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Article

Evaluation of time-dependent buffer effectiveness in open vs. sealed Long Island Sound microcosms

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SUMMARY

Rising atmospheric CO_2 concentrations disrupt the existing gas equilibrium between the atmosphere and the ocean, driving chemical reactions that lower seawater pH, a process known as ocean acidification. Ocean acidification threatens calcifying organisms such as shellfish whose shells dissolve before they can fully form. In coastal waters, acidification is further amplified by eutrophication and freshwater inputs. In response, shellfish industries actively use chemical, mineral, and biological buffering strategies to counteract seawater pH decline. This study tested the impact of air-water gas exchange on pH recovery in acidified Long Island Sound (LIS) water treated with buffers. We hypothesized that air-water gas exchange would limit the effectiveness of buffering strategies. We compared pH recovery in open and sealed containers following a standardized acidification shock. We used two- and three-factor Type II ANOVAs to quantify the effects of air-water gas exchange and buffering on pH recovery over time. Air-water gas exchange had no detectable effect at one-hour post-acidification but became the dominant factor after eight days. Both experiments suggest that air–water gas exchange progressively dominates buffering over time. They also suggest that buffering strategies are more effective in low-exchange environments, which are characteristic of stratified bottom waters, enclosed hatchery systems, or hypoxic layers. This framework helps identify where chemical, mineral, or biological interventions are most likely to improve pH stability in coastal waters.

INTRODUCTION

Fossil fuel combustion and other human activities are causing CO_2 emissions that lead to climate change and ocean acidification (1,2). While atmospheric CO_2 concentration remained close to 280 parts per million for thousands of years, concentrations began rising with the Industrial Revolution and reached about 420 parts per million in 2025 (1,3). This corresponds to a 50% increase since the pre-industrial period, and this trend is projected to continue (3). Oceans absorb atmospheric CO_2 as concentrations in the atmosphere and seawater equilibrate (1). When CO_2 dissolves in seawater, it triggers a series of chemical reactions and forms carbonic acid (H_2CO_3) (1,4,5). Carbonic acid then dissociates into hydrogen ions (H^+) and bicarbonate ions (HCO_3^-) (1,5,6). The increased concentration in hydrogen ions results in a lower pH. Hydrogen ions combine with carbonate ions (CO_3^{2-}), reducing their availability (1,4,5). Shells are formed by a reaction between carbonate ions and calcium ions (Ca^{2+}). The loss in carbonate ions as they combine with hydrogen ions directly threatens the ability of shellfish to form shells (1,5,6). The aragonite saturation state (Ω_{ar}) is an indicator of whether seawater chemistry favors or inhibits shell formation. When Ω_{ar} values fall below ~1.5 calcification is difficult, and when Ω_{ar} values fall below 1.0, newly formed shells may begin to dissolve (4,5). By depleting carbonate ions, ocean acidification reduces Ω_{ar}, directly threatening shellfish (1,4,6).

Ocean acidification is intensified in coastal waters (5,7). First, nutrient inputs from agriculture and wastewater stimulate algal blooms. When the algae die, bacterial decomposition of the organic matter consumes O_2 and releases CO_2 into the water, lowering pH (7). Second, freshwater-dominated inputs, such as rivers or rainwater runoffs, dilute the natural alkalinity of seawater and reduce its natural buffering capacity (5). These factors make coastal waters more vulnerable to acidification than the open ocean (5,7).

The Long Island Sound (LIS) is a body of coastal water along the shores of Connecticut and New York. It receives saltwater from the Atlantic Ocean and freshwater from several rivers, mostly in Connecticut (8). The LIS is home to 120 fish species and includes many marine ecosystems and habitats (9). It faces multiple environmental threats, including coastal acidification caused by CO_2 absorption and nutrient pollution from nearby bodies of water (7,9,10). During the summer, pH values can drop from 7.9 to 7.0 or below in certain areas (7,9). In the western portion of the LIS during the summer of 2020, the average summer bottom-water Ω_{ar} dropped to 0.88 ± 0.22, below the threshold of 1.0 when shellfish shells begin to dissolve (10). These conditions reduce the growth and survival rate of shellfish, including oysters and clams which are economically significant to the region (7,9,11,12,13).

