EFFECTS OF SEDIMENT AND WATER COLUMN ACIDIFICATION ON GROWTH, SURVIVAL, AND BURROWING BEHAVIOUR OF MARINE INVERTEBRATES

by

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Bachelor of Science (Honours), University of New Brunswick Saint John 2016

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

Master of Science

in the Graduate Academic Unit of Biology

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This thesis is accepted by the
Dean of Graduate Studies

THE UNIVERSITY OF NEW BRUNSWICK

December, 2018

ABSTRACT

In coastal regions, sediment-dwelling animals are exposed to a high degree of variability in ocean and sediment pH which is expected to increase in the future due to anthropogenic effects. The present study examined the impacts of a 6-week exposure to reduced-pH (acidified) water on length, weight, and mortality of 2 species of molluscs and 1 species of crustaceans that inhabit mudflats: juvenile soft-shell clams (*Mya arenaria*), adult mud snails (*Tritia obsoleta*), and adult mud shrimp (*Corophium volutator*), and subsequently investigated the interactive effects of this exposure and sediment acidification on burrowing behaviour of these species. The predator-prey relationship between mud snails and mud shrimp was also examined by investigating the effects of water column acidification on mud shrimp mortality in the presence of mud snails. Acidified water increased mortality of mud shrimp held alone but not of those in the presence of mud snails, decreased shell length of mud snails, and had no significant effect on soft-shell clams. Sediment acidification reduced mud shrimp burrowing, and prior exposure to acidified water reduced mud snail burrowing, but neither affected soft-shell clam burrowing. These results suggest taxonomic variation in species response to ocean and sediment acidification with respect to growth, mortality, and burrowing behaviour.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my supervisor Heather Hunt for the tremendous support, advice, and guidance throughout this entire process. Thank you to my supervisory committee, Jim Kieffer and Myriam Barbeau, for insightful feedback and critical analysis of my experiment and written thesis. I am beyond grateful for all the help, assistance, and advice from Marie-Josée Maltais, especially with the experimental setup and design. A big thank you to the staff at the Huntsman Marine Science Centre, who were always so kind and helpful with any sort of problems in the lab. This research wouldn't have been possible without the help and assistance that was graciously provided by Jeff Clements, with everything from the experimental setup to alkalinity titration analyses, and instruction regarding the CO2Sys Program. Special thanks to the technicians at UNBSJ and to the graduate and undergraduate students who are part of the Rochette and Hunt labs for any kind of assistance with this project. The completion of this thesis would not have been likely without the endless support and encouragement of my dearest friends and family, being there for me every step of the way.

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Introduction

Over the past century, there has been a noticeable increase in the concentration of atmospheric carbon dioxide (CO_2) from anthropogenic activities, such as burning oil and fossil fuels (Cigliano et al. 2010). Approximately one-third of this excess atmospheric CO_2 is absorbed into ocean surface waters, altering seawater chemistry and reducing seawater pH, a process referred to as "ocean acidification" (Fabry et al. 2008; Kurihara 2008; Duarte et al. 2013; Chien et al. 2018). As the ocean absorbs atmospheric CO_2, the increasing concentration of dissolved CO_2 reacts with water molecules forming carbonic acid (H_2CO_3), which rapidly breaks down into hydrogen (H^+) and bicarbonate ions (HCO_3^-) (Cigliano et al. 2010):

$$CO_2 + H_2O \leftrightharpoons H_2CO_3 \leftrightharpoons H^+ + HCO_3^-$$

H^+ ions produced in this process then react with carbonate ions (CO_3^{2-}) already present in the water column to form HCO_3^-:

$$H^+ + CO_3^{2-} \leftrightharpoons HCO_3^-$$

This lowers the concentration of CO_3^{2-} in the water column, resulting in a reduced calcium carbonate saturation state and leading to the dissolution of calcite and aragonite (calcium carbonate minerals) from the shells and skeletons of calcifying organisms (such as molluscs, corals, echinoderms, and crustaceans). As the concentration of H^+ ions in the water column increases, the dissolution of CO_3^{2-} ions from organisms' calcium carbonate ($CaCO_3$) shells or skeletons increases as well (Fabry et al. 2008; Cigliano et al. 2010; Green et al. 2013):

$$CaCO_3 \leftrightharpoons Ca^{2+} + CO_3^{2-}$$

Overall, the absorption of atmospheric CO_2 into the ocean ultimately results in an increased water column concentration of H_2CO_3, HCO_3^-, and H^+ ions, and a decreased concentration of CO_3^{2-} ions. Consequently, the accumulation of H^+ ions results in a decreased water column pH ($pH = -\log[H^+]$), causing the water column to become more acidic (Fabry et al. 2008; Kurihara 2008; Duarte et al. 2013). Through the process of ocean acidification, it is expected that by the end of the century, the average pH of ocean surface waters will drop by 0.3 – 0.4 pH units from pre-industrial values (Caldeira and Wickett 2005; Fabry et al. 2008; Hagens and Middelburg 2016). Consequently, the threat that ocean acidification poses on marine organisms is a growing concern (Kroeker et al. 2010; Clements and Hunt 2015; Hoegh-Guldburg et al. 2014).

Unlike ocean surface waters, the processes involved in lowering pH in coastal areas are more complex (Duarte et al. 2013; Green et al. 2013) as ecosystem structure, human impacts, and community metabolism are all factors (among others) that drive pH variability in the water and sediments of these regions (Duarte et al. 2013; Waldbusser and Salisbury 2014; Wallace et al. 2014). In comparison to the open ocean with a relatively constant pH of ~8.0, coastal waters have a weaker buffering capacity and are more susceptible to drastic and often abrupt change in pH, having a natural range fluctuating between 7.4 and 8.0 (Wootton et al. 2008; Cornwall et al. 2013; Duarte et al. 2013). This is because coastal regions are repeatedly exposed to acidifying sources, both natural and anthropogenic, that result in decreases in pH (Duarte et al. 2013; Waldbusser and Salisbury 2014). For instance, eutrophication and agricultural runoff (anthropogenic processes), and freshwater input (natural process; e.g. rivers) can all lead to decreased pH in coastal waters. Previous research has demonstrated that acidification is not only

restricted to the ocean's water column, but can be transmitted down through the porewater of underlying sediments, especially in areas along the coast (Dashfield et al. 2008; Widdicombe et al. 2009; Gazeau et al. 2014). The porewater of underlying sediments often has a lower pH and availability of carbonate ions than the overlying water column due to natural ecosystem processes such as microbial decomposition of organic matter deposits, making it a harsh environment for sediment-dwelling organisms (Green et al. 2013). Moreover, decreased water column pH can affect underlying sediment, decreasing sediment porewater pH further, through a process called "sediment acidification". Sediment acidification occurs through the same chemical reactions as ocean acidification (explained above); it is the result of alterations in porewater chemistry and can have negative impacts on sediment-dwelling organisms (Green et al. 2009; Gazeau et al. 2013; Clements and Hunt 2014).

Low pH and carbonate chemistry conditions in the water and sediment imposes kinetic stress on many marine species. Organisms with calcium carbonate shells or skeletons are particularly affected, such as crustaceans and molluscs (Green et al. 2009; Flynn and Smee 2010; Kurihara et al. 2008; Kroeker et al. 2010; Duquette et al. 2017). As these calcifying organisms are exposed to reduced pH and carbonate availability conditions, their shells or skeletons become increasingly susceptible to dissolution (as explained above), which ultimately inhibits them from effectively supporting their living tissue and protecting themselves against predators (Green et al. 2009; Gazeau et al. 2013; Clements and Hunt 2015; MacLeod and Poulin 2015; Duquette et al. 2017). Decreased pH levels have also been found to decrease reproductive success, survival, and growth of some species of crustaceans (Long et al. 2013; Borges et al. 2018). Additionally, low pH

conditions can lead to the development of acidosis of internal body fluids of exposed animals, resulting in a lower than normal blood pH, leading to problems associated with physiological processes such as respiration and excretion (Gazeau et al. 2013). The challenge of ocean and sediment acidification that many benthic organisms experience in their environment today is expected to increase in the future due to increasing anthropogenic CO_2 absorption into the ocean.

Previous work has also suggested that acidified conditions negatively alter marine invertebrate behaviour. For example, studies have shown alterations in gastropod predator avoidance behaviour (Manriquez et al. 2014; Watson et al. 2014) and self-righting behaviour (Manriquez et al. 2013) under acidified conditions, as well as increased activity and altered defensive behaviours in cephalopods under similar conditions (Spady et al. 2014). Moreover, recent studies on crustaceans have demonstrated that water column acidification alters crab foraging behaviour (Dodd et al. 2015) and snapping loudness and frequency of snapping shrimp (Rossi et al. 2016). Additionally, bivalve burrowing behaviour has been shown to be altered under acidified sediment conditions (Green et al. 2013; Clements and Hunt 2014; Clements et al. 2016). Particularly, burrowing behaviour of juvenile soft-shell clams (*Mya arenaria*) is reduced in acidified sediment conditions (Clements and Hunt 2014; Clements et al. 2016), which has been postulated to be an adaptive response to avoid the lethal effects of low-pH sediment conditions, such as increased physiological stress, shell dissolution, and risk of mortality (Clements and Hunt 2018). Research regarding the effects of ocean and sediment acidification on marine invertebrate behaviour is expanding, and more studies

continue to report evidence that low-pH conditions alter behaviour patterns of different taxonomic groups.

Presently, there is a lack of understanding regarding the impacts of sediment and water column acidification on sediment-dwelling invertebrate species. Studies concerning sediment acidification remain relatively scarce, and it is unclear whether the effects of sediment acidification on sediment-dwelling organisms are restricted to bivalves (Green et al. 2013; Clements and Hunt 2014; Clements et al. 2017) or affect a variety of taxonomic groups. Moreover, meta-analyses have revealed significant variation among broad taxonomic groups in physiological and behavioural responses of invertebrates to water column acidification (Kroeker et al. 2010; Kroeker et al. 2014). Additionally, knowledge is limited regarding the interactive effects of multiple environmental factors, and the combined effects of water and sediment acidification on invertebrates have never been assessed. Therefore, the objective of the present study was to examine and compare the effects of water column acidification on growth and survival measures (body length, body weight, and mortality) among 3 species of marine invertebrates inhabiting the Bay of Fundy mudflats: juvenile soft-shell clams (*Mya arenaria*), adult eastern mud snails (*Tritia obsoleta*), and adult mud shrimp (*Corophium volutator*), and subsequently investigate the interactive effect of water column and sediment acidification on the burrowing behaviour of species. These species were chosen as they serve important ecologic roles in intertidal mudflats of the Bay of Fundy. Soft-shell clams and mud shrimp are primary consumers and are predated by several species such as polychaetes, green crabs, and other invertebrates (Raffaelli and Milne 1987; Coffin et al. 2012; Cheverie et al. 2014; Clements and Hunt 2018), while mud snails predate on mud shrimp

among other resources (Coffin et al. 2012). Additionally, to further investigate the effects of water column acidification, predator-prey interactions between mud snails and mud shrimp were investigated, to determine whether predation of mud snails on mud shrimp can be altered by a reduced water column pH. Overall, this study allowed the investigation of the potential variation in response to acidification among different taxonomic groups currently inhabiting the Bay of Fundy mudflats.

Meta-analyses reveal that calcifying organisms (such as bivalves and gastropods) are generally more susceptible to the effects of acidification as their calcium carbonate shells are prone to dissolution under such conditions. On the other hand, more active animals (such as crustaceans) are generally found to be more tolerant to the effects of acidification, which has been postulated to be due to their increased metabolic rates (Kroeker et al. 2010; Kroeker et al. 2014). Therefore, based on these meta-analyses, I predicted that soft-shell clams and mud snails would show a similar response to water column acidification as they are both calcifying organisms, possessing calcium carbonate shells. I also predicted that mud shrimp would show a greater tolerance to the effects of acidification than soft-shell clams and mud snails, since they are crustaceans, and this seems to be the general trend for animals within this Superclass (Kroeker et al. 2010; Kroeker et al. 2014).

