820) were obtained from the FWS Genoa National Fish Hatchery, Genoa, Wisconsin, USA, and *L. siliquoidea* (Barnes, 1823) were obtained from the U.S. Geological Survey Upper Midwest Environmental Sciences Center, La Crosse, Wisconsin, USA. Before placement into tanks, we took digital images of individuals (0.75×) with a Nikon CoolPix500 camera mounted on a Nikon SMZ1500 microscope. Shell length, defined as the maximum anterior-posterior axis parallel to the hinge line, was measured on digital images using ImageJ software (nearest 0.02 mm). Mean (standard deviation, SD) initial shell length was 15.0 (2.0) and 10.2 (1.8) mm for *L. cardium* and *L. siliquoidea*, respectively. We placed 10 *L. cardium* and 20 *L. siliquoidea* into one container per tank (15 cm, diameter × 10 cm, depth) and provided ~ 7.5 cm depth of washed river sand for mussels to orient and bury (Figure S2).

At the midpoint (day 40) and end (day 75) of the study, juvenile mussels were removed from containers and assessed for survival and growth. We measured shell length with a digital caliper (Mitutoyo CD-6" ASX, Mitutoyo manufacturing, Tokyo, Japan) (nearest 0.01 mm) because mussels were too large to photograph under the stereomicroscope. At the conclusion of the study, all *L. siliquoidea* ($n = 20$) and two *L. cardium* per tank were sacrificed for determination of dry weight. The remaining *L. cardium* ($n = 8$) were preserved for biochemical and transcriptomic analysis as part of a companion study. We separated soft tissue and shell, dried samples at 60 °C to a constant mass and measured dry mass (0.001 mg) on an analytical balance (AT200, Mettler, Columbus, Ohio, USA). We calculated condition indices to compare the effects of CO₂ on shell and soft tissue. Tissue condition (TC) was calculated as: $TC = (\text{dry tissue mass}/\text{shell length}) \times 100$. Shell condition (SC) was calculated as: $SC = (\text{dry shell mass}/\text{shell length}) \times 100$.

Carbon dioxide and water quality

We measured CO₂ concentration in test tanks by three methods: (1) the partial pressure of CO₂ (PCO₂) was calculated using tank pH and temperature, and headbox alkalinity values (Robbins et al. 2010), (2) %CO₂ was measured

with a CO₂ probe (Vaisala BMP220 and GMT221, Vaisala Corp., Helsinki, Finland) and converted to PCO₂ based on temperature and barometric pressure, (3) CO₂ concentration was measured daily in each tank by titration with 3.636 N sodium hydroxide (NaOH) to a pH endpoint of 8.3 (APHA 2018a).

We measured water quality (dissolved oxygen, pH, temperature) and flow rate at least once a day in each test tank throughout the study. Dissolved oxygen (DO) and pH were measured with LDO101 and pHC101 probes, respectively, connected to a Hach HQ40d meter (Hach Company, Loveland, Colorado, USA). Temperature was measured with a digital thermometer. We placed HOBO® pH and temperature loggers (MX2501, Onset Computer Corporation, Bourne, Maryland, USA) in select test tanks and in the effluent tank for continuous measurement of temperature and pH. Total ammonia nitrogen was measured once a week in each test tank by spectrophotometric methods (Hach DR 3900, Hach, Loveland, Colorado, USA); un-ionized ammonia concentration was calculated using total ammonia nitrogen (NH₄-N mg/L), temperature, and pH of each tank (Emerson et al. 1975). Alkalinity and hardness of the incoming water to the headbox were measured in triplicate at least once a week. Total alkalinity (mg/L as CaCO₃) was determined by spectrophotometric method (Hach DR 3900, Hach, Loveland, CO, USA) or by titrimetric method to a pH endpoint of 4.5 (APHA 2018b). Total hardness (mg/L as CaCO₃) was determined by spectrophotometric method (Hach DR 3900, Hach, Loveland, Colorado, USA) or EDTA titrimetric method (APHA 2018c).

Statistical analysis

We used the Statistical Analysis Software package (SAS, Ver 9.4, Cary, North Carolina, USA) for all statistical analyses, except macroinvertebrate and periphyton community comparisons (see below). We report P values for statistical tests that were conducted. The experimental unit was the tank. Means, ranges, standard deviation (SD), and 95% confidence intervals (CIs) were calculated for water quality measurements (DO, temperature, alkalinity, hardness, ammonia, specific conductance), and biomass on settlement plates. Data were checked for normality and homogeneity of variance (Shapiro-Wilk's and F-max; Sokal and Rohlf 1981). Periphyton biovolume was log transformed before model fitting. Periphyton cell count, biovolume, and native mussel growth (daily rate) were analyzed with a general linear model (Proc GLM) for main effects of CO₂ treatment. Least square means with Bonferroni adjustment identified differences among treatments. Native mussel condition indices were analyzed using a mixed model (Proc mixed). The model included the fixed effect of CO₂ treatment and tank nested within treatment as a random effect. Biomass (excluding zebra mussels) on settlement plates was analyzed using a repeated

measures mixed model with simple covariance structure. Because samples were only collected from two of the four replicate tanks per treatment each week, the data from the first sampling periods for each tank comprised time period one (T1), and from the second sampling period for each tank comprised time period two (T2). Time period three (T3), which occurred on the final day of the experiment, comprised samples from all treatments and test tanks. The model included fixed effects of CO₂ treatment, sampling period, and the interaction of treatment sampling period with repeated measure on the tank. In all mixed models, differences among treatments were compared using least-squares means comparison with Bonferroni adjustment for multiple comparison.

Response of the macroinvertebrate and periphyton community to the CO₂ treatments was analyzed with Permutation Multivariate Analysis of Variance (PERMANOVA+ Prime-E version 7 software, Anderson et al. 2008). Twenty taxa groups were identified in the benthic biofilm on the settlement samples, representing three time periods during the CO₂ exposures. Data from the first sampling periods for each tank comprised time period one (T1), and from the second sampling period for each tank comprised time period two (T2). Time period three (T3), which occurred on the final day of the experiment, comprised samples from all treatment/replicate test tanks. The macroinvertebrate community count data were square-root-transformed before applying Bray-Curtis similarity measures to determine the effects of treatment and time on communities within the tanks. Zebra mussels were found only in the C_{amb} tanks and therefore, were not included in the community analysis. We conducted a repeated-measures mixed model PERMANOVA+ analysis of the Bray-Curtis similarities measure for main effects of CO₂ treatment and sampling period as fixed effects and tank nested within treatments as a random effect. Pairwise comparisons of treatment levels or sampling periods used a Monte-Carlo test with permutation of residuals under a reduced model. We report exact Monte-Carlo *P*-values unadjusted for number of comparisons. Similarity percentages (SIMPER) analysis was conducted to determine taxonomic group(s) driving the dissimilarities observed among treatments and/or time periods. Periphyton community analysis followed that of the macroinvertebrate analysis with the exception that the taxonomic resolution (48 groups) in the data was reduced to six divisions (Cyanophyta, Bacillariophyta, Chlorophyta, Haptophyta, Euglenophyta, and Cryptophyta) to reduce the number of unique taxa (1 observation per taxa) in the data matrix.