To counteract ocean acidification, many shellfish hatcheries actively monitor water chemistry and add chemical (sodium bicarbonate) or mineral (crushed oyster shell) buffers, which raise pH and allow shellfish to develop properly (11). Buffers can also be biological, as eelgrass and macroalgae raise pH through photosynthetic CO_2 uptake (13,15). In co-farming strategies, shellfish are raised alongside seaweeds to exploit this effect (16). These chemical, mineral, and biological buffering interventions have demonstrated effectiveness in controlled hatchery settings (6,7).

Shellfish hatcheries are an integral component of the LIS region, producing oyster, clam, and scallop larvae that support the coastal economy and restoration efforts. These hatcheries typically pump seawater directly from Long Island Sound into flow-through culture systems, with continuous water circulation and therefore air–water gas exchange. Hatchery configurations differ in how much contact the water

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maintains with the atmosphere. Flow-through systems draw water continuously from the LIS, leading to high exchange rates, whereas recirculating systems restrict the amount of exchanges (12). Hatchery water chemistry is closely linked to the carbonate chemistry and physical exchange dynamics of Long Island Sound, making atmospheric CO_2 exchange an important factor when considering acidification mitigation in hatcheries (5,16,18).

However, few studies have examined the interaction between gas exchange and buffering, and more specifically how this interaction evolves over time. Air-water gas exchanges are the continuous transfer of gases, especially CO_2, across the boundary between the water surface and the atmosphere. It is driven by the difference in CO_2 partial pressure between air and water, and it proceeds until equilibrium is reached. Continuous CO_2 exchanges between the water surface and the atmosphere can counteract pH gains achieved by buffering. Understanding how much and how quickly air-water gas exchange undermines buffering effectiveness can have direct implications for hatchery design and for their choice of buffering strategy (5). The level of air-water gas exchange can vary considerably in practice, from totally open water environments to low-exchange systems such as enclosed hatchery tanks, so it is relevant to assess the impact and importance of such exchanges. This study aims to compare pH evolution (i.e., the temporal trajectory of pH) in acidified Long Island Sound seawater subject to buffering treatments, using open and sealed containers which model high and low air-water gas exchange environments, respectively. We hypothesized that air-water gas exchange would limit the effectiveness of buffering strategies.

Our findings indicate in what context buffering strategies are effective. Ultimately, because buffering effectiveness is dictated by the extent of air-water gas exchanges, buffering effort may be better directed toward enclosed systems with limited exchange, such as hatchery pipes, stratified bottom layers, and areas with long water residence times, than toward open water. Because shellfish, especially oysters, are so economically and environmentally significant to the LIS, assessing the conditions under which buffers are most likely to counteract acidification could help strengthen restoration efforts and the overall Northeast aquaculture industry (7,9,11,12).

RESULTS

To test the impact of air-water gas exchange on buffering, in Experiment 1, we subjected the LIS seawater containers to an initial homogeneous artificial acidification shock, meant to mimic in a consistent and replicable manner the acidification of oceans caused by increasing CO2 concentration. Immediately after the acidification shock, we applied the following buffering treatments: no buffer, sodium bicarbonate, and crushed oyster shell. We then compared pH evolution across these buffering treatments in open containers and in sealed containers over the course of eight days.

In open containers, pH increased consistently and across treatments. pH of open unbuffered containers increased from 7.22 immediately after the acidification shock, to 7.90 at Day 8. The addition of sodium bicarbonate immediately raised the pH from 7.22 to 7.81. The pH subsequently continued to increase, reaching 8.40 by Day 8 in open containers buffered with sodium bicarbonate. The addition of crushed oyster shells raised pH gradually, from 7.22 immediately after the acidification shock to 7.42 after 1 hour, 7.53 after 24 hours and 7.95 by Day 8 (**Figure 1**).

