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8 *In situ* perturbation experiments: natural venting sites, spatial/temporal gradients in ocean pH, manipulative *in situ* p(CO_2) perturbations

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8.1 Introduction

The objectives of *in situ* experiments studying ocean acidification vary widely, with most recent studies centering on the effects of changing carbonate chemistry on the biology and ecology of organisms and ecosystems. The advantages of *in situ* observations are that they offer a completely independent approach to laboratory experiments, one that is based on looking directly at how organisms and communities and ecosystems react to high/low pH and saturation state (Ω) in the real world, replete with all its biodiversity, ecosystem interactions and adaptation to the ambient chemistry. Studies have included measurements of faunal patterns, calcification, dissolution, growth, survival, metabolic rate, physiological responses, behaviour, community interactions, and other processes, in relation to spatial and/or temporal changes in pH, p(CO_2), or aragonite and calcite saturation states. Here, we focus principally on the approach and methods of *in situ* experimental studies, using examples of recent work and developing techniques. Two major types of *in situ* experiments are used for ocean acidification research including:

- ***In situ* observational studies** that compare patterns or processes between areas that differ *naturally* in seawater acidity and/or carbonate saturation states.
- ***In situ* perturbation experiments**, where researchers manipulate conditions to compare patterns or processes between *artificially* acidified and control conditions.

8.1.1 *In situ* observational studies

Observational studies, also termed natural experiments, allow researchers to exploit gradients in ocean chemistry that exist at sites such as hydrothermal or other CO_2 vent sites, across changes in pH with depth or among sites, or even between ocean basins. Natural differences in carbonate chemistry between sites can differ by as much as those associated with a doubling of atmospheric CO_2 (Rost *et al.*, 2008). Researchers typically have little or no control over treatments in *in situ* observational studies, and measure differences between parameters of interest (e.g. calcification, abundance etc.) along local gradients in pH or other carbonate system parameters, or between sites or depths that differ in ocean chemistry. The main weakness of *in situ* observational studies is the potential for significant, but hard to detect effects of confounding factors that vary among locations with (or independent of) seawater carbonate chemistry. Waters emanating from deep-sea vent sites, for example, are rich in carbon dioxide, but often have high concentrations of methane, sulfide, heat and other parameters. Each of these factors may affect the physiology and performance of individual organisms in the community, with cascading effects on community patterns and processes. In addition, treatments in observational studies are usually segregated in space (as well as variable over space and time), making it difficult to intersperse replicates among treatments. For research on ocean acidification, however, the great advantage of *in situ* observational studies is their increased realism, both in terms of long duration and inclusion of all elements of the ecosystem. The long-term nature of environmental conditions examined in most *in situ* observational studies allows sufficient time for the development of both the direct effects of chronic ocean acidification on organisms, and the emergence of any cascading indirect impacts (if any) on ecological patterns and processes. These time scales are much longer than is typically possible for manipulative ocean acidification experiments. See chapter 4 for further discussion on the design of ocean acidification experiments.

Unlike *in situ* observational studies, most recent studies of the effects of ocean acidification have been based on acute exposure in laboratory and mesocosm experiments whereby CO_2 levels were manipulated over short timescales – see chapters 6 and 7 for further information on these methods. However, attempts to determine whether these experimental results and related models provide realistic predictions of future impacts of chronic ocean acidification have been hindered by the difficulty of simulating ocean acidification *in situ* over long periods. Thus, it is possible that the responses to increased CO_2/acidity observed in short-term experiments are related principally to acute exposure, and may not represent the effects of chronic ocean acidification that may include long-term acclimation, evolutionary adaptation, changes in the composition of the species assemblage, and also complex feedbacks and indirect effects occurring within a natural marine system.

