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Abstract

Resource managers need for effective methods to prevent the movement of silver (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*) from the Mississippi River basin into the Laurentian Great Lakes. In this study, we evaluated dissolved carbon dioxide (CO_2) as a barrier and deterrent to silver (278 ± 30.5 mm) and bighead (212 ± 7.7 mm) carp movement in continuous-flow outdoor ponds. As a barrier, CO_2 significantly reduced upstream movement but was not 100% effective at blocking fish passage. As a deterrent, we observed a significant shift away from areas of high CO_2 relative to normal movement before and after injection. Carbon dioxide concentrations varied across the pond during injection and reached maximum concentrations of 74.5±1.9 mg/L CO_2; 29 532 – 41 393 µatm at the site of injection during three independent trials. We conclude that CO_2 altered silver and bighead carp movement in outdoor ponds and recommend further research to determine barrier effectiveness during field applications.

Keywords

Carbon dioxide; Asian carp; invasive species; barrier

Introduction

Range expansion of the invasive silver (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*) throughout the Mississippi River basin has been documented by resource agencies since the 1970's (Kelly et al. 2011). These prolific fish cause harm to native aquatic ecosystems by competing with native mussels, early life-stage fish, and other filter-feeding fish for food resources from a planktivorous diet overlap (Irons et al. 2007; Sampson et al. 2009; Walleser et al. 2014). This increased competition has been demonstrated through major shifts in zooplankton communities on the Illinois River where silver and bighead carp are in high abundances (Sass et al. 2014). Recent detections using eDNA and physical captures place the upper range of these invasive carp at tributaries adjacent to the southern Great Lakes (Jerde et al. 2013; Parker et al. 2015). With direct hydrological connection of these tributaries to the Great Lakes, research evaluating potential barriers to prevent further upstream movement has become increasingly important.

Physical barriers are not an option on many navigational rivers due to economic losses from the movement of goods and services. Closure of navigational locks for invasive species control on the Chicago Area Waterway System (CAWS), the shipping channel connecting the Mississippi River basin to the Great Lakes basin, was estimated to cost consumers and commercial traffic $1.3 billion USD annually (Schwieterman 2010). With obvious economic concerns, resource managers have started exploring non-physical barriers that aim to prevent invasive species movement while concurrently allowing water-body navigation (Noatch and Suski 2012).

Fisheries professionals have described a number of potential non-physical strategies for preventing the spread of invasive carps in North America, but most agree that a general lack of data addressing the effectiveness of these techniques will ultimately delay management actions (Wittman et al. 2014). In response, recent studies have begun to address this gap in published literature. Experiments using acoustic barriers, bubble barriers, hydro-guns, carbon dioxide (CO_2), and electricity have all shown varying levels to efficacy to deter invasive carps (Taylor et al. 2005; Kates et al. 2012; Ruebush et al. 2012; Romine et al. 2015). Realizing the need for continued research, the Asian Carp Regional Coordination Committee (ACRCC) prioritized promising technologies (ACRCC MRW 2014) and dissolved CO_2 was identified as a strategy that requires further testing.

A review of non-physical barriers by Noatch and Suski (2012) highlights the potential for CO_2 to work as a barrier for invasive fishes. Further, reviews by Ishimatsu et al. (2005) and Perry and Abdallah (2012) describe a suite of negative physiological responses by fish when exposed to hypercapnia. Most fish sense CO_2 through chemoreceptors located on the gills (Ishimatsu et al. 2005; Perry and Abdallah 2012) and the potential to exploit CO_2 sensitivity in fish suggests that this technique may also work to deter invasive carp movement (Noatch and Suski 2012). In laboratory tests with silver carp, Kates et al (2012) observed avoidance behavior and irregular movements at 70 – 90 mg/L CO_2. Further, recent tests in a static pond confirmed that silver and bighead carp avoided approximately 60 mg/L CO_2 (Donaldson et al. 2016). With evidence growing, the effectiveness of CO_2 as a barrier to invasive carp movement needs to be tested.

The objective for our study was to evaluate silver and bighead carp movement in outdoor ponds in response to a CO_2 barrier over three independent trials. We hypothesized that CO_2 would reduce upstream passage and subsequently deter fish to seek refuge in areas of lower CO_2. Barrier effectiveness was determined by quantifying upstream passage through the injection site. Deterrence effectiveness was determined by quantifying shifts in fish locations relative to changes in CO_2. Using these behavioral metrics, we compared fish movement collected before, during, and after CO_2 injection. Results are focused on the applicability of CO_2 to deter silver and bighead carp passage with implications for reducing the risk transfer between the Mississippi River and Great Lakes basins.

Materials and Methods

Study Animals – We obtained silver carp (n = 15; total length: 278 ± 30.5 mm; wet weight: 229.2 ± 81.9 g; mean ± standard deviation) and bighead carp (n = 15; total length: 212 ± 7.7 mm; wet weight: 101.4 ± 12.3 g; mean ± standard deviation) from the Missouri River near Columbia, Missouri, USA and a private aquaculture farm in Osage Beach, Missouri, USA . Fish were held at the U.S. Geological Survey (USGS) Upper Midwest Environmental Sciences Center (UMESC) Invasive Species Complex (La Crosse, WI USA) until the time of testing. During the week preceding each trial, five bighead and five silver carp were removed from the 1500-L source tanks and placed in a single 130-L flow-through tank at 12±1°C where diet was converted from dry feed to an algal feed mixture. Algal mix contained equal parts of 3.6 g/L Chlorella (Yaeyama Shokusan Co. Ltd., Shiraho, Ishigaki Island, Japan) and Spirulina (Stakich, Inc., Royal Oak, MI, USA) and was the same feed administered during the outdoor trials. Approval from UMESC Animal Care and Use Committee (Protocol Number: AEH-12-PPTAC-01), using similar guidelines as described in Guide for the Care and Use of Laboratory Animals (1996), was gained before study initiation.

Pond Setup - Trials were conducted in outdoor concrete ponds at UMESC. Pond dimensions were 10.0 m long x 4.9 m wide x 1.2 m deep with a volume of approximately 58 700 L. Influent water was supplied directly to the pond from two on-site wells and effluent water was discharged from the pond by spilling over at the top of the opposite wall. Setup was similar to a large choice chamber, with the pond divided into two symmetrical halves by a partial concrete block wall and a 3.1 m opening at the effluent end (Figure 1). Each half contained similar water volumes and a continuous supply of inflowing water (300 L/min) down each side. Wood lattice was affixed to all pond walls, including the brick partition, to reduce reflection of acoustic telemetry signals during fish tracking.

Three micro-bubble diffusers (Model: Point Four™ MBD 300, Pentair Aquatic Eco-Systems™, Apopka, FL, USA) were placed at an equal distance across the opening to one half of the pond and were used to inject CO_2 into the pond. To evaluate changes in fish locations, the pond was divided into two areas (Figure 1). The side where CO_2 was injected was termed "High CO_2 Area" and the opposite side "Low CO_2 Area". Names for each area also describe relative CO_2 concentrations found during CO_2 injection.

