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CORRESPONDENCE

The importance of accurate CO_2 dosing and measurement in ocean acidification studies

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Chung et al. (Chung et al., 2014) recently reported the results of a study investigating the effect of dissolved CO_2 level on the retinal response of a marine fish. The two CO_2 concentrations tested represented different ocean acidification scenarios. The lower concentration represented a near-future CO_2 concentration (466 µatm), and the other a 2100 scenario with elevated CO_2 due to anthropogenic inputs (944 µatm). Such studies are timely given the high probability that atmospheric CO_2 concentration will continue to increase. The results will likely be incorporated into future Intergovernmental Panel on Climate Change reports, and may form part of future action plans designed to preserve species and ecosystem viability. For this reason, it is crucial to ensure that any testing of future ocean acidification scenarios is carried out with a high degree of certainty about the CO_2 levels tested.

The methods Chung et al. used to both quantify and dose CO_2 in their study lack the necessary accuracy and precision to definitively assert that the target CO_2 test levels were correct. The key problem is their dependence on pH measurements made with a glass potentiometric electrode and low ionic concentration calibration buffers (a method commonly employed by a number of the authors on this paper), which is not recommended best practice for the accurate determination of pH in waters of high ionic concentration (Dickson, 2010), such as seawater. A cross-laboratory comparison of the accuracy of glass pH electrodes by Illingworth (Illingworth, 1981) found a mean measurement error of 0.2 pH units for high ionic concentration solutions, with many probes performing worse. Recalibration does not help, as many electrodes will apparently calibrate perfectly well in the low ionic concentration NBS (National Bureau of Standards) buffers (probably the most commonly used pH calibration buffers), but when used in saltwater will give erroneous measurements. Sources of error for seawater pH measurements based on glass electrodes and NBS buffer calibration include sodium ion error (e.g. Na^+ is detected as H^+), differences in ionic diffusivity between the reference electrode solution and the sample solution, and the fact that NBS reference buffers are not directly related to hydrogen ion concentration (which is necessary to compute dissolved CO_2) (Dickson, 1993; Grasshoff, 1983; Millero et al., 1993). Small errors in pH measurement will have significant effects on the computed dissolved CO_2 concentration. For example, for seawater (34.5 ppK salinity, 30°C, total alkalinity 2269 µmol kg^{-1} SW) with pH 7.9, the computed pCO_2 is 604 µatm, and at pH 7.8 pCO_2 is 788 µatm. For reference, the range of CO_2 concentrations typically used in ocean acidification scenarios (400 versus 1000 µatm) are equivalent to a pH difference of between 0.3 and 0.4 pH units. In addition, when glass potentiometric electrodes are continually submersed in saltwater, they quickly drift and lose measurement span, limiting the value of pH meters as a method to control the dosing of pure CO_2 into experimental tanks. In light of the known limitations glass electrodes present for measuring seawater pH, and the total dependence of the Chung et al. study on pH to determine test pCO_2 levels, the dose–response effect reported in their paper should be treated with caution.

Researchers and manuscript reviewers need to be aware of the difficulty of accurately measuring and dosing CO_2 when approaching studies such as that of Chung et al. (Chung et al., 2014). Best practice guidelines for ocean acidification research have been developed (Riebesell et al., 2010), and there is plenty of information available on methods for accurate dissolved CO_2 measurement (Dickson et al., 2007). In order to ensure ocean acidification studies of aquatic biota are valid and repeatable, we must ensure that the test CO_2 concentrations are both accurate and precise. The solution is to adopt the measurement methods used by ocean chemists, which not only rely on techniques with a high degree of measurement certainty, but are also subject to quality control and assessment (i.e. the use of reference material to validate measurements) (Dickson et al., 2007). Ideally, direct pCO_2 measurements should be made via nondispersive infrared measurement (NDIR), where dissolved gases are equilibrated with a carrier gas which then passes through an infrared analyser (the Alliance for Coastal Technologies has evaluated some of the main manufacturers of this technology, www.act-us.info). If pH is to be used for pCO_2 calculation (together with either total inorganic carbon or alkalinity), then it is necessary to have an absolute measurement of H^+ concentration that is free of the errors introduced by potentiometric glass electrodes and non-specific calibration buffers. The most accurate and precise pH measurement method is spectrophotometry plus an indicator dye (Millero et al., 1993), which can be automated to monitor seawater pH continuously (McGraw et al., 2010). Total alkalinity (TA) can be measured with a high degree of precision using potentiometric electrodes and commercially available acid standard solutions, and together with pH, TA can be used to calculate pCO_2 [as was done by Chung et al. (Chung et al., 2014)]. However, there is an assumption that the components contributing to the alkalinity are known and accounted for, which allows for the calculation of HCO_3^- and CO_3^{2-} concentration ($[HCO_3^-]+[CO_3^{2-}]=TA-[\text{non-carbonate buffers}]$). In oceanic seawater with a low organic loading, the carbonate alkalinity represents almost all of the TA, but in waters with a significant organic loading, the contribution of non-carbonate buffers to total alkalinity may be substantial. In experiments where fish are kept for days in the same body of water [as is common in experiments such as that of Chung et al. (Chung et al., 2014)], the contribution of metabolic waste products (ammonia, nitrates, phosphates, organic acids, fatty acids and proteins) to alkalinity and pH balance increases significantly. As CO_2 is a small percentage of total inorganic carbon (at atmospheric CO_2 concentrations), small errors in $HCO_3^- + CO_3^{2-}$ measurement will result in substantial pCO_2 calculation error. For waters with complex alkalinity compositions, total inorganic carbon is a better carbonate parameter to measure (via water sample acidification plus NDIR analysis of carrier gas CO_2 concentration). One method of ensuring accurate

