https://doi.org/10.5194/sp-2023-7
Preprint. Discussion started: 13 July 2023

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Laboratory experiments in ocean alkalinity enhancement research

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Abstract. Recent concern about the consequences of continuing increases in atmospheric CO_2 as a key heat-trapping agent (USGCRP, 2017; IPCC, 2021) have prompted ocean experts to come together to discuss how to provide science-based solutions. Ocean alkalinity enhancement (OAE) is being considered not only as a ocean carbon dioxide removal (CDR) approach, but also as a potential way to mitigate ocean acidification. Over the last two decades, inter-laboratory comparisons have proven valuable in evaluating the reliability of methodologies associated with sampling and analysis of carbonate chemistry parameters, which have been routinely used in ocean acidification research (Bockmon and Dickson, 2015). Given the complexity of processes and mechanisms related to ecosystem responses to OAE, consolidating protocols to ensure compatibility across studies is fundamental for synthesis and upscaling analysis. This chapter provides an overview of best practice in OAE laboratory experimentation and facilitates awareness of the importance of applying standardized methods to promote data re-use, inter-lab comparisons, and transparency. This chapter provides the reader with the tools to (1) identify the criteria to achieve the best laboratory practice and experimental design; (2) provide guidance on the selection of response variables for various purposes (physiological, biogeochemical, ecological, evolutionary) for inter-lab comparisons; (3) offer recommendation for a minimum set of variables that should be sampled and propose additional variables critical for different types of synthesis and upscaling; and (4) identify protocols for standardized measurements of response variables.

1. Introduction

Laboratory studies on ocean alkalinity enhancement (OAE) are intended to be reproducible, consistent and transparent to provide the scientific community and regulators with useful information to move the field forward and facilitate the development of safe guidelines. The current focus is on understanding ocean carbon dioxide removal (CDR) potential through the addition of various alkali *via* direct carbonate chemistry analysis and measuring impacts at various levels of biological organization (ecological, physiological, biochemical, molecular) of OAE approaches. The field of OAE faces a great diversity of challenges given the continuously evolving experimental methods, diverse approaches and emerging data availability that will undoubtedly provide new information and ideas to optimize best practice in laboratory experimentation.

The rich insights obtained in ocean acidification research are key to supporting OAE studies. However, as crucial as it is to follow guidelines when designing laboratory experiments, it is equally important to acknowledge that there may be potential confounders and challenges which may not be accounted for in the guidelines. Being able to conduct quantitative laboratory intercomparisons will be critically dependent on identifying recommendations regarding experimental design, sample collection and data analysis. Important considerations include the source of alkalinity, rate of alkalinity addition, testing air-CO_2-equilibrated *versus* non-equilibrated seawater, and the effect of ancillary variables (e.g., temperature) in multifactorial experiments which are known to yield complex and variable results (see the interactive effects of ocean acidification and warming - Harvey et al., 2013). Offering guidelines provided in this chapter should significantly improve the quality and impact of the OAE research, which is required to meet the identified societal need for research on OAE and other types of ocean CDR (NASEM, 2021).

2. Lessons learned from ocean acidification research

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An exploration of procedures, patterns and challenges associated with ocean acidification research has offered ideas on how to design rigorous and reproducible laboratory experiments that enable measuring and monitoring carbonate chemistry shifts and biological responses to ocean acidification (Cornwall and Hurd, 2016). In their study, 95% of the experimental work between 1993 and 2014 had interdependent or lacked replication in clearly defined treatments, or did not report sufficient methodological detail. More broadly, results from Wernberg et al. (2012) from marine climate change experiments between 2000 and 2009, reported that ~49% of the experiments had identifiable issues with their experimental procedures, and 91% of the experiments reported showed a lack of treatment replication or pseudo-replication. Amongst the studies, 9% included extreme/unrealistic treatments of temperature or pH far beyond worst case scenario projections (Wernberg et al., 2012) although 'extreme' pH/alkalinity conditions may prove useful to define thresholds of tolerance and to constrain upper limits of alkalinity enhancement. Given the urgent need for laboratory data before conducting field trials, addressing these issues upfront is a necessary step.

Like in ocean acidification research, careful attention should be paid to the advantages and disadvantages that concern the choices of dissolved inorganic carbon species to measure, and how error propagation will affect the calculated parameters (Martz et al., 2015). Moreover, dissolved organic matter (DOM) is known to contribute to alkalinity (Kim and Lee, 2009; Koeve et al., 2010) and therefore, care should be taken to the design of experiments, particularly when using natural seawater.