Sealed containers all reacidified, albeit at different levels. The pH of sealed unbuffered containers continued to decline after the initial acidification shock, reaching 6.99 at Day 8 (**Figure 1**). Addition of sodium bicarbonate immediately increased pH to 7.81 in sealed buffered containers. Our sealed containers with sodium bicarbonate buffer then started reacidifying after 24 hours, with pH declining to 7.68 by Day 8 (**Figure 1**). Addition of crushed oyster shell raised pH to 7.51 after 12 hours (**Figure 1**). pH of sealed containers with crushed oyster shell buffer then decreased again to stabilize

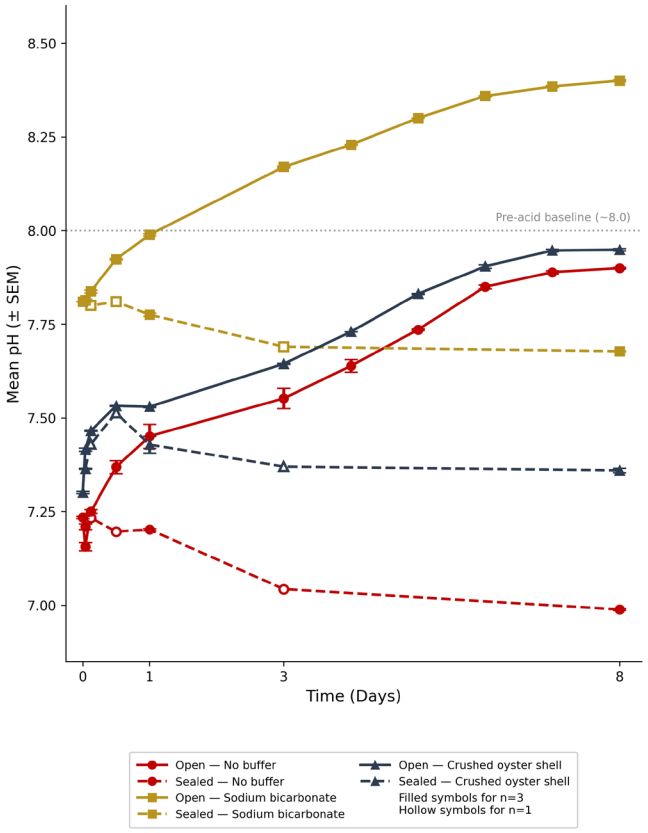

Figure 1: Effect of air-water gas exchange on water pH in open and sealed containers of acidified Long Island Sound (LIS) saltwater, immediately after being treated with different types of buffers (Experiment 1). Line graphs with mean pH (± SEM) measured over 8 days in acidified seawater under open and sealed conditions. Containers containing Long Island Sound water were manually acidified at day 0 using 10mL of 0.5% acetic acid. Resulting solution was then buffered using 7.5g of crushed oyster shell or using 10mL of a solution containing 10g of sodium bicarbonate for 100g of saltwater. Measurements were taken immediately after buffering, and then after one hour, three hours, twelve hours, one day, three days, four days, five days, six days, seven days, and eight days. *n*=3 at one hour, one day, eight days for sealed containers, and at all points for open containers. Each pH datapoint determined using three technical measurements. Average pH is then calculated from *n* =1 or *n* =3 datapoints (average of three datapoints for *n* =3). SEM bars indicated for *n* =3 (may not be clearly visible if SEM too small).

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at a lower level (7.37 at Day 3 and 7.36 at Day 8) (**Figure 1**). A comparison across containers and treatments at Day 8 showed that the relative impact of buffers was larger in sealed containers than in open containers. Sodium bicarbonate raised the pH by 0.69 pH units in sealed containers vs. 0.50 pH units in open containers, and crushed oyster shell raised pH by 0.37 pH units in sealed containers vs. 0.05 pH units in open containers (**Figure 1**).

We wanted to determine which was the strongest factor in pH recovery: air-water gas exchange or buffers. Therefore, we performed a two-factor ANOVA (air-water gas exchange x buffer). The two-factor ANOVA showed that air-water gas exchange (open containers vs. sealed containers) had the biggest impact on pH level at Day 8 (F=89,299, $p < 0.001$). Buffer type (no buffer vs. sodium bicarbonate vs. crushed oyster shell) had a smaller, albeit significant effect (F=19,722, $p < 0.001$). Interactions between air-water gas exchange and buffer had a smaller impact (F=1,422, $p < 0.001$). We wondered if air-water gas exchange also had the highest F-statistic at earlier timepoints. We also performed this two-factor ANOVA at earlier timepoints. It showed that air-water gas exchange had no detectable effect at 1 hour post-acidification ($F = 0.05$, $p = 0.82$), while buffers dominated ($F = 5{,}875$, $p < 0.001$). At 24 hours air-water gas exchange had become highly significant ($F = 203$, $p < 0.001$) with buffers still remaining dominant ($F = 632$, $p < 0.001$).