For water chemistry sampling, plastic tubing (4.8 mm ID, Python Products Inc., Milwaukee, WI, USA) was run from six locations near the pond bottom to a peristaltic pump (Model 7553-70, Cole-Parmer®, Vernon Hills, IL, USA) outside of the pond area to prevent the potential influence of sample collection on fish behavior (Figure 1). All tubing was placed before fish were stocked into the pond. In the High CO_2 Area, shade was created near the inflowing water as refuge to entice carp to pass through the area of elevated CO_2. Once-daily, 15 L of the algal feed mixture was injected using a small water pump (Little Giant®, Oklahoma City, OK, USA) under the shaded area and served as an additional attractant for fish to cross the High CO_2 area. Diffusers, shade and feeding were placed on the west half of the pond during Trials 1 and 2. Trial 3 was run as a mirror-image of the first two trials; with diffuser, shade, and feeding moved to the opposite (east) pond half. Study events were identical for all three trials.

Acoustic Telemetry - Fish positions were continuously monitored and recorded using an acoustic telemetry array. One Hydroacoustic Technology Inc. Model 290 acoustic tag receiver (HTI, Seattle, WA, USA) was connected to 16 individual HTI Model 590-series hydrophones. Hydrophones were top-mounted at the water's surface by suspending each hydrophone from a grid of cable strung above the pond supported with small foam floats. Locations for each hydrophone were manually surveyed from an origin at the bottom of the southwest pond corner. All hydrophones were secured in the same fashion.

Ten modified HTI Model 795LD acoustic tags were used for each trial. To eliminate the need for surgical implantation of acoustic tags, tags were modified to allow for external attachment using a T-bar whisker (Floy Tag, Seattle, WA, USA) from methods described in Romine et al. (2015). Modifications included a T-bar whisker, foam insulation (approximately 6 mm diameter) and an acoustic tag inserted into heat shrink tubing (BuyHeatShrink.com, Deerfield, FL, USA) with the ceramic end of the acoustic tag and T-bar whisker protruding from the tubing at the same end. Heat was carefully applied to the heat shrink tubing until all components were securely held in place. Modified tags were placed in water and the foam trimmed to achieve near neutral buoyancy. Individual tags were programmed using a HTI Model 490-LP Acoustic Tag Programmer (ATP) with ping rates ranging from 2059 to 2675 ms. Once activated, presence and persistence of pings were verified using the HTI Model 492-B Acoustic Tag Detector before attachment to fish.

During post-processing of acoustic data, valid signal returns were filtered from ambient noise and signal multipath using USGS-developed (public) software, FishCount. Position estimates were then created using USGS–developed (public) software, GeneticFish, from the previous valid detections. Logic filters (i.e., pond borders, distance between detections, velocity, vector angles, and absence of signal) were used to identify and remove any unreasonable position estimates. We determined the accuracy of our telemetry array to be < 0.2 m by comparing known tag locations to post-processed position estimates.

CO_2 Trials - Feed was withheld 48 h before transfer and tagging of fish in the outdoor pond as recommended when sedating fish. All fish were under light sedation using 50 – 100 mg/L AQUI-S®20E (5 – 10 mg/L active ingredient eugenol; AQUI-S New Zealand Ltd., Lower Hutt, New Zealand) during transfer (Cupp et al. *in press*). Modified acoustic tags were disinfected for 15 min using a solution of 3% (v/v) Nolvasan® (active ingredient: chlorohexidine diacetate [2%], Ford Dodge Animal Health, Ford Dodge, IA, USA) and rinsed with deionized water. Five bighead and five silver carp were used during each trial. Fish were removed from the sedative solution and the tag was inserted with a T-bar anchor

tagging gun at a 45° posterior angle into the dorsal musculature approximately 1 cm lateral and posterior to the mid-line of the dorsal fin. This allowed streamline tag protrusion while swimming and ensured that the T-end was securely implanted (Wydoski and Emergy 1983). All fish appeared to recover in <1 min from the light sedation when placed in the pond. However, fish were given until the following day to fully recover from sedation and handling. Water temperature during the stocking of fish was typical of UMESC well water at 12±1°C. During experimentation, recorded pond temperature was 13.0 ± 3.0°C (Trial 1), 11.3 ± 0.4°C (Trial 2), and 8.2 ± 2.5°C (Trial 3) corresponding with air temperatures at the time of testing. Pond water alkalinity measured throughout testing was 137 ± 1 mg/L $CaCO_3$.

After recovery, the first trial began and fish positions were continuously recorded for 72 h. All trials were discretized into three sequential 24 h periods: (1) pre-; (2) during; and (3) post-CO_2 injection. No human influence on fish behavior occurred at any time during these 72h trials. Fish positions collected pre- and post-CO_2 were used to determine baseline occupancy and movement of fish throughout the pond in the absence of CO_2. Study events across all three time periods were identical, except for the date of CO_2 injection. No stimuli (e.g. injection of compressed gas) was added during the pre- or post-injection.

During CO_2 injection, gas was supplied directly from the port on a single 180-L liquid CO_2 tank (Airgas Inc., La Crosse, WI, USA) connected to a manifold of three flow meters (AngelAqua®, Busan, Korea). Diffusers delivered CO_2 at a rate of 3 L/min. Two peristaltic pumps (Model 7553-70, Cole-Parmer®, Vernon Hills, IL, USA) continuously extracted water through the plastic tubing from six locations (Figure 1). Water samples were collected from pumps and analyzed immediately for carbon dioxide by a modified HACH® Method 8205 digital titration method using sodium hydroxide. Briefly, the 100 mL water sample was poured into a 500-mL glass-beaker and placed on a stir plate with a magnetic stir bar. Titrant (0.3636 ± 0.00200 N NaOH) was added to the sample using the HACH® Digital Titrator until an endpoint of pH 8.3 (Beckman Coulter Model 410 pH meter, Beckman Coulter Inc., Chaska, MN, USA) was reached. Partial pressures of CO_2 were calculated from pH, temperature, and alkalinity using USGS CO2Calc (http://pubs.usgs.gov/of/2010/1280/). Alkalinity was measured using the pH 4.5 titrimetric method with 0.02 N H_2SO_4 at various times throughout all three trials from the water samples (APHA 1995). Atmospheric carbon dioxide levels were intermittently measured during each trial using a handheld pSense Model AZ-0001 meter (CO2 Meter Inc., Ormond Beach, FL, USA) to ensure human safety.

At the conclusion of the trial, the pond was drained and fish were euthanized using an overdose (200 mg/L) of MS-222. Trials were repeated using the same methods previously described, except for the injection site, shade, and feed being placed on the opposite pond half during Trial 3. All trials began at exactly 07:00 CDT and subsequent transitions between study phases occurred at exactly 24h thereafter. Trials were conducted October 15, 2014 to November 1, 2014.

Statistical Analysis –Exploratory data analysis approaches was used to visualize data (Tukey 1977). Fish locations were visualized using density plots for pre-, during and post-CO_2 injection. Fish location counts were also visualized for the same three time periods. Carbon dioxide and pH trends were visualized through time. Fish crossings per hour were visualized using box-and-whisker plots before and after CO_2 concentrations stabilized. All data visualization was done with the ggplot2 package (Wickham 2009) in R (R Core Team 2015). Confirmatory statistics were used for parameter estimation and to test the research hypotheses. Generalized linear mixed models (GLMM) with Poisson error terms were used for all statistical comparisons because the three exposure tanks created a nested experimental design and individual fish are pseudo-replicates (Bolker 2008). Trial number and fish ID were included as a random effect. Species and time period (pre, during, and post- injection) were included as fixed effects. For the crossing per hour per fish GLMM, trial had an interaction with CO_2 concentration within the model and was included as a fixed effect. The glmPQL package from the MASS (Venables and Ripley 2002) in R was used for all GLMM's. Statistical significance was declared at $\alpha < 0.05$.

