

Ghost Factors of Laboratory Carbonate Chemistry Are Haunting Our Experiments

A. W. E. GALLOWAY^{1,*}, G. VON DASSOW¹, J. B. SCHRAM¹, T. KLINGER²,
T. M. HILL³, A. T. LOWE⁴, F. CHAN⁵, R. M. YOSHIOKA¹, AND K. J. KROEKER⁶

¹Oregon Institute of Marine Biology, University of Oregon, 63466 Boat Basin Road, Charleston, Oregon 97420; ²School of Marine and Environmental Affairs, University of Washington, 3707 Brooklyn Avenue NE, Seattle, Washington; ³Department of Earth and Planetary Sciences, and Bodega Marine Laboratory, University of California, Davis, California 95616; ⁴Tennenbaum Marine Observatories Network, Smithsonian Institution, 647 Contees Wharf Road, Edgewater, Maryland 21403; ⁵Department of Integrative Biology, Oregon State University, 3029 Cordley Hall, Corvallis, Oregon 97331; and ⁶Department of Ecology and Evolutionary Biology, University of California, Santa Cruz, California 95064

Abstract. For many historical and contemporary experimental studies in marine biology, seawater carbonate chemistry remains a ghost factor, an uncontrolled, unmeasured, and often dynamic variable affecting experimental organisms or the treatments to which investigators subject them. We highlight how environmental variability, such as seasonal upwelling and biological respiration, drive variation in seawater carbonate chemistry that can influence laboratory experiments in unintended ways and introduce a signal consistent with ocean acidification. As the impacts of carbonate chemistry on biochemical pathways that underlie growth, development, reproduction, and behavior become better understood, the hidden effects of this previously overlooked variable need to be acknowledged. Here we bring this emerging challenge to the attention of the wider community of experimental biologists who rely on access to organisms and water from marine and estuarine laboratories and who may benefit from explicit considerations of a growing literature on the pervasive effects of aquatic carbonate chemistry changes.

Introduction

The recognition of ocean acidification (OA) and associated changes in seawater carbonate chemistry as a threat to marine ecosystems has motivated more than a decade of experimental work aimed at elucidating the effects of changing carbon-

ate chemistry on marine organisms (*e.g.*, Kroeker *et al.*, 2010; Padilla-Gamiño *et al.*, 2016; Frieder *et al.*, 2018) and ecosystems (Fabry *et al.*, 2008; Campbell and Fourqurean, 2014; Silbiger and Sorte, 2018). Experimental methods for OA research have advanced quickly, driven by collaborations between aquatic chemists and organismal biologists, widespread adoption of best practices for OA research (Dickson *et al.*, 2007), and technological innovation. The rapidly growing body of experimental evidence highlights the effects of OA across a range of fish and invertebrate species' life-history stages and critical life processes, including growth, survivorship, reproduction, and behavior (Kroeker *et al.*, 2010; Clements and Hunt, 2015; Nagelkerken and Munday, 2016; but also see Clark *et al.*, 2020). Other examples of the surprising breadth of biological consequences of seawater carbonate chemistry include changes in toxin production across unrelated groups of marine algae (Fu *et al.*, 2012; Raven *et al.*, 2020), biological sound production (Rossi *et al.*, 2016), and low-frequency sound transmission (Ilyina *et al.*, 2010).

Biologists working to describe fundamental processes of physiology and development, and others focused on microbiology, ecology, toxicology, or disease, may still not be fully aware of the influence of uncontrolled variations in carbonate chemistry (*e.g.*, $p\text{CO}_2$, pH, total alkalinity, carbonate ion concentration, and carbonate saturation state) on the response variables of interest in their experiments. Not only do these factors depend on oceanic drivers, but also they can be influenced by processes within laboratories—for example, biological respiration. Other factors, including nutrients and temperature, also influence water quality in experimental facilities

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* To whom correspondence should be addressed. Email: agallow3@uoregon.edu.

Abbreviation: OA, ocean acidification.

and can interact with changing carbonate chemistry. While we recognize the complexity imparted by a suite of interacting water chemistry variables, our purpose here is to bring attention to the issue of uncontrolled carbonate system variables as ghost factors affecting experimental marine research. We (the authors) have all experienced challenges controlling seawater carbonate chemistry in our laboratory experiments, even when biological response to OA is a primary objective of the research. Our goal is to encourage biologists and ecologists who are not already thinking about seawater carbonate chemistry to consider how this suite of variables could affect both current research and interpretation of past experiments, and to embrace this variability as an opportunity in future work. While our discussion mainly focuses on the marine systems in which we work, we note the broader relevance of this issue. Acidification resulting from human activities affects water types on the continuum from marine to estuarine and freshwater (Phillips *et al.*, 2015).