The opportunities to observe the long-term consequences of elevated $p(CO_2)$ or acidity using *in situ* observational studies has prompted a number of recent studies investigating processes such as calcification, photosynthesis, growth and community structure. Tyrrell *et al.*, (2008) reported that coccolithophores are common in the Black Sea, but absent from the Baltic, which undergoes seasonal aragonite undersaturation. In a similar study, Marshall *et al.*, (2008) found that shell dissolution of marine gastropod populations (*Thais* sp.) varies in concert with pH in Southeast Asian estuaries. However, because salinity, temperature, light, or some combination of these factors varied with pH among sites in each study, it is difficult to disentangle the effects of these potentially confounding factors. Another interesting recent report of an *in situ* observational study on ocean acidification is an examination of variation in community composition along a natural pH gradient in a shallow subtidal community around the small island of Ischia off the Italian coast (Hall-Spencer *et al*., 2008). Dramatic changes in faunal and floral composition with pH (7.4 - 8.2) indicate that long-term exposure to high-CO_2/low-pH waters benefits some seagrasses, but is detrimental to other species, such as corals and coralline algae (Hall-Spencer *et al.*, 2008; Martin *et al.*, 2008). Although the strong correspondence between gradients in faunal patterns and pH are striking, the limited spatial scale of the venting site provides only one venting area on each side of the island, and temporal variation in pH may be large. The limited potential for replication (n = 2 sites) increases the likelihood of confounding – site to site variation in factors other than pH may drive faunal gradients, particularly if pH variation is great. Natural venting sites have also provided evidence concerning the influence of pH and/or $p(CO_2)$ levels on deep-sea community patterns (Vetter & Smith, 2005). Echinoderms are known in general to be rare or absent from hydrothermal vent sites (typically low in pH) where many chemosynthetic communities thrive (Grassle, 1985; Van Dover, 2000). Tunnicliffe *et al.* (2009) observed clusters of the vent mussel *Bathymodiolus brevior* growing (albeit with poorly-formed shells) in extremely acidic conditions (pH values between 5.4 and 7.3) near deep hydrothermal vents where predatory crabs are absent. In addition, recent surveys of biological patterns in the vicinity of CO_2-rich vent sites in the Okinawa Trough (Boetius *et al.*, unpubl.) indicate strong coupling of faunal patterns and pH. However, most observations to date from deep-sea vents have been influenced not only by high CO_2 but also by the effects of heat and H_2S or CH_4.

Spatial gradients have been used to assess the sensitivity of cold-water corals (>95% live where aragonite saturation state (Ω_a) is currently ≥1; Guinotte *et al.*, 2006) and warm-water corals (most live where Ω_a was >3.5 in 1750, Kleypas *et al.*, 1999) to carbonate saturation, their calcification strength (Manzello *et al.*, 2008) and their rates of dissolution (e.g. Andersson *et al.*, 2007). Temporal changes in carbonate saturation, pH, or $p(CO_2)$ that occur over diurnal cycles, through the boom and bust of plankton blooms, over seasons, and longer time scales (Bensoussan & Gattuso, 2007; Findlay *et al.*, 2008; Tyrrell *et al.*, 2008; Wootton *et al.*, 2008) also provide opportunities for ocean acidification studies. Changes in carbonate chemistry have now been documented over decadal and longer scales at several sites, yet few reports of corresponding changes in community structure or function are available (but see Cooper *et al.*, 2008). Most studies of temporal changes in ocean chemistry or associated faunal patterns or processes have examined carbonate sediments preserved over long (i.e. thousands to millions of years) periods (e.g. Hönisch & Hemming, 2005). Recent work by

Iglesias-Rodriguez *et al.* (2008), however, detected changes in coccolithophore calcification in the sediment record over only two centuries, though the cause of these changes remains unknown.

Areas where p(CO_2) levels are high, and pH and carbonate saturations are low, provide systems for studies of the long-term effects of ocean acidification because the acidified seawater conditions at such sites occur on sufficiently large spatial and temporal scales to integrate ecosystem processes such as production, competition and predation (Hall-Spencer *et al.*, 2008). However, there are considerable differences between such systems and that of global-scale ocean acidification caused by rising atmospheric CO_2. For example, temporal and spatial variability in CO_2 and pH perturbations around vent sites or over short distances over oceanographic gradients complicate the determination of a reliable dose – response relationship (Riebesell, 2008). Global-scale acidification will almost certainly be more stable in time and space. In addition, other important factors, such as natural temperature or salinity variation among sampling sites, or emigration and immigration of organisms from venting sites or other treatment locations can complicate the interpretation of observed effects of high CO_2 at naturally acidified sites. However, evidence demonstrating the ability to survive at low pH/saturation provides important information about the consequences of ocean acidification, regardless of values of other factors. Natural study sites will therefore need to be used in conjunction with more controlled approaches to help predict the future effects of ocean acidification. See chapters 6 and 7 concerning some pH control experiments in laboratory and field settings.

Natural venting sites and spatial/temporal gradients in ocean pH can also be exploited as laboratories for manipulative experiments concerning the effects of ocean acidification. Although treatment levels may not be controlled, it is possible to manipulate conditions within ocean acidification treatments (e.g. translocation experiments - moving organisms in and out of natural sites that differ in ocean carbonate chemistry). By exposing trapped amphipods to CO_2-rich fluids venting from the Loihi Seamount, Vetter & Smith (2005) observed the onset and recovery from CO_2-induced torpor (inactivity). Likewise, numerous manipulative experimental studies are in progress at the shallow CO_2 venting sites off Ischia (e.g. Rodolfo-Metalpa *et al.*, in press), where the effects of ocean acidification on animal biology, ecological interactions and succession, as well as other topics can be evaluated under naturally varying p(CO_2) treatments.