Results

Water Chemistry – CO_2 was stable at ambient levels before and after injection (range: 3.8 - 7.6 mg/L CO_2; 1 776 – 4 688 µatm). During injection, CO_2 increased and pH decreased (Figures 2-3). Highest CO_2 concentrations (max = 74.5±1.9 mg/L; 29 532 – 41 393 µatm) were found at the injection site relative to the rest of the pond. Lowest CO_2 concentrations after 24 h of injection (max: 34.1±8.6 mg/L; 16 683 – 20 352 µatm), were found farthest from injection and nearest to the inflowing fresh water. CO_2 stabilized at maximum levels approximately 4 – 6 h after injection began (Figure 2).

Barrier and Deterrence Efficacy– Signal transmission stopped from one tag in Trial 2 (n=9) and two tags from Trial 3 (n=8) early in each trial and those tags were subsequently removed from the dataset. No signals were lost during Trial 1 (n=10). Physical retention of tags by fish was 100% at the completion of all three trials. After applying filters to the raw telemetry data, 679 248 (Trial 1), 777 807 (Trial 2), and 637 665 (Trial 3) valid fish positions were included in analyses. There were no mortalities during any of the trials.

Silver and bighead carp were found significantly more times in the High CO_2 Area during pre- (t =8.40, $p < 0.0001$, df = 52) and post-injection (t = 7.20, $p < 0.0001$, df = 52) compared to when CO_2 was injected (Figure 4). Detections in the High CO_2 Area did not differ between species (t = -1.329, p = 0.19, df = 23). Conversely, silver and bighead carp were detected significantly more times in the Low CO_2 Area during CO_2 injection compared to pre- (t = -20.63, $p < 0.0001$, df = 52) and post-injection (t = -20.80, $p < 0.0001$, df = 52; Figure 4). This did not differ by species (t = -0.444, p = 0.66, df = 23). Silver and bighead carp crossed upstream through the injection site significantly fewer times during CO_2 injection compared to pre- ($t = 14.46$, $p < 0.0001$, $df = 52$) and post-injection ($t = 6.76$, $p < 0.0001$, df = 52; Figure 4). Density plots visualized the change in fish locations for all three trials (Figures 5,6,7). Fish crossed the injection site in both directions and there were no instances where a single fish remained upstream of the CO_2 injection site. Silver carp crossed fewer times than bighead carp (t = -2.10, p = 0.046, df = 23). Carbon dioxide levels began to stabilize at the injection site around 60 mg/L CO_2 (Figure 2). When CO_2 levels were > 60 mg/L (approximately > 24 000 µatm), silver and bighead carp passed through the injection site significantly fewer times per hour relative to concentrations ≤ 60 mg/L CO_2 (t =-9.22, p < 0.0001, df = 652; Figure 8). Silver carp also passed through fewer times than bighead carp (t = -2675, p = 0.016, df = 16). Trial 3 had the lowest number of crosses (t = -6.48, p < 0.0001, df = 652).

Discussion

The potential for CO_2 to function as a non-physical deterrent has been recently described in the literature (Noatch and Suski 2012; Kates et al. 2012), and our study was the first to quantify the effectiveness of a CO_2 barrier to invasive carps movement. We found that that CO_2 injection was effective to reduce the upstream movement of silver and bighead carp, but did not completely stop fish passage. Similarly, we observed a significant shift in fish movement away from areas of high CO_2. Most notably, results were consistent across all three trials, including when CO_2 was injected into the opposite side of the pond during Trial 3, providing evidence that CO_2 modified silver and bighead carp behavior. This study demonstrates the potential use of CO_2 to deter silver and bighead carp movement and results contribute to a growing body of research evaluating the use of CO_2 as a deterrent to invasive fishes.

Many fish species have been shown to avoid CO_2 during laboratory experiments. For instance, Clingerman et al. (2007) used 60 – 120 mg/L dissolved CO_2 for non-physical transfer of rainbow trout (*Oncorhynchus mykiss*) between large aquaculture tanks with 100 percent effectiveness. A similar approach was conducted by Kates et al. (2012), where avoidance responses were documented from silver carp, bluegill (*Lepomis macrochirus*), and largemouth bass (*Micropterus salmoides*) at concentrations ≥ 100 mg/L CO_2. Further, blacknose dace (*Rhinichthys atratulus*) and brook trout (*Salvelinus fontinalis*) exhibited avoidance behavior when exposed to 120 mg/L CO_2 in small laboratory tanks (Ross et al. 2001). When in our outdoor pond, we observed similar avoidance behavior of silver and bighead carp at concentrations around 70 mg/L CO_2 and found that this technique could potentially be used to deter upstream movement.

The sensitivity of fish to elevated CO_2 warrants continued investigation into the use of this non-physical method. However, with any non-physical deterrence strategy, less than 100% deterrence must be an acceptable outcome (Noatch and Suski 2012; Wittman et al. 2014). During periods of hypercapnia, fish undergo physiological detriments such as ion imbalance (Claiborne et al. 2002), increased stress response (Kates et al. 2012), acidosis (Ishimatsu et al. 2005), hyperventilation (Perry and Abdallah 2012), hypoventilation (Kates et al. 2012), loss of sensory function (Nilsson et al. 2012), and changes in protein composition (Dennis et al. 2015). In conjunction with elevated CO_2, a corresponding decrease in pH occurs due to the hydration equilibrium of CO_2 to form bicarbonate (HCO_3^-) and carbonic acid (H_2CO_3). This decrease in pH may play a role in behavioral avoidance during hypercapnia due to the influence of pH on biochemical processes, however, several studies have shown that fish specifically sense and avoid CO_2 (Ishimatsu et al. 2005; Perry and Gilmour 2006; Perry and Abdallah 2012).

Consideration should be given to the methods used to quantify and units reported of dissolved CO_2, for comparisons with previous literature. Our study quantified CO_2 using methods described in Clingerman et al. (2007), Kates et al. (2012), Dennis et al. (2015), and Donaldson et al. (2016) to allow direct comparisons of the results. Free CO_2 can be easily related with pH, which may also facilitate transition to the field using common water chemistry data loggers (Kates et al. 2012). However, CO_2 gas saturation is dependent on temperature, pressure, alkalinity, biological status and pH. Common units for carbon dioxide are reported or derived in terms of pressure (e.g. PCO_2, mmHg, μatm). While there is a general lack of uniformity in units quantifying CO_2 across current research, recognizing units are important for comparison of results.