Drivers of Laboratory Carbonate Chemistry

In coastal ecosystems, carbonate system chemistry can vary sharply across both space and time. Spatial variation can occur at the scale of millimeters, for example, in diffusive boundary layers (Noisette and Hurd, 2018) to the scale of kilometers or more, while temporal variation can occur over scales of minutes to decades (Takeshita *et al.*, 2015). Two major processes (and many minor processes) contribute to this variation. First, biological processes can drive variation in coastal and estuarine carbonate chemistry (Lowe *et al.*, 2019). Photosynthesis (which consumes CO₂) and respiration (which produces CO₂) can cause large oscillations in carbonate system variables over the day-night cycle (*e.g.*, Silbiger and Sorte, 2018). Similarly, seasonal algal blooms and the subsequent decay of algal biomass can cause sharp seasonal declines in pH (Wallace *et al.*, 2014). Second, physical factors controlling the mixing or stratification of coastal waters, such as temperature and upwelling, bring low-pH, high-*p*CO₂ water to the surface for periods of days to months (Harris *et al.*, 2013; Kapsenberg and Hofmann, 2016).

Other processes influence variability in seawater carbonate chemistry at varying scales. For example, tides can cause substantial variation in seawater chemistry as a result of horizontal displacement of water masses. Internal waves can cause vertical displacement of density layers, with attendant changes in seawater chemistry. Freshwater discharge from rivers can, in some locations, reduce seawater pH and influence total alkalinity (Xue and Cai, 2020). Importantly, these processes act to modify the expression of the global OA signal in coastal waters, often amplifying the extremes of exposure on top of the secular trend of CO₂ enrichment. Where measured, many, if not most, coastal ecosystems worldwide exhibit seasonal and finer-scale variations in carbonate chemistry that easily exceed the range of values associated with the change in sur-

face open ocean conditions from the pre-industrial levels of 280 ppm to the present-day 415-ppm CO₂ in the atmosphere (Yu *et al.*, 2011; Carstensen and Duarte, 2019).

For experimental biologists, the wide spatial and temporal variation in carbonate chemistry presents a key challenge in marine laboratories. Depending on the laboratory, the day, or even the time of day, one may be conducting studies that differ substantively in pH or other aspects of carbonate chemistry that cross thresholds for biological responses. Not knowing whether studies are conducted at opposite ends of hidden functional response curves or reaction norms can introduce uncertainties that dampen the ability to resolve dynamics of interest.

Unintended carbonate chemistry modifications can inadvertently be imposed on marine biological experiments that are conducted at field stations as a result of external and internal factors that affect seawater chemistry (Fig. 1). Most experiments conducted at coastal field stations involve a stationary intake pipe providing seawater to an entire laboratory. The pipe draws in whatever water flows by that place at that moment, independent of the physical and biological processes that have influenced the chemistry of that water. Once water is drawn into the seawater system, respiration continues and may even increase as a result of biofouling and temperature affects in the laboratory. Photosynthesis may or may not continue, depending on the setting. Often, seawater from the system is held in secondary containers for extended periods of time, potentially intensifying these effects. Thus, the mechanics of seawater delivery frequently cause seawater carbonate chemistry to deviate substantially from natural conditions. Except in dedicated OA experiments, the carbonate chemistry of the seawater that ends up in experimental chambers is rarely measured or adjusted. This is especially true when the treatment variable of interest is, for example, temperature, light, nutrients, or biological interactions, such as competition and predation. As a consequence, investigators may not be aware of seawater carbonate chemistry conditions that could influence experimental results and challenge replicability.