Results

Zebra mussels

Veligers were most abundant in the incoming water during the first week of July (mean test tank density = 13.4 veligers/L, SD 3.9). Veliger density

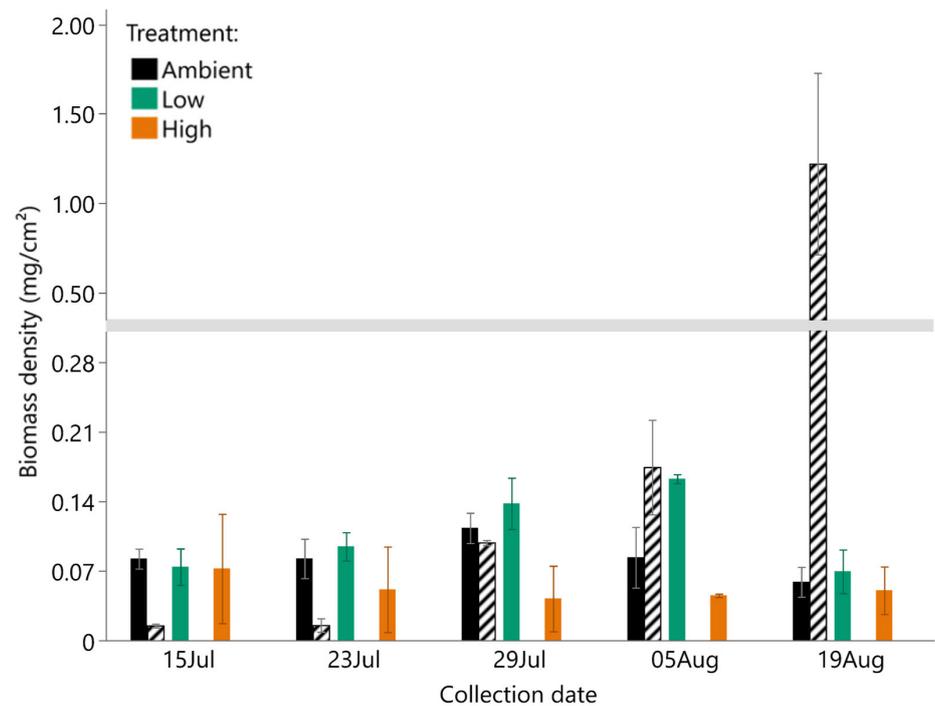


Figure 1. Mean dry biomass density of all macroinvertebrates (solid bars) and zebra mussels (dashed bars) on settlement plates in ambient, low, and high carbon dioxide (CO_2) tanks. No zebra mussels were collected from low and high CO_2 tanks. Settlement samplers were deployed on 06 June 2019 and four plates per tank were removed weekly from two tanks per treatment, except on 19 Aug 2019 when all tanks were sampled. Ambient = 2478 μatm PCO_2 ; Low = 35,094 μatm PCO_2 ; High = 66,685 μatm PCO_2 ; $n = 4$ tanks per treatment.

declined to < 10 veligers/L at subsequent sampling periods (Figure S3, Table S1). There was no detectable difference in veliger density in the inflow to each tank ($F_{2,9} = 0.72$, $P = 0.512$) or in the percentage of veligers retained (inflow-outflow density) among tanks ($F_{2,9} = 0.31$, $P = 0.741$) (Figure S3, Table S1).

We found zebra mussels on settlement plates in C_{amb} tanks starting on July 15 and each week thereafter until the end of the study (Figure S4). We found no settlement on plates in CO_2 treatments except for a single zebra mussel in a C_{high} tank. Cumulative mean (SD) settlement in C_{amb} tanks ranged from 28.9 (3.9) to 67.1 (7.1) mussels per plate (0.10 to 0.23 mussels / cm^2) (Figure S4). At the end of the experiment, the total zebra mussel count in whole tank samples (minus settlement plates) ranged from 552 to 1239 in C_{amb} tanks, 0 to 4 (6 total) in C_{low} tanks, and zero in C_{high} tanks. When the infusion manifolds were dismantled at the conclusion of the study, the C_{amb} manifold had zebra mussels encrusting the inside while the elevated CO_2 manifolds had no attached zebra mussels.

Total biomass on settlement plates in C_{amb} tanks increased concomitant with the number and size of zebra mussels (Figure 1). Total zebra mussel biomass ranged from mean (SD) of 15.5% (3.3%) at the first sampling period to 94.9% (2.0%) at the last sampling period in C_{amb} tanks. No zebra mussel biomass was found in C_{low} and C_{high} tanks. Overall, biomass (excluding zebra mussels) differed by treatment (degrees of freedom, $df_{2,9}$

