***Trajectory and character of oyster growth under altered total alkalinity and salinity***

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***Abstract—*** Sessile organisms in dynamic coastal and estuarine environments can experience both gradual transitions in seawater properties, as well as sudden, step-like changes that then persist for long periods. Seawater carbonate system parameters like total alkalinity (TA) and salinity are examples of parameters that vary in this fashion. Less is known, however, about how organisms experiencing such variation will respond over the course of a short term exposure (36 d). In particular, it remains unclear whether physiological responses to changes in TA and salinity manifest quickly or slowly, and whether such responses are transient or persist for extended periods. Understanding such details is essential to predicting how marine species might cope with increasingly perturbed shoreline environments. Here, we compare effects of a rapidly initiated and persistent modification (i.e., ‘step-change’) to TA and salinity on growth of juvenile Eastern oysters (*Crassostrea virginica*). We examine responses within two time windows: one immediately following the initiation of the treatment (days 0 – 18) and another that follows later (days 19 – 36). We employ six treatment levels of total alkalinity (spanning 792 to 2916 µmol kg-1) and two salinities (27 and 34 **psu**) that might be expected within coastal zones or estuaries that receive stream inputs. In addition to assays measuring growth in shell area (mm2), we also examine consequences of altered TA and salinity for indices of shell thickness (mg mm-2) and oyster condition index (mg mg-1). Results demonstrate that growth in shell area of oysters was higher during the earlier time window compared to the later window of exposure. Growth in shell area was unaffected by TA in the earlier window, but increased with higher TA during the later window. Oyster individuals of larger initial size grew faster in the earlier time window. Salinity had no effect on growth during either window. Over the full time period of the experiment spanning both time windows, neither shell thickness nor condition index varied with TA or salinity. The lack of trend over such a longer period resembles that observed in other well-fed calcifiers that increase energy expenditures to cope with stressful conditions, or could indicate a trade off in one axis of physiological response against another. This work begins to broaden our understanding of temporal dynamics and sensitivities of species to multiple types of altered seawater carbonate system parameters characteristic of nearshore habitats.

***Introduction—*** Coastal zones and estuaries exhibit variability in the seawater carbonate system, which have implications for shell-building inhabitants. Various factors, including biogeochemical processing and river inflows, can contribute to deviations in total alkalinity (TA) from open-ocean values (Hunt et al. 2011, Fassbender et al. 2016, Montagna et al. 2018, Savoie et al. 2022). Likewise, salinities can fluctuate depending on the physical and chemical mixing of coastal seawater with freshwater sources (Das et al. 2012, Geiger et al. 2013, Hollarsmith et al. 2020). Moreover, shoreline and estuarine conditions are temporally complex, with TA and salinity changing abruptly, often in conjunction with storms or seasonal shifts, and then persisting for days to months (Najjar et al. 2020, Ricart et al. 2021b, Bartoloni et al. 2023). Such dynamics require that sessile calcifiers conform to new conditions and then endure them for extended durations. Given the multi-faceted extent to which other coastal seawater properties dictate physiological and ecological performance of marine calcifiers (for an unexhaustive list, refer to reviews by Hofmann and Todgham 2010, Gaylord et al. 2015, Kroeker et al. 2016, Byrne and Fitzer 2019, Fernández et al. 2019, and Clements and Comeau 2019), investigating responses to variation in TA and salinity deserves detailed attention.

Global perturbations to the carbonate system will likely manifest differently at the local scale, in part, due to the consequences of variability in freshwater total alkalinity (Waldbusser and Salisbury 2014, Carstensen et al. 2018), with implications for calcification processes in individuals at regional or local scales (see Pérez et al. 2015, Fassbender et al. 2016). Globally, approximately a third of human-produced carbon dioxide (CO2) emitted to the atmosphere is absorbed into the oceans (Sabine et al. 2004). This absorption alters the relative abundance of multiple carbon-based ions of the seawater carbonate system, which can lead to a reduction in seawater pH (i.e. ‘ocean acidification, Caldeira and Wickett 2003). Several of these carbon-based ions have been identified as factors that at least partially control calcification and growth processes in bivalves, including pH and bicarbonate ions (HCO3-, a major component of total alkalinity) (Waldbusser et al. 2011, Dickinson et al. 2013, Ninokawa et al. In review), among other characterizations of carbonate system conditions (e.g., waldbusser omega, SIR , cyronak). global warmingaltering(Fowler and Hennessy 1995, Espinoza et al. 2018, Giorgi et al. 2019, González-Trujillo et al. 2023), and thus the seawater carbonate system,normal (Lorrain-Soligon et al. 2023). In locations where absorbed CO2 emissions are unbuffered by low TA freshwater, the seawater environment may become more chemically corrosive to CaCO3 shell material (see Zeebe and Wolf-Gladrow 2001). Consequently, both small- and large-scale processes governing variation in TA and salinity operate within coastal areas and estuaries and can impact the ability of shell-forming taxa to precipitate their calcium carbonate structures.

Substantial effort has been dedicated to understanding how the growth of calcifiers can be disrupted by an altered carbonate system, both within and across various life stages (for reviews see Gazeau et al. 2013, Clements and Hunt 2017, Byrne and Fitzer 2019, Ducker and Falkenberg 2020). A subset of this work has emphasized effects of temporal characteristics of exposure (e.g., static versus fluctuating conditions, often of pH or CO2) (Mangan et al. 2017, Bitter et al. 2021). However, gaps remain in our understanding of whether responses to sudden changes in the carbonate system manifest quickly or build up more slowly, and whether responses continue to hold or abate as perturbed conditions persist. Such ‘step-change’ exposures are not uncommon in coastal systems due to longer retention periods from reduced estuarine mixing (Bruner De Miranda et al. 2017) or slow mixing between fresh water and coastal seawater (Horner-Devine et al. 2015). Consequently, the durations of these exposures can vary across systems and locations. Therefore, information regarding the trajectory of calcifier responses across time is valuable. These points become especially relevant to dissecting potential physiological trade-offs in energy allocation, or, understanding the capacity to cope with conditions – for example among growth (shell or tissue), reproduction, movement, and maintenance – each of which has its own time course and metabolic pathway (e.g., Kroeker et al. 2014).

