Quantifying pCO$_2$ in biological ocean acidification experiments: A comparison of four methods

Sue-Ann Watson[1]*, Katharina E. Fabricius[2], Philip L. Munday[1]

1 Australian Research Council Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland, Australia, 2 Australian Institute of Marine Science, Townsville, Queensland, Australia

* sueann.watson@jcu.edu.au

Abstract

Quantifying the amount of carbon dioxide (CO$_2$) in seawater is an essential component of ocean acidification research; however, equipment for measuring CO$_2$ directly can be costly and involve complex, bulky apparatus. Consequently, other parameters of the carbonate system, such as pH and total alkalinity (A$_T$), are often measured and used to calculate the partial pressure of CO$_2$ (pCO$_2$) in seawater, especially in biological CO$_2$-manipulation studies, including large ecological experiments and those conducted at field sites. Here we compare four methods of pCO$_2$ determination that have been used in biological ocean acidification experiments: 1) Versatile INstrument for the Determination of Total inorganic carbon and titration Alkalinity (VINDTA) measurement of dissolved inorganic carbon (C$_T$) and A$_T$, 2) spectrophotometric measurement of pH$_T$ and A$_T$, 3) electrode measurement of pH$_{NBS}$ and A$_T$, and 4) the direct measurement of CO$_2$ using a portable CO$_2$ equilibrator with a non-dispersive infrared (NDIR) gas analyser. In this study, we found these four methods can produce very similar pCO$_2$ estimates, and the three methods often suited to field-based application (spectrophotometric pH$_T$, electrode pH$_{NBS}$ and CO$_2$ equilibrator) produced estimated measurement uncertainties of 3.5–4.6% for pCO$_2$. Importantly, we are not advocating the replacement of established methods to measure seawater carbonate chemistry, particularly for high-accuracy quantification of carbonate parameters in seawater such as open ocean chemistry, for real-time measures of ocean change, nor for the measurement of small changes in seawater pCO$_2$. However, for biological CO$_2$-manipulation experiments measuring differences of over 100 µatm pCO$_2$ among treatments, we find the four methods described here can produce similar results with careful use.

Introduction

Since the beginning of the Industrial Revolution, the oceans have absorbed about a third of all anthropogenic carbon dioxide (CO$_2$) emissions released into the atmosphere [1, 2]. In seawater, CO$_2$ reacts to form carbonic acid (H$_2$CO$_3$) which dissociates into hydrogen (H$^+$) and

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bicarbonate ions (HCO$_3^-$). This process, known as ocean acidification, results in increased concentrations of CO$_{2(aq)}$, H$^+$ and HCO$_3^-$, and reductions in carbonate ion (CO$_3^{2-}$) concentration and the saturation state of seawater with respect to calcite and aragonite. As a result of ocean acidification, surface oceans are now approximately 0.1 pH units lower and 30% more acidic than 250 years ago [3]. Ocean chemistry is changing faster than any time during the last 65 million years [4], and possibly the last 300 million years [5]. Under current CO$_2$ emissions rates (Representative Concentration Pathways, RCP 8.5 scenario), atmospheric CO$_2$ levels are projected to exceed 900 ppm by the end of this century [6] and seawater pH projected to decline a further 0.14–0.43 units [3].

In the surface ocean, pCO$_2$ is rising at the same rate as atmospheric CO$_2$ [7]. Recent models suggest seasonal pCO$_2$ cycles will be amplified as atmospheric CO$_2$ levels rise, which means that pCO$_2$ in the surface ocean may be considerably higher than in the atmosphere for many months each year and open ocean regions could exceed 1000 µatm pCO$_2$ before the end of the century [8]. Coastal waters exhibit particularly large seasonal and diel variation in pH and pCO$_2$ (e.g. [9, 10]), and consequently, anthropogenic amplification of the pCO$_2$ cycle in coastal waters is likely to be even more pronounced [11].

Seawater pCO$_2$ can be assessed: 1) by direct measurement of CO$_2$ in a gas volume equilibrated with seawater using gas analysers equipped with non-dispersive infrared (NDIR) sensors, or 2) indirectly by measuring two parameters of the seawater carbonate chemistry system and then calculating pCO$_2$. Direct NDIR measurement of CO$_2$ is often conducted using equilibrators that are specifically designed for the continuous measurement of CO$_2$, such as on ships (e.g. [12]), or modified to measure CO$_2$ in a small volume of air in a closed loop that is equilibrated with CO$_2$ in water. Commonly, seawater pCO$_2$ is calculated using any pair of carbonate chemistry parameters. Frequently used parameters include pH, total alkalinity (A$_T$), and dissolved inorganic carbon (C$_T$).