Biological Context: Marine Laboratories Are Hotspots for Biological Discovery

The main reason to conduct laboratory studies at field stations is for access to wild organisms as close to their natural context as possible—including the environmental variation they experience. For this reason, among many others, marine laboratories have always been, and remain, vital resources for all of biology, not just explicitly marine science. Biologists owe a deep and continuing debt to field stations for a staggering breadth of discovery, ranging from the basics of fertilization, development, and cytology in the nineteenth century, through the central discoveries of neurophysiology and cell physiology in the mid-twentieth century, to the cytoskeleton, molecular motors, and green fluorescent protein (GFP) in living memory—all

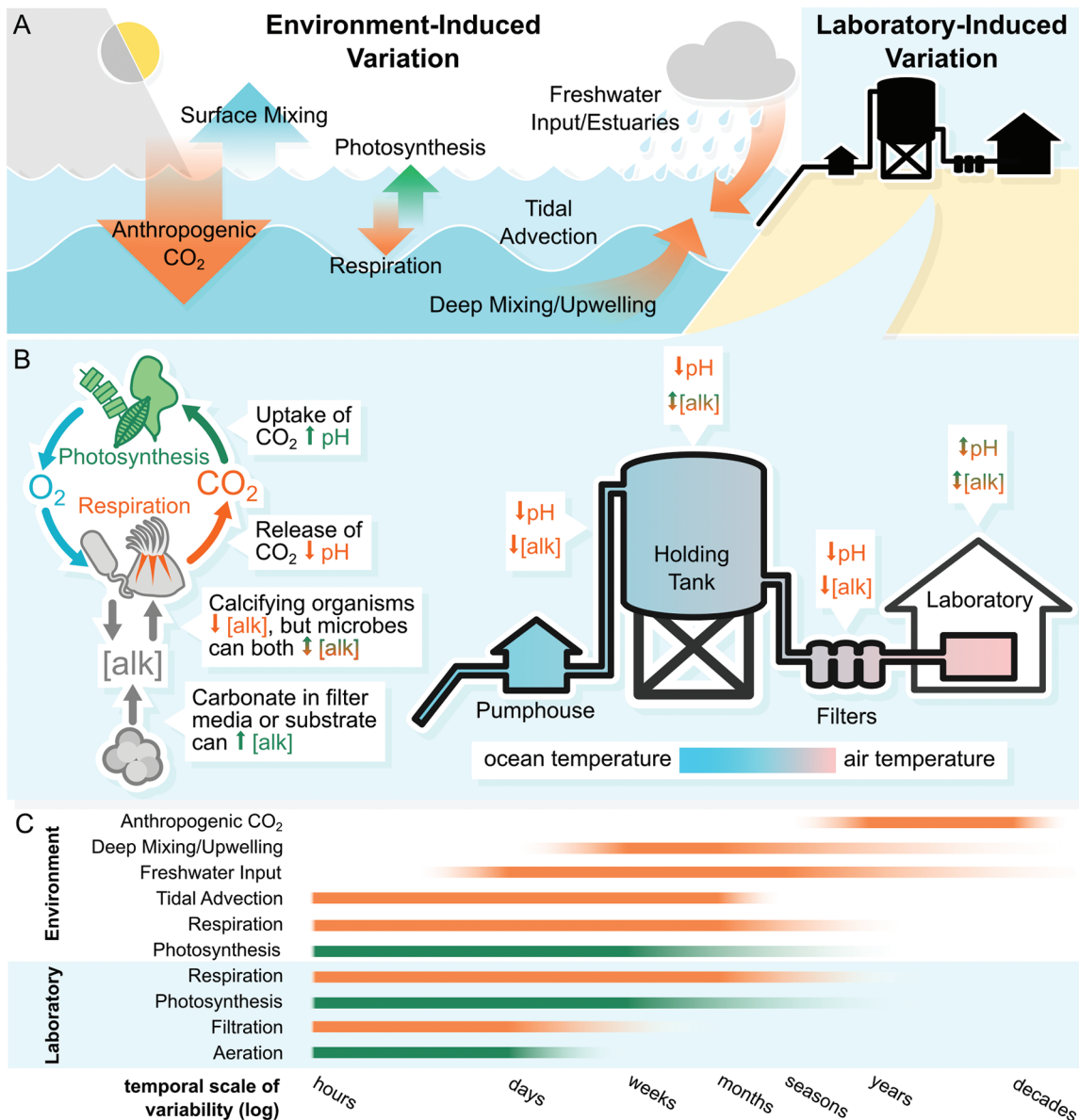


Figure 1. Factors contributing to unintended carbon-system modification during experiments in laboratory settings. (A) Processes influencing environmental carbonate chemistry in the ocean and estuaries. (B) Factors influencing carbonate chemistry of the seawater within the plumbing of the lab, with emphasis on how pH and alkalinity (alk) are affected by temperature, photosynthesis, and respiration by organisms in the system. (C) Predominant time scales at which these processes affect pH in natural and laboratory settings.

seemingly far removed from climate change and its biological consequences. Yet it is not hard to imagine how such research might be skewed by water quality, whether to alter outcomes or to impede replication.