While it is fairly straightforward to determine how individual changes in parameters influence chemical and biological responses, understanding impacts of multiple parameters (e.g., increased alkalinity and warming) can be challenging as they can interact in complex ways. Indeed, ocean acidification research revealed antagonistic, synergistic, and additive responses when studying ocean acidification and warming (Byrne and Przeslawski, 2013; Kroeker et al., 2013a; Harvey et al., 2015; Pistevos et al., 2016). Identifying tipping points and interactive effects when other parameters (e.g., temperature) are altered in seawater, in addition to alkalinity, is critical given the capacity to drive (otherwise unpredictable) shifts in species abundances, biodiversity and community composition (Crain et al., 2008; Darling and Côté, 2008; Galic et al., 2018).

Box 1. Good standard practices

- ***Reproducibility***. From the emerging OAE research (e.g., regarding the formation of secondary precipitates - see Montserrat et al., 2017 versus Fuhr et al., 2022; and Moras et al., 2022) and the ocean acidification literature (e.g., see Ridgwell et al., 2009), we have learned that similar approaches can lead to conflicting and unresolved outcomes. Without appropriate reporting of sample collection, methodology and data processing, it is challenging to re-analyze the data and reconcile the discrepancies. As the field emerges and evolves, it will be required to reevaluate early experiments and possibly re-analyze results with updated protocols.
- ***Defining inclusion and exclusion criteria.*** In order to reduce confounding covariates, attention must be paid to factors affecting flocculation, aggregation of particles (e.g., possibly impacted by dissolved organic matter increases after phytoplankton blooms), fluctuations in temperature, which affect mineral dissolution rates, and biological and physiological properties, including stage during the life cycle, trophic state, and seasonality, that affect the susceptibility of organisms to OAE (e.g., see Vandamme et al., 2015; Subhas et al., 2022).
- ***Establishing experimental controls.*** In OAE experimental designs, controls must be appropriately selected. These could include seawater without added alkalinity, seawater ± nutrients/food, treatments with and without the organisms tested. When mineral dissolution is too slow, an alternative analog that reproduces the basic chemistry is encouraged (for example, the use of salts and alkali; e.g., $CaCl_2$ and Na_2CO_3 to mimic the effect of limestone-based mineral dissolution). Controls could also contain an alternative form of alkalinity that alters the seawater carbonate chemistry solely, without adding carbon or metals (e.g., NaOH).
- ***Basic biological responses.*** Studies on organisms' physiological responses (e.g., growth, respiration, size, reproduction, photosynthesis and calcification) are recommended. These responses can be measured directly; for example, as uptake rates of solutes using traditional assays, mass spectrometric methods for indirect assessment of changes in elements, or molecular responses using markers of functional processes. For organisms that undergo development one must determine which stage of development (e.g., larval vs adult; vegetative vs gamete stage) to target. Also, when altering

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more than one parameter, particular attention must be paid to potential confounding effects. Multi-factorial experiments can be used as controls to explore the weight of each parameter.

3. Seawater media preparation

The different steps in experimental design are outlined in Table 1. The process starts with natural or artificially made seawater with or without nutrient additions. One must consider whether adding nutrients/food/prey is required; for example, whether exploring OAE impacts is intended in conjunction with specific scenarios, e.g., nutrient fertilization, specific stages of growth or population development, and the extent to which nutrient additions or any other basic manipulation of the environmental conditions might impact the interpretation of results. For OAE manipulations, autoclaving is discouraged given the alterations in carbonate chemistry, including loss of CO_2, leading to a decrease in dissolved inorganic carbon and alterations in alkalinity (increase with increasing salinity/decrease with precipitation of carbonate) triggered by autoclaving. Filter-sterilization of seawater through small pore size filters (e.g., 0.22 µm filters) is required to remove particles and most bacteria, and produce the stock media where different manipulations are applied to create different alkalinity treatments.

There are several approaches to simulating the addition of alkalinity that capture different components of any manipulation experiment. The first could be viewed as testing the impact of instantaneous addition of alkalinity to seawater to mimic the impact on ecosystems at the point of deployment. The second involves aeration and equilibration with the atmosphere to explore the physico-chemical response to an equilibrium scenario. In the latter instance, the medium is aliquoted out to the experimental vessels/tanks where aeration is applied to promote air equilibration. Monitoring carbonate chemistry through time enables determining when equilibration of seawater with air occurs.

4. Sources of alkalinity

As yet, it is unclear what the optimal method or source of alkalinity enhancement may be in order to simulate the desired chemistry in seawater media. Proposed sources of alkalinity include silicate minerals (olivine, basalt), brucite, limestone and its derivatives (quicklime and portlandite), NaOH and mine tailings (NASEM, 2021; Nawaz et al., 2023). Given the slow dissolution kinetics of the minerals, generating alkaline solutions artificially is acceptable. For example, Gately et al. (2023) simulated alkalinity enhancement via a limestone-inspired solution by adding Na_2CO_3 and $CaCl_2$ or its hydrated form ($CaCl_2H_4O_2$) to seawater. Adding Na_2CO_3 raises TA and DIC in a 2:1 ratio, with 2 moles of TA added by 2 conservative Na^+ ions in Na_2CO_3, and 1 mole DIC added by CO_3^{2-}. $CaCl_2$ does not raise alkalinity because it adds equal amounts of positive and negative conservative charge to the solution from Ca^{2+} and 2 x Cl^-. However, it does raise the Ca in solution.