8.1.2 *In situ* perturbation experiments

In situ perturbation experiments are those where researchers control one or more factors and replicate observations for comparisons between control and treatments groups or along gradients in ocean chemistry. *In situ* perturbation experiments, also called "field experiments", have greater control on experimental parameters and can usually include a more fully factorial and replicated design (see chapter 4) than observational studies. However, because most variables in field experiments are not controlled, conditions are inherently more variable than in laboratory experiments, with potentially large effects on the variance within and among groups. Nevertheless, measuring performance of organisms or other processes in a natural setting provides more realism than is typically possible in laboratory experiments.

Field experiments where researchers have controlled pH or p(CO_2) have been performed in deep-sea environments, with ongoing efforts in various habitats. Studies to date have ranged from releases of liquid CO_2 or CO_2-rich fluids to examine effects of environmental hypercapnia on deep-sea fauna (e.g. Tamburri *et al.*, 2000; Barry *et al.*, 2005; Thistle *et al.*, 2005), and the deployment of chamber systems capable of modifying pH/p(CO_2) in enclosed samples of seabed (Ishida *et al.*, 2005). Recent developments to enable *in situ* perturbation experiments concerning ocean acidification include more advanced chamber systems and open or semi-enclosed systems that emulate the Free Air CO_2 Enrichment (FACE; Ainsworth & Long, 2005) facilities used for decades to evaluate the effects of elevated atmospheric CO_2 levels on terrestrial communities. Free Ocean CO_2 Enrichment (FOCE) systems may allow long-term (weeks to months) experiments with controlled pH treatments in marine communities without isolating the study area from input (currents, plankton etc.) from the surrounding area (Walz *et al.*, 2008).

8.2 Approaches and methodologies

8.2.1 Design of experiments

Although a more thorough treatment of issues to consider in designing any experimental study is available in chapter 4, *in situ* experiments (natural or manipulative) have special considerations to maximise inferential power. For *in situ* observational studies, replication, randomisation, and interspersion of replicates among treatments are often difficult. Natural venting sites are relatively rare, at least in shallow waters, limiting opportunities for replication of sites. Persistent natural gradients in ocean chemistry typically may be linked to other processes, such that treatment levels for pH or other ocean acidification parameters positioned along the gradient may overlap gradients in confounding factors. In addition, venting sites may be concentrated in a single area, reducing opportunities for replication and interspersion of treatments. Manipulative ocean acidification experiments that use natural venting sites or gradients in ocean chemistry as treatments into which subjects (i.e. animals, carbonate etc.) are placed also suffer from problems of replicate interspersion and potentially confounding factors. Thus, to exploit natural patterns in ocean chemistry for ocean acidification studies, researchers have made compromises that violate, to some degree, the principles of experimental design, but which nonetheless have great value in ocean acidification research. Inference gained from a weak experimental design can ideally be supplemented using controlled *in situ* or laboratory experiments to examine more closely patterns or processes suggested to be linked with ocean acidification parameters from field studies. This approach has been used effectively by Hall-Spencer *et al.* (2008) as an independent test of the role of ocean acidification in faunal patterns (Fabry *et al.* 2008). For controlled *in situ* experiments, interspersion of replicates among treatments should be less problematic, but control of pH and p(CO_2) may be logistically difficult, limiting studies to short periods.

8.2.2 *In situ* observational studies

Observational studies along natural gradients in ocean chemistry

The general approach in studies exploiting natural CO_2 venting sites or other natural gradients in pH has been to examine the relationship among samples of interest (e.g. faunal structure, carbonate dissolution rates) along a gradient in or zones of pH or other carbonate system parameters. Hall-Spencer *et al.* (2008) made careful measurements of community structure and ocean pH, alkalinity, temperature and other factors around CO_2 vents off the Mediterranean island of Ischia, providing strong evidence that pH was a driver of community structure (Figure 8.1), though the range and variability in pH was greater than is expected under most future climate scenarios. Likewise, Inagaki *et al.* (2006) documented patterns in microbial community structure along gradients in sediment pH and p(CO_2) in the Yonaguni Knoll hydrothermal vent field in the Okinawa Trough. In each case, researchers measured variation in ocean acidification parameters and the ecological measures of interest (abundance and distribution of organisms).

The approach for controlled field studies varies depending on the goals of the study and the potential for control of seawater carbonate system parameters. For exploiting natural vent sites or gradients in pH or p(CO_2), treatment levels may be determined by placing samples of study organisms in locations with the desired levels of pH or other parameters, preferably replicated among several sites. As for observational studies, ocean acidification parameters, as well as other potentially important factors, must be characterised carefully. Vetter & Smith's (2005) study of the effects of low pH waters on deep-sea amphipods (*Eurythenes* cf. *obesus*) exploited natural pH variation at the Loihi Seamount vent site. Amphipods were captured in baited traps and placed for short periods (15-60 min) in the hypercapnic (pH ~ 6.0) and slightly warmer (~8°C) vent waters from the Loihi Seamount. Comparisons of their behaviour prior to and after exposure, coupled with observations during ascent into warmer waters, indicated that torpor was induced by exposure to the hypercapnic plume. Amphipods were otherwise active until reaching depths during ascent where temperatures were above 10 to 11°C. Although only a single site was available, the experiment was repeated several times with consistent results.