Explorations of how growth rate responds to altered TA and salinity represent a valuable extension of prior work. Extensive studies have documented disrupted growth in bivalves under ocean acidification (for reviews see Gazeau et al. 2013, Shi and Li 2023), and specifically in *C. virginica* (Miller et al. 2009, Beniash et al. 2010, Waldbusser et al. 2011, Dickinson et al. 2012). However, few studies in bivalves have deliberately examined effects of variations in TA (with exceptions including Thomsen et al. 2015, Sanders et al. 2021, Ninokawa et al. In review). Similarly, there are limited studies on the experimental manipulation of TA and how that impacts crustaceans and echinoderms, which have demonstrated variable and broad tolerance to OA conditions, respectfully (see reviews in Dupont et al. 2010, Whiteley 2011, Byrne et al. 2013). Strong decreases in salinity are likewise known to affect growth (Ko et al. 2014, Pourmozaffar et al. 2020), but also see (Méthé et al. 2015, Sanders et al. 2021). However, when modifying seawater with distilled or deionized freshwater, TA undergoes linear dilution, due to negligible contributions from the freshwater. Correlations such as these can obfuscate the potential import of freshwater TA contributions in natural systems on oyster growth (Waldbusser et al. 2011, Hollarsmith et al. 2020), especially in scenarios where additional seawater conditions influenced by TA, such as seawater pH, are tested (e.g., Dickinson et al. 2012). Therefore, explicit tests examining the effects of TA and salinity on calcification abilities of coastal and estuarine taxa are needed.

Oysters contribute significant ecological and economic value to coastal populations of humans and wildlife alike. Because many oyster species create structure-forming reefs, they often provide habitat for other estuarine taxa (Coen et al. 2007). Moreover, they help protect against shoreline erosion from waves (Wiberg et al. 2019). Commercially, when reared and harvested for human consumption, they contribute to a globally-expanding diet of shellfish (Santeramo et al. 2017). This industry is substantial in the United states, with a value exceeding $1 billion annually, and globally surpassing $25 billion each year (Van Der Schatte Olivier et al. 2020). In such contexts, relative and absolute amounts of shell and tissue mass become pertinent considerations.

Here we explore how TA and salinity affect growth of juvenile Eastern oysters (*Crassostrea virginica*), with special attention to the trajectory of response to step changes in seawater TA and salinity. In particular, we examine how growth in shell area differs between an earlier time window of exposure, starting immediately after initiating treatment conditions, and a second time window two weeks later. In these efforts we also account for the size of oysters at the beginning of each time window. At the end of the experiment, we additionally compare a metric of oyster shell thickness (shell mass per area) and condition index (tissue mass per shell mass) across different TA and salinity exposures. Examinations such as these lend insights into ways in which calcifying coastal and estuarine species may respond across time to abrupt and persisting changes in seawater carbonate chemistry.

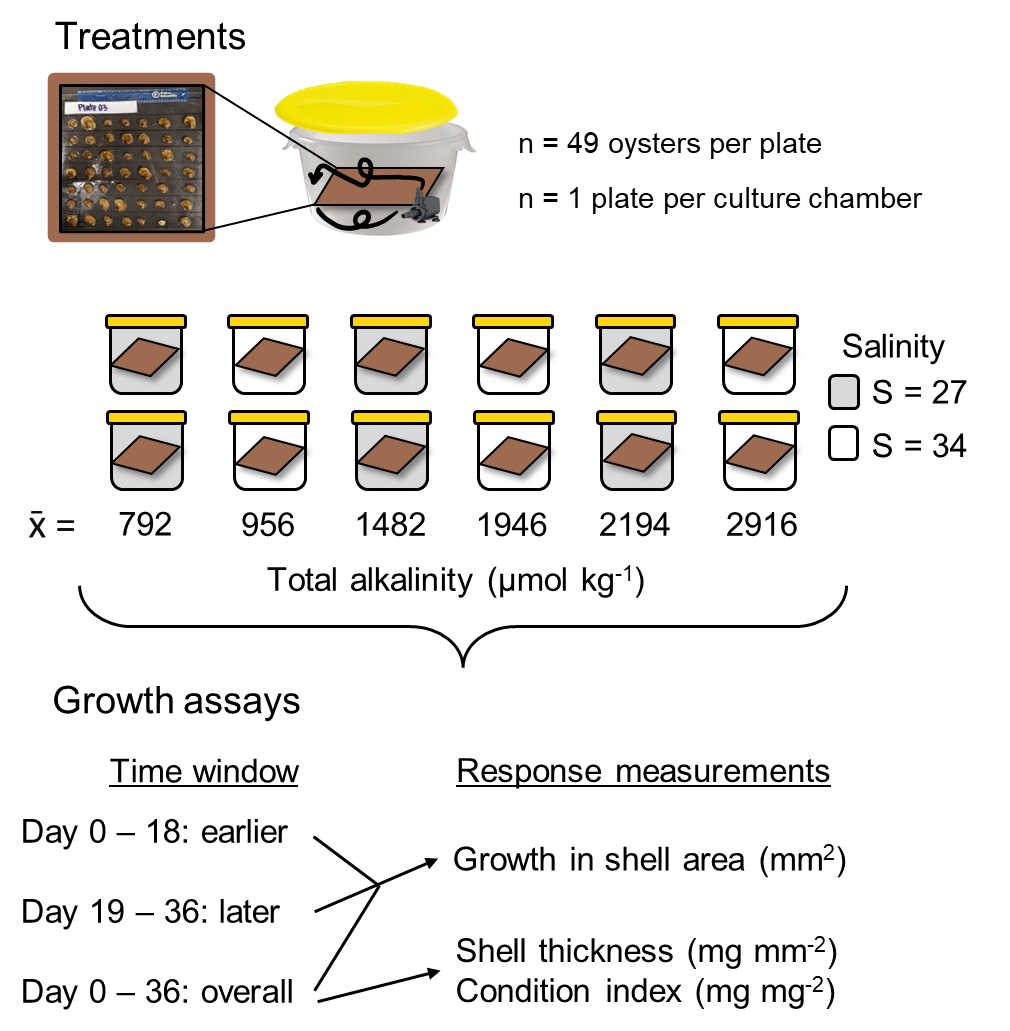
***Methods—***

**Study species—** The Eastern oyster, *Crassostrea virginica*, is native to estuaries of eastern North America, from the Gulf of St. Lawrence to the Gulf of Mexico. Due in part to its wide salinity and thermal tolerance, this species is also grown commercially elsewhere, including the west coast of the United States and Hawaii. For our experiments, we sourced juvenile oysters from a local aquaculture farm in Tomales Bay, California, USA (Hog Island Oyster Company; 38.162327, -122.893489). On 22 July 2022, we transported oysters in cool seawater from Tomales Bay to the University of California, Davis’ Bodega Marine Laboratory (BML; Bodega Bay, California, 60 min transit time), and placed them immediately into flow-through, continuously bubbled seawater drawn from the adjacent ocean. We fed the oysters with slow-release mixed algal diet (5% of their wet mass with thawed whole-cell concentrate of *Tetraselmis sp.*, *Thalassiosira* *weissflogii*, *Thalassiosira pseudonana*, and *Schizochytrium sp.*; Reed Mariculture) once every two days, throughout a 30-d lab acclimation period. At the end of the acclimation period, we glued the oysters (left valve) to plastic plates using Loctite brand marine epoxy (n = 49 per plate, n = 12 plates). We then returned the plates with attached oysters to the acclimation tanks, and three days hence began a 36-d growth experiment (Fig. 1).