We wanted to see if the dominance of air-water gas exchange over buffering after several days also applied to biological buffers. To that end, in Experiment 2, we subjected Long Island Sound seawater to an initial artificial acidification shock immediately followed by application of the following buffering treatments: macroalgae, sodium bicarbonate, combination of sodium bicarbonate and macroalgae, and no buffering. We measured pH after nine days in open containers and in sealed containers. The addition of one day from Experiment 1 was made under the assumption that macroalgae's action might be slower than that of sodium bicarbonate and crushed oyster shell.

We measured the pH of open containers throughout Experiment 2. pH increased in all open containers, with final pH highest in containers containing sodium bicarbonate (pH on Day 9 of 8.40 with macroalgae and 8.41 without macroalgae), followed by containers containing only macroalgae but not sodium bicarbonate (pH of 8.20 on Day 9), followed by containers containing neither sodium bicarbonate nor macroalgae (pH of 7.90 on Day 9, i.e. close to, but below, the base pH of 8.0 pre-acidification) **(Figure 2**). Measurements in macroalgae-only open containers displayed higher variability, with tendency for lower pH in morning close to sunrise versus tendency for higher pH in evening close to sunset (**Figure 2**).

Upon unsealing on Day 9, the pH of sealed unbuffered containers was measured at 7.17, i.e., 0.05 lower than immediately post-initial acidification. In sealed containers, pH levels were between 7.65 for the sodium bicarbonate buffered containers and 7.68 for macroalgae buffered containers. Their combined effect raised the pH to 7.85. Variability of pH across the three replicates was bigger for containers that contained macroalgae (**Figure 3**).

We wanted to determine which was the strongest factor in pH recovery, air-water gas exchange, sodium bicarbonate buffer, or macroalgae, therefore we performed a three-factor ANOVA (air-water gas exchange x buffer x macroalgae). The three-factor ANOVA showed that air-water gas exchange (open containers vs. sealed containers) had the biggest impact on pH level at Day 9 ($F = 367.89$, $p < 0.001$). Buffer type (sodium bicarbonate vs no buffer) had a smaller, albeit significant effect ($F = 101.80$, $p < 0.001$). Macroalgae (macroalgae vs. no macroalgae) had a smaller impact than buffer treatments ($F = 55.63$, $p < 0.001$). Two significant two-way interactions were also detected: Air-water gas exchange × Macroalgae ($F = 9.69$, $p = 0.007$) and Buffer × Macroalgae ($F = 21.88$, $p < 0.001$), indicating that the effect of macroalgae on pH was stronger in sealed than in open containers, and that macroalgae and sodium bicarbonate combined together produced a greater pH elevation than either treatment alone (**Figure 4**). The three-way interaction and the Air-water gas exchange × Buffer interaction were not significant ($p > 0.05$), suggesting that the effects of air-water gas exchange and buffer operated independently of each other.

Across both experiments (Experiment 1 and Experiment 2), buffers produced an average pH uplift in sealed containers of +0.55 units above unbuffered controls, compared to an average pH uplift of +0.37 in open containers. The uplift produced by buffers was 0.17 pH units larger in sealed containers, compared to open containers, on average across the experiments (**Figure 1**).

DISCUSSION

In our study, we compared effectiveness of buffers in sealed versus open containers and sought to assess to what extent air-water gas exchange limits the effectiveness of buffering strategies. Our question is relevant for shellfish hatcheries that implement buffering strategies in coastal environments. Open containers model zones with free air-water gas exchange. Sealed containers model low-exchange environments such as hypoxic bottom waters, stratified