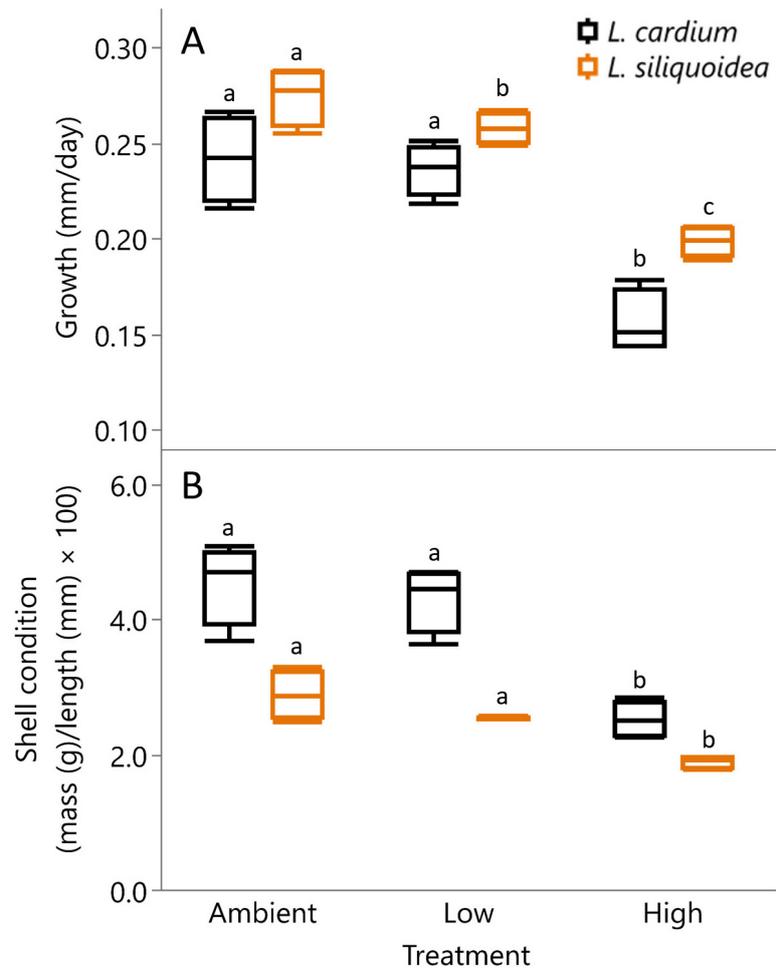


Figure 2. (A) Daily mean growth (shell length) and (B) shell condition (SC) of juvenile *Lampsilis cardium* ($n = 10$ /tank for shell growth) and *L. siliquoidea* ($n = 20$ /tank) in ambient, low, and high carbon dioxide (CO_2) treatments. Ambient = 2478 μatm PCO_2 ; Low = 35,094 μatm PCO_2 ; High = 66,685 μatm PCO_2 . Within a species, plots with the different letters are significantly different ($P < 0.05$). Boxplot rectangle spans first to the third quartile, median is the solid horizontal line, maximum is upper line (whisker), minimum is lower line; $n = 4$ tanks per treatment.

$F = 13.19$, $P = 0.002$) and sample period ($df_{4,9}$ $F = 7.75$, $P = 0.004$); a treatment \times sample period interaction was detected ($df_{4,9}$ $F = 4.46$, $P = 0.011$). Across sample periods, non-zebra mussel biomass was greater in C_{amb} and C_{low} tanks compared to C_{high} tanks (C_{low} vs C_{high} $t = 5.812$; adj $P = 0.002$; Figure 1) and did not differ between C_{amb} and C_{low} tanks ($t = 2.22$; adj $P = 0.160$). Non-zebra mussel biomass decreased at the end of the experiment compared to the midpoint sample period ($t = 3.93$, adj $P = 0.003$).

Native mussels

Native mussel survival was 100% in all tanks except for mortality of a single *L. siliquoidea* in a C_{high} tank at the end of the study. At the end of experiment, *L. cardium* shell length (SD) averaged 34.2 (2.2), 34.3 (3.1), and 27.4 mm (4.5) in C_{amb} , C_{low} , and C_{high} , respectively. Daily mean growth in shell length of both *Lampsilis* species was reduced by CO_2 exposure ($F_{2,9} = 29.21$, $P = 0.001$) especially in C_{high} (Figure 2A, Table S2). Daily mean shell growth of

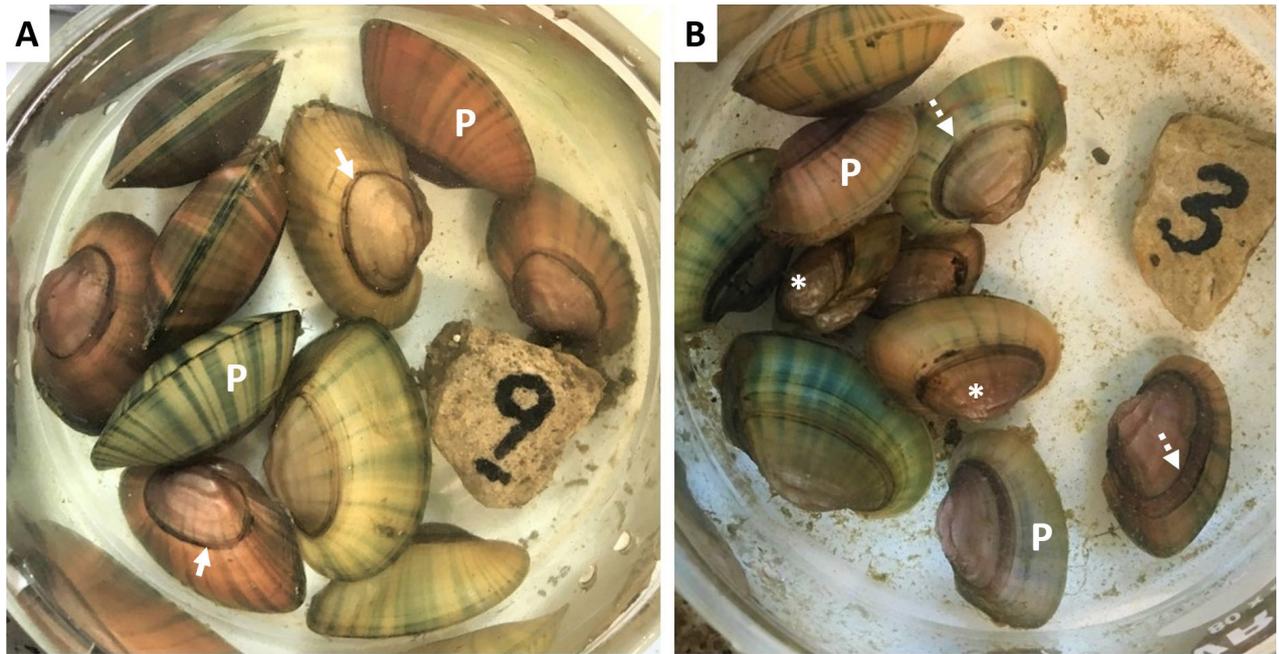


Figure 3. *Lampsilis cardium* from (A) ambient (mean 2478 μatm PCO₂) and (B) high CO₂ treatment tank (mean 66,685 μatm PCO₂) at the end of the study. Note dull pigmentation of the periostracum (P) and erosion on the beak sculpture (*) in mussels exposed to CO₂. New shell growth started at dark line (solid arrow in A) and band (dashed arrow in B). The wide band on shells in high CO₂ (B) indicates interrupted or reduced growth. Photographs by D. Waller.

L. cardium was similar in C_{amb} and C_{low} treatments ($t = 0.44$, adj $P = 1.000$), but was reduced in C_{high} (C_{low} vs C_{high} $t = 6.39$, adj $P = 0.002$; Figure 2A, Table S2). *Lampsilis siliquoidea* shell length averaged (SD) 31.8 (2.9), 30.9 (2.8), and 26.0 (3.7) mm in C_{amb}, C_{low}, and C_{high} treatments, respectively. Daily mean growth of *L. siliquoidea* was also reduced by CO₂ exposure ($F_{2,9} = 60.70$, $P < 0.001$). Growth differences were detected between C_{amb} and C_{high} ($t = 10.48$, $P < 0.001$) and C_{low} and C_{high} ($t = 8.19$, $P < 0.001$) but not between C_{amb} and C_{low} ($t = 2.28$, adj $P = 0.145$) treatments (Figure 2A, Table S2).