**Design of experiment—** Our primary aim was to determine effects of total alkalinity (TA) on oyster shell growth. We established six treatment levels of TA, spanning conditions characteristic of estuaries with substantial rainwater input (low TA, 800-1500 µmol kg -1), through more typical alkalinity conditions (intermediate TA, 2000 – 2200 µmol kg -1), to conditions observed in estuaries supplied by watersheds of high-carbonate geology (high TA, > 2200 µmol kg-1). Because streams and rivers that deliver low- to high-TA fresh water can simultaneously decrease estuarine salinity, we also established two treatment levels of salinity, an ambient level (S = 34) and a reduced salinity level (S = 27). The resultant experimental design linked treatments of both lower and higher TA with both low and ambient salinity. Although the treatment conditions were not fully orthogonal (Fig. 1), this design enabled exploration of independent effects of TA and salinity across a spectrum of environmental conditions relevant to *C. virginica*. Each TA and salinity treatment combination (6 total) was replicated across two static culture chambers, each containing one plate with 49 attached oysters. The overall configuration thus summed to 49 oysters x 2 cultures x 6 treatments = 588 oyster individuals across the experiment.

We were additionally interested in whether oysters might respond in a different way immediately following exposure to a novel set of TA and salinity conditions, compared to a response later on after the exposure had continued for multiple days. We therefore sampled growth in shell area initially, and then every 18 days throughout the 36-d experiment, focusing especially on growth across two exposure time windows, one earlier (days 0 – 18) and one later (days 19 – 36).

**Oyster growth—**We tracked changes in shell surface area throughout the experiment, taking photos of shell area on day 0, day 18, and day 36. We used ImageJ software (v.1.53t) to analyze the photos and determine projected surface area of each oyster’s top valve, ensuring a scale bar was visible in each image. Acknowledging the potential for greater human error in measurement than from software error in this type of analysis, we conducted a repeat set of shell area measurements on a subset of the images (25% of total n) to ensure replicability and estimate error (size measurements were within 5% of original area measurement in all cases). We quantified the growth in shell area (difference in shell area between start and end dates) within earlier and later time windows, and the overall shell growth across the full 36 days of the experiment. We additionally measured condition index at day 36, which we quantified as dry tissue mass per dry shell mass, after separating the tissue from the shells and drying each at 60°C for 48 hr. We then divided shell mass by shell area to develop a rough metric of shell thickness.



**A**

**B**

Fig. 1. Schematic of A) experimental culture conditions and B) growth responses that were measured through time. We employed six TA conditions and two salinity levels, replicating each TA-salinity combination. One oyster plate was assigned to each of the resulting 12 culture chambers for the 36-day experiment. We measured growth in shell area in an earlier and later window (days 0 - 18 versus days 19 - 36). We also measured overallshellgrowth across the full experimental period (36 days), and quantified shell thickness (mg mm-2) and condition index (mg mg-2) at the end of the experiment.

**Culture conditions—** Each static culture during the experiment included an aquarium pump to ensure adequate water motion. The continuous stirring allowed gas exchange at the water’s surface to keep oxygen levels at >80% saturation . The only exceptions were two cultures that dropped to ~70% on one occasion each, due to pump failure. The resulting episodes of decreased oxygen lasted less than 8 hr. Oysters were fed daily with shellfish mixed algal diet(Reed Mariculture, approximately 4g dry algal biomass (whole-cell concentrate: *Tetraselmis* sp., *Thalassiosira weissflogii, Thalassiosira pseudonana*, and *Schizochytrium* sp.) per animal), and were held in the dark to minimize the influence of shadows on activity (Bamber 2023). Complete water changes were done every three days, and the sides of the culture chambers, and pumps, cords, and tubing were cleaned of any fouling organisms and debris. The experimental cultures had lids but were not tightly sealed due to a gap created by the pump power cord, which resulted in minor drifts in seawater temperature (1-2 °C) and TA (60-140 µmol kg-1) between water changes (Fig. 2). Despite this drift, salinity and TA chemical conditions across treatments remained distinct, and temperature fluctuated similarly in all treatments (Supplementary Table 5). Any mortality of oysters was recorded at the same time as water changes, and shells of deceased oysters were promptly removed from the cultures and discarded (< 15% mortality in any given culture chamber).

Before and after each water change during the experiment, we measured seawater temperature, salinity, pH, and dissolved oxygen concentration with a handheld multi-parameter sensor (Yellow Springs Instrument ProQuatro). In 50% percent of pH measurements, we collected discrete bottle samples for spectrophotometric determination of pH (calibrated on the same day with m-cresol dye standards, Easley and Byrne 2015), and used the latter data to translate all YSI pH data to the total scale (estimated instrument precision 0.01 pH units). We also collected and immediately froze 250 ml seawater samples before and after each water change for later TA determination. We quantified seawater TA in triplicate using a Metrohm 855 Titrosampler, correcting titration acid concentration daily with certified reference materials from the laboratory of Dr. Andrew Dickson (Scripps Institute of Oceanography). The median TA value was used to characterize seawater conditions (average standard error of samples = 1.05 µmol kg-1). Finally, we used measurements of seawater TA (µmol kg-1) and pH (total scale) at specified salinities and temperatures to estimate the remaining seawater carbonate system parameters with the *seacarb* package in the software R (version 3.3.1). In our *seacarb* estimates, we used equilibrium constants from Lueker et al. 2000 (K1 and K2), Perez and Fraga 1987 (Kf), and Dickson 1990 (Ks). For simplicity, we refer to the seawater carbonate system in terms of TA, though additional carbonate system parameters vary in conjunction with shifts in alkalinity (Table S5), some of which may influence oyster growth separately from salinity and TA.