test pCO_2 levels is to equilibrate water with a known gas composition, which can be derived from a gas-mixing pump, mass flow mixer or pre-purchased from a gas cylinder supplier.

For good reasons, ocean acidification research and its possible biological impacts are targeted areas for funding and are of considerable public interest. The failure to carry out accurate CO_2 dose–response experiments will limit our ability to find general patterns between ocean acidification studies. It can potentially waste research resources as the results may be uninterpretable, and there is the real possibility that the media, public and stakeholders will lose confidence in our societal value if we fail to predict the effects of climate change accurately.

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Response to ‘The importance of accurate CO_2 dosing and measurement in ocean acidification studies’

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In his comment on our paper, Moran (Moran, 2014) raises the important issue of ensuring that experiments investigating the responses of marine organisms to future ocean acidification scenarios are carried out with a high degree of certainty about the CO_2 levels being tested. We agree wholeheartedly, which is why we take considerable care in measuring and validating pCO_2 in our field-based and laboratory experiments. Here we explain, more fully than was possible in a Short Communication, the procedures used to measure and cross-validate pCO_2 in Chung et al. (Chung et al., 2014) and other studies we have conducted over recent years (in which the methods have been reported). These techniques are in accordance with those mentioned by Moran and detailed descriptions are already available in the literature (e.g. Hari et al., 2008). We also correct a number of factual errors and incorrect assumptions made by Moran in his comment.

Moran states that because of the ‘dependence of the Chung et al. study on pH to determine test pCO_2 levels, the dose–response effect reported in the paper should be treated with caution’ and that ‘we cannot assert that the target CO_2 levels were correct’. The author proposes that: ‘Ideally, direct pCO_2 measurements should be made via nondispersive infrared measurement (NDIR), where the dissolved gases are equilibrated with a carrier gas which passes through an infrared analyser.’ Indeed, that is exactly what was done in our experiment. While we did not have room in Chung et al. (Chung et al., 2014) to report on the method, we have previously reported that we cross-validate our estimates of pCO_2 from seawater carbonate chemistry using NDIR (e.g. Munday et al., 2010; Munday et al., 2013; Simpson et al., 2011; Watson et al., 2014). As described by Hari et al. (Hari et al., 2008), we used a Vaisala GMP343 infrared CO_2 probe (accuracy ±5 ppm CO_2 + 2% of reading over the range of our experimental manipulations) in a closed loop to measure xCO_2 in the experimental treatment. Temperature-, pressure- and humidity-corrected pCO_2 by NDIR was 946±14 µatm (mean ± s.e.m., N=9), which is within 2 µatm of the value estimated by seawater chemistry (944±19 µatm). This confirms the reliability of our methods and the accuracy of the pCO_2 treatment reported in the paper.

Moran mistakenly refers to our current-day control (466±15 µatm CO_2) as a near-future treatment. It is well known that pCO_2 of seawater in coral reef lagoons is elevated above atmospheric levels in summer because of enhanced calcification by corals and the reduced flushing of lagoons. For example, the daily average pCO_2 on a Hawaiian coral reef was 431–622 µatm (Shamberger et al., 2011). This is the environment inhabited by coral reef fish, and thus the appropriate current-day control. It is not a near-future treatment.