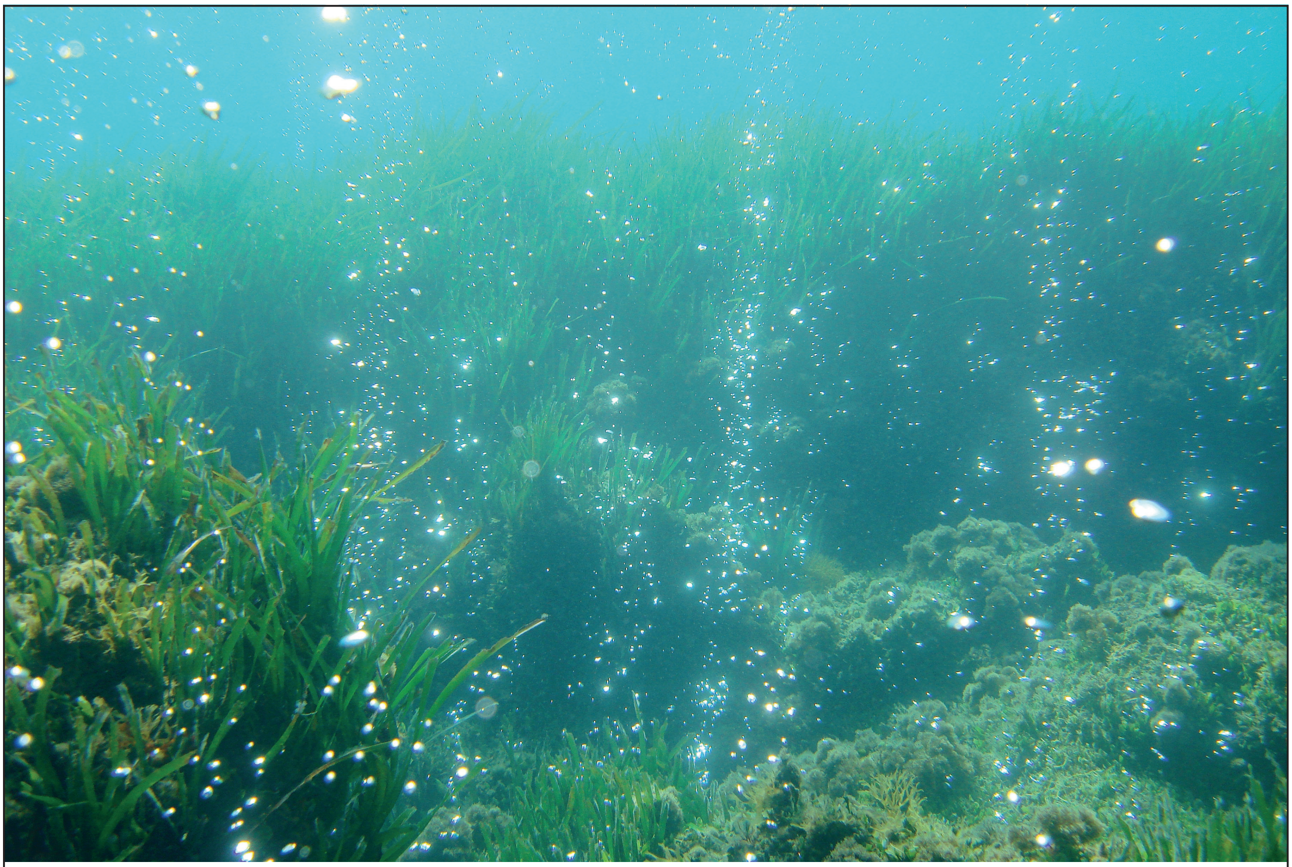

Figure 8.1 Sparkling seawater. Venting of volcanic CO_2 near the Mediterranean island of Ischia provides a natural experiment to observe changes in a rocky shore ecosystem along gradients of decreasing pH close to the vents (photo credit: J. Hall-Spencer).

8.2.3 *In situ* perturbation experiments

Field experiments in which researchers perturb carbonate system conditions to examine the performance of organisms (e.g. calcification, survival, growth, metabolism) or communities (e.g. community structure, succession), or a process of interest (e.g. dissolution rates), are based on comparisons among treatments, typically using a factorial design for ANOVA comparisons, or a regression design. Treatments to study the effects of ocean acidification can be created artificially or may occur naturally, as described above for *in situ* observational studies.