Qualitatively, we saw loss of shell coloration and integrity in C_{high} compared to C_{amb} and C_{low} treatments. Shells in C_{amb} and C_{low} tanks had saturated pigmentation of the periostracum and shiny nacre, while those in C_{high} tanks appeared bleached and dull (Figure 3). In C_{high} tanks, we saw loss of periostracum from the umbo, a wide growth band (rather than a line), and increased shell brittleness. The relative loss of shell mass was quantified with the calculated shell condition index (SC). We saw a treatment effect on SC of both *L. cardium* ($df_{2,9}$, $F = 22.56$, $P < 0.003$) and *L. siliquoidea* ($df_{2,9}$, $F = 22.72$, $P < 0.003$; Figure 2B, Table S2). Shell condition of *L. cardium* was reduced in C_{high} compared to C_{amb} ($t = 6.14$, adj $P < 0.001$), but not in C_{low} ($t = 0.71$, adj $P = 1.000$). We detected differences in SC of *L. siliquoidea* between all treatments which was greatest between C_{amb} and C_{high} ($t = 6.63$, adj $P < 0.001$), followed by C_{low} and C_{high} ($t = 4.40$, adj $P = 0.005$). Shell condition in *L. siliquoidea* was similar in C_{amb} and C_{low} treatments ($t = 2.23$, $P = 0.159$) (Figure 2B, Table S2). We detected a treatment effect on tissue

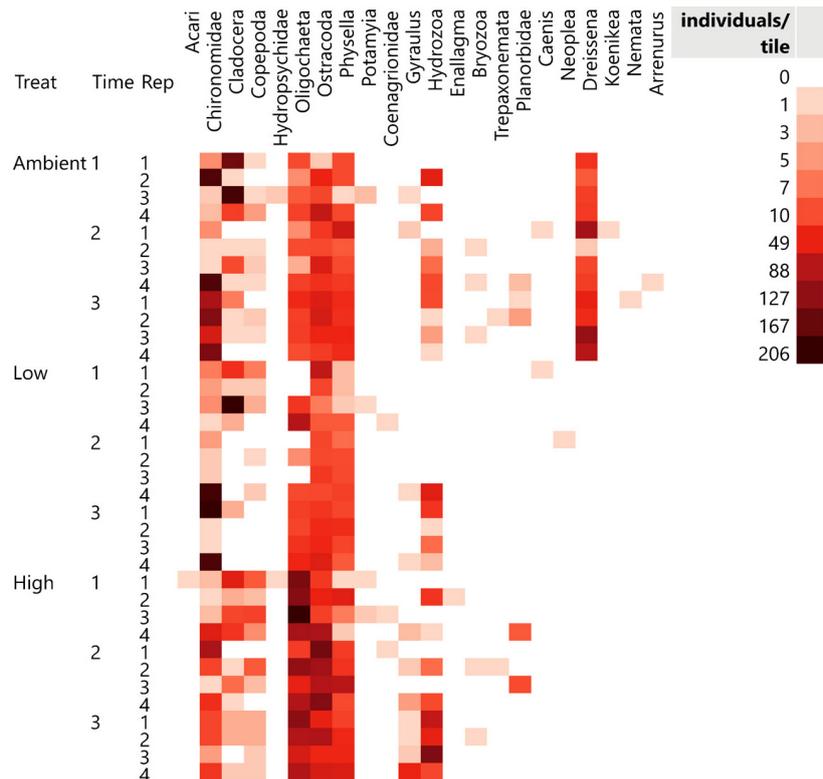


Figure 4. Macroinvertebrate abundance (individuals/tile) on settlement plates in ambient, low, and high carbon dioxide (CO_2) at sample periods T1, T2, and T3. Settlement samplers were deployed on 06 June 2019 and four plates per tank were removed weekly from two tanks per treatment, except on 19 Aug 2019 when all tanks were sampled. Ambient = 2478 μatm PCO_2 ; Low = 35,094 μatm PCO_2 ; High = 66,685 μatm PCO_2 ; $n = 4$ tanks per treatment; Rep = replicate test tank.

condition (TC) of *L. siliquoides* ($df_{2,9}$, $F = 11.82$, $P = 0.003$). *Lampsilis siliquoides* TC differed between C_{amb} and C_{high} ($t = 4.04$, $\text{adj } P = 0.009$) and between C_{low} and C_{high} ($t = 4.37$, $\text{adj } P = 0.005$), but not between C_{amb} and C_{low} ($t = -0.34$, $\text{adj } P = 1.000$; Table S2). Likewise, we found a treatment effect on TC of *L. cardium* ($df_{2,9}$, $F = 60.91$, $P < 0.001$) but the analysis was limited to two mussels per tank. We detected differences in TC of *L. cardium* between C_{amb} and C_{high} ($t = 9.56$, $\text{adj } P < 0.001$) and C_{low} and C_{high} ($t = 9.56$, $\text{adj } P < 0.001$), but no difference between C_{amb} and C_{low} ($t = 0.00$, $\text{adj } P = 1.000$; Table S2).

Benthic community

Twenty taxonomic groups, excluding *D. polymorpha*, were identified in the macroinvertebrate samples. The macroinvertebrate community across all treatments and times was dominated by Oligochaeta (16.3%), Ostracoda (16.8%), and Chironomidae (44.0%) (Table S3). Taxa that contributed 5 to 10% or more of total abundance included Cladocera, Hydrozoa, and Physella. Macroinvertebrates found at < 1% occurrence were Acari, Copepoda, *Potamyia*, Coenagrionidae, *Gyraulus*, *Enallagma*, Bryozoa, Trepaxonemata, Planorbidae, *Caenis*, *Neoplea*, *Koenikea*, Nemata, and *Arrenurus* (Figure 4, Figure S5, Table S3).

Benthic community composition was assessed based on Bray-Curtis similarity measure and differed among the CO₂ treatment levels (Permanova-CO₂, $df_{2,35}$ Pseudo- $F = 12.933$; $P(\text{perm}) = 0.001$), and among sampling periods (Permanova-Period, $df_{2,35}$ Pseudo- $F = 6.091$; $P(\text{perm}) = 0.001$) but did not indicate a treatment \times time interaction (Permanova-CO₂ \times Period, $df_{4,35}$ Pseudo- $F = 0.718$; $P(\text{perm}) = 0.774$). Because pairwise tests between treatment levels indicated the number of unique permutations was < 100 (unique permutations $n = 35$), pairwise Monte-Carlo (MC) tests (999 permutations) were used which indicated differences between the C_{high} and C_{low} treatments ($t = 4.96$, $P(\text{MC}) = 0.001$), C_{high} and C_{amb} ($t = 3.51$, $P(\text{MC}) = 0.001$), and C_{low} and C_{amb} ($t = 1.89$, $P(\text{MC}) = 0.023$). Similarity between C_{amb} and C_{low} was 68.04%, C_{low} and C_{high} was 53.57%, and C_{amb} and C_{high} was 59.3%. Similarity within CO₂ treatments was above 69.5% showing good agreement among replicate test tanks. Taxonomic groups consistently driving differences among treatments were Oligochaeta and Chironomidae with 13 to 26% of dissimilarities; Oligochaeta were commonly found in greater abundance within the C_{high} treatments and in lesser abundance in C_{amb} and C_{low} treatments. Chironomidae were most common in C_{low} treatments followed by C_{amb} and C_{high} treatments in order of abundance (Figure 4). Cladocera was at 19.9% dissimilarities for C_{low} and C_{amb} treatments; however, dissimilarities to standard deviation ratio was less than 0.9 indicating high variability relative to contribution to the dissimilarities. Hydrozoa and Ostracoda each contributed 11 to 12% of the dissimilarities among treatments. Differences in the macroinvertebrate community between sampling periods used pairwise comparisons which indicated differences between T1 and T2 ($t = 2.48$, $P(\text{perm}) = 0.004$), T1 and T3 ($t = 3.10$, $P(\text{perm}) = 0.006$), and T2 and T3 ($t = 2.15$, $P(\text{perm}) = 0.017$). Similarity in macroinvertebrate community composition between sampling periods was as follows: 58.7% T1 vs T2, 67.3% T2 vs T3, and 59.7% T1 vs T3. Similarity within sampling periods was 60.9, 65.8, and 72.4% for T1, T2 and T3, respectively (Figure S5).