**Chemical manipulation of seawater—** Seawater chemical conditions at the beginning of the experiment and at each water change were established as follows. We first depleted seawater TA to negligible concentrations in large sumps (n = 4 sumps/water change) by adding hydrochloric acid (HCl) to drive the carbonate system reactions towards CO2, which then off-gassed over two days in conjunction with strong bubbling with air. We then mixed the TA-depleted seawater with distilled fresh water and premade solutions of NaHCO3 (sodium bicarbonate) and Na2CO3 (sodium carbonate) with HCl to adjust the carbonate system back to desired salinity and TA levels (Waldbusser et al. 2015, Ninokawa et al. in review).

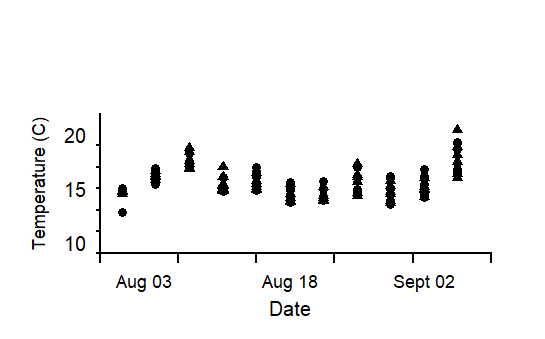
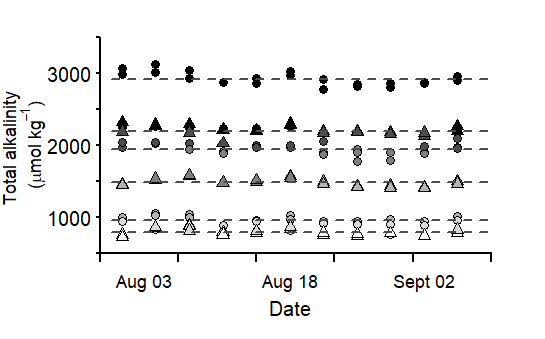
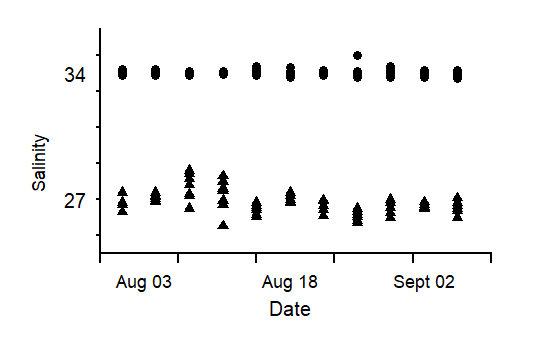


Fig. 2. Daily average A) total alkalinity (TA), B) salinity, and C) temperature conditions from culture chambers (n = 2 per TA-salinity combination, for details see Fig. 1), sampled every three days over the 36 days of the experiment. In panel A, dashed lines show the average TA value of the two replicate chambers, each with a single plate of oysters (n = 49 individuals).



**C**

**A**

**C**

**B**

**Statistical analysis—** All statistical tests were performed in R Studio (ver. 2022.07.02). We used a mixed effects model (*nlme*, *lme4*) to explore how TA affects growth in shell area over the course of an exposure, across two salinity levels, and as a function of initial oyster size. We explored growth responses during two temporal periods: an earlier response window (days 0-18), and a later response window (days 19-36). The initial size corresponded to the projected surface area at the beginning of the time window under consideration. Total alkalinity and initial oyster size were treated as continuous, fixed effects, whereas salinity (ambient versus low) and response window (earlier versus later) were included as categorical effects. We incorporated oyster individual and culture chamber as random intercepts to account for the lack of independence associated with repeated sampling of the same oysters and the lack of independence of oysters within a given culture. In the resulting analysis output, the effect of the earlier time window is considered the ‘default’, and as such, reported model estimates of the intercept and fixed effects (slopes) refer to effects within the earlier window. The effect of the second time window appears via the interaction terms between TA or salinity and the response window. We used similar models to test the influence of TA, salinity, and initial oyster size on overall growth over the full 36 days of the experiment. The influence of these latter three predictors on shell thickness (shell mass per area, mg mm-2) and condition index (tissue mass per shell mass, mg mg-2) were also tested, including culture chamber as a random intercept. We added a weighted variance term to models that failed the Breusch-Pagan test (*lmtest*) for residual heteroscedasticity, which specifies that the weight of each data point is equal to the proportional variance across bins. Assumptions of normality were visually assessed with qqplots and histograms of model residuals. We employed backwards step-wise model selection to test the effect size of parameters found significant in the model, running ANOVA comparisons between a full model and one with a given parameter omitted. The computed L-ratios, shown in Tables 1-4, indicate a proportional effect size relative to other predictors in the model, at a given p-value.

***Results—***

**Growth in shell area—** Growth in oyster shell area was higher during the earlier (0-18 d) response window than the later (19-36 d) response window (compare panels in Fig. 3). This difference was appreciable, with average oyster growth across all treatments during the second 18 d time window dropping from 11 to 2 mm2 compared to the first 18 d window, representing an almost ~80% decline. Total alkalinity did not influence growth in shell area during the earlier response window, but TA had a positive effect during the later response window (Fig. 3B; Table 1). The pattern during the later window may indicate that TA is more important in situations where rates of calcification of oysters are reduced by other factors. Salinity did not affect growth in shell area in either time window (Fig. 3, Table 1). In the earlier response window, there was a positive relationship between initial size and growth, while the relationship was reversed in the later window (Fig. 4, Table 1).

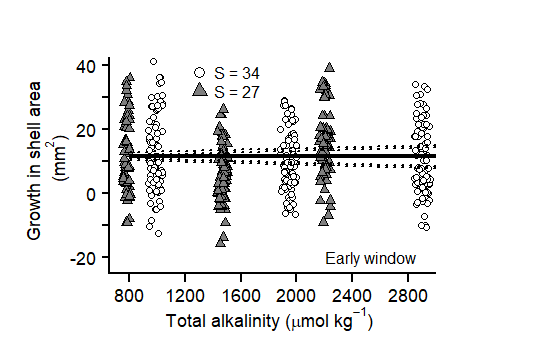


Fig. 3 Growth in shell area of juvenile *C. virginica* oysters during an A) earlier time window (days 0-18) and B) later time window (days 19-36) following initiation of treatment conditions. Growth in shell area depended on TA in the later window but not the first and was not affected by salinity (grayscale). Model predicted intercepts and slopes (line) and SE borders (dashed line) were taken from a mixed-effects model, and therefore incorporate the effect of other model predictors (Table 1).