This study helps to increase our understanding of ocean and sediment acidification and how it affects growth, mortality, and behaviour of marine infauna. The combination of acidification stressors tested in this study is faced by sediment-dwelling invertebrates in their natural environment today. This study will thus help to provide

information on the vulnerability of coastal infauna to both present-day variability and future impacts of ocean and sediment acidification.

Methods

Experiment Part I: Water Column Acidification

Animal collection and care

The laboratory experiment took place at the Huntsman Marine Science Centre in Saint Andrews, N.B., during the months of July and August 2017. Effects of water column pH on growth and mortality were compared among 3 species: the soft-shell clam *Mya arenaria*, the mud shrimp *Corophium volutator*, and the mud snail *Tritia obsoleta*. A fourth animal treatment (mud snail + mud shrimp) was used to determine if water column acidification had an impact on the survival of mud shrimp in the presence of mud snails. Mud shrimp for the mud shrimp alone treatment were collected from the outer Bay of Fundy (see below). Since the mud snail only inhabits the inner Bay of Fundy, and mud shrimp from this area are exposed to this predator in their natural environment, mud shrimp for the snail + mud shrimp treatment were collected from a site in the inner Bay of Fundy (see below), an area where mud snails are a known predator of mud shrimp. Furthermore, there are differences in population dynamics (Gratto et al. 1983; Barbeau et al. 2009) and genetic differentiation (Einfeldt and Addison 2015) of mud shrimp between the inner and outer Bay of Fundy.

Mud snails (*T. obsoleta*; 1.6 $\pm$ 0.4 cm) were collected at low tide from Mary's Point in N.B., Canada (Mary's Point Road, 45°43'30.1"N 64°40'14.3"W) during the first week of July 2017. They were picked off the surface of the sediment, placed in buckets, and transported to the lab.

Juvenile soft-shell clams (*M. arenaria*; 6.0 ± 2.6 mm) and mud shrimp (*C. volutator*) for the mud shrimp alone treatment were collected from sediment obtained from the outer Bay of Fundy, at a mudflat in Little Lepreau, N.B., Canada (Cassidy Lane, 45°07'28.82"N, 66°28'17.97"W) in June and July, 2017, and maintained in the lab for approximately 2 weeks before the experiment began. Mud shrimp used in the snail + mud shrimp treatment were collected from sediment at a site in the inner Bay of Fundy: Peck's Cove in Sackville, N.B., Canada (Lower Rockport Road, 45°44'56.3"N, 64°28'58.8"W), and maintained in the lab for approximately 1 week before the experiment began. After collecting sediment at each of these sites, it was transported in buckets to the lab to be sieved (1 mm mesh) and juvenile clams and mud shrimp of any size were picked out from the sieved sediment under a microscope.

Animals maintained in the lab prior to the experiment were held in seawater with an average temperature and salinity of 14°C and 33 ppt, respectively. Soft-shell clams were held in a Nitex mesh basket container (42 cm diameter, 19.5 cm deep). The Nitex basket sat inside a larger 18.9 L bucket which was filled with approximately 8 L of seawater aerated with an air stone. The clam bucket was cleaned regularly (every 48-72 h), using a spray bottle of autoclaved seawater, to remove any waste or algal buildup. The clams were fed every 24-48 h with ~250 mL of diluted Instant Algae®, Nannochloropsis sp. (200 µL algae: 1000 mL seawater).

Mud shrimp were held in a plastic container (33 cm long x 20 cm wide x 11.5 cm deep) with flow-through seawater and sediment (~5 cm deep) covering the bottom. Two holes (1 cm diameter) were drilled into the container on opposing sides (1 hole on each side), ~2 cm from the brim. A plastic tube (50.8 cm long, 1 cm diameter) was inserted

into one of these holes which to allow seawater to flow into the container. Another tube was covered with 0.5 mm mesh (to prevent any mud shrimp from going down the drain) and inserted into the other hole, allowing water to exit the container.

Snails were maintained in a large plastic container (56 cm long x 34 cm wide x 17 cm deep) with flow-through seawater and sediment (~8 cm deep) covering the bottom. The container was covered with a lid and two holes (1 cm diameter) were drilled into the container on opposite sides. Tubing was inserted into each hole to allow water to flow into the container on one side, out through the other, and down the drain.

Experimental setup

To determine if exposure to water column acidification over 6 weeks influences juvenile soft-shell clam, adult mud shrimp, and adult mud snail length, weight, and mortality, and mud shrimp survival in the presence of mud snails, two water column treatments (control and acidified water) contained in separate header tanks were set up using a flow-through seawater system at the Huntsman Marine Science Centre in St. Andrews, N.B. Over the course of the experiment, average pH ($\pm$ SD) was 7.64 $\pm$ 0.19 in the acidified (i.e. reduced-pH) water treatment, and 7.89 $\pm$ 0.08 in the control (ambient) water treatment. pH of water treatments was chosen based on the typical pH range found in the water of coastal regions (pH = 7.4-8.0), which can fluctuate quite drastically within a 24 h period as the result of exposure to acidifying sources, both natural and anthropogenic. Additionally, expectations of future declines in pH in coastal waters (Duarte et al. 2013; Clements and Hunt 2015; Waldbusser and Salisbury 2014) were also taken into considerations when determining pH of water treatments of this study.

Sand-filtered seawater (14.2°C; 33.5 ppt) flowed continuously into the header tank (137.2 cm long x 114.3 cm wide x 86.4 cm deep, 1353. 9 L) of each water treatments. In the header tank containing acidified water, a *PINPOINT*® pH monitoring system was used to maintain a water column pH between 7.5-7.7 over the duration of the experiment. A *PINPOINT*® pH probe floating in the acidified water treatment was connected to the monitoring system. When the pH of the water rose above 7.7, a CO_2 cylinder connected to the unit would turn on and CO_2 would be released slowly into the header tank, decreasing the pH of the water column. A large air stone was connected to a *PINPOINT*® CO_2 regulator on the cylinder by an air house and helped diffuse the CO_2 being released into the header tank. When the pH of the water fell below 7.6, the cylinder shut off to avoid a further drop in pH. If the pH did reach the lower set point of 7.5, an air pump that was also connected to the unit would turn on, allowing air to disperse in the water through two air stones, helping to eliminate excess CO_2.

To buffer the pH in both treatments, *Mya arenaria* shell hash collected from the Little Lepreau Basin, N.B., Canada (Little Lepreau Road, 45°07'56.1"N, 66°28'22.4"W) and chicken grit purchased from a local store were sprinkled along the bottom of the header tanks of both the acidified and control water treatments. This helped to maintain a relatively stable pH in the water column due to shell dissolution and the basicity of the chicken grit.

Seawater was pumped from both header tanks into to a single holding tank (360.7 cm long x 119.4 cm wide x 43.2 cm deep) that housed the different animal treatments (see *Experimental Procedure*). Two Supreme® Classic submersible pumps (1892.71 LPH maximum flow) in each header tank pumped water through a plastic tube (292.1 cm

long, 2 cm diameter) to a PVC pipe frame (95 cm long x 34 cm wide x 88 cm high) sitting in the holding tank (Fig. 1). The frame was used to help support and organize tubing, as well as to evenly distribute water to the 80 containers housing the animal treatments (see *Experimental procedure*). Tubing coming from each header tank was connected into valves on all four corners of the frame. Tubes from the acidified header tank were connected to the PVC pipe running along the front edge of the frame, while tubes from the control header tank were connected to the PVC pipe running along the back edge of the frame. A total of 40 valves (20 on each side) came out of opposite sides of the PVC frame. Tubing (50.8 cm long, 1 cm diameter) hooked up to each of those valves and connected to a splitter valve, where two other tubes (142.2 cm long, 1 cm diameter) were connected. Each of those tubes were inserted through the lid of an animal container, allowing either acidified or control water to flow throughout the animal replicate.

Experimental design and procedure

This experiment was made up of two water column treatments (control and acidified) and four animal treatments. The four animal treatments consisted of: mud shrimp alone (50 individuals from the outer Bay, 25 female: 25 male), clams alone (25 individuals), and snails alone (20 individuals). A fourth animal treatment (snail + mud shrimp) was composed of 5 snails and 37 mud shrimp (31 female: 6 male) from the inner Bay of Fundy. Densities and sex ratios of animals in each experimental unit were determined based on the total amount of individuals collected from sediment prior to experiment, preliminary experiments, as well as the natural density of animals on

mudflats in the Bay of Fundy (Peer et al. 1986; Wilson 1988; Curtis 2005). There were 10 replicates for each animal and water treatment combination; therefore, a total of 80 animal containers inside the holding tank (2 water treatments x 4 animal treatments x 10 replicates of each = 80 containers). All 80 containers holding animal replicates were evenly and randomly interspersed amongst one another when placed inside the holding tank.

80 Rubbermaid 1.6 L TakeAlongs® (11 cm long x 11 cm wide x 13.5 cm deep) were used to house the animal treatments. A dremel rotary tool was used to drill holes (9.5 cm x 2 cm) into all four sides of each container, ~1.5 cm from the brim. Subsequently, 1 mm mesh pieces were cut and glued to the container to cover each opening. Additionally, a small hole (1 cm diameter) was drilled into the middle of the lid of each container. This allowed water to enter each container, and then flow out through the mesh. Water ultimately exited the system through a drain located in the holding tank.

Approximately 24 h before beginning the experiment, sediment was collected from Little Lepreau and sieved (1 mm sieve) to remove other invertebrates. The day the experiment began, sieved sediment was put into each of the 80 containers to a depth of ~5 cm.

To begin the water column acidification experiment, the animals for each replicate were counted out. A combined wet weight of all individuals in a replicate was recorded using a Denver Instrument Model XP-300 digital scale. Shell length (maximum measurement along central axis) of each snail was measured using a set of calipers (to the nearest 0.1 mm). Shell length (maximum distance on the anterior-posterior axis) of each clam was measured using an ocular micrometer on a dissecting microscope. Body lengths

(end of second antennae to telson) of mud shrimp were only measured at the end of the experiment (see Experiment Part II: Experimental procedure and animal behaviours) due to their mobility and to avoid any physical harm it may have caused.

For clam, mud shrimp alone, and snail alone treatments, exposure to water column acidification began on July 11 (clams and snails) and 12 (mud shrimp), 2017, and ended on August 21, 2017, lasting a total of 6 weeks. For the snail + mud shrimp treatment, exposure to water column treatments began on July 17 and ended on August 14, 2017, lasting a total of 4 weeks.

Approximately 3-4 days a week, ~150 mL of diluted Instant Algae®, Nannochloropsis sp. (200 μL algae: 1000 mL seawater) was added to each clam replicate for food. Snails and mud shrimp were assumed to be feeding on biofilm growing on the sediment in their respective containers. Biofilm growth was enhanced by the installation of 10 Philips fluorescent plant lights (40W, 121.9 cm long) in the ceiling of the lab ~2 weeks after experiment began. These lights were set to a 16 h: 8 h photoperiod beginning on July 24, 2017. Before the plant lights were installed, regular lights in the lab were generally on during the day and turned off for the evening and night.

At the end of the water column experiment (4 weeks for snail + mud shrimp replicates and 6 weeks for other animal replicates), replicates were sieved (1 mm mesh) and the number of animals remaining in each was tallied. Animals in the snail + mud shrimp replicates were preserved by freezing. All other animals were weighed and measured the same way as the beginning of the experiment and were then used for the sediment acidification experiment (see Experiment Part II: Experimental procedure and

animal behaviour). Body lengths of mud shrimp in the mud shrimp only treatment were not recorded until after Experiment Part II.

Abiotic conditions

Over the course of the experiment, measurements of several water column conditions were made 5 days a week: temperature (using a Fisherbrand™ glass thermometer), salinity (using a salinity refractometer) and pH (using a handheld *PINPOINT*® pH probe and monitor). Prior to recording pH measurements, the *PINPOINT*® probe was standardized to pH 4 and 7 buffers (Ferris Chemicals). All abiotic measurements were recorded from water treatments in each header tank and for the overlying water in animal containers in the holding tank (5 random containers for the first half of the experiment; all containers for the second half of the experiment). Towards the end of the experimental period, water samples (~500 mL) were taken from each header tank for alkalinity titration analysis and preserved with 200 μL of mercuric chloride.