Measurements of seawater carbonate chemistry parameters vary in 1) measurement time, 2) accuracy, 3) cost, and 4) the time lag to obtain results (e.g. zero if results are obtained immediately, or potentially months later in the case of water sample batch processing; Table 1). For example, pH is commonly measured immediately *in situ* or *in vitro* using a relatively low-cost pH meter and electrode, or spectrophotometrically *in vitro* after addition of a pH indicator dye. A$_T$ and C$_T$ are measured *in vitro*, usually from mercuric chloride poisoned water samples, and generally require more complex, customised, bulky and costly laboratory equipment such as an automatic titrator or Versatile INstrument for the Determination of Total inorganic carbon and titration Alkalinity (VINDTA), respectively; although it is possible to perform titrations manually with a lower-cost pH meter and electrode (or pH indicator), and burette. Systems such as VINDTA are complicated but very precise, while other methods that are easier to use may not reach the same accuracy.

Table 1. Summary table of methods used in this study. Sample measurement time refers to measurements made during this study, with the upper end of the time range allowing for machine warm-up.

Method	Time lag to obtain results	Approx. sample measurement time	Approx. cost of equipment (USD in 2016)
VINDTA C$_T$	Often long as samples are usually batch processed, often after the experiment	10–60 min	$75,000
Spectrophotometric pH$_T$	Shortly after real-time	10–45 min	$3,000–10,000
Electrode pH$_{NBS}$	Real-time	1–3 min	$1,000
NDIR CO$_2$ equilibrator	Shortly after real-time (corresponding to equilibration time)	30–60 min	$4,000

Choosing appropriate measurement techniques to achieve the required precision and accuracy for carbonate chemistry parameters should be the primary consideration; however, the number and frequency of measurements required for a study is also an important consideration in measurement technique choice, and will relate to the number of treatment levels and replicates within the experimental design. For biological ocean acidification experiments, measurement accuracy will vary depending on the research question addressed and target $p\text{CO}_2$ treatment levels employed, and accuracies of ±50 μatm $p\text{CO}_2$ are likely commonly suitable for biological CO_2-manipulation experiments measuring differences of more than 100 μatm $p\text{CO}_2$ among treatments. Furthermore, the measurement of some carbonate chemistry parameters requires sophisticated equipment, which is not always accessible, particularly at remote field sites. Many biological ocean acidification studies are now conducted in more remote locations such as field research stations, and submarine CO_2 vents and seeps (e.g. [13–16]), and include large, highly-replicated ecological studies. In such field based environments where access to specialist chemical oceanography equipment is limited, researchers need to repeatedly monitor seawater carbonate conditions during their experiments, often with multiple treatments and upwards of 50 or more replicates running simultaneously that require monitoring on a daily or more frequent basis. Field researchers therefore need techniques that can provide reliable, cost-effective, real-time estimates of $p\text{CO}_2$ to maintain their experiments.

Here we assess a range of methods commonly available to determine seawater carbonate chemistry in biological ocean acidification experiments. We consider four parameters that are commonly measured to constrain the CO_2 system in seawater: A_T, C_T, pH, and $p\text{CO}_2$ [17], and we compare four different methods to determine the $p\text{CO}_2$ of seawater: 1) C_T and A_T, 2) spectrophotometric pH_T and A_T, 3) electrode pH_{NBS} and A_T, and 4) a portable CO_2 equilibrator with a NDIR gas analyser to measure CO_2 directly *in situ*. We assess measurement time, accuracy, costs and the time lag to obtain results for the four methods. We focus particularly on $p\text{CO}_2$ determination firstly, because quantifying $p\text{CO}_2$ is particularly important in designing biological manipulation experiments relevant to emissions trajectories such as the Intergovernmental Panel on Climate Change's Representative Carbon Pathways (IPCC RCPs) and secondly, because $p\text{CO}_2$ is very sensitive to small changes in other carbonate parameters making it a useful measure for this comparative approach. We also expand on the CO_2 equilibrator technique by describing a simple method for the direct, *in situ* measurement of CO_2 in seawater using a portable CO_2 equilibrator coupled to a NDIR gas analyser.