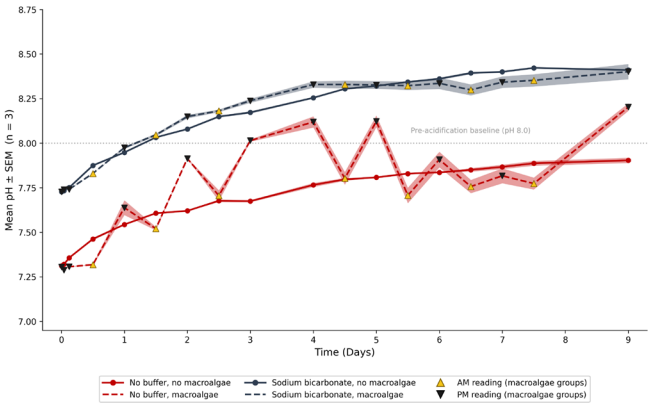

Figure 2: Daily reading of pH in open containers with macroalgae and with or without buffer (sodium bicarbonate) over nine days (Experiment 2). Mean pH (± SEM) measured over 9 days in acidified seawater under open conditions across four treatment combinations: no buffer without macroalgae, no buffer with macroalgae, sodium bicarbonate without macroalgae, and sodium bicarbonate with macroalgae ($n = 3$ containers per group). Solid lines = no macroalgae; dashed lines = macroalgae present. SEM plotted as shaded areas. For macroalgae groups, individual AM (▲) and PM (▼) readings are shown to illustrate diel variation.

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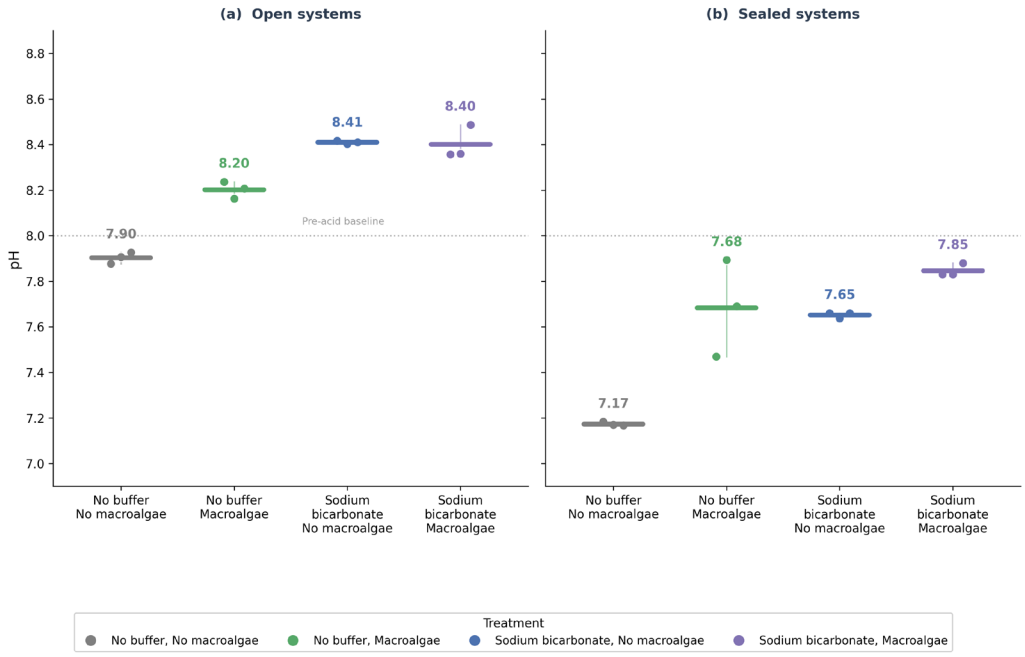

Figure 3: Comparison of water pH in all open and sealed containers nine days after acidification and addition of buffer treatments (Experiment 2). Scatter plot with error bars showing mean pH ± SD (*n*=3).

summer layers, and enclosed hatchery containers. Across this study, several conclusions emerged consistently.

In open containers, pH consistently rebounded toward or above pre-acidification levels regardless of buffer treatment. That rebound across containers is consistent with equilibration of CO2 between open water and the atmosphere through continuous air-water gas exchange.

Conversely, sealed containers re-acidified (**Figure 1**). This re-acidification was visible even when pH had been raised by sodium bicarbonate or crushed oyster shell additions (**Figure 1**). This finding is consistent with microbial respiration: CO_2 forms through the respiration of microbial organisms contained in the Long Island Sound saltwater and cannot escape the sealed containers. Trapped CO_2 dissolves and progressively lowers pH. In open containers, that CO_2 would immediately escape back into the air; in sealed containers, it accumulates.