Consider a story told about T. H. Morgan, whose fame as the leading *Drosophila* exponent only eclipsed his first reputation as an experimental embryologist. Like most embryologists in his time, Morgan worked primarily with marine animals. Among other topics, he studied fertilization in solitary ascidians, hermaphrodites that deploy self-incompatibility

mechanisms of various efficacy. One day, he had before him several dishes, each containing both eggs and sperm dissected from one individual *Styela* (an ascidian). Into one dish he squirted juice from a lemon otherwise meant for his tea, with the effect that acidification overcame the block to selfing. Whether the lemon juice got into the watch-glass accidentally or on purpose varies in the telling (Morgan, 1942; Sturtevant, 2001; A. Whiteley [University of Washington], pers. comm. to GvD). The question implied is, how often have we, in effect, failed to notice that stray drop of lemon juice?

The story of Morgan's lemon is a leaf taken from more than a century's literature on fertilization biology that relies heavily on field station laboratory studies. It is hard to overstate the importance of fertilization biology to ecology, biogeography, and life-history evolution, in addition to its obvious relevance for development. It is also clear how quantitative traits like fertilization success, parthenogenesis, or hybridization rates, all of which are key determinants of incipient speciation barriers, might plausibly be sensitive to OA-like excursions or associated parameters. For a hypothetical example, the ctenophore *Beroë* is reported to be naturally polyspermic (Yatsu, 1912; Carré and Sardet, 1984). This observation, which is succeeded by highly credible descriptions of nuclear migration and electrical responses reported by skilled observers (Carré and Sardet, 1984; Goudeau and Goudeau, 1993), anecdotally seems difficult to reproduce (GvD, unpubl. data). Is the original observation doubtful? Almost certainly not. Instead, perhaps ctenophore egg physiology is sensitive to seawater chemistry such that only under exceptional circumstances (*e.g.*, a marine lab emplaced hardly a mile from the abyss) do investigators have access to suitable water for these organisms to perform as they do in nature. Indeed, Yatsu's (1912) paper, reporting this pioneering but almost-forgotten embryologist's visit to the Stazione Zoologica in Naples to study cell division in *Beroë*, includes a pointed quote:

As to experimentation, I wish to lay especial stress upon the following points. Great care was taken to secure good water quite far from the shore. The water taken near the city of Naples was so polluted that it was unfit for use . . . This is the indispensable condition for ctenophore experiments. The high mortality in Driesch and Morgan's work [the same lemon-wielding Morgan] seems to have been due to the neglect of this precaution. (Yatsu, 1912, pp. 1–2)

Another family of examples can be found in developmental plasticity, regeneration, and cloning by echinoderm larvae. These phenomena, which are linked mechanistically by indeterminacy of cell fate in echinoderm planktotrophic larvae, are profoundly significant to theories of life-history evolution and adaptation. Strathmann's landmark demonstration that urchin plutei alter developmental investment in larval *versus* juvenile structures in response to food regime (Strathmann *et al.*, 1992) has been repeated by many others (*e.g.*, Heyland and Hodin, 2004), with clear parallels emerging in OA-inspired studies on development under acidification (Byrne *et al.*, 2013). Similarly, larval regeneration and cloning are now well documented in planktotrophic larvae representing four echinoderm classes (Bosch *et al.*, 1989; Jaeckle, 1994; Balser, 1998; Vickery *et al.*, 2002; Eaves and Palmer, 2003). Clones routinely arise at low frequency in lab cultures of larvae, without apparent induction (Vickery and McClintock, 2000; Eaves and Palmer, 2003; Allen *et al.*, 2019). Since cloning appears prevalent in some natural plankton (notably, the Gulf Stream: Bosch *et al.*,

1989; Knott *et al.*, 2003), it seems implausible that cloning in lab culture is merely an artifact (*e.g.*, an aberrant outcome of wound-induced regeneration). Yet a definite inducer remains elusive; some lab culture studies relate cloning frequency to food quality or abundance (Vickery and McClintock, 2000; Allen *et al.*, 2019) or predator cues (Vaughn and Strathmann, 2008), but at least one study shows that OA-like conditions induce buds resembling incipient cloning events (Chan *et al.*, 2013). That seawater chemistry demonstrably modifies responses, whether to mimic or suppress relevant physiological pathways, implies the need for inclusion in experimental studies.