Results from our study identify many of the consideration with CO_2 as a control tool in fisheries management. For example, the U.S. Army Corps of Engineers operates and maintains an electrical barrier in the CAWS near Romeoville, IL, USA to inhibit further upstream movement of silver and bighead carp into the Great Lakes basin (Moy et al. 2011). While the electric barrier is currently the most important management tool to deter upstream movement of carp in the Great Lakes, there is a need for redundancy and multiple integrated deterrence mechanisms (Noatch and Suski 2012). Additionally, Noatch and Suski (2012) expressed concern that electricity loses effectiveness on smaller fish due to a reduction in total body voltage at smaller body sizes (Reynolds 1996; Dolan and Miranda 2003). The potential for size-related effectiveness with the electric barrier further supports the need for alternative and supplemental deterrence strategies. In our study, CO_2 deterred stock length silver carp and sub-stock length bighead carp (Phelps and Willis 2013). With branchial CO_2 receptors present throughout the life-history of most fish (Perry and Abdallah 2012), the absence of size-related effectiveness suggests the CO_2 could be used to supplement other barriers to deter smaller fish passage.

Carbon dioxide may be useful to supplement other barriers (e.g. lock and dams or electrical barriers). During scheduled usage or maintenance, CO_2 could be beneficial to deter silver and bighead carp until primary barriers are restored. For instance, CO_2 could be applied to reduce fish from entering or passing gates during planned vessel lockage. One important observation during our study was that adequate time must be allowed for CO_2 to accumulate to target concentrations to be most effective at deterring fish (e.g. Figures 5,6,7). We observed 4 -6 h for CO_2 levels to stabilize using a simple diffusion injection system and recommend exploring improved designs to reduce build-up time. Reducing the build-up time would expand the utility of CO_2 under different applications. Further, understanding operational efficiency of individual CO_2 injection systems under different scenarios will be important during rapid implementation (e.g. unexpected primary barrier failure) and for calculating operational costs. For rapid implementation, other deterrent strategies (e.g. mobile electrical barriers, sound-bubble-strobes, or acoustic noise) may be considered due to their on-and-off style of operation (Taylor et al. 2005; Ruebush et al. 2012; Vetter et al. 2015).

Carbon dioxide was not an absolute barrier to silver and bighead carp in our outdoor ponds. While the success of any barrier is binomial, it is possible that total crossings in our study may have been inflated due to the relatively small spatial size of our test pond. With recorded burst speeds of 1.28 – 1.66 m/s (Hoover et al. 2012), silver and bighead carp in our 10 m pond could have moved upstream through the injection site before sensing elevated CO_2. This hypothesis is further supported by the reduced time that fish spent in the High CO_2 Area. In our study, fish consistently avoided areas of elevated CO_2 when given an option of fresh-water. Further, when concentrations were greater than 60 mg/L CO_2 (~ 24 000 µatm) we also observed the most effective reduction in upstream movement relative to lower concentrations, suggesting a potential dose-response effectiveness (Figure 8). Higher concentrations may be considered for future research. Complementary to the statistical comparisons, visualized distributions of fish in the pond shown in Figures 5,6,7 demonstrate the clear shift in fish locations relative to CO_2 injection. Most notably, similar movement away from the injection site during Trial 3 when CO_2 was moved to the opposite side of the pond confirmed that CO_2 deterred fish. Testing on a larger spatial scale can likely address this shortcoming and application of CO_2 on a larger spatial scale may increase its effectiveness as a deterrent, especially when access to fresh water is made readily available. Locations such as the entrance to approach channels at lock and dams or tributaries on large rivers where fish can choose to stay out in the main channel should be considered for further testing.

Carbon dioxide may provide a safer alternative than other non-physical methods, primarily the electrical barrier in the CAWS. Measurements taken by the U. S. Coast Guard conclude that the electrical barrier can result in a lethal shock to humans if they fall into the water near the barrier (ADRSC 2011). Carbon dioxide exists naturally in the atmosphere as a byproduct of aerobic respiration and burning of fossil fuels at concentrations of 0.038 - 0.04 percent (NOAA 2015). Acceptable human exposure limits of atmospheric CO_2 established by the Occupational Safety and Health Administration (OSHA) were set at 5 000 ppm as a time weighted average (TWA) for an 8 h exposure and 30 000 ppm for acute exposures (OSHA 29 CFR 1910.1000). Atmospheric CO_2 measured before and during our trials were stable at a range of 433 – 498 ppm and did not increase during periods of CO_2 injection. While these concentrations are well below levels generating safety concerns, adequate monitoring should be incorporated when CO_2 is applied at a larger field-scale.

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Figure Captions

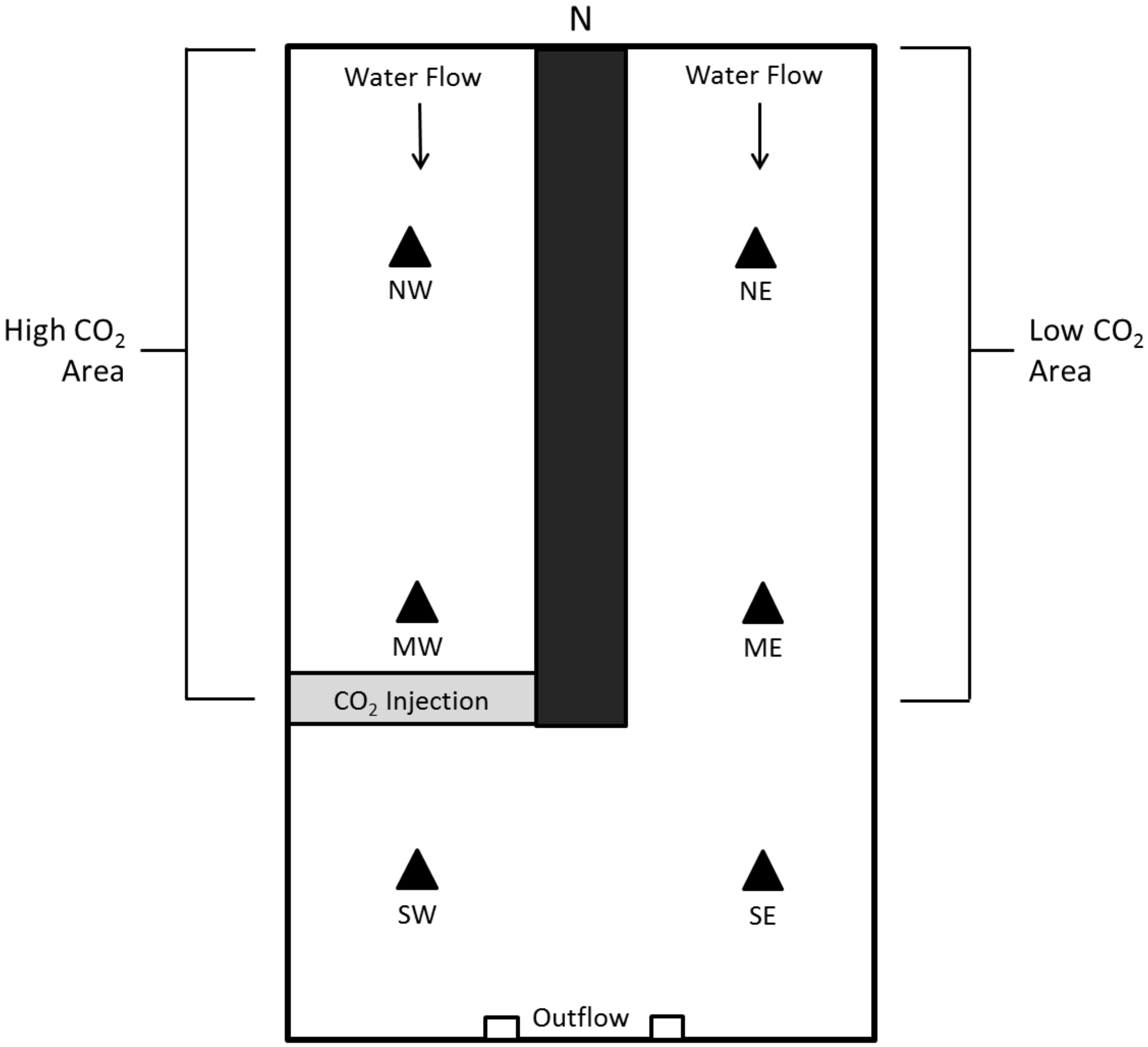
Figure 1: Outdoor concrete pond setup at the Upper Midwest Environmental Sciences Center in La Crosse, WI during the evaluation of CO_2 as a barrier and deterrent to silver (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*) conducted October 15, 2014 – November 1, 2014. Graphical illustration shows setup during Trials 1 and 2. Trial 3 was a reversed setup, with CO_2 injection moved to the opposite side of the center partition. Black triangles show approximate pH and CO_2 spatial sampling locations within the pond: NW (northwest), MW (middle-west), SW (southwest), NE (northeast), ME (middle-east), and SE (southeast).