The results of the ANOVA performed at Day 8 in Experiment 1 were consistent with our hypothesis that air-water gas exchange would limit the effectiveness of buffering strategies. The ANOVA showed that air-water gas exchange had the largest effect and was highly significant, far exceeding buffering contributions. This would suggest that, in real conditions with significant air-water gas exchange, buffer additions might not lead pH to meaningfully exceed the reference level determined by atmospheric equilibrium.

The temporal progression of ANOVA *F*-statistics provides additional insight. After one hour post-acidification, air-water gas exchange had no measurable statistical effect, and buffer chemistry dominated, consistent with CO_2 equilibration across the air–water interface operating on timescales of hours to days, not minutes (18). As exchange accumulated, it overwhelmed all buffer contributions. This has implications in real-life buffering strategies: benefits of buffering interventions may diminish rapidly with meaningful air–water contact. Conversely, more limited air-water gas exchange may be more favorable to buffer persistence.

A consistent observed pattern was that macroalgae raised pH, with a more visible effect in sealed containers (**Figure 4**). In open containers, the absorbed CO_2 can be replenished continuously from the atmosphere through air-water gas exchange, while in sealed containers, absorbed CO2 is not replenished. The lower pH increase in open containers is therefore consistent with photosynthesis absorbing CO_2.

Combining sodium bicarbonate with macroalgae showed strong additionality in sealed containers, but not in open containers. In sealed containers, combining sodium bicarbonate and macroalgae produced a higher pH than either alone, with alkalinity addition and photosynthetic CO_2 absorption combining to increase pH. In open containers, pH converged regardless of the presence of macroalgae. Air-water gas exchange could therefore be setting an upper limit (**Figure 2**).

The ANOVA performed on Day 9 on Experiment 2 supported the hypothesis that air-water gas exchange is the primary driver of pH evolution. Statistical analysis showed that air-water gas exchange had the largest effect and was highly significant, far exceeding buffering contributions.

The impact of biological buffers on pH was more dispersed than the impact of the other treatments, both between replicates and between different measurement timepoints (for open containers, which were measured repeatedly). With biological buffers, pH values tended to be higher for measures at the end of the day, and lower at the beginning of the day, consistent with photosynthetic capture of CO_2 during the day and emission of CO_2 via respiration at night (**Figure 2,4**).

There are limitations to the study, which open several avenues for future research. Acidification through the addition

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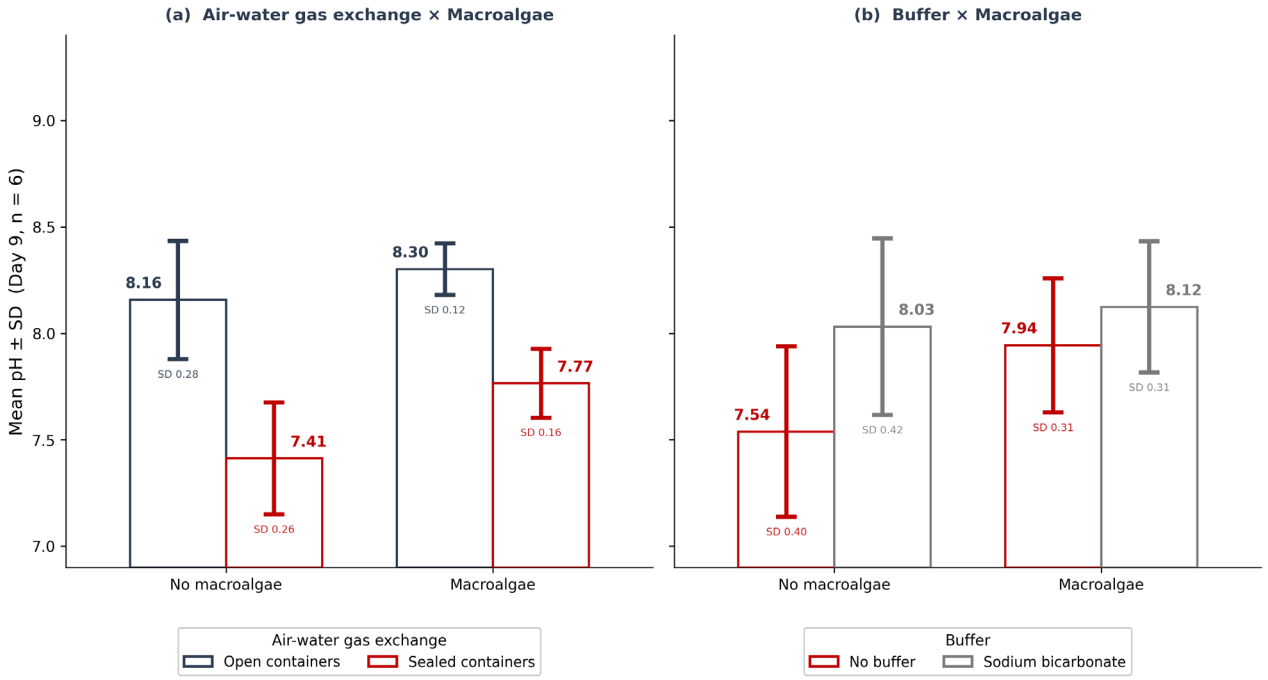