**B**

**A**



Earlier window

Later window

**Overall shell growth, thickness, and condition index—** When overall growth spanning both time windows (i.e., over the full duration of the 36-d experiment) was computed, effects of TA were no longer apparent (Fig. 5, Table 2). Likewise, salinity did not influence growth in shell area after 36 days. However, the overall growth in shell area did increase with the initial size of oysters, similar to what occurred during the earlier of the two time windows (but not the later window). Neither shell thickness nor condition index, both assayed at the end of the experiment at day 36, showed an influence of total alkalinity or salinity (Fig. 6). However, oysters with larger initial shell areas tended to have a higher condition index. Shell thickness exhibited no trends as a function of initial oyster size.

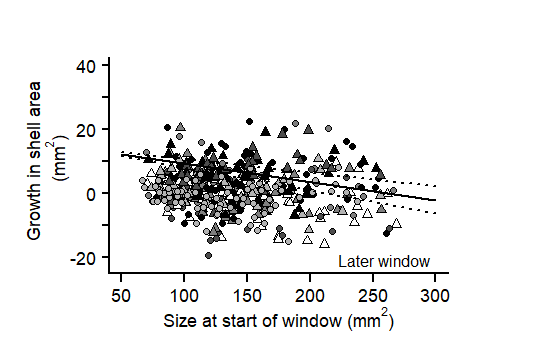
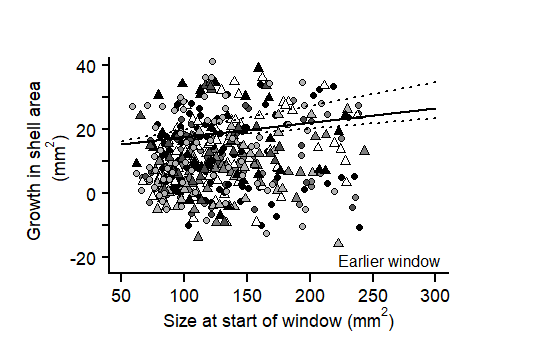


Fig. 4 Growth in shell area (mm2) exhibited different relationships with initial size between earlier (0-18 days) and later (19-36 days) exposure windows in juvenile *C. virginica* oysters. In the earlier window, growth was higher in larger oysters (positive slope), whereas larger oysters exhibited lower growth in the later window (negative slope). Intercept and slope model predictions (line) and SE borders (dashed lines) were taken from a mixed-effects model in Table 1.



**A**

**B**



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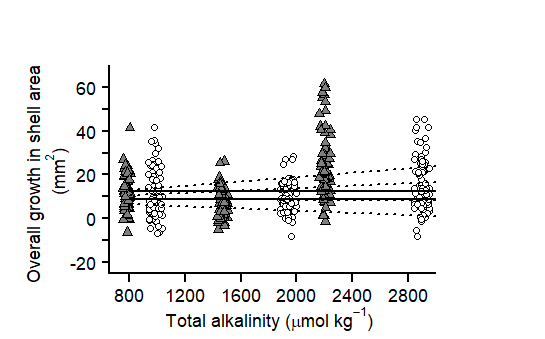
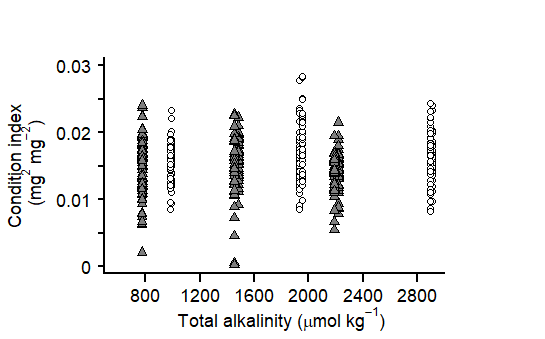
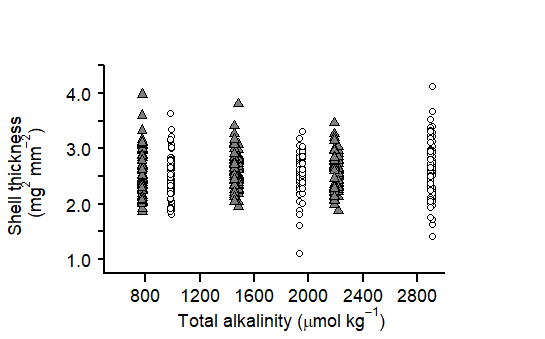


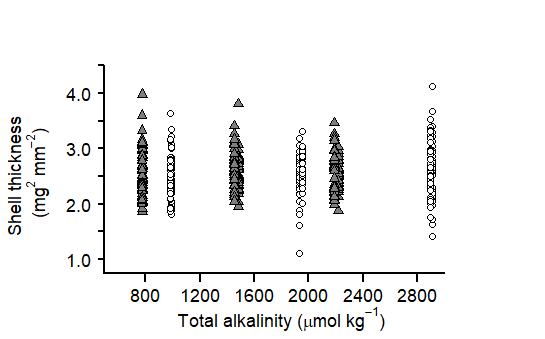
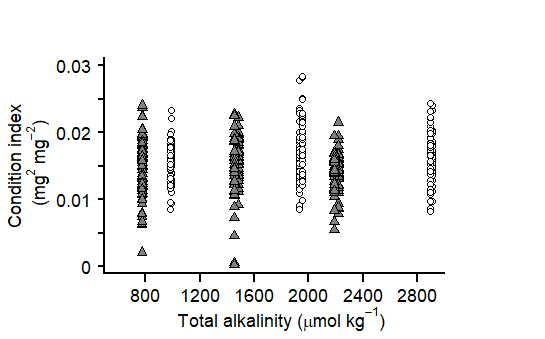
Fig. 5. Neither changes in total alkalinity (unit) nor salinity (greyscale) influenced the overall growth in shell area (unit) of juvenile *C. virginica* oysters after 36 days of exposure to altered conditions. Intercept and slope model predictions (line) and SE borders (dashed lines) were taken from a mixed-effects model in Table 2.

Fig. 6. Neither changes in total alkalinity (µmol kg-1) nor salinity (greyscale) influenced A) shell thickness (mg mm2) or B) condition index (mg mg2) of juvenile in *C. virginica* oysters.