Many possibilities for solid or liquid alkalinity additions are being considered (see chapter 3). While adding minerals as precursors of alkalinity can provide a source of potentially beneficial nutrients (e.g., silicate, iron, magnesium) (Hartmann et al. 2013), the possible toxic effect of metals such as nickel (Ni) (Montserrat et al., 2017), leached from olivine, is of concern. Therefore, cleaner sources such as NaOH might perhaps be preferable in that respect as it can be considered 'clean' (although its production generates HCl, which is currently processed as a source of hydrogen or as a cleaning agent - see Thiel et al., 2017). Indeed, the use of NaOH is currently gaining attention as a preferred choice of alkali given that (a) it does not contain residues and the amount of Na added to seawater is very small relative to the large background of NaCl in seawater; and (b) it does not require the environmental footprint of minerals proposed for OAE, which necessitate an expansion of mining operations, transportation, and industrial processing, which are energetically costly and can lead to air pollution.

The addition of NaOH and other forms of alkalinity to seawater cause initial spikes in pH and drops in CO_2 that can be balanced to a steady state via bubbling with air (Table 1). It may be that large manipulations of alkalinity are needed to elicit a measurable and reproducible response, and the required alkalinity concentrations will be refined with more detailed modeling but, based on current information, reasonable targets for alkalinity manipulations are 3000-4000 µmol kg^{-1} (Renforth and Henderson, 2017). ~4000 µmol/kg is the concentration of alkalinity expected at locations in the ocean where alkalinity is initially added, and ~3000 µmol/kg is the concentration of alkalinity expected once ocean circulation has dispersed the alkalinity over a larger area (Renforth and Henderson, 2017). Alkalinity thresholds for the formation of precipitates will need to be determined for each experimental approach and condition.

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Table 1. Experimental design considerations for OAE experimentation. **Medium preparation**: the seawater can be obtained from coastal or open ocean sites and supplemented with nutrients using f/2 or variations of f/2 media; see Guillard and Ryder, 1962). Alternatively, seawater media can be prepared from artificial recipes (e.g., Aquil medium; Morel et al., 1979). Media must be sterilized by filtration rather than through autoclaving and nutrients can be added, typically from stock solutions. When possible, moderate aeration should be applied. Types of alkali include adding pulverized mineral directly to the media and promote dissolution physically (e.g., by stirring); dissolving the mineral separately and filter out any particles remaining in the media before experimentation; dissolving salts to mimic the chemistry of the dissolved alkali (e.g., to mimic limestone dissolution, dissolve $CaCl_2$ and $NaCO_3$, which result in higher dissolution rates); and adding liquid alkali such as NaOH. Establishing time series prior to the experiment to determine time frames regarding length of experiment, frequency of sampling, etc. is recommended. **Experimental design**: in addition to optimizing reproducibility by designing enough replication and test the reproducibility of the method, researchers should remain engaged with respect to protocols and experimental design to avoid artifacts and undesirable side effects of methodology. When possible, ensure equilibration of seawater gasses with air and define experimental time frames to test impacts under conditions representative of the site of deployment (where limited gas exchange occurs) and those representative of steady state/equilibrated conditions. Although most laboratory experiments address short term impacts, chronic effects can be tested in long term incubations. **Sampling and analysis**: the **parameters to be considered should allow** inter-lab comparisons, address functional properties of organisms (e.g., calcification, silicification, particulate organic carbon) and fulfill needs to improve model parameterizations. It is important to establish well defined time windows for sampling as well as frequency of sampling to capture physical, chemical and biological properties of the studied system. It is advisable to limit the time of sample storage to minimize observations that might confound interpretation of results (e.g., reverse weathering during storage). Stock solutions (e.g., nutrient and alkalinity solutions) must be stored in the appropriate vessels to avoid contamination from leachates coming out of the vessel itself (e.g., silicate contamination from solutions stored in borosilicate containers). Detection limits and accuracy and precision should be offered for each protocol.