Figure 4: Interaction effects on pH nine days after acidification and addition of buffer treatments (Experiment 2). Panel (a): Air-water gas exchange × Macroalgae interaction showing mean pH (± SD). Panel (b): Buffer × Macroalgae interaction showing mean pH (± SD) . *n* = 6 containers per displayed group (3 containers per underlying treatment combination).

of acetic acid does not fully mirror the CO_2 absorption carbon chemistry in oceans, although it provides consistent and measurable acidification, which allows testing of buffering responses in various environments. Use of acidification via CO_2 bubbling would better mirror carbon chemistry. Another limitation is that the impact of microbial respiration was inferred but not directly measured. Future work should measure dissolved inorganic carbon or total alkalinity, and dissolved oxygen to confirm the mechanisms at play explicitly. Also, the study did not separate the relative contributions of microbial CO_2 production and absence of outgassing to pH decline in sealed containers. Finally, the daily photosynthesis/respiration cycle influenced results, with pH lower in the morning and higher in the evening, and this effect could be further studied and better isolated.

Although simplified in design, the findings of these experiments could provide useful insight for real-world buffering intervention, in particular by shellfish industries. First, this study suggests that gas exchange plays a dominant role and that any system with free air-water contact, as is the case of most hatcheries, should see pH heavily constrained by atmospheric CO_2. Second, it suggests that buffers matter mostly when air exchange is restricted. In the LIS and other coastal environments, such restricted air exchange environments include in particular tight bottlenecks, hypoxic bottom waters, stratified summer layers, hatchery pipes, and areas with long water residence time. Our findings therefore suggest that buffering interventions should be considered depending on the gas-exchange environment. In high-exchange settings, air-water equilibration dominates; in low-exchange settings, buffering becomes meaningful. This could mean that hatcheries and other shellfish farming installations should consider air-water gas exchange configurations, if they plan to use buffering.

MATERIALS AND METHODS

Two experiments were conducted to test the effects of air-water gas exchange and buffers on water pH. Experiment 1 was designed to assess the persistence of acidification and the impact of chemical and mineral buffers in open vs sealed containers. Experiment 2 was designed to study the impact of biological buffers and their interactions with chemical buffers in open vs sealed containers.

In both series, bottles were used as containers to allow for sealed groups. Zen Water Plastic Bottles (ZenWTR, Alkaline Water, 1 liter) were selected because of their wide caps, which facilitated the introduction of macroalgae and of a pH meter probe.

All bottles were rinsed and filled with 750mL of Long Island Sound saltwater. The Long Island Sound saltwater was collected off a pier in Mamaroneck, New York. Collection was done at high tide. All samples were collected the same day. They were mixed in a large recipient to ensure homogeneity of water across all bottles.

Artificial acidification was conducted using acetic acid (vinegar) to reduce pH. The solution used was a commercial five percent white vinegar. It was first diluted to 0.5% (using the same batch of Long Island Sound water and vinegar, in a 90mL to 10mL ratio). 10mL of that solution was then added to each bottle. The dosing had been calibrated, through a series of trials testing various doses, to lower pH by 0.7.