Preserved water samples from control and acidified water treatments were used to fill 10, 20 mL scintillations vials (5 vials filled with control water, and 5 filled with acidified water). Total alkalinity was then determined for each subsample by following the titration method employed by Edmond (1970). Titrations were performed using 0.01 M hydrochloric acid. Total alkalinity values obtained from titration analyses were entered into a CO_2SYS program (Pierrot et al. 2006) along with average salinity, temperature, and water sample pH (from time of collection). With these parameters, the CO_2SYS program calculated aragonite saturation (ΩAr), CO_2 partial pressure (pCO_2), and

bicarbonate concentration ([HCO_3^-]) in water samples. Since mud snail and soft-shell clam shells are primarily composed of aragonite (a carbonate mineral), aragonite saturation state of water samples indicated the likelihood of shell dissolution under treatment conditions; more acidic conditions decrease ΩAr and thus increases the likelihood of shell dissolution (Green at al. 2013; Clements et al. 2017).

A few days prior to ending Experiment Part I, a sediment pH profile was conducted for sediment in 10 animal containers (5 randomly chosen from each control and acidified water treatment). An Accumet AB15 pH meter (Fisher Scientific) fitted with a MI411 microelectrode from Microelectrodes Inc., was used to make sediment pH measurements and was standardized to pH 4 and 7 buffers (Ferris Chemicals). The pH probe was inserted into the sediment using a Velmex Unislide (Velmex Inc., New York, USA) to control vertical position of the probe tip in the sediment. For each animal container, sediment pH was recorded at the surface and then at 1 cm interval depths 1-4 cm below the surface, for 3 different locations on the sediment surface in each container. An average sediment pH for each depth interval was then calculated for each replicate from the 3 measurements obtained.

Experiment Part II: Sediment Acidification

Experimental design

Part II of the experiment took place on August 21, 2017. A 2×2 factorial design was employed to examine the potential combined impacts of sediment acidification and prior exposure to water column acidification on the burrowing behaviour of soft-shell clams, mud snails, and mud shrimp. Following the 6-week exposure to water column

acidification (described in Experiment Part I), animal replicates from control and acidified water treatments were exposed to control and acidified sediment conditions (with overlying control seawater, pH ~7.90) to determine if species' burrowing response to sediment pH treatments differed following exposure to control and acidified water column treatments. This yielded a total of 4 experimental treatment combinations: 1. control sediment, animals previously exposed to control water; 2. acidified sediment, animals previously exposed to control water; 3. acidified sediment, animals previously exposed to acidified water; and 4. control sediment, animals previously exposed to acidified water. Each treatment combination was replicated 5 times. All animals used for sediment acidification trials came directly from Experiment Part I; i.e., animals were not rearranged among replicates.

Sediment pH manipulation

Sediment was obtained from the Little Lepreau mudflat at low tide, 24 h prior to the experiment. Collected sediment was brought back to the lab and sieved (1 mm mesh) to remove larger infauna and rocks. For acidified sediment treatments, sediment pH was manipulated through the addition of CO_2 from a cylinder in the laboratory via a hose and air stone that was gently stirred through ~8 L of sieved sediment in a 15 L bucket for 30-60 s, allowing CO_2 to diffuse into the mud and ultimately lower the pH of the sediment. A stir-stick was then used to mix the sediment for 30 s to ensure a uniform diffusion of CO_2 throughout the mud. For both control and acidified sediment treatments, sediment pH was measured at 5 random depths in the top 2 cm. Because all sediment in a treatment started with the same pH, no gradient in sediment pH was expected over the short

duration (≤ 4 h) of experiment part II. pH measurements of the sediment were made using an Accumet AB15 pH meter (Fisher Scientific) fitted with a MI411 microelectrode from Microelectrodes Inc., standardized to pH 4 and 7 buffers (Ferris Chemicals). An average pH of surface sediment was calculated for the 10 replicates from the five measurements per container.

Following sediment manipulations, two samples of sediment from each of the acidified and control sediment treatments were transferred into 15 mL centrifuge tubes for alkalinity titration analysis and preserved with 6 μL of mercuric chloride. Centrifuge tubes containing preserved sediment samples were later centrifuged at 3000 rpm for 5 min to extract porewater, which was decanted into 3, 20 mL scintillation vials. Total alkalinity analyses were performed on the porewater and values obtained were entered into a CO_2SYS program (as described in Experiment Part I: Abiotic conditions). pH values entered into the program represented the average surface sediment pH of control and acidified sediments for mud shrimp trials of Experiment Part II.

For each animal species, sediment acidification trials were run at different times within the same day, with a different batch of sieved sediment (i.e., the amount of CO_2 added to acidified sediment treatments varied slightly among batches); for this reason, control and acidified sediment pH values differed between the trials for the different animal species.

Experimental procedure and animal behaviour

Following Experiment Part I, animal replicates were removed from the holding tank and the animals were then used for sediment acidification trials. Sediment in each

replicate was sieved and individuals were picked out and placed in a labeled Petri dishes containing control seawater (pH ~7.90). This continued until individuals from all 20 replicates for each species were isolated. This method allowed for replicates to remain consistent from Experiment Part I to Experiment Part II and provided time for individuals to acclimate to control seawater prior to sediment acidification trials.

For each species, sediment acidification trials were run for one species at time, throughout the same day. Of the 10 animal replicates for each water column treatment, 5 were randomly chosen to be used for the acidified sediment replicates, while the remaining 5 were used for the control sediment replicates. Rubbermaid TakeAlong® containers were used as experimental units for each species (clams: 284 mL, 8.5 cm diameter, 7.5 cm deep; snails: 3 L, 23 cm diameter, 13 cm deep; mud shrimp: 1.2 L, 11 cm long x 11 cm wide x 10 cm deep). For snail containers, a dremel rotary tool was used to create a small hole (1 cm diameter) into the middle of the lid and 4 rectangular holes (2 mm thick, 6 cm long) along the sides of each experimental unit. For each species, 10 containers were filled with control sediment, while the other 10 were filled with acidified sediment. Experimental units were filled with sediment to a depth of ~6 cm for snails, ~5 cm for mud shrimp, and ~7.3 cm for clams. For snails and mud shrimp, control seawater flowed into experimental units through a plastic tube (142.2 cm long, 1 cm diameter) inserted into each lid through the drilled hole. Clam replicates were submerged within 3 trays filled with control seawater. Before placing animals on sediment treatments, water flowed throughout experimental units for ~5 min to allow stirred-up sediment to settle.

Animals for each replicate were carefully placed on the surface of the sediment. After the last individual was placed in each replicate, the time was recorded to ensure an

equal amount of time to burrow among replicates. The length of sediment acidification trials differed between species: clams were allowed to burrow for 20 min, mud shrimp for 2 h, and mud snails for 4 h, upon which burrowing behaviour was assessed for all replicates. Each of these burrowing time periods were based on previous work (Clements et al. 2017) as well as preliminary experiments observing burrowing behaviour of species.

For each species, burrowing response to sediment acidification was recorded as completely burrowed, not burrowed, and partially burrowed (for snails only). Burrowing behaviour of animals was assessed at the end of each trial by visually counting the number of individuals remaining on the surface of the sediment and, in the case of snails, on the walls or lid of the container, which were classified as not burrowed. For mud shrimp, unburrowed individuals also included those swimming in the water column. Snails were considered to be partially burrowed if their shells were above the surface of the sediment while their bodies were burrowed into the sediment.

At the end of the experiment, clams, snails, and mud shrimp were preserved by freezing. Subsequently, the body lengths of mud shrimp individuals were determined by using a set of calipers (to the nearest 0.1 mm) so that comparisons could be made between replicates exposed to the control and acidified water column treatments. As previously mentioned, lengths of all other animals (i.e., soft-shell clams and mud snails) were determined immediately before sediment acidification trials began, while animals were still alive.

Statistical Analyses

Using R version 3.0.2 and a significance level of 0.05 (α=0.05), t-tests were performed to determine the effects of water column acidification on animal length, wet weight, mortality, and the survival of mud shrimp in the presence of mud snails at the end of Experiment Part I. Two-way ANOVAs were used to examine combined impact of sediment and water column acidification on the burrowing behaviour of soft-shell clams, mud snails, and mud shrimp for Experiment Part II. All factors (sediment/water pH) were fixed and had two levels (control and acidified sediment or water). Since experimental data were proportions, data were transformed using arcsine square-root transformation. Homogeneity of variances and normality of residuals were assessed with Levene's tests ($p<0.05$) and Q-Q plots, which showed that the data satisfied the ANOVA assumptions, both before and after transformation.

A post-hoc power analysis was conducted on the interaction of water and sediment acidification and its effects on burrowing behaviour of soft-shell clams in Experiment Part II. Given the large amount of variability in the data, I tested the power to detect a significant effect with a variety of critical effect sizes. In R, the function "power.anova.test" was used to conduct this analysis using 5 different critical effect sizes (0.1, 0.2, 0.3, 0.4, and 0.5), a sample size of 5, an alpha level of 0.05, and 4 levels (1. Control water, control sediment; 2. Acidified water, control sediment; 3. Control water, acidified sediment; and 4. Acidified water, acidified sediment).

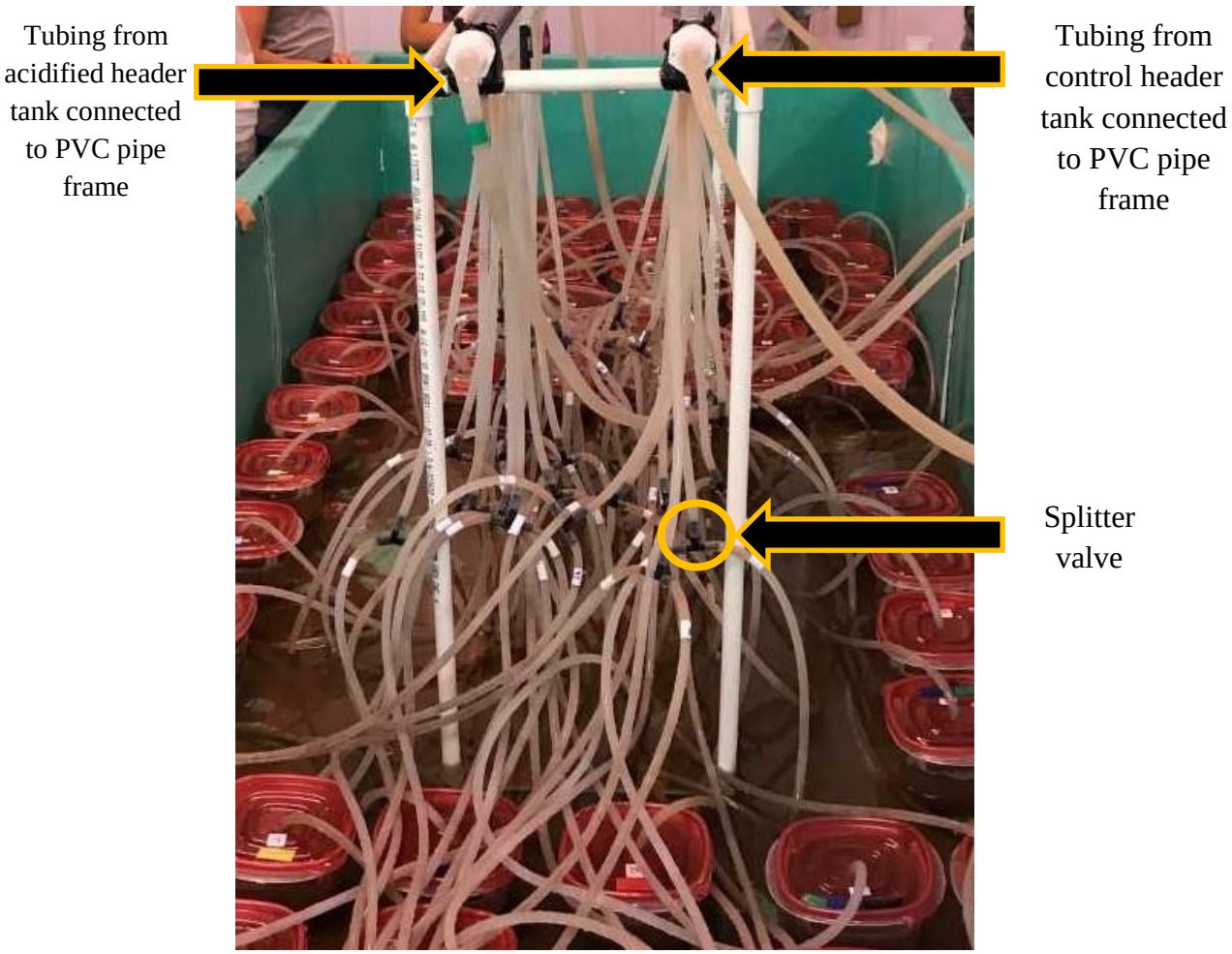

Figure 1 : PVC pipe frame in holding tank. It was used to help support and organize tubing and evenly distribute water to containers housing animal treatments exposed to control and acidified seawater treatments.