Medium preparation	Experiment design	Sampling and analysis
Natural/artificial seawater **Filter sterilization (e.g., 0.22 um)** **+/- nutrient addition** **+/- aeration** **Type of alkalinity treatments** • Pulverized mineral • Pre-dissolved mineral • Dissolved salts • Liquid alkali **Pre-equilibrated vs non-equilibrated seawater with air phase** • Carbonate chemistry • Flocculation/aggregation • Biology	**Best actions to maximize confidence** • Within study replication and pseudo-replication • Coordinated networks (teams sharing progress to decide on best protocols) **Preliminary time series of TA and carbonate chemistry** • Define experimental time frames • Assess TA upper limits • Expand the upper limits to address impacts at site of deployment **Abrupt *vs* chronic biology impacts** • Short-term tests (acclimation) • Long-term experiments (adaptation)	**Criteria for key parameters** • Inter-lab comparisons • Functional properties • Model parameterization **Sampling frequency and timing** • Select time window for sampling • Identify sampling frequency that captures key chemical, physical or biological features **Limit storage to minimize artifacts** **Identify and report key analytical parameters affecting error** • Detection limits • Measurement accuracy/precision • Identify any impact of experimental design on uncertainties

5. Impacts of impurities/metal leachates

An important consideration in OAE studies is the impact of metals leached from dissolving minerals and their ecotoxicological potential on marine organisms. For example, although some elements (e.g., Fe and Mg) leached out of minerals could be beneficial micronutrients, the potentially toxic effect of metals such as nickel (Ni) (Montserrat et al., 2017), leached from olivine, is of concern. Diverse responses have however been reported with respect to Ni and it appears that some cyanobacteria rely on Ni more than other photosynthetic organisms (see

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Dupont et al., 2008, 2010; Ho, 2013; Guo et al., in review). A recent laboratory study testing olivine leachates (containing Si, Ni, Mg, Fe, Cr and Co) in phytoplankton revealed either positive or neutral physiological short term responses in all treatments (Hutchins et al., 2023). However, one should consider the role of long-term experiments to examine organismal and population adaptation of metal exposure as well as potential bioaccumulation and biomagnification impacts in consumers.

Another important consideration is the effect of pH on metal speciation as pH and a decrease in the concentration of OH^- and CO_3^{2-} ions can affect the solubility, adsorption, toxicity, and rates of redox processes of metals in seawater thus affecting the interactions of metals with marine organisms (Millero et al., 2009). When dissolving minerals in seawater one must consider nonstoichiometry and incomplete dissolution perhaps as a result of dissolution of impurities, precipitation of secondary minerals, or preferential leaching of elements from the mineral surface (Brantley, 2008, NASEM, 2021). The formation of secondary precipitates has been observed in several studies exploring the dissolution of olivine (Fuhr et al., 2022), and limestone derivatives (Moras et al., 2022; Gately et al., 2023; Hartmann et al., 2023). Using an alkaline solution rather than reactive alkaline particles has been recommended to reduce carbonate formation unless seawater critical supersaturation levels are exceeded (Hartmann et al., 2023). In addition of runaway $CaCO_3$ precipitation, a condition where more alkalinity is removed than initially added, which reduces the OAE CO_2 uptake efficiency, more complex precipitates containing Fe, Si, and P were observed in a study using a limestone-inspired OAE approach revealing that mineral precipitation caused by seawater alkalinization can also remove inorganic nutrients from solution (Gately et al., 2023). Identifying thresholds of alkalinity addition (e.g., <1000 μmol kg^{-1}) and timely analysis of samples, i.e., avoiding long storage times has been recommended (Subhas et al., 2022).

Maintaining alkalinity following OAE is critically dependent on the carbonate saturation state, its temporal evolution, and particle surface processes (Hartmann et al., 2023). To minimize the loss of alkalinity and maximize alkalinity enhancement, Hartmann et al (2023) propose the application of an alkaline solution in CO_2 equilibrium with the atmosphere and/or solutions with tested saturation levels to avoid loss of alkalinity. A separate reservoir where alkaline solutions have been prepared is desirable for testing upper limits of alkalinity addition and identifying saturation thresholds to minimize precipitation.

6. Experimental replication

Several approaches are applied experimentally to address replication (Fig. 1). For example, simple replication involves an experimental unit (containing replicates) per treatment where responses to the treatment are measured [defined by Hurlbert (2009) as the "evaluation unit"] and each experimental unit is treated as independent. In temporal replication, multiple measurements are made through time (temporal trends) on the same experimental unit and treated as independent experimental units of a treatment. Sacrificial replication involves the use of multiple experimental units per treatment (for example, a time series) and multiple replicates within each experimental unit, but the replicates are treated as the experimental units during statistical analysis. Each approach has distinct strengths and limitations, and the choice of the approach depends on the scientific questions and the extent of the risk of error propagation. For example, one might choose sacrificial replication for certain chemical manipulations that require sampling from vessels with comparable volumes but choose instead temporal replication for monitoring the evolution of a microbial culture or the physiology of fish over time under certain alkalinity conditions. To improve comparability between future work, it may be useful to agree on a desirable minimum set of variables with the understanding that more variables might be added as new results emerge (Table 2).