Materials and methods

Experimental system and seawater manipulation

This study was conducted at Lizard Island, Great Barrier Reef, Australia (S 14° 41', E 145° 28') at the Australian Museum's Lizard Island Research Station flow-through aquarium facility. Water from the ocean was pumped into an environmentally-controlled room where seawater flowed into a 60 L header tank fitted with a powerhead to circulate the water. Seawater from the header tank was gravity-fed into a 32 L (38L x 28W x 30H cm) experimental tank at 1.5 L.min^{-1}.

Elevated-CO_2 seawater was achieved by dosing the header tank with 100% CO_2 to a set pH_{NBS} using a pH-controller (pH computer, Aqua Medic, Germany), following standard techniques [18]. A needle valve was used to regulate the flow of CO_2 into the powerhead intake to ensure a slow, steady stream of fine CO_2 gas bubbles during dosing. This slow dosing and rapid mixing in the header tank ensured that the experimental tank received a steady supply of well-mixed water.

The CO_2 dosing system was set at a series of different pH_{NBS} levels throughout the experiment. A range of seawater pH_{NBS} values (8.2 to 7.6) were used, corresponding to ambient and elevated pCO_2 of <400 to >1400 µatm, and measurements of seawater chemistry were taken in the experimental tank simultaneously using the four methods described below. Briefly, air equilibrated with seawater from the experimental tank passed across a NDIR CO_2 gas analyser until CO_2 levels had stabilised (c. 1 hr). Once CO_2 readings were stable, data on CO_2, pH_{NBS} and temperature were recorded. Water samples were taken for immediate spectrophotometric analysis of pH_T, and preserved for later analysis of C_T and A_T. Full details are described below.

Quantification of carbonate chemistry parameters

1) Determination of seawater dissolved inorganic carbon (C_T) and total alkalinity (A_T). Water samples taken from the experimental tank at the time of measurement were immediately poisoned with a saturated solution of mercuric chloride (at 0.05% of the sample volume) and later analysed *in vitro* for C_T and A_T at the Australian Institute of Marine Sciences (AIMS) on a Versatile INstrument for the Determination of Total inorganic carbon and titration Alkalinity (VINDTA 3C, MARIANDA, Kiel, Germany). The VINDTA 3C was configured with a UIC Coulometer (model 5015) and UIC Anode Reagent and Cathode Reagent (UIC Inc., Joliet, Illinois, U.S.A.) for C_T analysis and a Metrohm Titrino titrator (model 702, Metrohm, Switzerland) with 0.1M HCl (fortified with NaCl to the ionic strength of seawater) added in 150 µl steps for A_T analysis, calculated by Gran titration. The VINDTA was calibrated with certified reference material (CRM) consisting of sterilized natural seawater of known C_T and A_T preserved with mercuric chloride (Prof. A.G. Dickson, Scripps Institution of Oceanography, U.S.A., batch number 126, one-point calibration). CRMs and samples were water-jacketed at 24°C and sample results were adjusted for salinity of the sample compared with the CRM. Since the VINDTA samples a fixed volume and the CRM is certified in mass units (µmol.kg^{-1}), a small adjustment for the difference in the salinity of the sample compared with the salinity of the CRM at 24°C was required. Consequently, the raw VINDTA output of C_T and A_T was multiplied by seawater density at the CRM salinity, and divided by seawater density at the sample salinity. This adjustment reduced the raw VINDTA output of C_T and A_T by approximately 2–3 µmol.kg^{-1} to produce the final C_T and A_T measures.

A_T data were used as the second parameter in carbonate chemistry calculations for each of the four methods. Carbonate chemistry parameters derived from C_T and A_T were used to compare carbonate chemistry parameters determined from the other three methods. Reported measurement uncertainty for C_T and A_T using state-of-the-art methods with reference materials is 2–3 and 2–3 µmol.kg^{-1}, respectively [17].

2) Spectrophotometric determination of seawater pH_T. Seawater pH on the total hydrogen ion concentration scale (total scale, pH_T) was measured *in vitro* using a spectrophotometer following standard operating procedures (SOP 6b; [19]). The SOP was adapted for field use by using a compact, single-beam spectrophotometer (Spectronic 20 Genesys) and a spectrophotometric cell made of optical glass with a 10 mm path-length. This more compact system allowed transportation to the field site. Seawater pH determination was performed using the indicator dye meta/*m*-cresol purple (mCP) (*m*-cresol purple sodium salt 99%, non-purified, Acros Organic).