Periphyton cell density and biovolume were influenced by CO₂ (cell density $F_{2,9} = 21.40$, $P < 0.001$; logbiovolume $F_{2,9} = 6.49$, $P = 0.018$) with greatest values occurring in C_{high}, followed by C_{low} and C_{amb} treatments (Table 1, Figures S6 and S7). Periphyton measures did not differ between C_{low} and C_{high} treatments, but both differed from C_{amb}. Forty-eight taxonomic groups were identified in the periphyton collected in our study. Because of the substantial number of unique taxa in one or a few samples, the data were aggregated at the taxonomic level of division. Six individual divisions were represented in this aggregation with Cyanophyta (blue-green algae), Bacillariophyta (diatoms), and Chlorophyta (green algae) most observed in the samples (Table 1, Figures S6 and S7). Haptophyta, Euglenophyta (euglenids), and Cryptophyta (cryptomonads) were also represented in the samples but not consistently across treatments (Table 1, Figure S6; note this

Table 1. Periphyton taxa and abundance on settlement plates at the end of the study by treatment. Ambient = 2478 μatm PCO_2 ; Low = 35,094 μatm PCO_2 ; High = 66,685 μatm PCO_2 ; $n = 4$ plates per tank; 4 tanks per treatment.

Treatment	Division	Genera	Genera unique to treatment	Mean (SD) biovolume (mm^3/L)	Mean (SD) 1,000,000 cells/L	
Ambient	Bacillariophyta	<i>Aulacoseira</i> sp.	0	3.11 (1.99)	4.50 (3.08)	
		<i>Cyclotella</i> sp.				
	Chlorophyta	<i>Chlamydomonas</i> sp.	0	2.22 (3.40)	5.41 (3.22)	
		<i>Scenedesmus</i> sp.				
Low	Cyanobacteria	<i>Eucapsis</i> sp.	0	0.01 (*)	33.74 (*)	
	Euglenophyta	<i>Euglena</i> sp.	1	1.5 (*)	1.8 (*)	
High	Bacillariophyta	<i>Achnanthydium</i> sp.	4	5.95 (8.92)	12.37 (14.18)	
		<i>Actinocyclus</i> sp.				
		<i>Aulacoseira</i> sp.				
		<i>Cyclotella</i> sp.				
		<i>Navicula</i> sp.				
		<i>Nitzschia</i> sp.				
		<i>Rhoicosphenia</i> sp.				
		<i>Skeletonema</i> sp.				
		Chlorophyta	<i>Chlamydomonas</i> sp.	0	1.91 (4.41)	6.41 (3.71)
			<i>Crucigenia</i> sp.			
	<i>Dictyosphaerium</i> sp.					
	<i>Drepanochloris nannoselene</i>					
	<i>Scenedesmus</i> sp.					
	Cryptophyta		<i>Cryptomonas</i> sp.	0	1.23 (0.45)	1.8 (0.00)
			Cyanobacteria	<i>Eucapsis</i> sp.	4	0.29 (0.26)
	<i>Leptolyngbya</i> sp.					
	<i>Merismopedia</i> sp.					
	<i>Microcystis</i> sp.					
	<i>Phormidium</i> sp.					
	<i>Planktolyngbya</i> sp.					
<i>Planktothrix</i> sp.						
<i>Pseudanabaena</i> sp.						
Bacillariophyta	<i>Synechocystis</i> sp.					
	<i>Actinocyclus</i> sp.	7	6.80 (9.73)	14.24 (20.04)		
	<i>Aulacoseira alpigena</i>					
	<i>Aulacoseira granulata</i>					
	<i>Aulacoseira</i> sp.					
	<i>Cocconeis</i> sp.					
	<i>Cyclotella</i> sp.					
	<i>Melosira</i> sp.					
	<i>Nitzschia fruticosa</i>					
	<i>Nitzschia</i> sp.					
Chlorophyta	<i>Staurosirella</i> sp.					
	<i>Stephanodiscus</i> sp.					
	<i>Synedra</i> sp.					
	<i>Ulnaria</i> sp.					
	<i>Chlamydomonas</i> sp.	2	1.57 (1.90)	14.25 (15.96)		
	<i>Crucigenia</i> sp.					
	<i>Dictyosphaerium</i> sp.					
	<i>Drepanochloris nannoselene</i>					
	<i>Monoraphidium</i> sp.					
	<i>Scenedesmus</i> sp.					
Cryptophyta	<i>Tetraedron</i> sp.					
	<i>Cryptomonas</i> sp.	0	0.33 (*)	1.80 (*)		
Cyanobacteria	<i>Anabaena</i> sp.	5	0.19 (0.22)	23.53 (38.78)		
	<i>Aphanocapsa</i> sp.					
	<i>Aphanothece</i> sp.					
	<i>Chroococcus</i> sp.					
	<i>Leptolyngbya</i> sp.					
	<i>Limnothrix</i> sp.					
	<i>Merismopedia</i> sp.					

Table 1. (continued).