Technical variability amongst experimental methods ranging from sampling and sampling processing can propagate through the various steps before analysis; for example, chemical analysis and molecular work/sequencing can be significant and error-prone (e.g., Catlett et al., 2020). The use of blanks every time sampling is conducted is essential for detecting contamination originating from the experiment itself or from the adjacent environment (e.g., exogenous sources such as surface contamination, flagellates in droplets through aeration, etc.). When possible, several barriers to contamination are recommended (e.g., filters at various points of aeration). Additionally, for samples (other than those preserved for analysis of alkalinity, dissolved inorganic carbon analysis or pH) that are kept for further analyses, contaminants that grow during shipping or while samples are being stored can sometimes be reduced by freezing at -80 °C, when possible, or by using the appropriate preservatives when storing at ambient temperature is required (e.g., ethanol, paraformaldehyde, glutaraldehyde). Attention should be paid to the material of vessels where samples and solutions are stored; for example, avoid borosilicate bottles to store nutrients or alkalinity solutions as silicate can be leached into solution.

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7. Testing impacts on marine organisms

In addition to testing the biological responses to abrupt enhanced alkalinity exposure, marine organisms can be exposed to enhanced alkalinity conditions after equilibration of seawater pCO_2 with that in the air-phase following alkalinity addition. Ideally, aeration should be maintained to ensure O_2 levels required by marine animals and also maintain stable pCO_2 levels in the alkalinity perturbation experiments. Depending on the organism tested (a few organisms do not tolerate aeration in tanks), aeration might or might not remain for the duration of the experiment. The vessels used in OAE experiments might not be traditional tanks used in aquaria, but rather any type of container adequate for different type of organisms (e.g., culture flasks for bacteria, conical flasks, carboys for phytoplankton, open tanks for echinoderms and fish) with air lines to introduce aeration in the media. When running multifactorial experiments (e.g., temperature and alkalinity), designing an analysis plan and concrete experimental questions to interrogate can help determine the sample size and minimum number of treatments.

Standardizing technical details in protocols, sampling, sample processing and analyses are crucial to control for variation introduced by reagents, sample storage and other factors. The collection and curation of metadata associated with each sample are critical for data interpretation, inter-lab comparison and drawing conclusions to move forward with planning of research field deployments. For studies involving more than one level of biological organization; i.e., grazing experiments, competition experiments, particular attention should be paid to designing adequate controls.

The effects of OAE and its interactions with other parameters might differ depending on the duration of the experiments. Indeed, in ocean acidification studies, compensatory metabolic pathways appear to take longer to become established, depending on factors such as the exposure history (Calosi et al., 2013) and phase of the life cycle phase (Hettinger et al., 2012). In a study testing ocean acidification and warming, biological effects were not detectable in the short term, but were rather manifested over time (Godbold and Solan, 2013). It was suggested that species responses to seasonal variations in environmental conditions might explain these differences that, depending upon timing, can either exacerbate or buffer the long-term directional effects of climatic forcing (Godbold and Solan, 2013).

8. Choice of species

Criteria for selection of species should include whether the organism is amenable to laboratory experimentation, the amount of background knowledge on the organism's physiology and biogeochemistry, ecological importance of the organism, and local and global impacts. Considerations when selecting organisms should also include geographic origin (e.g., temperate/tropical/polar) and ecosystem type (e.g., benthic vs pelagic). Special attention should be paid to those species that (1) significantly impact or respond biogeochemically to chemical changes caused by alkalinity addition (e.g., possibly calcifiers, photosynthetic organisms); (2) keystone organisms (e.g., corals, salmon, sea stars, toxin-producing phytoplankton); and (3) organisms/functional groups of known vulnerability to climate change (corals, urchins).

Calcium carbonate producing organisms are particularly interesting because of their known sensitivity to changes in carbonate chemistry and because any alteration in their abundance or calcification rates could have implications in the CDR potential of alkalinization. Mineralogical composition of carbonate containing organisms might possibly be affected by alkalinization. For example, recent meta-analysis of studies exploring the effects of the carbonate chemistry shifts caused by ocean acidification revealed effects on shell state, development and growth rate (Figuerola et al., 2021). Biomineralization studies should explore species-specific responses driven by mineralogical composition (calcite, aragonitic, high/low Mg calcite) of their tests, shells and skeletons. Environmental and biological control on calcification particularly any changes in the Mg content in calcite driven by the use of brucite and other minerals potentially adding Mg to calcite must be reported as calcite with a high Mg content is less stable in aqueous solutions (Bischoff et al., 1987).