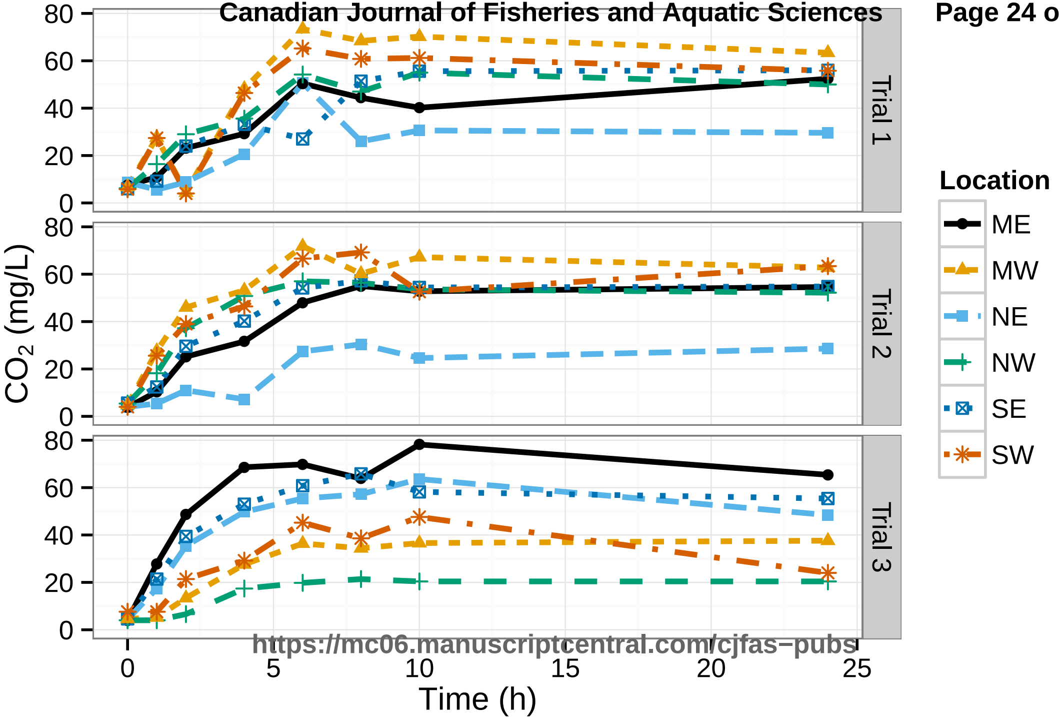
Figures 2,3: Carbon dioxide (CO_2) and pH levels in outdoor ponds during the evaluation of CO_2 as a barrier and deterrent to silver (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*) conducted October 15, 2014 – November 1, 2014 at the Upper Midwest Environmental Sciences Center in La Crosse, WI. We observed increases in CO_2 levels (Figure 2) and decreases in pH (Figure 3) throughout the entire pond during the entire 24h that CO_2 was injected into the pond. Injection was located at MW during Trials 1 and 2. During Trial 3, CO_2 was injected at ME. Locations where values were recorded in the pond are defined as: NW (northwest), MW (middle-west), SW (southwest), NE (northeast), ME (middle-east), and SE (southeast). Values describe a single measurement at each location and time.

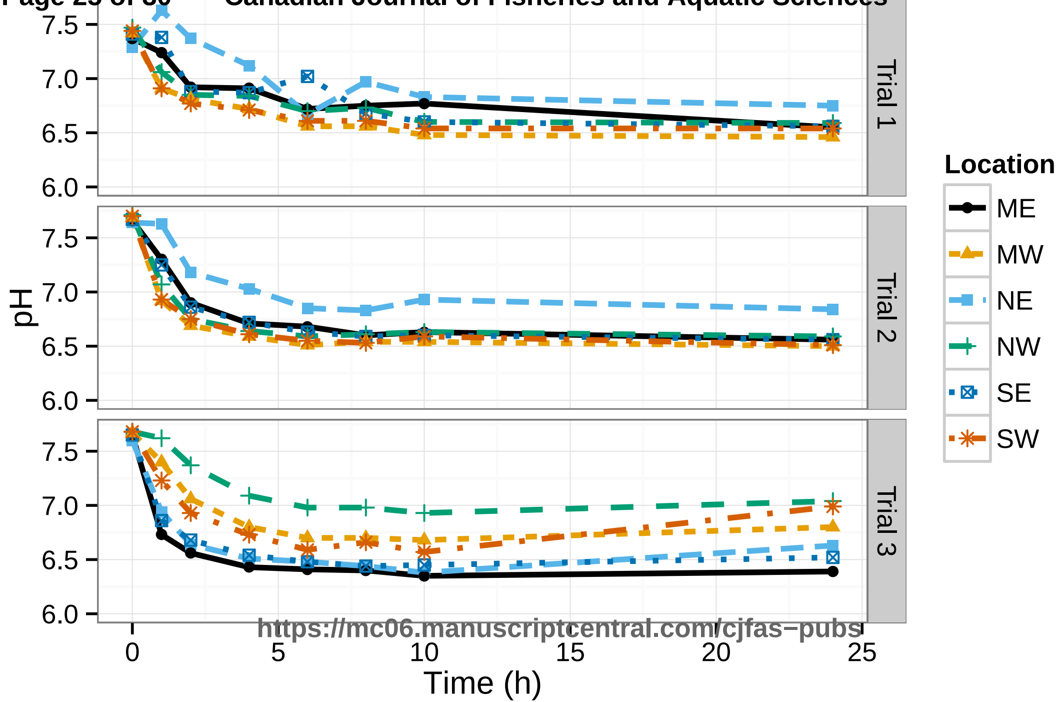
Figures 4: Occupancy and behavior of silver (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*) in outdoor ponds. Detections in the High CO_2 Area (top) decreased significantly while CO_2 was injected into the pond, while detections the Low CO_2 Area (middle) increased significantly. Crosses through the CO_2 injection site were significantly reduced during injection (bottom), but CO_2 was not 100% effective to stop upstream fish passage.