Treatment	Division	Genera	Genera unique to treatment	Mean (SD) biovolume (mm ³ /L)	Mean (SD) 1,000,000 cells/L
		<i>Microcystis</i> sp.			
		<i>Phormidium</i> sp.			
	Euglenophyta	<i>Trachelomonas</i> sp. 1	1	5.94 (*)	7.21 (*)
	Haptophyta	<i>Chrysochromulina</i> sp. 1	1	0.07 (0.02)	1.80 (0.00)

analysis is based on counts per cm²). Bray-Curtis similarity differed among the CO₂ treatment levels (Permanova-CO₂, $df_{2,11}$ Pseudo- $F = 5.55$; $P(\text{perm}) = 0.012$). Pairwise differences were observed between C_{amb} and C_{high} ($t = 2.61$, $P(\text{perm}) = 0.007$), C_{amb} and C_{low} ($t = 1.57$, $P(\text{perm}) = 0.008$), but not between C_{high} and C_{low} ($t = 1.09$, $P(\text{per}) = 0.347$). Similarity within treatments was 51.6, 83.3, and 71.3%, for C_{amb}, C_{low}, and C_{high}, respectively, indicating the greatest variability in C_{amb}. *Euglena* sp. was the only taxon that was unique to C_{amb} tanks, and Bacillariophyta and Chlorophyta comprised the greatest biovolume (Table 1). Cyanobacteria were absent from C_{amb} tanks except a single species (*Eucapsis* sp.) in one C_{amb} tank. Similarity between treatments was: 46.2% C_{amb} vs C_{low}, 74.3% C_{low} vs C_{high}, and 36.8% C_{amb} vs C_{high}. More than 70% of the cumulative dissimilarities between treatments were from three divisions, Cyanobacteria, Bacillariophyta, and Chlorophyta. In general, these divisions had the greatest mean abundance in C_{high} treatment, followed by C_{low}, and C_{amb} treatments (Table 1, Figure S6). The abundance of all divisions increased in elevated CO₂, accompanied by a shift in community composition that was primarily due to Cyanobacteria, Bacillariophyta, and Chlorophyta (Table 1, Figure S6). Cyanophyta were more consistently found in the CO₂ treatments compared to the controls (C_{amb}).

Carbon dioxide and water quality

Carbon dioxide levels were measured by three methods and are reported as PCO₂ (µatm) and CO₂ concentration (mg/L) for comparability to other reported CO₂ studies (Figure 5, Table S4); hereafter, we refer only to calculated PCO₂ because these values were derived from the most sampling points. PCO₂ levels ranged from 2,419 (SD 1,842) to 2,504 (SD 1,886) µatm in C_{amb} tanks; 33,739 (SD 9,254) to 36,341 (SD 8,925) µatm CO₂ in C_{low} tanks; and 65,289 (SD 14,761) to 68,715 (SD 16,069) µatm CO₂ in the C_{high} tanks (Figure 5, Table S4). Carbon dioxide levels fluctuated during the study but remained similar among replicate tanks within a treatment. The regression of logPCO₂ and pH showed strong correlation between the two parameters ($\log\text{PCO}_2 = 11.4 - 1.003 \cdot \text{pH}$, $R^2 = 0.985$). The effluent tanks in the test system were aerated to off-gas CO₂ and the PCO₂ of discharged water was ≤ ambient PCO₂ throughout the study.

Mean alkalinity and hardness of the source water was 198.1 (SD 54.7; range 134–286) and 276.5 (SD 62.3; range 212–379) mg/L as CaCO₃, respectively. A sharp decrease occurred from June 13 to 17, immediately

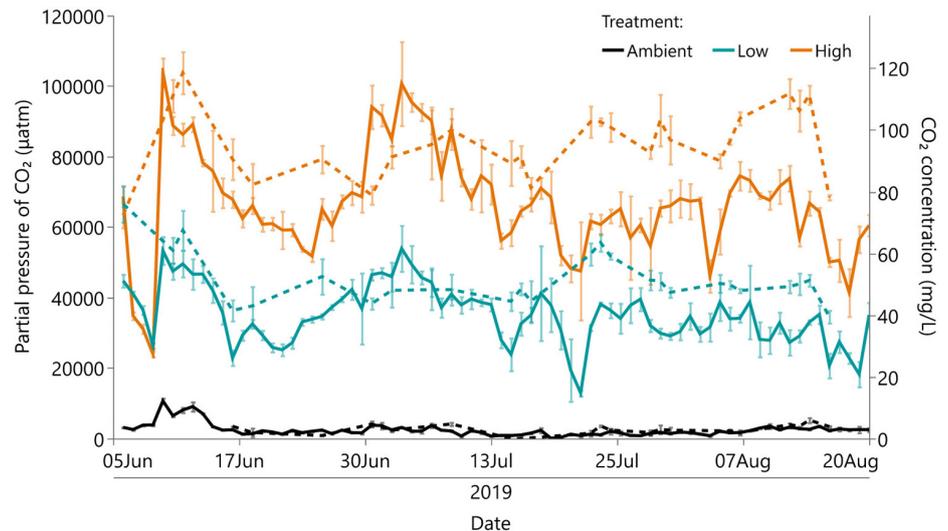


Figure 5. Mean partial pressure (solid line) and concentration (dashed line) of carbon dioxide (CO_2) in ambient, low, and high treatments during exposure period. Error bars indicate standard deviation. Concentration was measured by titration with NaOH; PCO_2 was calculated from pH, temperature, and alkalinity (Robbins et al. 2010). $n = 4$ tanks per treatment.

after opening of the harbor gate to the main channel of the Mississippi River on June 13. Water from the main channel of the river circulated throughout the harbor after this date. Mean (SD) alkalinity and hardness after June 13 were 170.3 (22.8) and 241.5 (21.3) mg/L as CaCO_3 , respectively (Figure S8).

Dissolved oxygen, temperature, and flow rate were similar among test tanks and treatments (Figure S9A–C, Table S5). Mean DO was slightly lower in CO_2 treatment tanks compared to controls, but there was no detectable CO_2 treatment effect. On occasion, debris (e.g., snail) temporarily blocked the inflow line to a test tank, which reduced DO for brief periods. A power failure occurred in week 9 and flow was stopped for ~ 8 h; DO decreased to < 3.0 mg/L for an unknown portion of that time. There was a persistent decrease in DO concentrations in all test tanks in August that was unrelated to the power failure. Mean (SD) DO in C_{amb} was 8.4 (1.3) mg/L in June and July and decreased to 6.3 (1.1) mg/L in August (Figure S9A–C, Table S5). A similar decrease occurred in C_{low} and C_{high} treatments. Mean temperature increased only 0.8 °C during the same time. Mean (SD) pH in C_{amb} tanks was 8.07 (0.26) compared to 6.84 (0.11) in C_{low} and 6.56 (0.13) in C_{high} tanks.

Ammonia is reported as total ammonia-nitrogen (TAN) and un-ionized ammonia ($\text{NH}_3\text{-N}$). We normalized TAN to pH 8 (TAN_{Eq}) for comparison to water quality criteria. Ammonia concentrations were greater in control tanks compared to the CO_2 treatment tanks (Table S6); however, concentrations in all tanks were below chronic aquatic water quality criteria (USEPA 2013). At mean water temperature 25 °C and pH 8.1, the chronic criterion magnitude (CCC) is 0.49 mg TAN/L (USEPA 2013), about four-fold greater than mean concentrations in C_{amb} tanks.