Results

Experiment Part I: Water Column Acidification

Abiotic conditions: water column pH and alkalinity

Sediments in animal replicates were found to have a lower pH than the overlying water treatment they were exposed to (control or acidified water; Fig. 2). While water treatments were maintained at an average pH ($\pm$ SD) of 7.89 $\pm$ 0.08 (control) and 7.64 $\pm$ 0.19 (acidified), the average pH ($\pm$ SD) of surface sediment was found to be 7.50 $\pm$ 0.39 (with an overlying control water column) and 7.24 $\pm$ 0.22 (with an overlying acidified water column). Additionally, in both water treatments, sediment pH decreased linearly with depth into the sediment (Fig. 2). Overall, at each depth interval, the average sediment pH was lower in acidified water treatments as compared to control water treatments (Fig. 2). Sediment pH ($\pm$ SD) at 4 cm, the last depth interval measured, was 7.25 $\pm$ 0.31 (with an overlying control water column) and 7.00 $\pm$ 0.23 (with an overlying acidified water column), illustrating the drop in sediment pH with depth in both water pH treatments (Fig. 2).

Through titration analyses, total alkalinity of the water column was determined to be higher in the control treatment than in the acidified water treatment (Table 1). Values calculated from the CO_2SYS program estimated that HCO_3^- concentration and aragonite saturation were higher and CO_2 partial pressure was lower in the control treatment than in the acidified water treatment (Table 1).

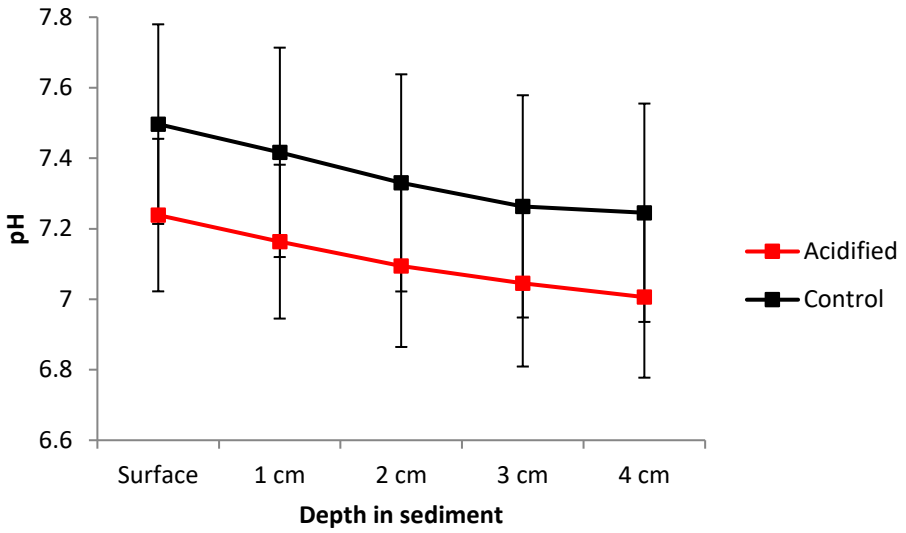

Figure 2 : pH profile (mean ± SD, n = 5 experimental units (from each water treatment)) of sediment in animal treatments with an overlying control (pH = 7.89 ± 0.08) or acidified (pH = 7.64 ± 0.19) water column, measured at 1 cm intervals to a depth of 4 cm. Sediment pH measurements were recorded a few days prior to ending the 6-week water column acidification experiment (Experiment Part I).

Table 1 : Carbonate chemical conditions (mean ± SD, n = 5) of water and sediment (sed) samples from control and acidified water treatments in Experiment Part I, including average pH, total alkalinity (TA: $\mu molkg^{-1}SW$), CO_2 partial pressure (pCO_2: µatm), HCO_3^- concentration ($[HCO_3^-]$: $\mu molkg^{-1}SW$) , and aragonite saturation (ΩAr, where ΩAr<1 indicates undersaturation and thus increased dissolution of aragonite under these conditions).

Treatment	pH	TA	pCO_2	$[HCO_3^-]$	ΩAr
Control water	7.89 ± 0.08	2709.35 ± 164.41	721.55 ± 44.65	2402.74 ± 148.67	1.96 ± 0.12
Acidified water	7.64 ± 0.20	2394.92 ± 242.64	1191.47 ± 122.29	2231.11 ± 229.01	1.02 ± 0.11
Control sed	7.51 ± 0.12	7899.84 ± 1146.29	5434.15 ± 790.84	7543.44 ± 1097.82	2.57 ± 0.37
Acidified sed	6.50 ± 0.13	5933.88 ± 2347.15	43539.06 ± 17227.95	5906.31 ± 2337.07	0.20 ± 0.08

Mortality, length, and wet weight of animals

The animals in each replicate that were not recovered after the 6-week experimental period were assumed to reflect mortality throughout the course of the experiment. By the end of the experimental period, mortality was low for clams (7.2% in control water; 10% in acidified water) and snails (0.5% in both water treatments), but much greater for mud shrimp (46.2% in control water; 65.4% in acidified water) (Fig. 3). Mortality of mud shrimp was 1.4 times greater in the acidified water treatment and differed significantly between the acidified and control treatments ($t_{15.3} = 2.50$, $p=0.024$), while there was no significant difference in mortality of soft-shell clams ($t_{16.9} = 0.61$, $p=0.55$) and mud snails between water treatments (Fig. 3).

Following the 6-week exposure to both control and acidified water treatments, soft-shell clams increased slightly in weight and shell length (Fig. 4); however, water column acidification did not have a significant impact on the proportional change in shell length or weight of soft-shell clams (weight: $t_{15.3} = 0.62$, $p = 0.54$; length: $t_{14.0} = -1.26$, $p = 0.23$; Fig. 4). Mud shrimp change in weight and final body length did not differ significantly between the two water treatments (weight: $t_{9.3} = 0.038$, $p = 0.97$; length: $t_{11.8} = -0.038$, $p = 0.97$; Fig. 5). Mud snail shell length decreased significantly in acidified water treatments after the 6-week experimental period ($t_{18.0} = -2.99$, $p=0.0079$; Fig. 6), while wet weight was not significantly affected by water column acidification ($t_{18.0} = 0.50$, $p=0.63$; Fig. 6).

For snail + mud shrimp treatments, the amount of mud shrimp not recovered from each replicate was assumed to be the amount that died through consumption by mud snails or from other causes. At the end of the experiment, 83.6 $\pm$ 13.3% and 83.3 $\pm$

10.6% of mud shrimp were missing from the control and acidified water treatments, respectively (Fig. 7). There was no significant difference ($t_{17.1} = -0.090$, $p = 0.94$) between control and acidified water column treatments in the amount of mud shrimp missing after 4 weeks in the presence of mud snails (Fig. 7).

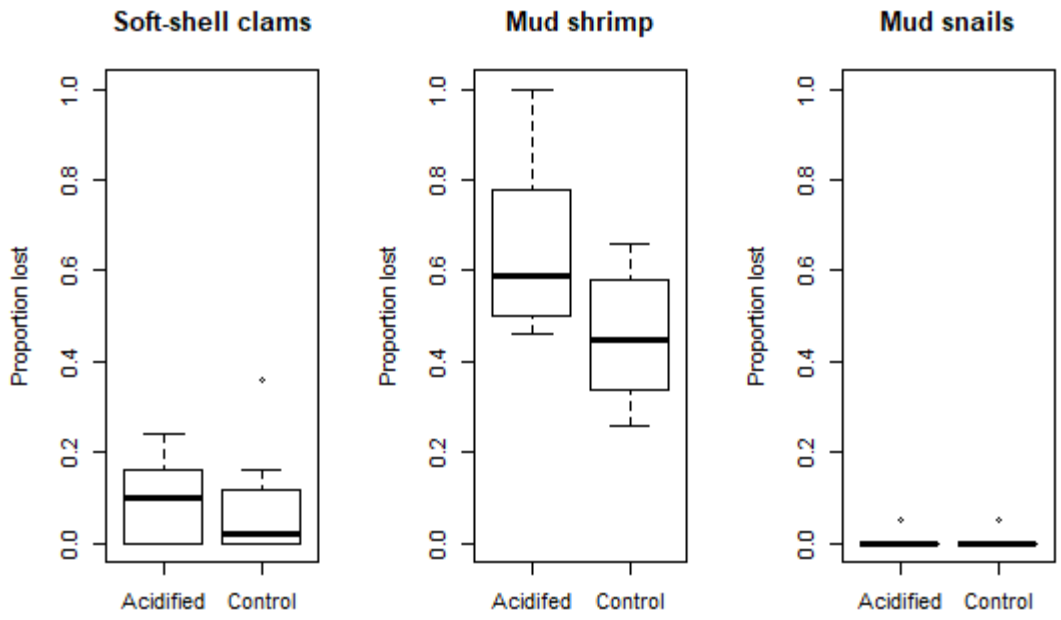

Figure 3 : Box plots of proportion of soft-shell clams (*Mya arenaria*, n = 10 experimental units), mud shrimp (*Corophium volutator*, n = 8 experimental units), and mud snails (*Tritia obsoleta*, n = 10 experimental units), not recovered after a 6-week exposure to control (pH = 7.89 $\pm$ 0.08) and acidified (pH = 7.64 $\pm$ 0.19) water column treatments.

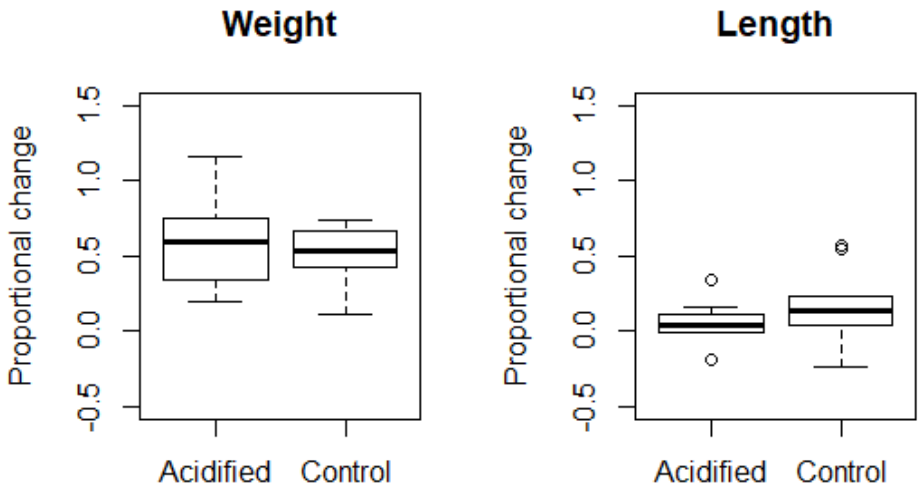

Figure 4 : Box plots of proportional change in wet weight and shell length of soft-shell clams (*Mya arenaria*, n = 10 experimental units) after a 6-week exposure to control (pH = 7.89 $\pm$ 0.08) and acidified (pH = 7.64 $\pm$ 0.19) water column treatments.