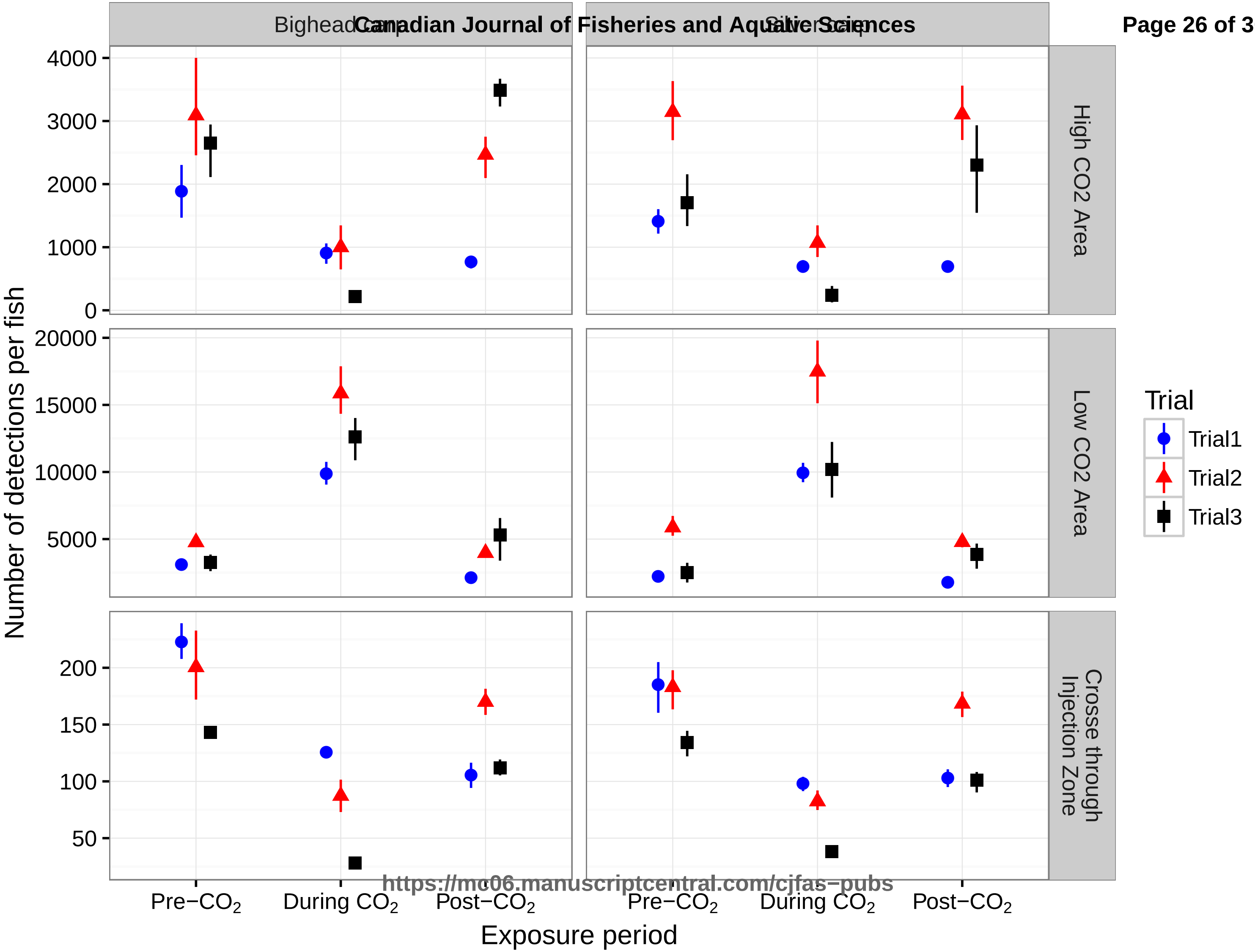
Figure 5,6,7: Density plots illustrating the spatial distribution of silver (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*) during the evaluation of carbon dioxide (CO_2) as a barrier and deterrent in outdoor ponds. Independent trials 1 (Figure 5), 2 (Figure 6), and 3 (Figure 7) were discretized into three 24h periods: before (PreCO2), during (DuringCO2), and after (PostCO2) CO_2 injection. The red line represents the approximate location of the CO2 barrier.

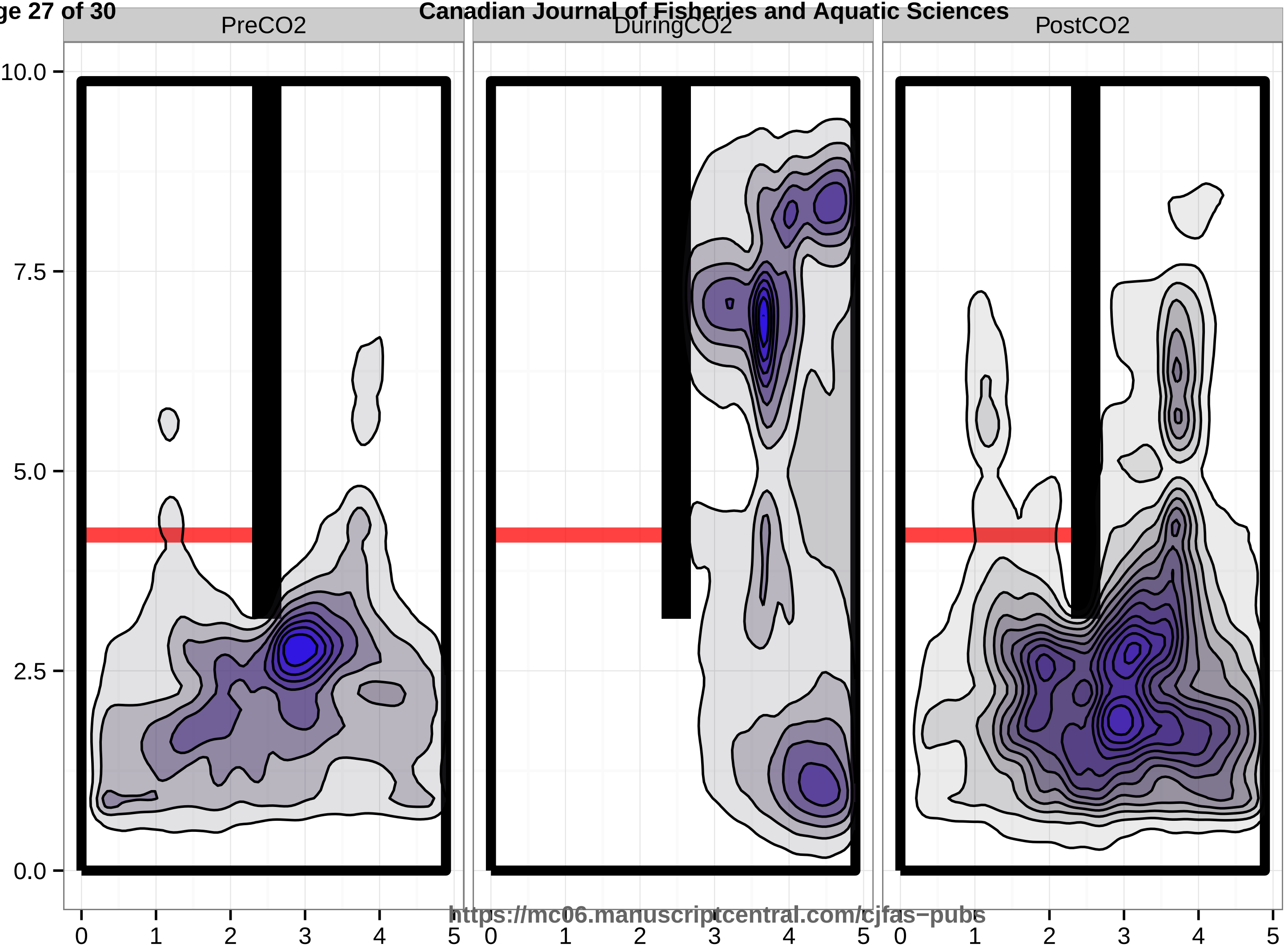
Figure 8: Box-and-whisker plots describing the movement of silver (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*) upstream through a CO_2 injection site in outdoor concrete ponds. The effectiveness of lower concentrations ($\leq$ 60 mg/L CO_2) to impede upstream movement was compared to higher concentrations (> 60 mg/L CO_2). Carp moved upstream significantly fewer times when CO_2 was $\geq$ 60 mg/L CO_2 ($p < 0.01$) relative to lower concentrations. No differences between species were observed ($p = 0.17$).