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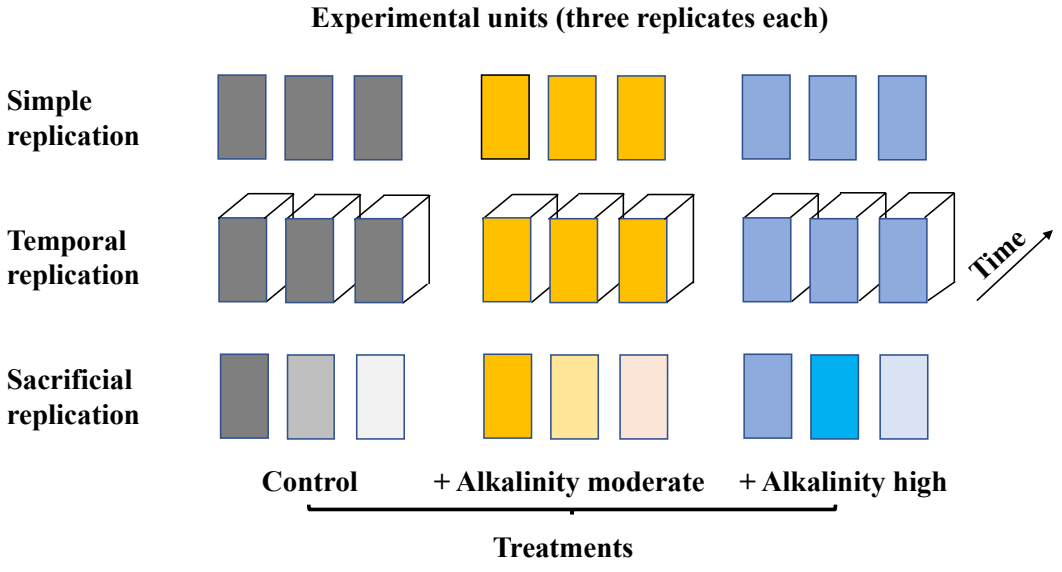

Figure 1. Examples of experimental laboratory design with regards to replication. Each experimental unit contains replicates and different treatments are represented by colors. Each experimental unit is treated as an independent experiment except in the sacrificial replication approach, where each replicate is treated statistically as an experimental unit.

9. Species interactions

For the most part, laboratory experiments are aimed at elucidating the physiological performance and biogeochemical responses of organisms (rather than communities) to physical or chemical alterations in the environment although responses in ecological fitness could be drawn from laboratory experiments (Table 2). Importantly, environmental change can affect species differently and interactions between species that are sensitive to environmental change can function as ecological leverage points through which modest changes in abiotic conditions are amplified into large changes in marine ecosystems (see Kroeker and Sanford, 2022). These interactions can be measured as competition, predation, and symbiotic relationships (mutualism, commensalism and parasitism) that can vary along environmental gradients that cause stress (Stachowick, 2001; Bruno et al., 2003; Ma et al., 2023).

Results from ocean acidification mesocosm experiments revealed that nutrient-limited phytoplankton communities appeared to be more responsive to changing carbonate chemistry than those having access to high inorganic nutrient concentrations (see Paul et al., 2015; Sala et al., 2015; Bach et al., 2016). These observations indicate that trophic state might play a role in the susceptibility of organisms to the changes in carbonate chemistry driven by alkalinization. Also, competition between species has been found to be altered under various carbonate chemistry conditions (see Kroeker et al., 2013b), which merits a focus on experiments that address preferential selection of taxonomic groups under different alkalinity conditions. Although applying nutrient-limiting conditions is experimentally challenging and requires complex experimental design, understanding how species succession and community composition might respond to alkalinization could in part be addressed in a laboratory context.

It should be noted that laboratory experiments can provide insights into the short and long term physiological responses of selected marine biota to OAE which can provide mechanistic insight into immediate and long-term impacts on particular biological pathways and key cellular/organismal attributes. Community scale and ecosystem natural selection responses can only be afforded by experiments in the natural environment either from manipulated perturbation or observations of responses to known events.

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10. Stress responses

Central to OAE laboratory experimentation is our ability to measure any possible stress induced by alkalinization and learn about underlying mechanisms behind acclimation to the chemical alterations of seawater caused by OAE. In addition to measuring basic functions (growth rates, size, reproductive success), sensitivities to alkalinization might be organism-specific and possibly trophic level-specific (e.g., Voigt et al. 2003, Gilman et al. 2010) although most laboratory experiments do not address trophic levels. Similarly, measuring adaptation and diversity in acclimation between and within related organisms is a challenge and the ocean acidification literature revealed how important it is to pay attention to diversity of responses (see Kroeker et al., 2010).

Stress is often measured as a reduction in organismal performance or fitness caused by environmental change (Schulte, 2014). In addition to these general physiological or behavioral responses, markers of stress such as oxidative stress can be used as a measure of stress. It is well established that the production of reactive oxygen species (ROS) can increase due to environmental stress including ocean acidification (Lesser, 2006; Lushchak, 2011). Many biomarkers are commonly used for studying oxidative stress in marine organisms (Cailleaud et al., 2007; Vehmaa et al., 2013) and an increase in ROS and superoxide dismutase and catalase activities have been reported in marine animals under stress (von Weissenberg et al., 2022). Heat shock proteins (HSPs) are also used as molecular markers of stress because of their abundance, high sensitivity to stress and being ubiquitously expressed (Gross, 2004). Among all HSPs, HSP70s are the most studied as a strong up-regulation of HSP70 production has been demonstrated broadly with the exception of *Hydra oligactis* (Bosch et al., 1998), and some Antarctic animals (La Terza et al. 2001; Place and Hofmann, 2005).