Discussion

Zebra mussels

Carbon dioxide prevented settlement of zebra mussel veligers at $\sim 35,000 \mu\text{atm}$ PCO_2 in our test system. Tanks that received CO_2 -infused water had zero or minimal zebra mussel settlement. Additionally, when the system was dismantled at the end of the study, we found that the two CO_2 infusion chambers were lined with a layer of organic biomass while the control manifold was completely encrusted with zebra mussels. In the whole tank contents, we found no live zebra mussels in C_{high} tanks and a total of six live mussels in C_{low} tanks. We did not determine whether CO_2 killed veligers at this level or only prevented settlement. Veligers flowed out of test tanks to effluent tanks at similar rates (Table S1); however, live zebra mussels were only found in effluent tanks receiving water from the C_{amb} treatment, indicating that CO_2 was lethal and not just a temporary deterrence to veliger settlement. Even lower CO_2 levels may be effective on veliger settlement. Claudi et al. (2012) reported that veligers did not settle below pH 7.1 in water acidified with phosphoric acid. Based on our pH- CO_2 regression, we predict that settlement could be prevented near $20,000 \mu\text{atm}$ PCO_2 in our microcosm.

Mussel control programs that use chemicals such as chlorine and copper employ a range of treatment schedules from continuous to intermittent to single application depending on the targeted life stage and the objectives of the treatment (Mackie and Claudi 2010; Chakraborti et al. 2014). Here, we assessed continuous infusion of CO_2 and did not determine minimum exposure time to prevent settlement. Short-term exposure (96 h) of adult mussels to $\sim 110,000 \mu\text{atm}$ PCO_2 at 20°C reduced attachment by 75% (Waller et al. 2020). Likely, short, intermittent exposure to lesser CO_2 levels would be effective for preventing biofouling by the more sensitive veliger stage. Additional data on minimum lethal concentration and exposure period for veligers would be needed to develop alternative application schedules for CO_2 , like those for chlorine and copper compounds.

Native mussels

Chronic exposure to CO_2 was not lethal to juvenile native mussels at the levels that we tested. Other studies report varying degrees of mortality to juvenile mussels depending on the CO_2 level and exposure duration. For example, in ~ 4 -d exposure to $100,000$ to $120,000 \mu\text{atm}$ PCO_2 at 20°C there was minimal mortality of juvenile *L. cardium* (5%) and *Leptodea fragilis* (7%). In contrast, Jeffrey et al. (2018b) reported $\sim 30\%$ mortality of juvenile *L. siliquoidea* after 14-d exposure to $58,000 \mu\text{atm}$ PCO_2 at 25°C . Survival was dependent on size (shell length), and juveniles > 12.1 mm in length (range 7 to 16 mm) experienced no mortality. In 28-d exposure, the estimated lethal levels to 20% (LC20) of juvenile *L. higginsii* (Lea, 1857) and *L. siliquoidea*

were 31,800 and 58,200 $\mu\text{atm PCO}_2$, respectively (Waller et al. 2019) while we found 100% survival at these levels. We tested larger *L. siliquodea* (mean shell length = 10.2 mm, range 5.6 to 14.9 mm) than were tested by Waller et al. (2019) (mean shell length = 7.2 mm, range 4.2 to 9.2 mm) which may account for some of the lethality differences between studies. Test systems and holding condition may also influence juvenile response to CO_2 . Earlier studies used laboratory water and fed juveniles an artificial diet (Jeffrey et al. 2018b; Waller et al. 2017, 2019), which may not be optimal for their growth and condition. In the present study, juveniles were held in river water and received a constant supply of a natural food source. The microcosm was more similar to the river environment, thus juveniles may have been in better condition and had greater tolerance to a stressor, like CO_2 , than those in a laboratory setting.

Although CO_2 was not lethal to juvenile mussels in our study, sublethal effects on growth and condition were detected in the high treatment. The decline in tissue condition indicated that CO_2 adversely affected tissue growth. Energy (caloric) demands of juvenile mussels are especially high to support tissue growth. Increased metabolic demands of acid-base and ion regulation (Hannan et al. 2016a; Jeffrey et al. 2018a, b) may have diverted energy resources for tissue growth and maintenance in *L. siliquodea* in C_{high} . Like previous studies, we found that CO_2 reduced mussel shell growth and mass, particularly in C_{high} (66,685 $\mu\text{atm PCO}_2$). Waller et al. (2019) reported that shell growth was reduced at half this level (median effective concentration, $\text{EC}_{50} = 28,6000 \mu\text{atm PCO}_2$) in 28-d exposure of the same species. As previously discussed, differences among studies may be due to use of smaller animals (~ 4 mm) by Waller et al. (2019) compared to the present study. The shell is the source of carbonate for acid-base and ionic balance in mussels and a demonstrated consequence of elevated CO_2 in freshwater mussels is reduced biomineralization and shell formation (Jeffrey et al. 2017; Waller et al. 2019). The shell is the primary protection for mussels and reduced shell mass and integrity may increase their susceptibility to injury and predation.

Overall, the reported data on effects of CO_2 to native mussels show that low levels ($\sim 20,000$ to $35,000 \mu\text{atm PCO}_2$) are not lethal to juvenile mussels and would prevent zebra mussel settlement. However, if CO_2 infusion occurs continuously for several weeks, sublethal effects on juvenile mussel growth and condition are likely, even at this low range. Preferably, short and/or intermittent treatment levels can be developed that could reduce biofouling without adversely affecting juvenile mussels. Until more data are developed on size-related toxicity of CO_2 to native mussels, CO_2 use could be considered only for larger juveniles (> 12 mm shell length) and adult mussels. Furthermore, the existing data on CO_2 toxicity to juvenile native mussels is limited to lampsiline species. Additional CO_2 toxicity data are needed, especially for thin-shelled (i.e., Tribe Anodontini) species, to determine its safety more broadly to unionid mussels.

Benthic community

Overall benthic invertebrate biomass was similar between C_{amb} and C_{low} tanks and trended lower in C_{high} tanks (Figure 1). From late July to early August, biomass (excluding zebra mussels) declined in C_{amb} tanks as zebra mussel density increased. At the same time, biomass increased in C_{low} tanks in the absence of zebra mussel competition. However, this same increase did not occur in C_{high} tanks, despite the absence of zebra mussels, indicating an effect of CO_2 on benthic colonization at the higher level. Biomass was similar among all treatments in late August, despite a sharp increase in zebra mussel density in C_{amb} tanks, indicating that macroinvertebrate reproduction and settlement likely peaked in early August. For example, we observed episodes of chironomid emergence in August that likely contributed to decreased biomass on the last sampling date.