A small number of *in situ* studies have controlled carbonate chemistry treatments directly to examine their effects on animal biology or CO_2 chemistry. The approach of these studies has been to release liquid CO_2 or seawater saturated with CO_2 to produce pH/p(CO_2) perturbations, which are used as treatments to assess animal survival or performance. CO_2-saturated seawater is prepared by bubbling reagent-grade carbon dioxide through seawater within gas tight containers at a known temperature. Brewer *et al.* (2001) first developed a Remotely Operated Vehicle (ROV) based system to release liquid CO_2 in the deep-sea for studies of ocean carbon dioxide storage, which has now been used to investigate the effects of ocean acidification in general. Tamburri *et al.* (2000) released a CO_2-saturated seawater solution alone or in tandem with a fish odour (homogenised tuna extract) to evaluate the response of benthic scavengers. Although a single system was used and the experiment was repeated only twice, they showed that several scavengers displayed no avoidance to CO_2-rich odour plumes released near the seabed. Barry *et al.* (2004) created pools of liquid CO_2 on the abyssal seabed in a series of experiments to evaluate the impacts of environmental hypercapnia on deep-sea biota. Pools (20 to 100 l) of liquid CO_2 were released into small PVC corrals using an ROV-based CO_2 delivery system. The CO_2 dissolved into the bottom waters over 2 to 4 weeks, producing a CO_2-rich dissolution plume that drifted with ambient bottom currents over local sediments and experimental animals held in cages. For ANOVA-design experiments, corrals were arranged haphazardly on the seabed, with control corrals interspersed among those filled with CO_2. Animal survival and abundance was measured near control and treatment corrals. Regression designs with a larger CO_2 pool positioned centrally were also used to document pH and animal survival versus distance (~ΔpH). Local variation in currents, temperature and pH was measured with *in situ* sensors throughout each experiment to document spatial and temporal variation in pH. These experiments (e.g. Figure 8.2) demonstrated the sensitivity of various deep-sea organisms to large (ΔpH ~ 0.5 to 1.0 units; Barry *et al.*, 2004) and relatively modest (ΔpH < ~0.3 units; Barry *et al.*, 2005; Thistle *et al.*, 2005; Fleeger *et al.*, 2006) reductions in deep-sea pH. Although these CO_2 release experiments effectively perturbed the $p(CO_2)$/pH of abyssal waters for these experiments, variation in bottom turbulence, current speed and direction resulted in large variation in pH through the experiments, complicating interpretation of results (Barry *et al.*, 2005).

Because plankton move with ocean currents, their study via *in situ* perturbation experiments requires different techniques. An established technique is to transfer a volume of *in situ* seawater into a tank on the

Figure 8.2 Photograph of manipulative deep-sea experiment to evaluate the response of deep-sea organisms to low pH / high $p(CO_2)$. Liquid CO_2 (~60 l) was released into PVC tubes (~45 cm diameter) placed on the seafloor at 3600 m depth off central California. The liquid CO_2 dissolved into seawater, producing a high CO_2, low pH plume that drifted over the seabed and organisms in the sediment or held in cages at different distances from the CO_2 pools (photo credit: J.P. Barry).

deck of a research vessel, and then subject the natural plankton assemblage to different manipulated CO_2 levels. This technique has been used for a number of studies, including the response to elevated CO_2 of coccolithophore calcification (Riebesell *et al.*, 2000), phytoplankton carbon uptake (Tortell *et al.*, 2008) and pteropod shell formation (Orr *et al.*, 2005). Nutrients in the on-deck incubations are transferred with the ambient seawater. Light levels and temperature are higher on deck, which is typically compensated for by the use of blue-coloured light filters around the incubations, and by pumping ambient water around the tanks to keep them cool.

8.2.4 *In situ* chamber systems for ocean acidification studies

The development of *in situ* ocean acidification experiments with greater control over experimental conditions have made progress recently, with technically sophisticated designs for chamber systems and partially-enclosed pH control systems. A metabolic chamber system capable of pH perturbations by injection of CO_2-rich seawater was developed by Ishida *et al.* (2005). The system, including 3 replicate chambers, logs chamber pH and O_2, has been deployed repeatedly as a free vehicle to measure changes in microbial and meiofaunal abundance and metabolism under environmental normocapnia and hypercapnia.

Free Ocean CO_2 Enrichment (FOCE) systems are under development to mimic Free Air CO_2 Enrichment (FACE) facilities that have been used for over a decade to study the effects of increased atmospheric CO_2 on terrestrial communities (Ainsworth & Long, 2005). Similar to FACE systems, the FOCE design (Brewer *et al.*, 2005) is capable of injecting CO_2-rich seawater along the up-current margin of the enclosed lattice system to maintain a specified pH/p(CO_2) perturbation within the enclosed experimental section (Figure 8.3). FOCE systems will be automated to allow for long-term manipulative experiments in various habitats, such as coral reefs, soft-sediment benthos, kelp beds, intertidal zones, and potentially deep-sea coral communities. FOCE systems presently exist only as prototypes (Walz *et al.*, 2008) with a small control area (1 × 1 × 0.5 m) enclosed within a flume (Figure 8.2).