11. Effect of OAE on the uptake rates of nutrients

The uptake rate of carbon and other nutrients that results in the observed standing stocks of particulate matter involve many physiological processes that are sensitive to changes in inorganic carbon chemistry and pH (Matsumoto et al., 2020). Chemical changes following addition of alkalinity might alter physiological processes that represent sources (calcification, respiration) and sinks (photosynthesis) of CO_2. One should also pay attention to the reciprocal interactions between these physiological processes and the chemically altered environment as even minor changes in biological processes, or in the balance between them, can have implications for the CDR potential and biodiversity.

One of the most unknown effects of OAE is the fate of biological fixation rates of different elements (e.g., carbon and N_2 fixation rates). Such rates are measured in batch cultures and bioassay (mixed natural community) incubation experiments (LaRoche et al., 2010). While the objective of culture experiments is to understand the effect of environmental parameters on the elemental uptake by particular species in a lab, bioassay experiments have to deal with a rather complex species interaction in the field or after subsampling of mesocosms in a lab (Hutchins et al., 2007; Paul et al., 2016). Labelled/enriched (~99%) stable isotope tracers are the most used method for rate estimation these days. The rate calculation is based on isotopic mass balance equation (Montoya et al., 1996):

$$\text{C or } N_2 \text{ fixation rate} = [\text{POM}] \left(\frac{Af-A0}{Ae-A0}\right) \quad (1)$$

where, [POM] is the concentration of element of interest (C or N) at the end of the incubation. Likewise, *Af* = atom% in *POM* at the end of incubation, *A0*= atom% in *POM* at the start of the incubation, *t* is time of incubation, and *Ae* = isotopic enrichment in the dissolved form after the tracer addition at the start of the incubation

This equation/method is sensitive to analytical protocols in routine incubations (White et al., 2020), and might be even more sensitive in OAE incubations due to the issue of gas equilibration in tightly capped bottles. While the C substrate-based incubations are supposedly straightforward in incubations, N_2 gas incubation face a challenge of under-equilibration leading to underestimation of rates. But OAE incubations can produce larger errors in the C fixation estimates as well. This is because $NaHCO_3$ is generally used as a C substrate. To estimate ^{13}C isotopic enrichment after tracer addition (term in equation 1), a DIC value is normally assumed (as it does not change much at a given region). But OAE will have increased (or fluctuating) DIC during the experimental period, and thus a measured DIC value should be used in the enrichment factor calculation. Likewise, slow dissolution of N_2 gas poses a challenge to accurately estimating isotopic enrichment factor (*Ae*), and it is advisable to measure this term.

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Although the analytical precision of C and N isotopes is of order of sub permil levels, many times the low reported rates (<0.1 nmol N L^{-1} d^{-1}) are questionable (Gradoville et al., 2017). Therefore, the detection limit of rate measurements and its proper reporting is a major concern. To overcome this, following the prorogation of analytical and statistical errors in each term of mass balance equation (1), Gradoville et al. (2017) have proposed to report minimal quantifiable rates (MQR) and the limit of detection (LOD) in triplicate samples. We ought to follow these protocols in the rates measured in OAE. In addition, we must make sure to sample/filter sufficient water to achieve 35 µg N and 150 µg C in the sample for reliable mass spectrometric measurements.

12. Portable Incubation Experiments

Incubation experiments that simulate regional in situ alkalinity deployments will be an important step in understanding the potential impacts of alkalinization on marine organisms prior to field testing. These incubation experiments, which simulate alkalinity additions under diverse local in situ parameters (e.g., temperature, irradiance, nutrients), can be accomplished using portable incubators onboard research vessels (i.e., deck incubations) or outdoors, at coastal research facilities (Fig. 2).

When designing a portable incubator, one should use durable, clear acrylic (or plexiglass) – the thickness of the acrylic should be considered in relation to the volume of seawater to be contained within the incubator. If one is interested in studying photosynthetic organisms at specific depths, high-quality light filters should be attached to the acrylic to adjust photosynthetically active radiation (PAR) within the incubator (e.g., Fig. 2). To maintain in situ seawater temperatures, an inflow port can supply seawater to the incubator. Effort should be taken to ensure movement of seawater quickly through the incubator to maintain a uniform temperature, as well as to reduce biofilm buildup on the outside of culture vessels (e.g., polycarbonate carboys). An approach that one may use to accomplish this is to install a false bottom within the incubator to promote conveyor-like flow between the seawater inflow and outflow ports [e.g., see design in (Marcel et al., 1994)].