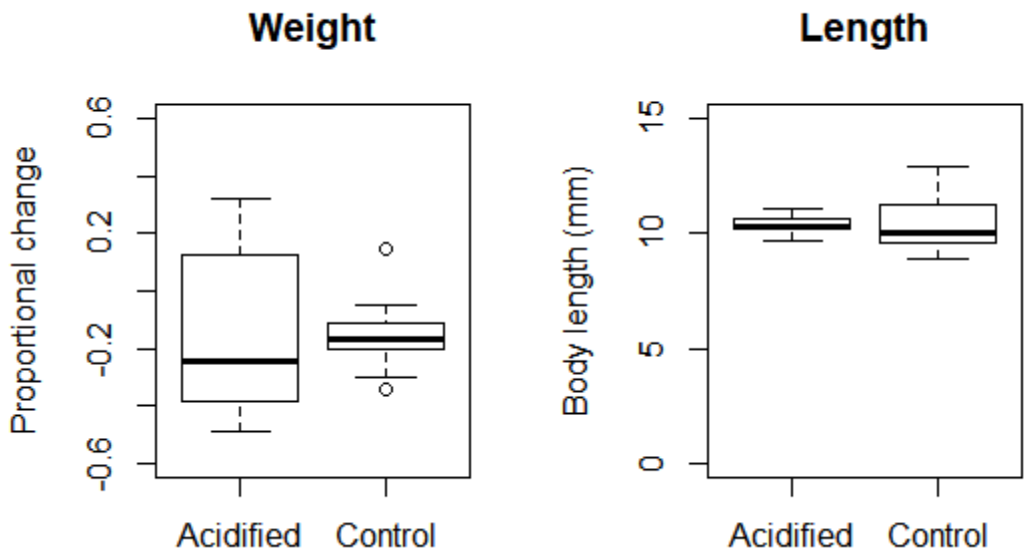

Figure 5 : Box plots of proportional change in wet weight (adjusted as per individual due to mortality throughout the experiment) and final body length of mud shrimp (*Corophium volutator*, n = 8 experimental units) after a 6-week exposure to control (pH = 7.89 $\pm$ 0.08) and acidified (pH = 7.64 $\pm$ 0.19) water column treatments.

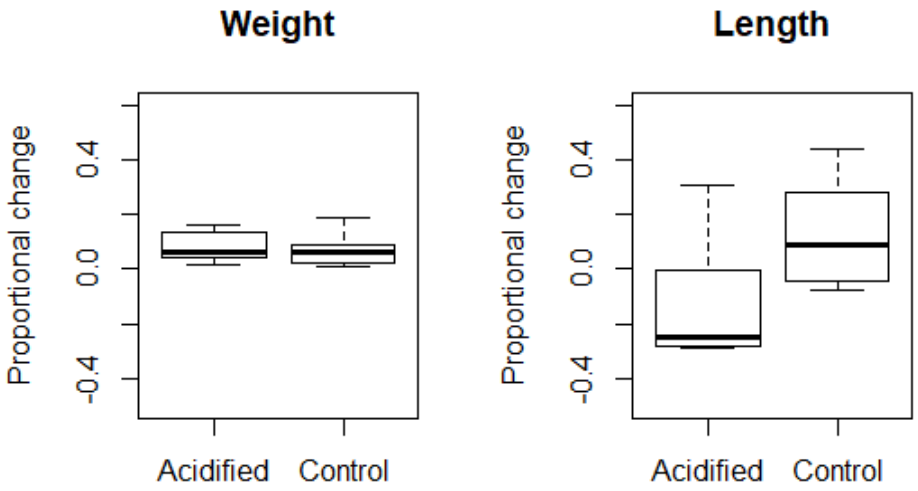

Figure 6 : Box plots of proportional change in wet weight and shell length of mud snails (*Tritia obsoleta*, n = 10 experimental units) after a 6-week exposure to control (pH = 7.89 $\pm$ 0.08) and acidified (pH = 7.64 $\pm$ 0.19) water column treatments.

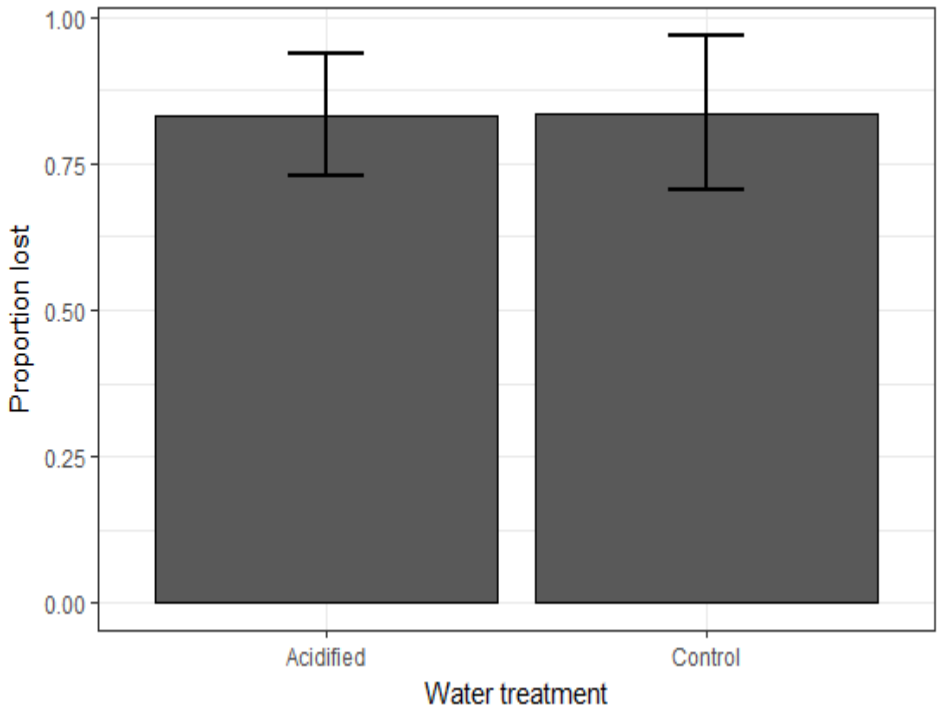

Figure 7 : Mean proportion (± SD, n = 10 experimental units) of mud shrimp (*Corophium volutator*) not recovered after a 4-week exposure to control (pH = 7.89 ± 0.08) and acidified (pH = 7.64 ± 0.19) water column treatments in the presence of mud snails (*Tritia obsoleta*).

Experiment Part II: Sediment Acidification

After the 6-week experimental period of water column acidification, animals were exposed to control and acidified sediment conditions to determine whether sediment acidification and prior water column acidification had an interactive effect on the burrowing behaviour of soft-shell clams, mud shrimp, and mud snails.

Abiotic conditions: sediment pH and alkalinity

pH of control and acidified sediment treatments fell within the natural range of sediment pH observed in Bay of Fundy mudflats (Clements and Hunt 2018): control 7.35 $\pm$ 0.074 (clams), 7.32 $\pm$ 0.20 (snails), and 7.51 $\pm$ 0.055 (mud shrimp); acidified 6.53 $\pm$ 0.20 (clams), 6.82 $\pm$ 0.070 (snails), and 6.50 $\pm$ 0.059 (mud shrimp).

Through titration analyses, total alkalinity of sediment porewater was determined to be higher in the control sediment treatment than in the acidified sediment treatment (Table 1). Values calculated from the CO_2SYS program estimated that HCO_3^- concentration and aragonite saturation were higher and CO_2 partial pressure was lower in the control sediment treatment than in the acidified sediment treatment (Table 1).

Burrowing behaviour

Following exposure to control and acidified water treatments, the proportion of soft-shell clams that burrowed into sediment treatments was the highest in control sediment for clams previously exposed to the control water treatment, as compared to the 3 other treatment combinations (acidified sediment, previously exposed to control water; control sediment, previously exposed to acidified water; acidified sediment, previously exposed to acidified water) (Fig. 8). However, ANOVA did not support a combined

impact of sediment acidification and prior exposure to water column acidification on the burrowing behaviour of clams, as there was no significant interaction between the two factors (Table 2). Additionally, there was no significant effect of either water column or sediment acidification on the burrowing behaviour of clams (Table 2). A greater proportion of soft-shell clams burrowed into control sediment conditions. The average difference in proportion burrowed between control and acidified sediment treatments was 33.3% for individuals previously exposed to the control water treatment and 2.3% for those previously exposed to the acidified water treatment. A power analysis revealed a high probability (≥0.81) power to detect an effect with a critical effect size ≥20% (Table 3).

The proportion of mud shrimp that burrowed was found to be lower in acidified sediment, but this was not affected by prior exposure to water column acidification (Fig. 9). There was no significant effect of previous exposure to control and acidified water treatments, nor an interaction between the effects of water and sediment pH on burrowing behaviour of mud shrimp (Table 4). However, acidified sediment conditions significantly reduced the proportion of mud shrimp that burrowed ($p < 0.001$) by an average proportion of 0.45 and 0.55, following exposure to a control and acidified water column treatments, respectively (Table 4, Fig. 9).

Most mud snails did not burrow into sediment in either sediment treatment (Fig. 10). A greater proportion of snails exposed to the control water column treatment burrowed completely into both sediment treatments as compared to individuals previously exposed to the acidified water treatment (Fig 10). The proportion of snails that burrowed completely into sediments was significantly affected by their prior exposure to

water column treatments, not affected by sediment acidification, and there was no interaction between water column and sediment acidification (Table 5). The proportion of snails considered to be partially burrowed was not affected by either water column or sediment acidification, and there was no significant interaction between prior exposure to water column acidification and subsequent exposure to sediment acidification (Table 5).

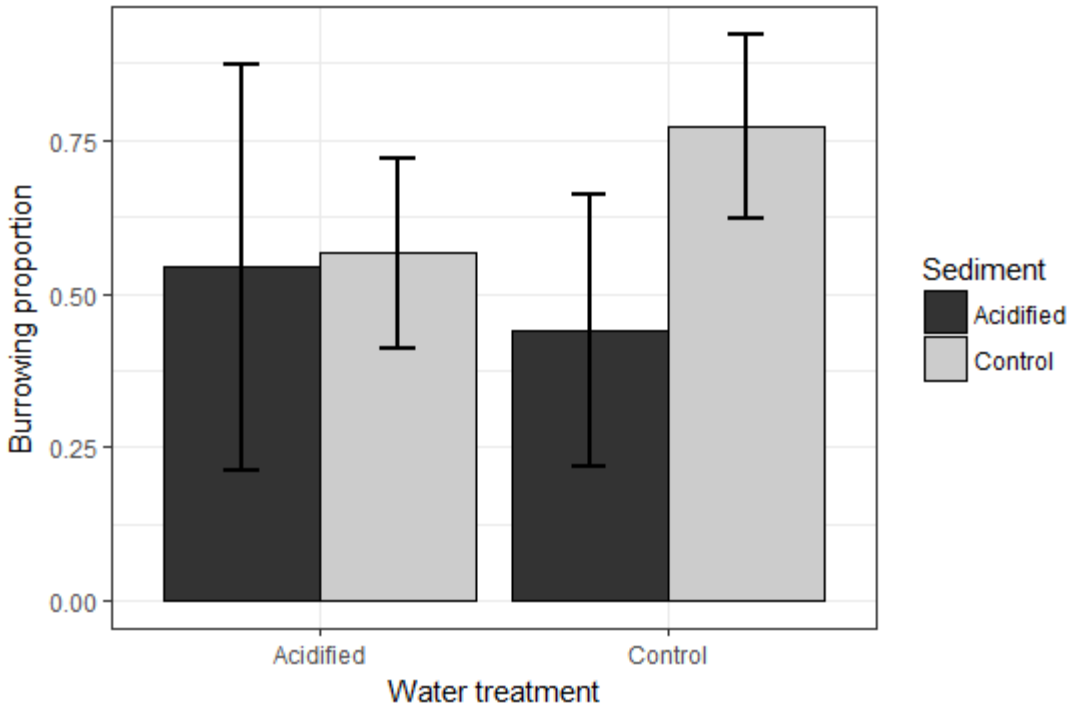

Figure 8 : Mean proportion (± SD, n = 5 experimental units) of soft-shell clams (*Mya arenaria*) burrowed in control (pH = 7.51 ± 0.12) and acidified (pH = 6.50 ± 0.13) sediment treatments after a 6-week pre-exposure to control (pH = 7.89 ± 0.08) and acidified (pH = 7.64 ± 0.19) water column treatments.