**A**

**B**



***Discussion—*** Growth in shell area of juvenile *C. virginica* oysters responded to altered alkalinity and salinity in a complex fashion. Greater increases in shell area during the first 18 days following imposition of treatment conditions, compared to days 19-36, suggested that oysters possess the physiological capacity to respond quickly but may reallocate energetic resources away from growth toward other body functions over longer exposures to altered water quality. A potential role for energy stores is supported by the tendency for individuals of larger initial size to exhibit greater growth in shell area during the first time window. In contrast, positive effects of increased TA on surface area growth emerged in the second time window, perhaps in conjunction with diversion of energetic resources to other needs, or the depletion of those resources. Salinity did not affect growth in shell area during either time window, indicating that oysters are less sensitive to changes in salinity. Also notable is the finding that, over the course of the full 36 days, there was no overall effect of TA in overall shell growth. Similarly, there was no detectable consequences of low TA for shell thickness or condition index. Together, the obscuring of shorter-term patterns as time progressed is consistent with the hypothesis that early and late responses operated at least partially in opposition, resulting in a “canceling out” of effects of those responses over longer durations. That said, an influence of initial oyster size on overall growth and condition index remained apparent at day 36.

The apparent capability of Eastern oysters to acclimate quickly and grow new shell in low TA conditions applied even to seawater conditions that are corrosive to shell material. Corrosivity of seawater to shell material can be represented by the omega saturation state of seawater conditions, which is a measure of the thermodynamic tendency of calcium carbonate minerals that form shells, to dissolve (Ω < 1) or form (Ω > 1) in those conditions. In *Crassostrea virginica* oysters, calcite is the mineral form comprising the majority of the shell. The two lowest TA treatments of our study were undersaturated with respect to calcite (Ωcalcite < 1, Supplementary Table 5). One possible explanation for their initially higher growth is that the oysters were able to compensate for shell loss due to dissolution because they were well fed and had the necessary energy resources, a trend that has been highlighted previously in a number of studies involving bivalves (Hettinger et al. 2012, Thomsen et al. 2013, Sanders et al. 2018, Schwaner et al. 2023). An alternative explanation is that higher shell area growth arose in oysters that were initially larger in size due to increased surface area available as a substrate for additional calcification. However, this concept appears less than fully likely given that there was a negative effect of oyster size on growth in the later time window, implying that at least one relevant driver must have shifted in the second part of the experiment compared to the first.

In our experiments corrosive conditions arose due to low TA. Undersaturation can also derive from more classic ocean acidification scenarios. The latter causes elevated CO2 concentrations in seawater but leaves TA unchanged. However, both decreases in TA and increases in dissolved CO2 lead ultimately to a reduction in pH. Given that other coastal calcifiers have shown disruptions to shell growth under altered pCO2 conditions and decreased pH (Ries et al. 2009), it would be useful to test whether the sensitivity of juvenile Eastern oysters to corrosive seawater manifests under low TA even when pCO2 remains at modest levels.

As was mentioned above, declines in oyster shell growth during the later of the two time windows (days 19-36) coincided with an observed response to TA, and both growth effects and responses to TA could have arisen from the oysters diverting energetic resources towards physiological processes other than shell formation (Bayne and Newell 1983). Our data are unfortunately unable to offer insight into which, if any, alternative processes might have been prioritized. Conducting follow-up experiments that explore a wider range of growth metrics and responses under varied TA and salinity would, therefore, offer valuable insights.

Other calcifying species have demonstrated greater resistance to dissolution when the shell periostracum (an organic surface layer) is present and minimizes shell contact with the overlying seawater. Although little is known about the effect of the periostracum on oyster shells, work by us with mussels has demonstrated a significant reduction in shell dissolution when this organic layer is present (see Chapter 1). Although the periostracum of Eastern oysters appears superficially rougher and thinner than in other bivalves, Zuykov et al. (2012) measured low porosity in this covering, suggesting it may indeed retard contact of seawater with vulnerable nacre beneath it. In our study, although we did not measure periostracum coverage, we did note a strong discoloration in the shells through time as a function of TA condition (Fig 7). Such bleaching of the shell exterior under low pH conditions may indicate periostracum loss and potentially even dissolution of surface shell. Future work may benefit from closer attention to the periostracum and its interaction with seawater TA. After 36 days of exposure, there was no significant variation in oyster shell thickness or condition index in response to changes in TA or salinity, which may suggest that oyster shell growth occurred without a discernible energy trade-off over this exposure duration. Measured growth rates of individuals were a bit higher, but similar, to those of a juvenile congener species (*C. gigas*; Ricart et al. 2021a), and were within the range of adult *C. virginica* along an estuarine gradient (Méthé et al. 2015). Given that we were only able to assess shell thickness and condition index at the start and end of the experiment, and not at intermediate time points, there are multiple scenarios that could have led to the same overall pattern in growth. First, oysters could have instead changed the cup shape and shell height (see Hajovsky et al. 2021), two metrics we were unable to record from aerial photograph angles that were used here. Second, it is theoretically possible that oysters first directed resources toward expanding the surface area of their shells, at the expense of shell thickness and condition index. Then, once larger in shell area, the oysters could have redirected resources toward thickening their shells and bolstering their tissue mass (e.g., Clark et al. 2020). Such a mode of response would not be discernible in our data. This scenario may be somewhat unlikely given that other oyster studies have shown increases in both shell thickness and condition index in high-food environments (Sokolova 2021), along with initial declines in condition index left uncompensated following recovery conditions (Lutier et al. 2023). Future research that decouples naturally shifting metabolic pathways from disruptions of altered seawater conditions in *C. virginica* and other calcifying species would be beneficial.

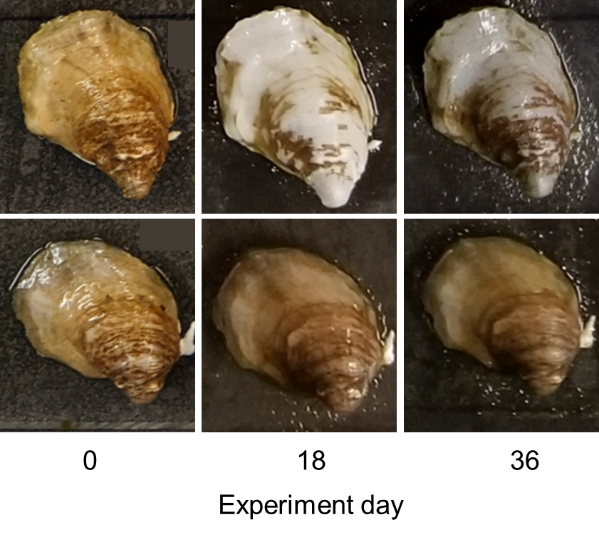


Fig. 7. Changes to shell color through time in two representative oyster individuals, one from A) low TA and one from B) high TA conditions. Photos were taken at the beginning, mid-point, and end of the 36-day experiment (0 = initial, 18 = end of earlier window, 36 = end of later window).