8.2.5 Assessing the effects of ocean acidification

Because the focus of ocean acidification studies varies widely, it is beyond the scope of this section to provide a comprehensive treatment of approaches and methods for quantifying effects of ocean acidification on response variables. For both natural and manipulative field experiments, however, response variables (e.g. survival, growth etc.) have typically been measured in parallel with measurements of carbonate system parameters to allow for regression or ANOVA analyses. For observational studies it is important to make high quality measurements of potential confounding factors (e.g. temperature, salinity, nutrient levels, light intensities) as well as of carbonate chemistry, so that their ability to explain observed changes in a variable(s) of interest (e.g. size-normalised foraminiferal weight) can be compared statistically. The appropriate statistical techniques to be used will depend on the details of the study, but techniques to be considered include calculation of correlation statistics, multiple regression analyses and analysis of variance. Important aspects include estimation of the percentages of variance that can be explained by different environmental factors, and the coefficients of correlation and statistical significance (P-value) of the different relationships. The statistical tests to be used should be taken into account at the stage of designing the fieldwork programme – regression designs are often more powerful than ANOVA designs. Numbers of replicates should be maximised in order to strengthen the statistical power of the conclusions that can be drawn from the data collected.

8.3 Strengths and weaknesses

The major strength of *in situ* studies is the inclusion of natural environmental variability that is difficult or impossible to capture in laboratory experiments. Thus, the performance of organisms or processes of interest measured during *in situ* experiments may represent more accurately natural patterns that may not be evident