Flushing the Pipes: What We Can Do Moving Forward

Despite the widespread and increasing understanding of the importance of OA on organisms and ecosystems, it remains underappreciated as an underlying environmental influence on biological processes. Both historically and currently, foundational work on development, physiology, and behavior of marine organisms is undertaken at marine laboratories where OA has been and is currently operating as a ghost factor in experiments. This underlying influence has changed over time, in part due to accelerating anthropogenic changes in climate and land use that affect the coastal and estuarine carbonate chemistry (Carstensen and Duarte, 2019) where marine laboratories are located. We encourage even biologists who are not actively pursuing OA research to account for the effects of carbonate chemistry in laboratory seawater systems. Seawater carbonate chemistry should be considered, measured, and controlled in laboratory studies as a critical, yet often overlooked, environmental factor. In closing, we offer a few constructive recommendations.

Monitor, describe, or control lab water chemistry

Awareness of carbonate chemistry as a factor should motivate marine laboratories and users to adopt new practices suitable to their aims. At the simple end of a spectrum, we urge monitoring and reporting key parameters for the sake of replication. Everyone already does this for temperature by using a cheap thermometer; increasingly affordable solutions include installing automated sensors (at the station level) or monitoring chemistry with benchtop salinity and pH probes (at the end-user level). Samples can be collected and analyzed in-house, following established best practices in Dickson *et al.* (2007); or, for more demanding needs, they can be sent for analysis by other laboratories.

Next, experimenters ought to consider intervening to limit unwanted variation. These interventions may involve only simple actions, such as providing aeration to equilibrate water to atmospheric CO₂ concentrations or adjusting the timing of experiments to avoid predictable excursions in ambient conditions (*e.g.*, avoiding seasonal blooms or estuarine input). More

focused applications might need to include carbonate chemistry variables as controlled factors in experimental design. We recognize, though, that measuring and controlling these variables can be both challenging and expensive. Depending on the setting, productive collaborations can be forged between experimentalists and carbonate chemists to reduce costs and transfer expertise. Such collaborations have allowed OA science to train a new generation of experimental biologists who are well versed in measuring and manipulating carbonate chemistry.

We need to think carefully about those not-so-obvious experimental conditions that can influence our results. For example, depending on their position in an experimental chamber, individuals may be exposed to millimeter-scale boundary layer effects that cause significant variation in carbonate chemistry. Other potentially insidious factors include the introduction of naive animals into treatment conditions (Suckling *et al.*, 2014) and unintended variation in densities (or biomass) of organisms between treatments, which can affect respiration and cause changes in seawater alkalinity for calcifying organisms (Mos *et al.*, 2016; Suckling *et al.*, 2020).

Finally, any factor that shapes lab outcomes implies a priority to validate results from laboratory studies with relevant evidence from natural populations wherever possible—that is, establishing field cognates for observed effects. As always, this is a powerful adjunct to experimental research and lends confidence to interpretations.

Critically evaluate earlier work for unknown variation in carbonate chemistry parameters

Scientists give each other little credit for repeating classic results or replicating others' work. However, the realization of a ghost factor in historical experiments implies that many experimental studies might have been either conducted near reaction norm extrema, resulting in misleadingly large effects, or subject to background variation along an unknown reaction norm, misleadingly obscuring real effects.

Embrace the variation and put it to work

The growing number of studies that intentionally address variability inherent in seawater carbonate chemistry (Murray *et al.*, 2014; Kapsenberg and Hofmann, 2016) suggest the rich information that is contained in this hidden variation. Experimental science relies on willing subjects: some organisms readily take to laboratory environments, hence the heavy reliance on, say, famously robust sea urchins instead of more sensitive ctenophores in developmental biology. Yet laboratory intolerance might be a clue to the very responses that reveal causes at the physiological level (as in the Morgan anecdote) or that shape large-scale outcomes at the ecological level. More broadly, plasticity reflects an adaptive genome, and response variation reflects population- to clade-level genetic diversity. As we seek to explain, predict, and shape systems-level re-

sponses to environmental change, biologists may now be ready to cope with variability in both environmental parameters and physiological responses, rather than continuing to depend on the predictable uniformity of inbred lines, hyper-robust model organisms, and, ideally (or presumably), stable lab conditions.

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