**A**

**B**

Although we observed no adverse impacts on shell growth in low TA with a salinity reduction to 27, it is worth considering that more severe declines in salinity, coinciding with forecasted extreme precipitation events (Donat et al. 2016), could exposure organisms to even lower salinity and TA conditions than tested in this study. The salinity reduction used in this tolerated by this species (Calvo 1999, Waldbusser et al. 2011), and in particular, is likely within ranges previously experienced by the individuals tested, at the oyster farm (Hollarsmith et al. 2020). Especially along the Atlantic and Gulf coasts of the United States, where *C. virginica* provides vital habitat and economic services, hurricanes are becoming more frequent (Knight and Davis 2009), which can rapidly lower salinity to levels below the species’ tolerance threshold, potentially resulting in shellfish mortality, as observed in extreme cases (Munroe et al. 2013). Even along the U.S. West Coast where hurricanes are rarer, extreme precipitation events have resulted in mass mortality of native oyster populations (Cheng et al. 2016), though *C. virginica* is likely more tolerable to low salinity conditions than other oyster species (Calvo 1999). The extent to which *C. virginica* oysters may be able to handle shifts in TA when coupled with more drastic reductions in salinity, like those corresponding to extreme storms, could provide insight into whether lower salinities have the potential to interact more strongly with variable TA than observed in our study.

The temporal influence of TA and salinity on oyster growth could influence inter-specific species interactions. For example, oysters that rapidly adjust to new conditions might fare better in staving off less-tolerant, invasive competitors (McFarland et al. 2015, Green et al. 2017). A later lower tolerance to corrosive conditions could coincide with predator sensitivity to altered conditions, which could weaken foraging pressure on oyster reefs broadly (Dodd et al. 2021), in addition to reduced predator activity in low salinity (Pusack et al. 2019).Oyster shells primarily use calcite, which make them less chemically vulnerable to dissolution than species with aragonite mineral forms, which could lead to separate response trajectories in windows of low shell building activity in species with more soluble shells, regardless of predator pressure. Moreover, a change in pH as a result of TA and salinity could have implications for parasitic infection outbreaks, to the extent that parasite outbreaks respond in similar ways to low pH from elevated pCO2 (Martinelli et al. 2020).

Understanding the trajectory and character of growth responses of coastal and estuarine calcifiers to changes in TA and salinity remains an area for future exploration. Our results illustrate that effects of TA on oyster shell growth manifest in a complex manner that can change over time and likely operate in tandem with other environmental stressors. As more extreme changes to conditions continue to emerge, it will become increasingly important to understand how growth in oysters and other calcifiers responds to ongoing and future shifts.

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***Tables—***

Table 1. Results of a mixed effects, linear regression model testing the independent effects of total alkalinity (TA), salinity (factor, 2 levels) and size at start of window (mm2) and their interaction with time window (factor, 2 levels) on growth in shell area (mm2) of juvenile *Crassostrea virginica* oysters. L-Ratios (for significant parameters) and p-values were recorded during backward stepwise model selection and refer to the ANOVA test output between the full model and a model with the specified interaction omitted. Bolded values denote a significant effect, determined by alpha < 0.05. The final model: Growth in shell area ~ total alkalinity + factor(salinity) + factor(time window) + initial size + interaction (TA: time window) + interaction (salinity + time window) + random intercept(individual) + random intercept(culture chamber), accounted for ~ 26% of the variation. In model estimates below, the intercept refers to the effect of ambient salinity, whereas the salinity term refers to the effect of low salinity, both in the earlier time window. The TA term refers to the earlier window effect of total alkalinity, whereas the interaction term between TA and window describes the effect of TA in the later window. Similarly, the second interaction term, ‘Salinity: window’, describes the effect of salinity during the later window.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Fixed Effects**  marg. r2 = 0.26  cond. r2 = 0.26 | **Estimate** | **Std. Error** | **t-value** | **df** | **L. Ratio** | **p-value** |
| Intercept | 13.6740 | 4.1776 | 3.2732 | 460 |  | **0.0011** |
| Salinity | 0.6498 | 0.9105 | 0.7137 | 460 |  | 0.4758 |
| Size at start of window | 0.0432 | 0.0104 | 4.1711 | 452 |  | **< 0.0001** |
| Total alkalinity (TA) | 0.0004 | 0.0006 | 0.7036 | 460 |  | 0.4820 |
| Time window | -0.4918 | 0.1461 | -3.3657 | 452 |  | **0.0008** |
| Interaction (TA: window) | 0.0032 | 0.0009 | 3.5938 | 452 | 12.9160 | **0.0003** |
| Interaction (Salinity: window) | 1.8572 | 1.2829 | 1.4476 | 452 |  | 0.1481 |
| Interaction (Size: window) | -0.0570 | 0.0140 | -4.0839 | 452 | 16.6780 | **< 0.0001** |
| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
| Individual | 0.0003 | -- |  |  | 37.5120 | < 0.0001 |
| Culture chamber | 0.0003 | 9.1748 |  |  |  | 0.9996 |

Table 2. Results of a mixed effects, linear regression model testing the independent effects of total alkalinity (TA), salinity (factor, 2 levels) and initial size (mm2) on overall shell growth (mm2) in juvenile *Crassostrea virginica* oysters after 36 days. L-Ratios (significant parameters) and p-values were recorded during backward stepwise model selection and refer to the ANOVA test output between the full model and a model with the specified predictor omitted. Bolded values denote a significant effect, determined by alpha < 0.05. In the final model: Overall shell growth ~ total alkalinity + factor(salinity) + initial size + random intercept(culture chamber, the estimate for the intercept refers to the effect of ambient salinity, whereas the salinity term refers to the effect of low salinity.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Fixed Effects**  marg. r2 = 0.12  cond. r2 = 0.42 | **Estimate** | **Std. Error** | **t-value** | **df** | **L. Ratio** | **p-value** |
| Intercept | -1.9521 | 5.5711 | -0.3504 | 450 |  | 0.7262 |
| Salinity | 3.4740 | 3.6491 | 0.9520 | 9 |  | 0.3660 |
| Initial size | 0.0517 | 0.0084 | 6.1657 | 450 | 22.3020 | **< 0.0001** |
| Total alkalinity | 0.0040 | 0.0025 | 1.5992 | 9 |  | 0.1442 |
| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
| Culture chamber | 5.7455 | 8.0515 |  |  | 86.7583 | **< 0.0001** |