Acidification from strong acids is widely reported to cause reductions in macroinvertebrate abundance and richness (e.g., Zischke et al. 1983; Allard and Moreau 1987; Feldman and Connor 1992). Together with reduced biomass, we expected a similar decrease in taxa richness in CO_2 treatments. We found that most riverine taxa in the microcosms tolerated extended exposure to hypercapnia. Only three taxa were unique to the C_{amb} tanks, including two water mite genera, each with a single occurrence. Although reported pH tolerances of macroinvertebrate taxa vary widely, most are below the levels produced by CO_2 infusion in the microcosms. Moreover, the mean pH in C_{high} (6.52 to 6.58) was above the minimum EPA water quality criteria of pH 6.5 (USEPA 2020). We might have detected shifts in species composition within groups with finer taxonomic resolution. More sensitive bioindicator taxa, such as Ephemeroptera, Plecoptera, Trichoptera, were represented by only two genera in the microcosm (Figure 4, Figure S5). *Potamyia* (Trichoptera) and *Caenis* (Ephemeroptera) were found in both C_{amb} and CO_2 tanks, but in low abundance, so we were unable to detect a response of these taxa to CO_2 treatments.

Differences in macroinvertebrate community composition among treatments were driven primarily by Chironomidae, Oligochaetae, Cladocera, Ostracoda, and Gastropoda (i.e., *Gyraulus*, Planorbidae, *Physella*). These groups of invertebrates, particularly crustaceans, oligochaetes, and chironomids, can comprise a major portion of consumer biomass in aquatic habitats and are major food sources for larger invertebrates and vertebrates. Significant shifts or reduction in their abundance that might occur from CO_2 infusion could adversely affect higher trophic levels and nutrient cycles. The two dominant macroinvertebrate taxa, Chironomidae and Oligochaetae, are adapted to a wide range of environmental conditions including hypoxia and organic pollution (Beck 1977; Lindegaard 1995; Rodríguez and Reynoldson 2011). Both taxa have respiratory pigments that provide buffering capacity in low pH water and resistance to the hypoxia

which occur with elevated CO_2 . Therefore, the relatively high abundance of these taxa in the CO_2 treatments was not surprising. Chironomids were less abundant than oligochaetes in C_{high} treatment which indicates greater tolerance of the latter to chronic hypercapnia.

The effects of CO_2 on primary producers in freshwater has focused on phytoplankton, and generally demonstrated increased growth in response to elevated CO_2 (Low-Décarie et al. 2011, 2014; Verschoor et al. 2013; Shi et al. 2015, 2017). We measured chlorophyll *a* to monitor phytoplankton concentration but we did not detect a change because of the short retention time in the test tanks. We also assessed the effects of CO_2 on the periphyton component. Periphyton cell density was greater in CO_2 treatments than the ambient but was not different between CO_2 treatments. The abundance of all divisions increased in CO_2 , but there was a shift in composition from predominately diatoms and chlorophytes in ambient tanks to a greater abundance of cyanobacteria in CO_2 treatment tanks. Cyanobacteria were found in only one C_{amb} tank but were relatively abundant in all CO_2 treatment tanks (Figures S6 and S7). A potential increase in cyanobacteria abundance as a result of CO_2 infusion could adversely affect aquatic invertebrates by reducing food quality (Müller-Navarra et al. 2000; Basen et al. 2012) and producing harmful toxins (Sandrini et al. 2016). In general, nutrients and light being the same, additional CO_2 at levels used in this study would increase periphyton abundance and may favor less desirable groups such as cyanobacteria (Visser et al. 2016), while short-term treatments of CO_2 would likely result in only temporary increase in these groups.

Conclusions

We demonstrated that CO_2 is an effective tool for preventing zebra mussel settlement at $\sim 35,000 \mu\text{atm PCO}_2$. Further investigation is needed to establish minimum effective CO_2 concentrations and exposure duration to develop alternative application schedules that would maximize effectiveness, reduce costs, and minimize nontarget effects. Infusion systems for CO_2 in closed systems could be readily adapted from ours or a similar design. In open water, a CO_2 curtain could be used to reduce settlement/biofouling on water infrastructure (e.g., sumps, trash racks), around marinas (e.g., docks) and in isolated ponds or bays. In closed or open water application, CO_2 can be off-gassed by aeration. Before implementing CO_2 to prevent biofouling around native mussels, more toxicity data are needed for smaller unionid juveniles and additional species. Efficacious concentrations of CO_2 were not lethal to juvenile native mussels, but chronic exposure to the levels tested here could reduce juvenile growth and long-term viability. Our study conducted during peak reproductive period for many freshwater taxa was unique in assessing the cumulative effects of chronic hypercapnia on the benthic community. Elevated PCO_2 did not eliminate any major

benthic groups but shifted abundance to more tolerant taxa. Periphyton abundance and richness increased across divisions, most notably in cyanobacteria, diatoms, and chlorophytes. As CO₂ is implemented in AIS control programs, more extensive investigations would allow managers and regulatory professionals to better understand the primary response of populations and secondary effects on aquatic communities from elevated CO₂ to avoid unintended adverse consequences of its use.

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Authors' contribution

LB, MB, and DW contributed to the study conception and design. TZ oversaw design and construction of the test system. MM, TS, DW conducted the study. MM and TS prepared data summaries, data review and release and prepared figures and tables. LB and DW analyzed data. All authors contributed to writing and critical review of the manuscript and approved the final manuscript.

Disclaimer

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by U.S. Government.

Data and code availability

The data and statistical code in support of this publication are available at Waller and Meulemans (2021). <https://doi.org/10.5066/P99QA1QO>

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Supplementary material

The following supplementary material is available for this article:

Figure S1. Schematic of test system.

Figure S2. Carbon dioxide infusion chamber and test tanks.

Figure S3. Veliger density in inflow to test tanks.

Figure S4. Cumulative zebra mussel settlement in ambient carbon dioxide tanks.

Figure S5. Boxplots of six most abundant macroinvertebrate taxa in ambient, low and high carbon dioxide treatment at three sampling periods.

Figure S6. Boxplots of benthic algae abundance by division in test tanks.

Figure S7. Heatmap of benthic algae abundance by division in ambient, low and high carbon dioxide treatment.

Figure S8. Mean alkalinity and hardness of source water during carbon dioxide exposure period.

Figure S9. Daily mean measurements of (A) dissolved oxygen, (B) temperature, and (C) water flow rate in ambient, low, and high carbon dioxide treatment during the exposure period.

Table S1. Estimated mean, standard deviation, range, and the percent of retained zebra mussel veligers in test tanks.

Table S2. Mean (standard deviation) shell growth and condition indices of juvenile mussels in ambient and carbon dioxide treatments.

Table S3. Macroinvertebrate taxa total count and percent occurrence.

Table S4. Mean, standard deviation, and range of carbon dioxide concentration and partial pressure in treatments during the study period.

Table S5. Mean, standard deviation, and range of dissolved oxygen, pH, temperature, and flow rates in ambient and treatment tanks.

Table S6. Mean, standard deviation, and range of total ammonia-Nitrogen, un-ionized ammonia and TAN equivalent during study period.

This material is available as part of online article from:

http://www.reabic.net/journals/mbi/2021/Supplements/MBI_2021_Waller_etal_SupplementaryMaterial.pdf