Natural seawater should be used when simulating *in situ* alkalinization. When collecting natural seawater, one must consider how biological interactions (e.g., grazing) could confound results and filter accordingly. Portable incubation experiments require instantaneous alkalinity additions; thus, careful consideration should be given to the method of alkalinity addition used. Filter-sterilized stock solutions (e.g., 1 M NaOH) are easy to transport, but flocculation commonly occurs upon alkalinity addition (Subhas et al., 2022). Another option is to add pulverized minerals directly to the treatment vessels; however, this method may be inefficient as mineral dissolution rates can be slow (e.g., Fuhr et al., 2022), leading some researchers to mimic mineral dissolution instead (Gately et al., 2023).

Once the vessels have been placed into the tank, they should be secured – especially for deck incubations at sea – to prevent damage (and potentially contamination) due to the motion of the vessel. Additionally, for deck incubations at sea, durable stainless-steel frames should be used to lift the incubator off the deck to allow ample water flow beneath it; doing so will minimize damage to, and the potential loss of, the incubator in heavy seas. One should also minimize the potential for vessel contamination while they are secured within the incubator: carefully wrap caps and vent ports with parafilm and avoid submerging carboys with spigots in running seawater.

As in the laboratory experiments described above, vessels within the incubator should ideally be aerated during experimentation. Careful attention should be given to securing the air supply including gas tanks and air pumps. In addition to chemical and biological parameters, PAR and temperature data should be collected throughout the experimental timeframe using applicable sensors and data loggers. The best practices outlined in Box 1 should be adhered to when planning portable incubation experiments.

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Figure 2. A, B: Portable incubator with blue filters (Lee Filters #068) to adjust photosynthetically active radiation (PAR). A scalar PAR sensor (LI-COR) can be observed within the incubator (A, right side). C: for reference, laboratory experiment using aeration and sacrificial replication. Images were taken by James Gately (A, C) and Sylvia Kim (B).

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449
450 **Table 2**. Examples of responses to ocean alkalinity enhancement to be measured in experimental
451 manipulation studies. Knowledge need (M=medium, H=high; measurement mode (MM=manual mode; S=sensor;
452 SD=sensor in development). A minimum variable set is highlighted in bold. Selected references are provided as
453 examples of protocols.
454
455

Type of response	Variable	Knowledge need	Measurement mode	Protocol reference
Basic chemistry variables	**Carbonate chemistry parameters {[HCO_3^-], [CO_3^{2-}], [CO_2], pCO_2, Ω}**	H	MM, S, SD	Dickson (2010); Bockmon and Dickson (2015)
	Dissolved organic matter	M	MM	Marañón et al. (2004); Sharp et al. (1995)
	Particulate organic matter (C, N, P)	H	MM	Verardo et al. (1990); Hilton et al. (1996); Pujo-Pay and Raimbault (1994); Fu et al. (2008)
	Trace metals (in solution and in aggregates)	M	MM	Guo et al. (2022); Hutchins et al. (2023)
	Biologically and biogeochemically relevant elements (e.g., Si, Mg:Ca)	M	MM	Brzezinski (1985); Lebrato et al. (2020)
Physiological	**Basic physiology (respiration, photosynthetic, growth rates; morphometric measurements)**	H	MM, S	Iglesias-Rodriguez et al. (2008); Kelly et al. (2013); Farrell et al. (2009)
	Functional group-specific physiology (e.g., calcification, silicification, nitrification/denitrification, toxin production)	H	MM, S	Cohen et al. (2017); DeCarlo et al. (2019)
	Stress physiology [e.g., heat shock proteins, oxidative stress-related	M	MM	O'Donnell et al. (2009); Moya et al. (2015); Trimborn et al. (2017)

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	proteins, photosynthetic stress (shifts in quantum yield), morphological alterations (e.g., cyst formation)			
	Incidence of pathogens and disease	H	MM	Asplund et al. (2014)
Reproduction	Spawning success	M	MM	Liu et al. (2011)
	Size of offspring	M	MM	Cao et al. (2018); Johnson (2022); Albright et al. (2010)
	Sperm motility	M	MM	Esposito et al. (2020); Havenhand et al. (2008)
	Epigenetic analysis	M	MM	Li et al. (2018); Lee et al. (2022)
	Fecundity	M	MM	Maranhão and Marques (2003); Thor and Dupont (2015)
	Hatching success	M	MM	Saigusa (1992)
Species interactions	Competition for resources	M	MM	Connell et al. (2013); Guo et al. (2022)
	Predation and species interactions	M	MM	Greatorex and Knights (2023); Bacus and Kelley (2023); Mitchell et al. (2023)
	Synergistic/antagonistic effects of other environmental parameters	M	MM, S	Gerhard et al. (2023); Khalil et al. (2023)

Acknowledgements

https://doi.org/10.5194/sp-2023-7
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This is a contribution to the “Guide for Best Practices on Ocean Alkalinity Enhancement Research”. We thank our funders the ClimateWorks Foundation and the Prince Albert II of Monaco Foundation. Thanks are also due to the Villefranche Oceanographic Laboratory for supporting the lead authors' meeting in January 2023.

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