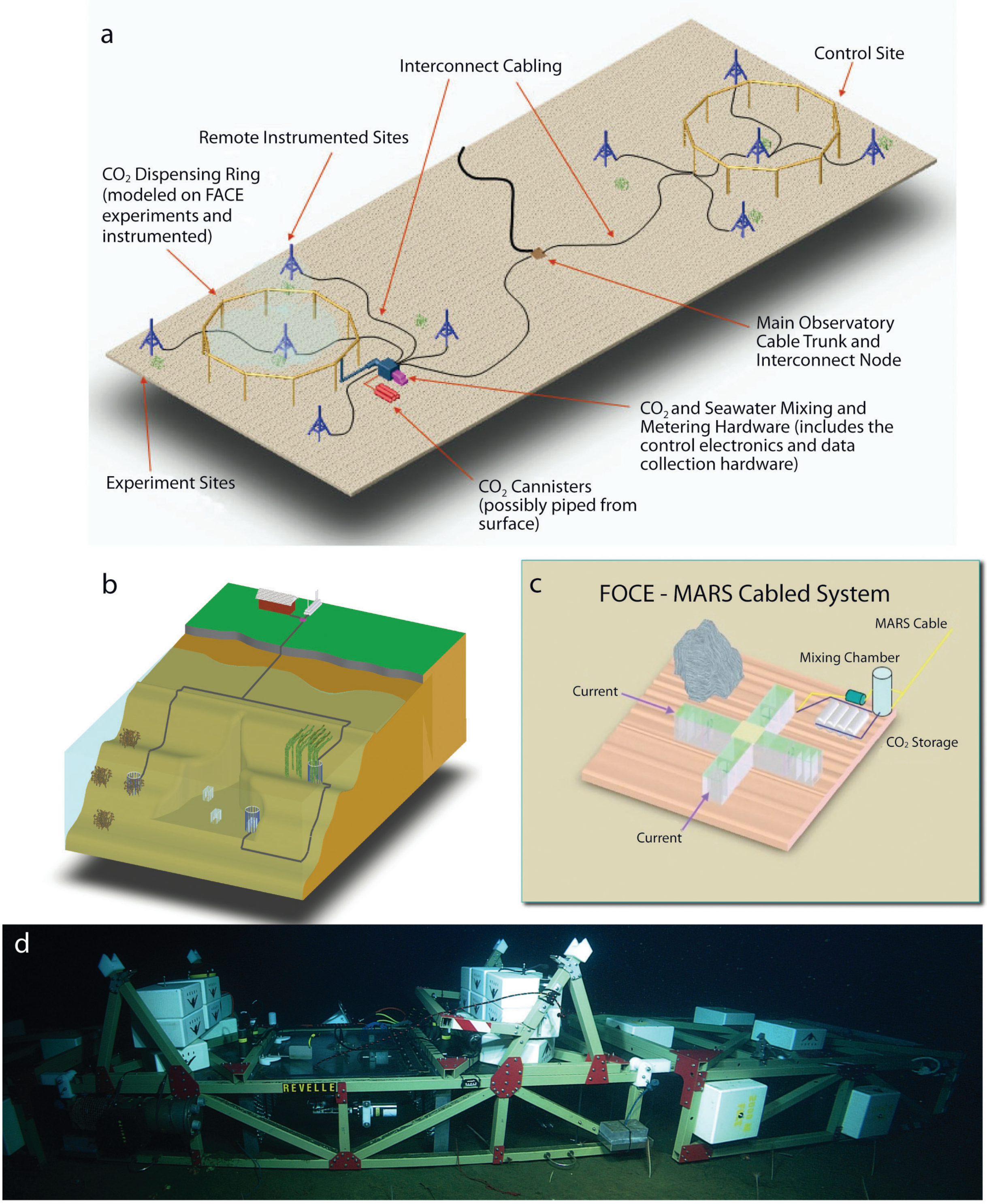

Figure 8.3 Free Ocean CO_2 Enrichment (FOCE) concepts and prototype. a. FOCE concept drawing with design analogous to FACE (Free Air CO_2 Enrichment) designs with coarse CO_2 plumbing and upstream CO_2 injection. b. FOCE concept for nearshore systems with shore-based supply and control stations leading to offshore FOCE plumbing frames. c. FOCE prototype design for connection to the Monterey Accelerated Research System (MARS) submarine cabled observatory. This system uses a flume design allowing flow into a control volume along 2 axes. d. Photograph of FOCE prototype at 900 m depth off Central California, attached for testing to the MARS cable system. Flow is along 1 axis, with a central control volume (behind "Revelle" label), flotation blocks (white blocks) for deployment/recovery, and a mixing zone in the upstream and downstream ends. (W. Kirkwood & MBARI).

in controlled laboratory experiments. For natural gradients or venting sites, the spatial and temporal scales can be very large, thereby allowing investigators to examine patterns and processes over large areas, and include response variables that require long-term responses to ocean acidification. For example, the nearly pure CO_2 venting sites bordering Ischia have persisted for long periods, allowing the natural assemblage to acclimate and adjust to the local effects of ocean acidification, resulting in the observed community patterns. Coupling such studies with controlled *in situ* or laboratory experiments can add greater inferential power to ocean acidification studies.

Another major strength is the ability of *in situ* studies to improve our understanding or gain information concerning the indirect and direct effects of ocean acidification. For instance, 8 years of monitoring of species occupancy at >1700 neighbouring rocky-shore locations found that most calcifiers fared poorly at low pH, but that some increased their presence (Wootton *et al.*, 2008). The authors speculated that some calcareous sessile species appeared to benefit from ocean acidification due to: (1) stronger effects of ocean acidification on the dominant calcareous species with which they compete for habitat space, or (2) suppression of calcareous predators (e.g. calcareous crabs preying on calcareous mussels). Another example of an indirect effect of acidification difficult to detect on a community level using laboratory experiments is the improved growth of seagrasses at low pH observed at Ischia (Hall-Spencer *et al.*, 2008), due to reduction in their epiphytic load (reduced amounts of crustose calcareous algae growing on their leaves).

The realism of *in situ* studies can also be a weakness; natural environmental conditions often lead to variation in response variables (e.g. survival, behaviour, growth etc.), due both to treatment effects and uncontrolled natural processes. See chapter 7 for a broader discussion of laboratory and shallow water mesocosm studies. In turn, higher variability may reduce the inferential power of *in situ* experiments, in which replication is often limited due to logistic constraints. And although *in situ* observational studies are capable of providing strong evidence concerning the indirect consequences of ocean acidification, the role of direct versus indirect effects may be obscure. For example, are the pH-related patterns in biological assemblages near Ischia (Hall-Spencer *et al.*, 2008) caused directly by the intolerance of larvae, juveniles, and/or adults among species, or perhaps indirectly through pH-related changes in densities of important predators and competitors? Nor may the effects of localised variation in pH and $p(CO_2)$ near venting sites reflect the eventual patterns arising from the global-scale acidification of the oceans (Riebesell, 2008). For instance, immigration of adult organisms into a vent site after passing their most sensitive early life stages elsewhere may give a false impression of tolerance. Only a subset of the regional population for each species will be impacted, placing in question the influence of ocean acidification on the population dynamics for the species. In addition, temporal variation in pH may lead to spurious conclusions concerning the effects of chronic, long-term, and relatively invariant changes in pH.

Confounding factors, particularly for observational studies where control of ocean acidification treatments is minimal, may have large effects that could be confused with or attributed to pH or $p(CO_2)$. For example, natural venting sites often have high levels of methane, sulfide, metals, and possibly hypoxic waters (Kelley *et al.*, 2002) as well as high temperatures, each with potentially important effects. For studies concerning the effects of ocean acidification along depth-related or other spatial gradients in pH, several other factors must also be considered (e.g. oxygen, pressure, temperature, nutrients, light).

Manipulative experiments have greater inferential power than observational studies and other natural experiments. These methods may best be used together to determine more explicitly the effects of ocean acidification. Finally, studies using natural gradients in carbonate chemistry, particularly localised CO_2 vents, may have limited opportunities for replication and interspersion of replicates among treatments. Some strengths and weaknesses of *in situ* experiments are listed in Table 8.1.