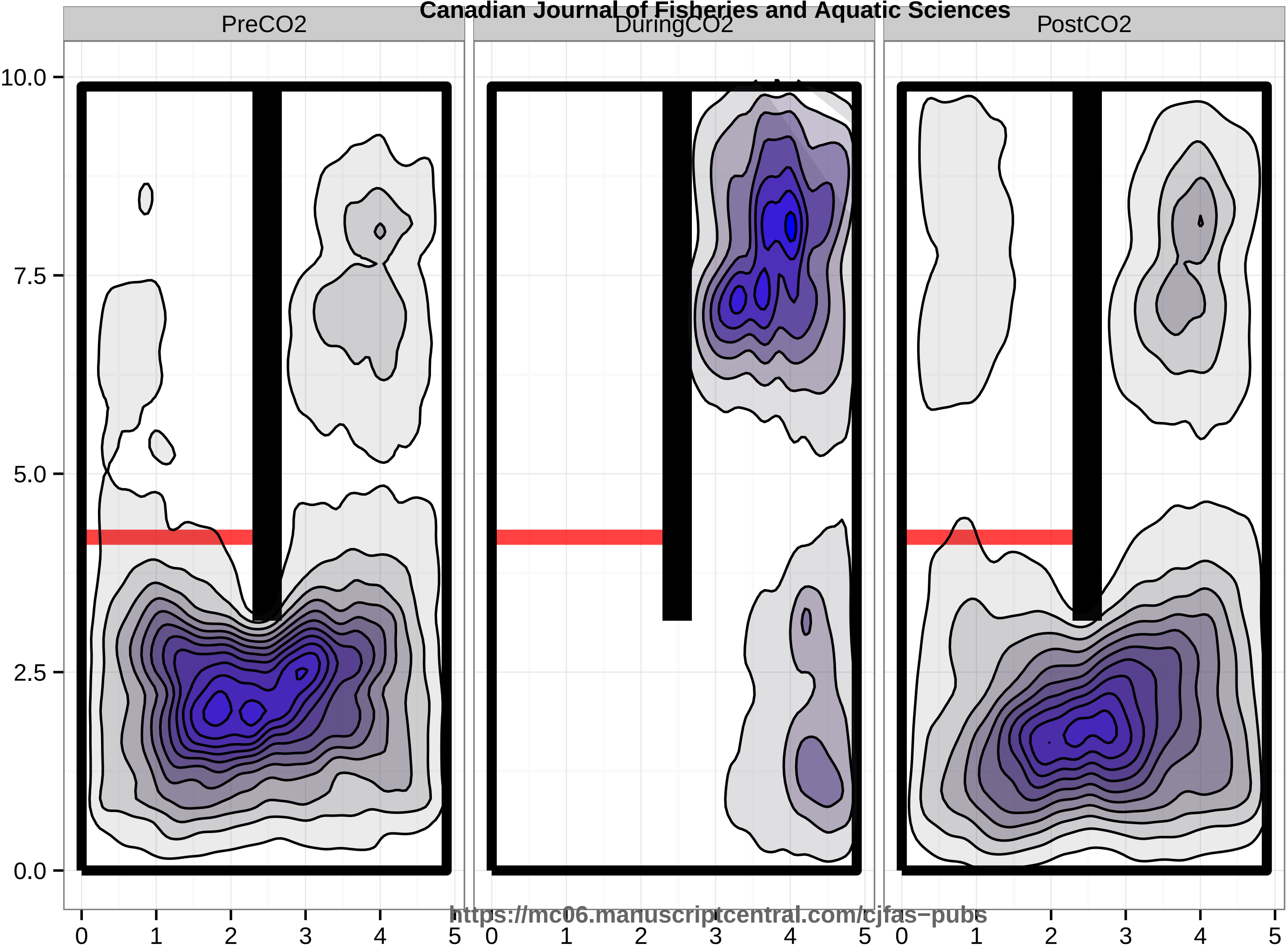

N
Water Flow
Water Flow
NW
NE
High CO_2 Area
Low CO_2 Area
MW
ME
CO_2 Injection
SW
SE
Outflow