Table 2 : Two-way ANOVA examining the effects of water column and sediment pH on the proportion of soft-shell clams (*Mya arenaria*) burrowed. Water column pH was a 6-week exposure to control (pH = 7.89 ± 0.08) and acidified (pH = 7.64 ± 0.19) water column treatments.

	df	SS	MS	F	*p*
Water pH	1	0.0133	0.01330	0.26	0.6167
Sediment pH	1	0.01584	0.15837	3.10	0.0937
Water pH×Sediment pH	1	0.1198	0.11981	2.35	0.1451
Residuals	16	0.8169	0.051006		

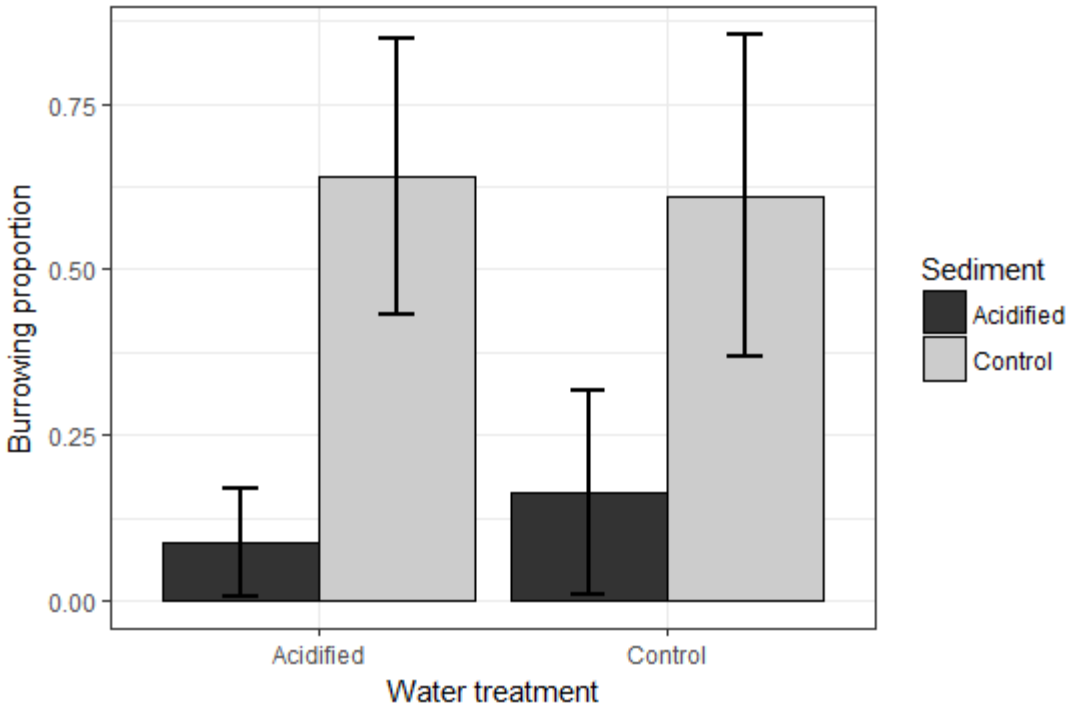

Figure 9 : Mean proportion (± SD, n = 5 experimental units) of mud shrimp (*Corophium volutator*) burrowed in control (pH = 7.51 ± 0.12) and acidified (pH = 6.50 ± 0.13) sediment treatments after a 6-week pre-exposure to control (pH = 7.89 ± 0.08) and acidified (pH = 7.64 ± 0.19) water column treatments.

Table 3 : Output of a post-hoc power analysis on the interaction of water and sediment acidification affecting burrowing behaviour of soft-shell clams (*Mya arenaria*) in Experiment Part II, following exposure to water column acidification for 6-weeks. Power was calculated using 5 different critical effect sizes (0.1-0.5 difference in proportion of clams burrowed among 4 treatment combinations), n = 5 experimental units, and an alpha level (α) of 0.05.

Critical effect size	n	α	Power
0.1	5	0.05	0.49
0.2	5	0.05	0.81
0.3	5	0.05	0.94
0.4	5	0.05	0.99
0.5	5	0.05	1.00

Table 4 : Two-way ANOVA examining the effects of water column and sediment pH on the proportion of mud shrimp (*Corophium volutator*) burrowed. Water column pH was a 6-week exposure to control (pH = 7.89 ± 0.08) and acidified (pH = 7.64 ± 0.19) water column treatments.

	df	SS	MS	F	*p*
Water pH	1	0.0095	0.0095	0.255	0.621
Sediment pH	1	1.0598	1.0598	28.560	<0.001
Water pH×Sediment pH	1	0.0117	0.0117	0.315	0.584
Residuals	14	0.5195	0.0371		

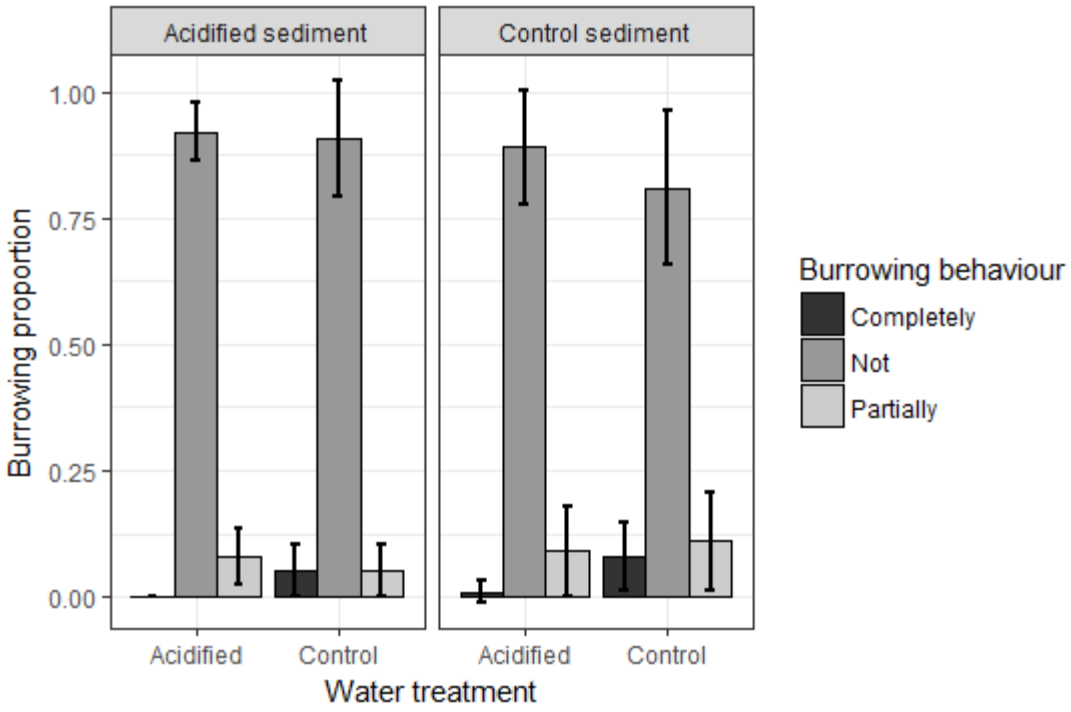

Figure 10 : Mean proportion (± SD, n = 5 experimental units) of mud snails (*Tritia obsoleta*) either completely, partially, or not burrowed in control (pH = 7.51 ± 0.12) and acidified (pH = 6.50 ± 0.13) sediment treatments after a 6-week pre-exposure to control (pH = 7.89 ± 0.08) and acidified (pH = 7.64 ± 0.19) water column treatments.

Table 5 : Two-way ANOVA examining the effects of water column and sediment pH on the proportion of mud snails (*Tritia obsoleta*) completely or partially burrowed. Water column pH was a 6-week exposure to control (pH = 7.89 ± 0.08) and acidified (pH = 7.64 ± 0.19) water column treatments.

		df	SS	MS	F	*p*
Completely burrowed	Water pH	1	0.01864	0.018637	5.600	0.0309
	Sediment pH	1	0.00180	0.001795	0.539	0.473
	Water pH×Sediment pH	1	0.00040	0.000400	0.120	0.733
	Residuals	16	0.05325	0.003328		
Partially burrowed	Water pH	1	0.00008	0.000080	0.014	0.908
	Sediment pH	1	0.00611	0.006107	1.058	0.319
	Water pH×Sediment pH	1	0.00288	0.002879	0.499	0.490
	Residuals	16	0.09238	0.005774		

Discussion

This present study investigated the impacts of a 6-week exposure to water column acidification on growth (change in length and weight) and mortality of juvenile soft-shell clams (*Mya arenaria*), adult mud shrimp (*Corophium volutator*), and adult mud snails (*Tritia obsoleta*), and their burrowing response to subsequent exposure to sediment acidification. Based on meta-analyses (Kroeker et al. 2010; Kroeker et al. 2014), I predicted that there would be differences between species with respect to their responses to water column and sediment acidification.

Basis of predictions

Meta-analyses revealed significant variation in species response to water column acidification among taxonomic groups (Kroeker et al. 2010; Kroeker et al. 2014). Water acidification has been shown to have strong negative effects on survival, growth, and calcification of marine species, and species' characteristics are believed to be factors in determining sensitivity to acidification. Calcified organisms, such as molluscs, and those in early life history stages are suggested to be more susceptible to acidification effects, while more active and mobile organism, such as crustaceans, are suggested to have a higher tolerance due to their increased metabolic rates and a greater ability to eliminate excess CO_2 during disturbances (Melzner et al. 2009; Kroeker et al. 2010; Kroeker et al. 2014). Consequently, I predicted that soft-shell clams and mud snails, which possess calcium carbonate shells, would display similar responses to water column acidification and would decrease in body length due to shell dissolution under reduced-pH (i.e. acidified) conditions. Additionally, I predicted mud shrimp to be more tolerant to

acidified conditions, as this has been the general trend observed in crustaceans (Kroeker et al. 2010; Kroeker et al. 2013; Kroeker et al. 2014). The results of my study indicated that impacts of exposure to water column acidification varied among species, but not in the manner predicted, as no significant effects were detected on juvenile soft-shell clams, while impacts were found on shell length of mud snails and mortality of mud shrimp. Prior exposure to acidified water significantly reduced mud snail burrowing behaviour in both control and acidified sediment treatments, while acidified sediment conditions significantly reduced burrowing behaviour of mud shrimp. Neither treatment significantly affected burrowing behaviour of juvenile soft-shell clams.