Table 8.1 Estimates of the strengths and weaknesses of *in situ* ocean acidification experiments. Plus symbols indicate strengths. Minus signs indicate weaknesses.

Strengths and weaknesses	Observational studies	Controlled experiments
Natural realism	++++	+++
Effects on individuals	+++	+++
Population and community effects	++++	+/-
Acute effects	+++	+++
Chronic effects	++++	---
Adaptation	+++	---
Direct effects	++	++
Indirect effects	++	?
Multiple stresses	---	+/-
Confounding factors	---	++
Randomisation	---	+++
Replication	+/-	+++
Control over carbonate system factors	+/--	+++

8.4 Potential pitfalls

In situ observations and experiments allow investigators to assess the effects of ocean acidification in natural ecosystems, but have limitations in both design and technology that should be considered carefully. These include:

- Stability of carbonate system parameters – for *in situ* observational studies and controlled manipulations variation in ocean acidification treatments (e.g. pH) may be considerable, thereby obscuring the effects of mean, versus extreme, changes in pH.
- For controlled experiments, the short-term response of organisms or populations may not represent gradual or chronic effects.
- Measurement and monitoring of carbonate system parameters during *in situ* experiments may be technically difficult.
- The effects of confounding factors with pH or other carbonate system parameters may be large.
- Scaling up from individual responses (or physiological effects) to communities/ecosystems may be difficult.

8.5 Suggestions for improvement

In situ ocean acidification studies will benefit from improved technology to enable controlled perturbation experiments, including FOCE-type or chamber systems with CO_2 control, sensors for accurate, long-term, *in situ* measurements of ocean carbonate system parameters (see chapter 1), and from the discovery of new natural vent sites releasing nearly pure CO_2. Presently, the technology to control pH or $p(CO_2)$ under field conditions is very limited, and restricts progress in understanding the effects of future ocean acidification under natural conditions. Recent advances in field effect transistor (FET) pH sensors (e.g. Honeywell durafet pH sensor) have promise for long term stability and accuracy, but are not yet widely available for *in situ* deployments. Other parameters of the carbonate system remain more difficult to measure *in situ*, particularly for long-term automated measurements. Observational studies should aim to measure more than 2 carbon parameters, so as to over-constrain the carbonate chemistry (chapter 1). See chapter 2 for a discussion of methods to control the seawater carbonate system parameters.

Considering the technical difficulty of performing CO_2 perturbation experiments under field conditions, the carbon dioxide venting areas off Ischia (Hall-Spencer *et al.*, 2008) and other natural gradients in ocean carbonate chemistry are important discoveries for ocean acidification studies, due to their value as natural laboratories. Though limited in spatial extent, conditions at the Ischia site will enable a variety of hypotheses concerning the effects of ocean acidification to be tested with controlled experiments. Discovery of additional venting sites or other natural gradients in carbonate chemistry in various ocean settings will also be useful for *in situ* observational studies, and can be combined with tests of specific hypotheses using manipulative experiments in the laboratory or *in situ* to advance understanding of the potential consequences of future ocean acidification.

8.6 Data reporting

Experimental studies concerning natural gradients in ocean pH or controlled *in situ* perturbations should include a clear explanation of the design, treatment levels for carbonate system parameters, potentially confounding factors, and response variables. It is beyond the scope of this chapter to consider the breadth of response variables that may be relevant for ocean acidification studies. However, the design of the experiment (e.g. ANOVA, regression etc.), including treatment levels for carbonate system parameters, layout of treatments and replicates, and a comprehensive list of all measurements (when and where), should be reported. For natural gradients in pH or other carbonate system parameters, potential limitations of the design (e.g. lack of interspersion or replication, temporal and spatial variability etc.) should also be reported and discussed. Seawater carbonate parameters in addition to pH should be reported, if possible, including the mean and range of variation for each parameter within each treatment. Where possible, potentially confounding factors (e.g. methane, sulfide, temperature, oxygen) should also be monitored and reported.

8.7 Recommendations for standards and guidelines

1. Consider the analytical design of the experiment before any fieldwork begins – regression designs are often more powerful than ANOVA designs.
2. Replicate treatments and intersperse replicates among treatments (see chapter 4).
3. Repeat experiments, if possible.
4. Measure multiple carbonate system parameters, if possible (see chapter 1).
5. Strive to reduce the variability in pH or other carbonate system parameters within treatments, to provide controlled, unambiguous treatment levels.

6. Monitor pH and other parameters throughout the experiment to determine their spatial and temporal variability within each treatment (see chapter 1).
7. Measure potentially confounding factors that may also influence response variables.
8. If possible, design experiments to include the influence and interaction among multiple factors in addition to carbonate system parameters (e.g. temperature, light, nutrients, hypoxia).

8.8 References

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