Table 3. Results of a mixed effects, linear regression model testing the independent effects of total alkalinity (TA), salinity (factor, 2 levels) and initial size (mm2) on shell thickness (mg mm2) in juvenile *Crassostrea virginica* oysters after 36 days. Bolded values denote a significant effect, determined by alpha < 0.05. In the final model: Shell thickness ~ total alkalinity + factor(salinity) + initial size + random intercept(culture chamber, the estimate for the intercept refers to the effect of ambient salinity, whereas the salinity term refers to the effect of low salinity.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Fixed Effects**  marg. r2 = 0.01  cond. r2 = 0.01 | **Estimate** | **Std. Error** | **t-value** | **df** |  | **p-value** |
| Intercept | 2.4537 | 0.0925 | 26.5246 | 450 |  | **< 0.0001** |
| Salinity | 0.0069 | 0.0403 | 0.1523 | 9 |  | 0.8823 |
| Initial size | 0.0002 | 0.0005 | 0.4743 | 450 |  | 0.6355 |
| Total alkalinity | 5.920e-05 | 3.1130e-05 | 1.9202 | 9 |  | 0.0895 |
| **Random Effects** | **Intercept** | **Residual** |  |  |  | **p-value** |
| Culture chamber | 2.5337e-05 | 0.4574 |  |  |  | 0.9997 |

Table 4. Results of a mixed effects, linear regression model testing the independent effects of total alkalinity (TA), salinity (factor, 2 levels) and initial size (mm2) on condition index (mg mg-2) in juvenile *Crassostrea virginica* oysters after 36 days. L-Ratios (significant parameters) and p-values were recorded during backward stepwise model selection and refer to the ANOVA test output between the full model and a model with the specified predictor omitted. Bolded values denote a significant effect, determined by alpha < 0.05. In the final model: Condition index ~ total alkalinity + factor(salinity) + initial size + random intercept(culture chamber, the estimate for the intercept refers to the effect of ambient salinity, whereas the salinity term refers to the effect of low salinity.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Fixed Effects**  marg. r2 = 0.16  cond. r2 = 0.32 | **Estimate** | **Std. Error** | **t-value** | **df** | **L. Ratio** | **p-value** |
| Intercept | 0.0116 | 0.0016 | 7.0949 | 450 |  | **< 0.0001** |
| Salinity | -0.0018 | 0.0011 | -1.7086 | 9 |  | 0.2205 |
| Initial size | 3.3018e-05 | 3.7727e-06 | 8.7517 | 450 | 65.1080 | **< 0.0001** |
| Total alkalinity | 2.080e-07 | 7.2360e-07 | 0.2877 | 9 |  | 0.4677 |
| **Random Effects** | **Intercept** | **Residual** |  |  | **L. Ratio** | **p-value** |
| Culture chamber | 0.0016 | 0.0033 |  |  | 49.1364 | **< 0.0001** |

Table S5. Full characterization of average seawater conditions of each experimental culture chamber. We used pH (total scale) and total alkalinity (TA; µmol kg-1) measurements together with salinity (S), temperature (T; °C), to estimate the remaining parameters of the carbonate system, employing the *seacarb* package in R (version 3.3.1). Column labels are as follows: Culture chamber (#), partial pressure of carbon dioxide (pCO2, µatm), bicarbonate ion concentration (HCO3-, µmol kg-1), carbonate ion concentration (CO32-, µmol kg-1), dissolved inorganic carbon (DIC, µmol kg-1), and saturation state of the calcite mineral form of calcium carbonate (Ωcalcite). A standard error term for each estimate is included within the table (± SE). Equilibrium constants used in *seacarb* were selected to match similar temperature and salinity ranges (K1 and K2 from Perez and Fraga 1987, Dickson 1990, Lueker et al. 2000).

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Ωaragonite** | 0.5 ± 0.1 | 2.6 ± 0.1 | 0.8 ± 0.1 | 2.8 ± 0.1 | 4.6 ± 0.2 | 2.6 ± 0.1 | 0.9 ± 0.1 | 2.7 ± 0.1 | 4.6 ± 0.1 | 1.6 ± 0.2 | 0.5 ± 0.0 | 1.3 ± 0.1 |
| **DIC** | 766 ± 17 | 1808 ± 14 | 923 ± 15 | 2094 ± 17 | 2688 ± 26 | 1798 ± 16 | 888 ± 15 | 2061 ± 26 | 2679 ± 21 | 1407 ± 15 | 782 ± 18 | 1417 ± 20 |
| **CO32-** | 20 ± 2 | 109 ± 5 | 35 ± 3 | 112 ± 6 | 192 ± 8 | 107 ± 4 | 38 ± 2 | 109 ± 4 | 189 ± 6 | 64 ± 10 | 20 ± 1 | 52 ± 2 |
| **HCO3-** | 729 ± 17 | 1682 ± 15 | 871 ± 15 | 1961 ± 18 | 2475 ± 22 | 1674 ± 15 | 837 ± 15 | 1931 ± 28 | S469 ± 21 | 1324 ± 20 | 744 ± 17 | 1345 ± 19 |
| **pCO2** | 478 ± 44 | 476 ± 29 | 451 ± 73 | 562 ± 46 | 582 ± 25 | 476 ± 23 | 363 ± 29 | 571 ± 46 | 582 ± 27 | 486 ± 53 | 473 ± 45 | 537 ± 32 |
| **TA** | 792 ± 15 | 1960 ± 14 | 980 ± 15 | 2230 ± 18 | 2929 ± 33 | 1946 ± 19 | 956 ±16 | 2194 ±21 | 2916 ±21 | 1492 ± 16 | 806 ± 15 | 1482 ±18 |
| **pH** | 7.60 ± 0.05 | 7.93 ± 0.02 | 7.70 ± 0.05 | 7.94 ± 0.03 | 8.01 ± 0.02 | 7.92 ± 0.02 | 7.75 ± 0.03 | 7.93 ± 0.02 | 8.00 ± 0.02 | 7.85 ± 0.05 | 7.61 ± 0.04 | 7.79 ± 0.02 |
| **T** | 17.0 ± 0.5 | 16.1 ± 0.3 | 15.9 ± 0.4 | 16.6 ± 0.4 | 16.3 ± 0.4 | 16.0 ± 0.3 | 17.1 ± 0.4 | 17.2 ± 0.4 | 16.1 ± 0.4 | 16.0 ± 0.3 | 16.4 ± 0.4 | 15.8 ± 0.3 |
| **S** | 26.5 ± 0.1 | 34.0 ± 0.0 | 34.1 ± 0.0 | 26.7 ± 0.1 | 33.9 ± 0.0 | 34.0 ± 0.0 | 34.1 ± 0.0 | 26.7 ± 0.1 | 33.8 ± 0.0 | 26.9 ± 0.2 | 27.1 ± 0.2 | 26.7 ± 0.2 |
| **Culture chamber** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |

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