Effects of acidification on growth and survival

This study found that mud snail (*T. obsoleta*) shell length was significantly reduced following the 6-week exposure to an acidified water column. The significant decrease in shell length was likely due to shell erosion that occurred in response to low-pH conditions. It has been documented that many shallow-water marine molluscs exhibit reduced rates of net calcification and shell growth with decreasing pH (Berge et al. 2006; Ries et al. 2009; Zhao et al. 2017; Kriefall et al. 2018). A recent study by Duquette et al. (2017) investigated whether chronic exposure to elevated CO_2 (low-pH conditions) alters mineralogy, structure, or strength of the shells of 4 species of gastropods (two limpets: *Patella rustica*, *P. caerulea*, a whelk: *Hexaplex trunculus*, and a top-shell snail: *Osilinus turbinatus*) located at 3 different sites near a natural CO_2 seep. It was discovered that whelk and top-shell snails displayed significant reductions in shell length with increasing proximity to the CO_2 seeps (Duquette et al. 2017). Similarly, MacLeod and Poulin (2015)

did a laboratory experiment to investigate the effects of reduced water-pH on shell growth and length of New Zealand mud snails. Their study consisted of three pH treatments: 7.4, 7.6, and 8.1, and found that acidified water caused significant decreases in shell growth and length. The findings support those of my study and suggest a similar trend in gastropods: that near-future ocean acidification could increase shell dissolution of mud snails. Ultimately, this could result in reduced tensile shell strength (Green et al. 2004; MacLeod and Poulin 2015), resulting in an increased risk of shell damage from variety of factors, such as wave action and abrasion, thus decreasing fitness and increasing risk of mortality.

Acidified water treatments did not have a significant effect on mud snail mortality in my study. A recent study done by Kriefall et al. (2018) on the Atlantic slippersnail (*Crepidula fornicata*) demonstrated a similar trend. In their study, slippersnail larvae were exposed to two extreme water acidification treatments (pH 7.5 and 7.6) for 4 days, which started within 12 hours of hatching. Kriefall et al. (2018) chose to investigate the larvae of this species as adult slippersnails are known to be resilient to environmental changes. Despite the extreme experimental levels of water column acidification used in their study, Kriefall et al. (2018) found that *C. fornicata* larvae were remarkably resilient and showed very low levels of mortality. The work by Kriefall et al. (2018) suggests that future research on mud snails should consider the larvae of this species, to see if low mortality is also observed in response to water column acidification, as it was for adult mud snails in my experiment. Furthermore, Shirayama and Thornton (2005) investigated water column acidification and its effects on mortality of juvenile (<1 year old) strawberry conch (*Strombus luhuanus*) throughout a 6-month experimental period.

Although their acidified treatment was maintained at a higher pH (7.9) than mine (7.64), Shirayama and Thorton (2005) found low mortality of conchs by the end of the experiment. Therefore, previous research on gastropods as well as my study on mud snails seem to show a general trend towards the consensus that certain species of molluscs may be resilient to mortality effects of ocean acidification.

Although previous work has demonstrated that water column acidification can alter growth of some species of crustaceans, such as juvenile marine shrimp (*Palaemon pacificus*, Order Decapoda) and tanner crabs (*Chionoecetes bairdi*, Order Decapoda) (Kurihara et al. 2008; Long et al. 2013), it was not found to affect the growth of mud shrimp (*C. volutator*, Order Amphipoda) in my study. Research on crustaceans has suggested variability in growth response to water column acidification, depending on the species and age of the individuals. In the present study, after exposing mud shrimp to acidified water for 6 weeks, body length and weight was not found to differ between control and acidified water treatments. Similarly, Almen et al. (2017) investigated the calanoid copepod *Pseudocalanus acuspes* (order Calanoida) and found that body size was not significantly affected following exposure to acidified water (pH ~7.7) for 2 months. A study done by Cooper et al. (2017) looked at the effects of elevated CO_2 concentrations on growth of the Pacific krill species (*Euphausia pacifica*, order Euphausiacea) following exposure to acidified water for 2 months and found that low-pH treatments significantly slowed growth of krill. Therefore, the present study is consistent with previous work showing the wide degree of species-specific variation in growth response to water column acidification within the subphylum Crustacea. However, since mud shrimp were initially collected using a sieve having a mesh size of 1 mm, it is likely that mostly adult

mud shrimp were obtained. Therefore, it is not surprising that mud shrimp were not found to grow significantly throughout the 6-week experiment, as adult mud shrimp do not grow much in comparison to juveniles and small adults. Additionally, body lengths of mud shrimp were measure in a non-typical way by including the second antennae (as compared to measurements of rostrum to telson typically used for crustaceans). As the second antennae has strong sexual dimorphism on *C. volutator* (Schneider et al. 1994; Barbeau and Grecian 2003), this may have confounded my results. Furthermore, sex ratios of mud shrimp were recorded prior to the 6-week exposure of mud shrimp to water column acidification but could not be determined after the experiment due to the method chosen to preserve animals (i.e., by freezing), which made it difficult to distinguish males form females once individuals were thawed. Considering the strong sexual dimorphism between male and female mud shrimp, this could have further confounded my results of body length measurements after the 6-week experimental period. Therefore, these confounding issues make it difficult to determine whether water column acidification affected mud shrimp body length.

This study is the first to suggest that water column acidification negatively impacts mud shrimp (*C. volutator*) survival. In my experiment, mortality of mud shrimp was 19% higher in the acidified water treatment than in the control. Previous work investigating different species of amphipods (*Peramphithoe parmerong* [Poore et al. 2013], *Gondogeneia antarctica* and *Paradexamine fissicauda* [Schram et al. 2016]) have also demonstrated a reduction in survival following exposure to acidified treatments. Although experimental durations of these studies were different from the 6-week experimental period of my study (Poore et al. 2013: 2 weeks; Schram et al. 2016: 3

months), pH levels of acidified treatments were comparable (mine: 7.64; Poore et al. 2013: 7.6-7.8; Schram et al. 2016: 7.6). Similarly, a study done by Kurihara et al. (2008) investigated a species from another Order of crustaceans (the marine shrimp *Palaemon pacificus*, Order Decapoda), and found that survival was reduced due to water column acidification. Their study evaluated the long-term (15-30 weeks) effects of increased seawater CO_2 concentration on survival, growth, feeding, and moulting of shrimp. Survival of *P. pacificus* was significantly suppressed in experimental groups that were exposed to acidified water (with the same pH as this study's acidified water treatments: 7.64) for a prolonged duration of time and suggests that future seawater CO_2 conditions could potentially reduce marine shrimp populations (Kurihara et al. 2008). My study corroborates these other studies and indicates that mortality of crustaceans can be adversely affected by exposure to acidified water within a matter of weeks.

Mortality of mud shrimp was high throughout my experiment in both acidified and control water treatments, which is likely due to the time of year that my experiment was conducted (June-August) and how it relates to the life cycle of mud shrimp. The annual life cycle of mud shrimp in the outer Bay of Fundy consists of a single generation per year (Gratto et al. 1983). During the month of June, a new cohort of mud shrimp typically begins to enter the population, while mortality of large adults increases in July (Gratto et al. 1983; Wilson and Parker 1996). Therefore, considering the natural life cycle of mud shrimp populations in the outer Bay of Fundy, it is not surprising that high mortality of mud shrimp was observed in both water treatments. Mud shrimp populations were likely undergoing generational transitions at the time of the experiment. Despite the high mortality of mud shrimp in both treatments, by the end of the 6-week experimental

period, mortality of mud shrimp was significantly greater in the acidified water treatment as compared to the control, indicating that mud shrimp survival is impacted by water column acidification.

My research found that exposing juvenile soft-shell clams (*M. arenaria*) to acidified water for 6 weeks resulted in low mortality and a non-significant difference in clam length and weight between water treatments, suggesting no impact of water column acidification on soft-shell clam growth and survival. This contrasts with past research on other species of bivalves that has found significant increases in mortality and decreases in weight and length under acidified conditions (e.g. Michaelidis et al. 2006; Bressan et al. 2014; Fitzer et al. 2015). However, these previous studies had much longer experimental periods than my study and exposed bivalves to acidified waters for durations ranging between 3-9 months. For example, Bressan et al. (2014) exposed Mediterranean mussels (*Mytilus galloprovincialis*) and striped venus clams (*Chamelea gallina*) to an acidified water treatment (pH = 7.4, lower than the pH of the acidified treatment in my experiment: pH = 7.64) for 6 months and analyzed the animals' survival, growth, and shell integrity. Throughout the experiment, mortality was found to be low, but significantly increased at the end of the experiment, especially the mortality of *C. gallina*. Additionally, Bressan et al. (2014) observed decreases in weight and shell length of *C. gallina* (but not *M. galloprovincialis*). Therefore, if soft-shell clams in my study had been exposed to the acidified treatment for a greater duration of time, perhaps a 6-month period as in the study by Bressan et al. (2014), then mortality of clams may have increased, and significant differences may have been observed in weight and shell length between acidified and control water treatments.

Studies have indicated that the risk of calcium carbonate shell dissolution of bivalves increases with decreasing pH of the water column and sediment (Ries et al. 2009; Melzner et al. 2011; Gazeau et al. 2013; Clements and Hunt 2015). Throughout the duration of the 6-week experiment, juvenile soft-shell clams were never observed to be on the surface of the sediment in direct contact with the overlying acidified water column and were assumed to have remained burrowed throughout the experiment. However, clams would still have been exposed to the acidified water as they would have been taking it in through their siphon. The sediment pH profile obtained towards the end of my experiment indicated that at each depth interval into the sediment, average sediment pH was lower in acidified water treatments as compared to control water treatments. Therefore, through exposure to acidified water and sediment, it would be expected that a decrease in shell length of clams because of dissolution would have been observed in this study. However, it was not, which could suggest the duration of this experiment was not long enough to observe such effects of water column acidification, or that soft-shell clams are not particularly affected by this level of acidification, given the drastic fluctuations in ocean and sediment pH their natural habitat (Clements and Hunt 2018). Moreover, effects of dissolution of juvenile bivalves is related in part to shell length; larger juvenile bivalves (>2 mm) are less susceptible to shell dissolution than smaller bivalves (<1 mm) (Green et al. 2004). Therefore, since the juvenile soft-shell clams used in this study (6.0 ± 2.6 mm)) were quite a bit larger than 2 mm in length, it is possible that the size of clams contributed to their ability to withstand the effects of shell dissolution throughout the 6-week exposure to acidified water. Future studies should increase the length of time that soft-shell clams are exposed to acidified water treatments,

investigate various size classes of juvenile soft-shell clams, and consider the range and variability of ocean and sediment pH that soft-shell clams experience in their natural habitat to more accurately predict the effects of future decreases in ocean pH.

Effects of acidification on predator-prey interactions

As it has been established that mud snails are active predators of mud shrimp (Coffin et al. 2012), my study also investigated whether water column acidification impacts the survival of mud shrimp in the presence of mud snails. The lack of a significant difference in the number of mud shrimp that remained in mud snail + mud shrimp replicates between control and acidified water treatments after the 4-week exposure to water column acidification suggests that predation of mud shrimp by mud snails is not affected by water column acidification. However, other factors need to be considered when interpreting these results. For example, given the duration of the experiment and the small containers holding animals, it is possible that enough time was allowed for mud snails to encounter and consume most of the mud shrimp present in the small space provided by the container, regardless of water treatment. Future work investigating the effects of ocean acidification on predatory behaviour of mud snails on mud shrimp should explore predator-prey interactions in more detail. For example, the study by Xu et al. (2017) looked at the effects of water column acidification on prey detection and handling and prey size preference of the muricid gastropod (*Thai clavigera*) on the mussel *Brachidontes variabilis*. Their study showed that the predator-prey interaction between muricid gastropods and mussels was altered under water column acidification; with decreasing pH, larger prey was preferred by snails, prey search time

increased, and handling time of mussels decreased (Xu et al. 2017). Such an approach would allow for a greater understanding of ocean acidification effects on the predator-prey relationship between mud snails and mud shrimp.