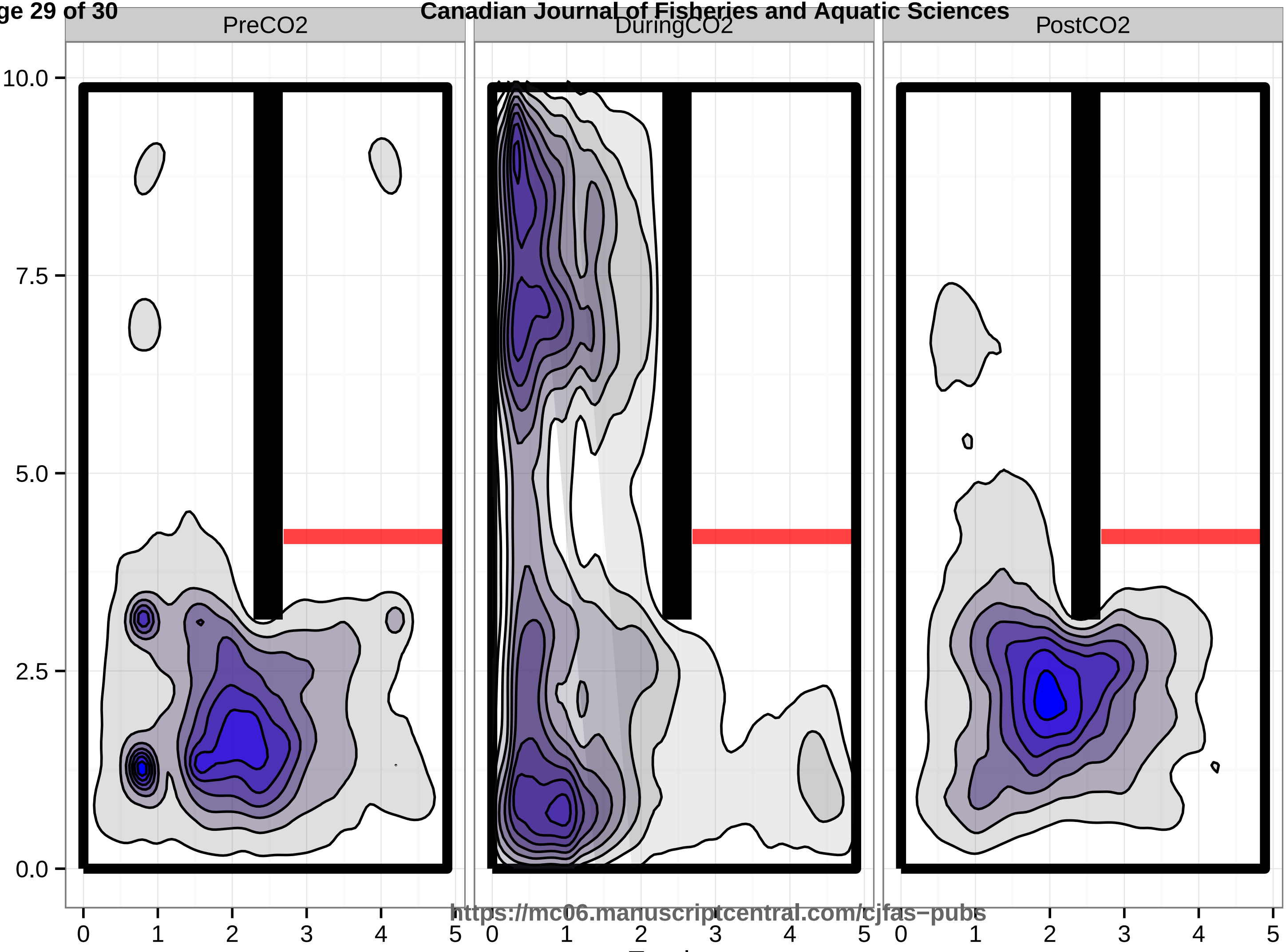

80
60
40
20
0
Trial 1
Trial 2
Trial 3
CO_2 (mg/L)
0
5
10
15
20
25
Time (h)
Location
ME
MW
NE
NW
SE
SW

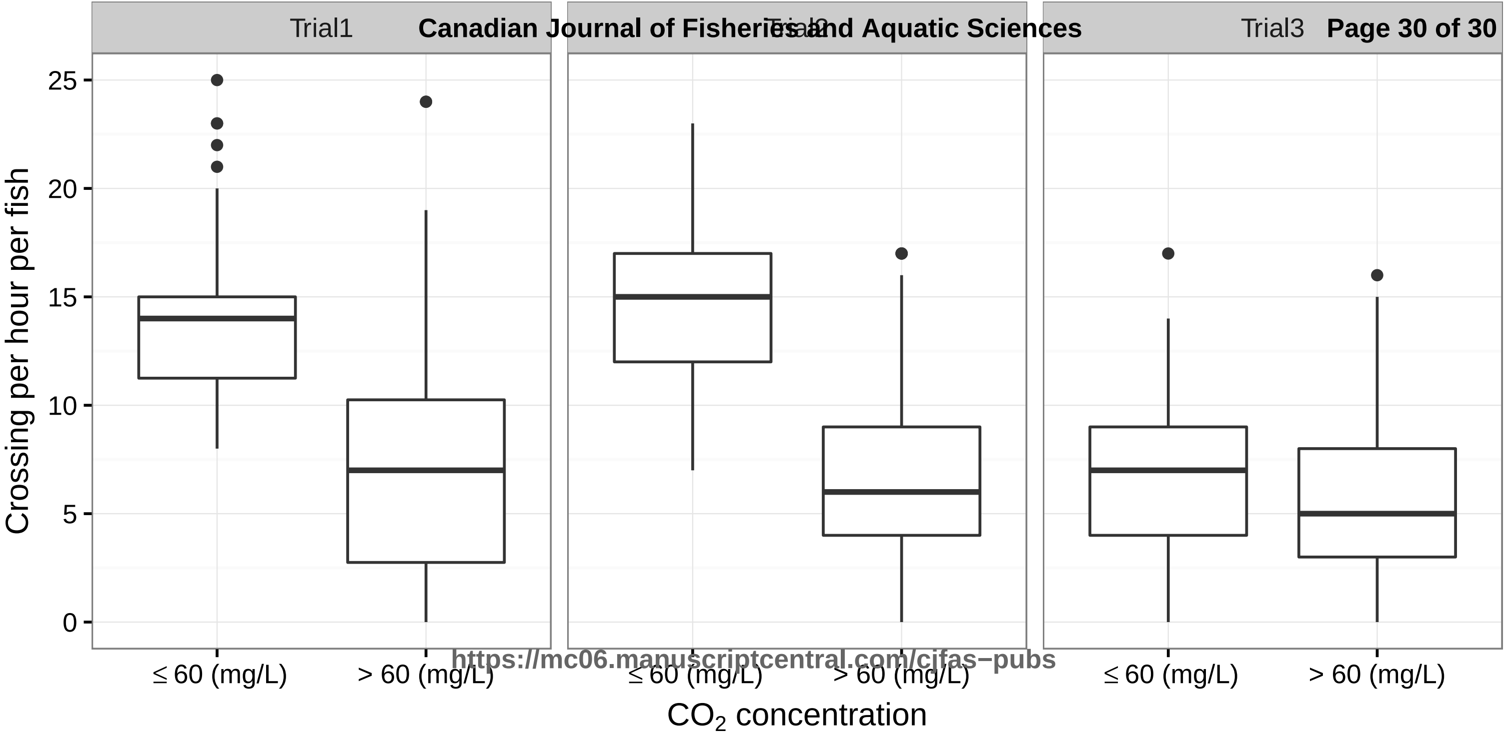

pH
7.5
7.0
6.5
6.0
Trial 1
Trial 2
Trial 3
0
5
10
15
20
25
Time (h)
Location
ME
MW
NE
NW
SE
SW


Bighead Carp
Silver Carp
High CO2 Area
Low CO2 Area
Crosse through Injection Zone
Number of detections per fish
4000
3000
2000
1000
0
20000
15000
10000
5000
200
150
100
50
Pre−CO$_2$
During CO$_2$
Post−CO$_2$
Exposure period
Trial
Trial1
Trial2
Trial3


PreCO2
DuringCO2
PostCO2
10.0
7.5
5.0
2.5
0.0
0
1
2
3
4
5
0
1
2
3
4
5
0
1
2
3
4
5


PreCO2
DuringCO2
PostCO2
10.0
7.5
5.0
2.5
0.0
0
1
2
3
4
5


PreCO2
DuringCO2
PostCO2
10.0
7.5
5.0
2.5
0.0
0
1
2
3
4
5
0
1
2
3
4
5
0
1
2
3
4
5


Trial1
Trial2
Trial3
Crossing per hour per fish
25
20
15
10
5
0
≤ 60 (mg/L)
> 60 (mg/L)
≤ 60 (mg/L)
> 60 (mg/L)
≤ 60 (mg/L)
> 60 (mg/L)
CO_2 concentration