Sodium bicarbonate ($NaHCO_3$) was used as the chemical buffer. The sodium bicarbonate was a food-grade product (It's Just). To improve dosing precision and mixing consistency,

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a stock solution was prepared by dissolving 10g of sodium bicarbonate in 100g of Long Island Sound water. 10mL of this solution was added to each chemically buffered container, corresponding to approximately 1g of sodium bicarbonate per container. The mineral buffer consisted of a pre-mix of crushed oyster shell (Manna Pro). 7.5g was directly added to each mineral-buffered container. Buffer quantities were calibrated in preliminary trials to raise pH toward the pre-acidification level, testing the effect on pH of various quantities ranging from 1g to 20g of crushed oyster shell. Live *Chaetomorpha* macroalgae for saltwater reefs, purchased from an aquarium store, were used as biological buffers. 4g of wet *Chaetomorpha* macroalgae was used for each sample. The weight of macroalgae had been calibrated through a prep experiment. All bottles were kept under natural indoor daylight near a window, with no artificial light source. Buffers were added immediately after the acidification shocks.

Time course experiments

Experiment 1 lasted 8 days and Experiment 2 lasted 9 days. Water pH was measured twice a day, close to sunrise and close to sunset, to coincide with photosynthesis/respiration cycles. Additional measurements were taken on the first day immediately after buffering, then after one hour, three hours, and 12 hours. pH was measured with a digital pH meter from Apera Instruments (PH60-WW Handheld pH Meter Tester Kit), with a precision of 0.01 units. The digital pH meter was calibrated using pH 4.0, 7.0, and 10.0 solutions. Bottles were gently swirled before each measurement. Salinity was not measured during the experiment, but all water originated from the same homogenized batch to ensure equivalent conditions across treatments.

For all sealed containers, containers were single-use and were eliminated from the experiment after being unsealed once for measurement (destructive sampling). Each setup (treatment and time of unseal) was replicated across three different containers, for any data used for ANOVA computation. To limit the overall number of bottles used, sealed containers that were used only in the context of time series (but not for the purpose of ANOVA) were not replicated. In total, 56 bottles were used for Experiment 1 and 24 bottles were used for Experiment 2.

At each datapoint, three technical measurements were taken to minimize experimental errors and approximations. Room and water temperatures remained constant throughout all experiments (around 15.5ºC) and were checked at each measurement session.

Experiment 1: air-water gas-exchange in chemical buffers

Experiment 1 tested how air-water gas exchange (open containers vs sealed containers) and buffer type (no buffer vs. sodium bicarbonate vs. crushed oyster shell) affected pH of a solution of acidified seawater over eight days. Three independent replicate bottles were included for each treatment.

A two-factor ANOVA with interaction was performed using the following model: pH Day 8 ~ Air-water gas exchange × Buffer. Residuals were examined visually to confirm approximate normality and homogeneity of variance. No severe deviations from ANOVA assumptions were detected. To examine the temporal evolution of effect, a two-factor ANOVA was also performed at 1 hour and 24 hours post-acidification.

Experiment 2: air-water gas exchange in biological buffers

Experiment 2 tested the combined effects of air-water gas exchange (open containers vs sealed containers), macroalgae (macroalgae vs no macroalgae), and chemical buffer (sodium bicarbonate vs no sodium bicarbonate) on the pH of a solution of acidified seawater over nine days. Only the pH measured at Day 9 was used in the ANOVA. Three independent replicate bottles were included for each treatment.

In Experiment 2, a full three-factor ANOVA was conducted using the following model: pH Day 9 ~ Air-water gas exchange × Macroalgae × Buffer. Residuals were examined visually to confirm approximate normality and homogeneity of variance.

Data analysis of pH changes

For both experiments, all statistical analyses were executed in Google Colab (Python 3.12.3) (19). Data were collected in Excel spreadsheets and imported into pandas DataFrames v3.0.2 (20). ANOVAs were fit as ordinary least squares models, statsmodels.formula.api.ols, with Type II ANOVA tables using statsmodels v0.14.2 (21) . Figures were generated with matplotlib v3.10.8 (22). A significance threshold of $\alpha = 0.05$ was used for all tests.

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