Variability in pH

Numerous studies have documented the high degree of variability in pH and carbonate geochemistry in coastal regions throughout the world (e.g. Hauri et al. 2009; Gagliano et al. 2010; Shaw et al. 2012; Johnson et al. 2013; Lienweber and Gruber 2013; Chien et al. 2018). Consequently, coastal organisms inhabit a highly variable pH environment where daily fluctuations are often driven by biological activity (e.g. photosynthesis, respiration, and sedimentation), seasonal upwellings, and CO_2 absorption (Wootton et al. 2008; Hauri et al. 2009; Cornwall et al. 2013). In some coastal regions, water pH fluctuates between 8.0 and 7.7 during the day (Cornwall et al. 2013) but can decrease as low as 7.4 in some areas (Wootton et al. 2008), exceeding the projected pH decline of the ocean by the end of the century (~7.65, Cornwall et al. 2013). Therefore, pH values used in my study were chosen to be within the range of present-day conditions in coastal regions. The acidified water treatment was set to remain within a pH range of 7.5-7.7, while water for the control treatment came in directly from the Bay of Fundy. The average pH of the acidified treatment over the 6-week duration of this study was 7.64 and that of the control was 7.89. However, it is important to acknowledge that the effects of daily fluctuations in water column and sediment pH are poorly understood, and therefore, may have different impacts on marine infauna than constant pH treatments

such as those used in my experiment and most other acidification experiments (e.g., Cornwall et al. 2013; Frieder et al. 2014; Dufault et al. 2012).

Carbonate geochemistry of sediments (i.e., sediment pH) is also known to have a high degree of variability both temporally and spatially (Wenzhöfer et al. 2001; Yates and Halley 2006), including in the Bay of Fundy mudflats (Clements and Hunt 2018). This variability is strongly associated with amount of rainfall, with increasing amounts of rainfall accompanied by decreases in sediment pH (Doney et al. 2007). Therefore, organisms inhabiting intertidal mudflats such as soft-shell clams, mud shrimp, and mud snails, are naturally exposed to abrupt and extreme environmental changes regularly. Presently, sediment pH of Bay of Fundy mudflats typically varies within the range of 6.0-8.0 (Clements and Hunt 2018). Sediment pH values used in my study (acidified sediment: 6.50-8.82, control sediment: 7.32-7.51) were chosen to be within the range of present-day porewater pH conditions to be comparable to the natural pH range that species are exposed to in their habitat.

Limitations in my study

The experimental setup for the water column acidification experiment included only one header tank per treatment, raising the issue of pseudo-replication. Therefore, I cannot conclude with certainty that my header tanks holding the control and acidified water treatments differed only in pH. However, to help manage this flaw in the experimental design, seawater conditions in each header tank were regularly recorded, which included temperature, salinity, and pH. Temperature and salinity remained relatively constant between water treatments, while pH was markedly different

throughout the experiment. Additionally, animal treatments were randomly placed in the holding tank so that those exposed to each water treatment were distributed amongst one another. Conditions of overlying seawater in animal replicates were not found to differ from water treatments in header tanks. Furthermore, I have subsequently repeated the experiment during the months of July-September 2018 with 4 header tanks for water treatments (2 control; 2 acidified) instead of 2. Although data analysis is still underway, the 2018 experiment shows similar trends to those of the 2017 experiment.

Effects of acidification on behaviour

After the water column acidification experiment, sediment acidification trials were performed to determine whether the 6-week exposure to water column acidification affected burrowing behaviour of species in response to sediment acidification. Although burrowing response of soft-shell clams to sediment acidification has been assessed in previous work (Clements and Hunt 2014; Clements et al. 2016; Clements et al. 2017), that of mud shrimp and mud snails has not been addressed. Moreover, all studies to date regarding the effects of sediment acidification has been done on bivalves (e.g. Green et al. 2013; Clements and Hunt 2014; Clements et al. 2016; Clements et al. 2017); therefore, there is a lack of knowledge regarding the responses of broader taxonomic groups to sediment acidification. Thus, the present study provides a unique perspective regarding potential combined impacts of water column and sediment acidification on burrowing response of species from different taxonomic groups.

Recent studies suggest some behaviours of amphipods can be altered under acidified water column conditions, such as feeding behaviour of *Orchestoidea*

tuberculata (consumption rates and preference; Benitez et al. 2016; Duarte et al. 2016). However, to date, studies have not investigated the effects of either water column or sediment acidification on the burrowing behaviour of mud shrimp (*C. volutator*). Burrowing behaviour of this species in response to other factors has been investigated in a few studies (Meadows and Reid 1966; Erdem and Meadows 1980; Limia and Raffaelli 1997). For example, it was found that mud shrimp burrowing behaviour is reduced in sediments with elevated levels of the contaminant mercury (Erdem and Meadows 1980). My study did not find a significant interaction between water column and sediment acidification on the burrowing behaviour of mud shrimp, but sediment acidification alone significantly reduced the proportion of mud shrimp that burrowed, similar to alterations in burrowing behaviour observed in soft-shell clams in previous work (Clement et al. 2017). Considering the projected drop in ocean and sediment pH that is expected to occur by end of the century (Caldeira and Wickett 2005; Fabry et al. 2008; Cornwall et al. 2013; Hagens and Middelburg 2016), this response to sediment acidification could have detrimental implications for mud shrimp populations. Crawling (i.e. unburrowed) mud shrimp are highly vulnerable to predation by benthic fish, polychaetes, mud snails, Semipalmated sandpipers (which stop in the Bay of Fundy during migration in late summer), and other intertidal invertebrates (Hicklin et al. 1987; Raffaelli and Milne 1987; McCurdy et al. 2005; Coulthard and Hamilton et al. 2011; Coffin et al. 2012; Cheverie et al. 2014; MacDonald et al. 2014). Therefore, future sediment acidification conditions projected to occur by the end of the century could potentially lead to declines in mud shrimp population densities, which could result in cascading effects throughout the food web of benthic communities.

This study is also one of the very few to establish that gastropod burrowing behaviour is altered under elevated-CO_2 conditions. My experiment demonstrated that complete burrowing of mud snails (*T. obsoleta*) below the sediment surface was significantly decreased after the 6-week exposure to water column acidification but was not affected by sediment acidification. A handful of studies have documented that low-pH conditions cause significant alterations of gastropod behaviours, such as anti-predator and predation behaviours (Watson et al. 2014; Jellison et al. 2016; Xu et al. 2017). However, very little research has been done to investigate the impacts of ocean acidification on gastropod burrowing behaviour. Recent work done by Watson et al. (2017) looked at burrowing behaviour of the cone snail, *Conus marmoreus*, following exposure to elevated CO_2 conditions for 2-3 weeks. Watson et al. (2017) found that exposing snails to acidified seawater resulted in a significant reduction in the proportion of snails that burrowed into sediment. Therefore, both my work and that of Watson et al. (2017) suggests that exposure to acidified water results in significant alterations in the normal burrowing patterns of gastropods.

This study demonstrated no significant effect of either water or sediment acidification on the burrowing behaviour of clams (*Mya arenaria*), and no significant interaction of water column and sediment acidification. This lack of an effect of sediment acidification was unanticipated as previous work has shown that exposing bivalves, including juvenile *M. arenaria*, to acidified sediment conditions significantly reduces the proportion of individuals that burrow (Green et al. 2013; Clements and Hunt 2014; Clements et al. 2016; Clements et al. 2017). However, my results were in the predicted direction, as a noticeably greater proportion of clams burrowed into control sediment

treatments as compared to acidified sediment treatments; the lack of a significant difference between treatments is likely due to the large amount of variability in the data.

Over the past few years, there has been a gradual increase in the amount of evidence suggesting that elevated CO_2 conditions interferes with normal functioning of the $GABA_A$ neurotransmitter receptor in fishes and invertebrates, leading to altered behaviour (Nilsson et al. 2012; Watson et al. 2014; Moya et al. 2016; Clements et al. 2017). For example, Clements et al. (2017) demonstrated that the reduced burrowing behaviour of juvenile soft-shell clams in acidified sediment was restored to normal (i.e. burrowing response in control sediments) when clams were treated with gabazine (an antagonist of the $GABA_A$ neurotransmitter receptor). Unpublished work for an Honours thesis demonstrated a similar result in mud shrimp: reduced burrowing behaviour under acidified sediment conditions, which was restored to normal proportions after treatment with gabazine (Walsh 2018). However, gabazine did not significantly affect the burrowing of mud snails in Walsh's study. Watson et al. (2014) demonstrated that alterations in conch snail escape behaviour during predator-prey interactions occurring under acidified conditions can be fully restored through treatment with gabazine. Therefore, during my study, it is possible that exposure to acidified water and subsequent exposure to acidified sediment conditions may have altered the $GABA_A$ neurotransmitter receptor functioning in mud shrimp and mud snails, leading to the observed atypical burrowing response in acidified sediment treatments. Future work should test whether the $GABA_A$ neurotransmitter is a physiological mechanism that drove the altered burrowing behaviour of mud snails under elevated CO_2 conditions.

Implications and conclusions

The reduced burrowing response of species to sediment acidification in my laboratory experiments suggests that the projected reductions in ocean and sediment pH conditions expected to occur by the end of the century (Caldeira and Wickett 2005; Fabry et al. 2008; Cornwall et al. 2013; Hagens and Middelburg 2016), as well as present-day variation in pH, may have ecological consequences. As previously mentioned, reduced burrowing behaviour of mud shrimp could increase their vulnerability to predation by benthic fish, polychaetes, mud snails, and other invertebrates (Raffaelli and Milne 1987; McCurdy et al. 2005; Coulthard and Hamilton 2011; Coffin et al. 2012; Cheverie et al. 2014). Reduced burrowing behaviour of mud snails could increase their risk of shell damage due a high water flow, as burrowing behaviour of mud snails has been postulated to be an adaptive response upon exposure to high water flow conditions to avoid dislodgement from the sediment surface (Levinton et al. 1995). Mud snail burrowing has also been suggested to be driven by sunlight as a response to avoid desiccation during low tide (Coffin et al. 2008). Although I did not find a significant difference in burrowing response to sediment acidification for soft-shell clams, previous work has demonstrated that soft-shell clams reduce burrowing upon exposure to acidified sediment conditions (Clements et al. 2016; Clements et al. 2017). Thus, this may indicate that soft-shell clams could also become vulnerable to predators such as green crabs and polychaetes (Clements and Hunt 2018).

In conclusion, this study helps increase our knowledge regarding the impacts of ocean and sediment acidification affecting growth, mortality, and burrowing behaviour of soft-shell clams, mud shrimp, and mud snails, and is consistent with consensus that there

is a wide degree of taxonomic variability in species response to water column acidification. This is the first study to investigate the combined effects of water and sediment acidification on marine infauna. Although I investigated only 3 species inhabiting the Bay of Fundy mudflats, response to water and sediment acidification varied extensively among species, highlighting the fact that the projected decrease in ocean and sediment pH will have greater impacts on certain taxonomic groups than others. Ultimately, such impacts of ocean acidification could initiate a cascade of effects amongst benthic communities, driving habitat restructuring, and altering biodiversity, species composition, and food webs. Future experiments should consider the natural variability in coastal water and sediment chemistry (Duarte et al. 2013; Waldbusser and Salisbury 2014; Wallace et al. 2014) and explore prolonged (lasting longer than 6 weeks) effects of water column acidification on other types of behaviour and physiological responses of benthic invertebrates, such as anti-predator behaviour, feeding behaviour, metabolic responses, etc. Future studies of burrowing behaviour should consider a more refined metric than burrowing proportion, such as burrowing depth. Furthermore, future water and sediment acidification studies should investigate the $GABA_A$ neurotransmitter and its role in the effects of reduced-pH conditions on invertebrate behaviour.

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Clements, J. C., **Bishop, M. M.**, and H. L. Hunt. 2017. Elevated temperature has adverse effects on GABA-mediated avoidance behaviour to sediment acidification in a wide-ranging marine bivalve. Mar Biol **164**: 1-11.

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Bishop, M. M., and H. L. Hunt. Atlantic Canadian Coastal and Estuarine Science Society, Montreal, QC. May 17-20, 2017. The combined impacts of sediment and water column acidification on behaviour and physiology of marine invertebrates. Awarded Honourable Mention for poster presentation.

Bishop, M. M. and H. L. Hunt. Science Atlantic Biology Conference, Halifax, NS. March 11-13, 2017. Testing the effects of sediment acidification and predator cues on the burrowing behaviour of juvenile *Mya arenaria*. Awarded Honourable Mention for poster presentation.