Moran incorrectly states that we kept fish for days in the same body of water, which could lead to significant organic loading that would affect the estimates of total alkalinity (TA) used to calculate pCO_2. As in our previous studies, fish were kept in replicate 30 l aquaria in a flow-through system, and the paper clearly states that each aquarium received a continuous flow of CO_2-equilibrated seawater at 1 l min^{-1}. A turnover time of 30 min is sufficient to flush dissolved metabolic waste from the tanks. Tanks were regularly cleaned of any solid waste. The close match between our estimates of pCO_2 from seawater chemistry and NDIR confirms that loading of non-carbonate buffers was not an issue in our experiment. Nevertheless, we agree with Moran that organic loading is an important issue to consider when designing experiments to study the biological effects of ocean acidification. Finally, as we report in our paper, we validated our measured values of TA against the relevant seawater standards. As we have reported elsewhere, our laboratory measures TA within 1% of the reference value.

The choice of methods to characterize seawater carbonate chemistry depends on the parameters of interest, the precision and accuracy required for studies of biological effects versus detecting ongoing changes in ocean chemistry, and other aspects of

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experimental design. Like many of our studies, Chung et al. (Chung et al., 2014) was conducted at a remote field station on the Great Barrier Reef, which imposes certain logistical constraints on the methodology. Spectrophotometry is a highly accurate method to measure pH; however, it is not practical to transport and operate a high-precision spectrophotometer at a remote field station. Similarly, it is often not practical to rely on dissolved inorganic carbon and TA estimations for replicated biological experiments at field stations because of the expense involved in transporting and analysing large numbers of seawater samples from numerous replicate tanks, and the impractical lag-time before results are returned to confirm experimental treatments. Provided is it carefully conducted, and preferably cross-validated with other techniques as we have done in our studies, pH can be a useful parameter for controlling experimental treatments and for characterizing seawater chemistry in biological experiments.

Moran correctly points out that glass pH electrodes may drift when continually immersed in seawater. That is one of the reasons we take daily measurements in the experimental tanks, so that we can cross-calibrate the dosing pH probes with the realised pH in our experiments and then adjust the pH set point of the dosing system as required. For seawater chemistry, we take daily, or twice daily, readings of pH in each aquarium using a high-quality pH meter and laboratory-grade electrode (Mettler Toledo). A fault of some other studies is that they rely on the continuously measured pH at the site where pCO_2 is manipulated, not in the tanks where the animals are being treated. We agree that has the potential to introduce errors in the reported seawater parameters, because of both electrode drift and CO_2 flux between the location of CO_2 dosing and the experimental tanks.

One advantage of using glass electrodes and careful calibration with NBS buffers, and cross-validation with NDIR, is that we can have large, appropriately replicated, experiments with multiple levels of CO_2 treatment. While it is important to ensure accurate seawater chemistry in ocean acidification experiments, there are many other issues of experimental design that can cause far greater bias in our understanding of the biological effects of environmental stressors. Inadequate replication, unrealistically high treatment levels, and insufficient duration of experiments are serious problems in many ocean acidification studies. We argue that, more so than chemistry methods that are already well established, this is an area that needs to be improved in ocean acidification research.

Toward the end of his comment, Moran proposes that one method of ensuring accurate test pCO_2 is to equilibrate water with a known gas composition derived from a gas mixing pump, mass flow mixer or pre-purchased gas cylinder. While this method can be successful for small volumes of static water that are continuously aerated, it can be difficult to achieve full equilibration in larger volumes of seawater, especially in a flow-through system. Good equilibration can be achieved with counter-current towers or purpose-designed diffuser membranes, but good equilibration is difficult without specialist equipment that would be difficult to deploy in a field-based situation. We warn the reader that this method is more challenging than it appears at face value, especially for experiments that require large volumes of flowing water, such as those with fish and other metazoans. One approach we have found successful is to first achieve the desired pCO_2 by treating to a set pH in separate header tanks or sumps and then aerating the experimental tanks with the desired gas composition.

Moran makes some useful comments for new researchers entering the field of ocean acidification research who may not be aware of the difficulties and challenges of accurately estimating seawater carbon parameters, including pCO_2. Perhaps the most important point, however, is the final comment about the need to accurately predict the effects of climate change. While achieving well-constrained chemistry in ocean acidification studies is part of that goal, we cannot hope to reliably predict the future impacts of ocean acidification on complex marine ecosystem without addressing the most challenging and important knowledge gaps in the field. Major knowledge gaps include the capacity for long-term acclimation and adaptation to ocean acidification (Sunday et al., 2014), interactive effects with other stressors, and how impacts on individual organisms scale up to affect ecological processes and ecosystem function (Hilmi et al., 2013). Having the vision to tackle these crucial questions, as some laboratories around the world are starting to do, is what will really drive ocean acidification research forward.

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