A seawater sample for spectrophotometric determination of pH_T was taken from the experimental tank underwater with no headspace, at the same time that all other seawater measurements and samples were taken. Absorbances of the cell + seawater were measured and recorded at the non-absorbing wavelength (730 nm) and at the dye absorption maxima (578 and 434 nm) as per SOP 6b [19]. Temperature of the sample during measurements was

maintained to within ±0.1˚C of 25.0˚C and confirmed with a temperature probe (C26, Comark, Norwich, U.K.) before and after each spectrophotometric measurement. A highly accurate thermometer (Traceable® Digital Thermometer 4000, Control Company, Texas, U.S. A.) was used to confirm the temperature probe reading was correct to within 0.1˚C. During measurement, temperature was maintained within ≤0.1˚C and any change in the non-absorbing wavelength at 730 nm was maintained within ≤0.001. These additional controls were employed to ensure maximum measurement quality at the field site. Additionally, spectrophotometer accuracy and stability were confirmed by replicate analysis of certified reference material (CRM) consisting of Tris buffer in synthetic seawater (Prof. A.G. Dickson, Scripps Institution of Oceanography, U.S.A., batch number 26, one-point calibration). Reported measurement uncertainty for pH using techniques with reference materials, other than state-of-the-art methods, is 0.01–0.03 pH units [17].

3) Electrode measurement of seawater pH$_{NBS}$. Seawater pH on the US National Bureau of Standards (NBS, an organisation now known as The National Institute of Standards and Technology) scale (pH$_{NBS}$) was determined *in situ* with a portable, hand-held pH meter (SevenGo Pro pH/Ion, Mettler Toledo) and glass electrode (InLab®413 S8, Mettler Toledo) calibrated with certified reference materials (CRMs) for NBS consisting of pH$_{NBS}$ 4 and 7 buffer solutions (Mettler Toledo, two-point calibration). Reported measurement uncertainty for pH using techniques with reference materials, other than state-of-the-art methods, is 0.01–0.03 pH units [17].

4) Measurement of seawater CO$_2$ with a non-dispersive infrared (NDIR) gas analyser. Seawater CO$_2$ was measured *in situ* with a portable CO$_2$ equilibrator with a high-resolution non-dispersive infrared (NDIR) gas analyser. This method for the direct measurement of CO$_2$ in seawater using a NDIR sensor, described in more detail below, is taken from Hari et al. [20]; see also Munday et al. [21]. The portable CO$_2$ equilibrator consisted of a NDIR CO$_2$ sensor (CARBOCAP® Carbon Dioxide Probe GMP-343, Vaisala, Helsinki, Finland, calibrated by Vaisala using certified reference materials (CRMs, six-point calibration) two months prior to the study) and data logger (Measurement Indicator MI70, Vaisala, Helsinki, Finland), air pump, gas-tight tubing, gas-permeable tubing and dehumidifying tubing (Fig 1). The NDIR CO$_2$ sensor range was pre-programmed from 0 to 5000 ppm CO$_2$ and the environmental settings on the data logger were set to 80.0% relative humidity, 1010.0 hPa ambient pressure and 21.0% oxygen. CO$_2$ data from the sensor were compared directly with estimated pCO$_2$ from the three other methods. The CO$_2$ sensor was connected to the data logger that also served as a data display and interface, allowing visualisation of real-time as well as recorded CO$_2$ data. Both the CO$_2$ sensor and display interface were enclosed in a water-resistant plastic container.

A gas-permeable membrane (medical silicone tubing ID 3.0 mm, OD 3.8 mm, length 12.2 m) was coiled around rigid plastic mesh and connected to the CO$_2$ sensor via gas-impermeable tubing (length 2.1 m, ID 4 mm, OD 6 mm) in a closed loop. A 60 cm length of Nafion® membrane tubing (ID 2.18 mm, OD 2.74 mm, ME-110-24BB, Perma Pure LLC, Lakewood, NJ, U.S. A.), selectively permeable to only water vapour, in-line between the gas-permeable membrane and the CO$_2$ sensor removed moisture from the air in the closed loop, if the humidity was greater than ambient, before it reached the sensor. A small 12 V AC closed circuit diaphragm pump (Rietschle Thomas miniature rotary vane pump, model G 01-K) was used to circulate air around the closed-loop system at a flow rate of 1.1 L.min^{-1}. Once the circuit was closed, the gas-permeable membrane was submerged in seawater in the experimental tank and the in-line air pump turned on. This allowed the air inside the closed loop to equilibrate with seawater CO$_2$ over time (S1 Fig). Including the water-resistant housing, the total system weighed 1.4 kg. Adding the 1.4 kg 12 V AC power transformer gave a combined total weight of 2.8 kg, and compact packed size of 26L x 23W x 17H cm.
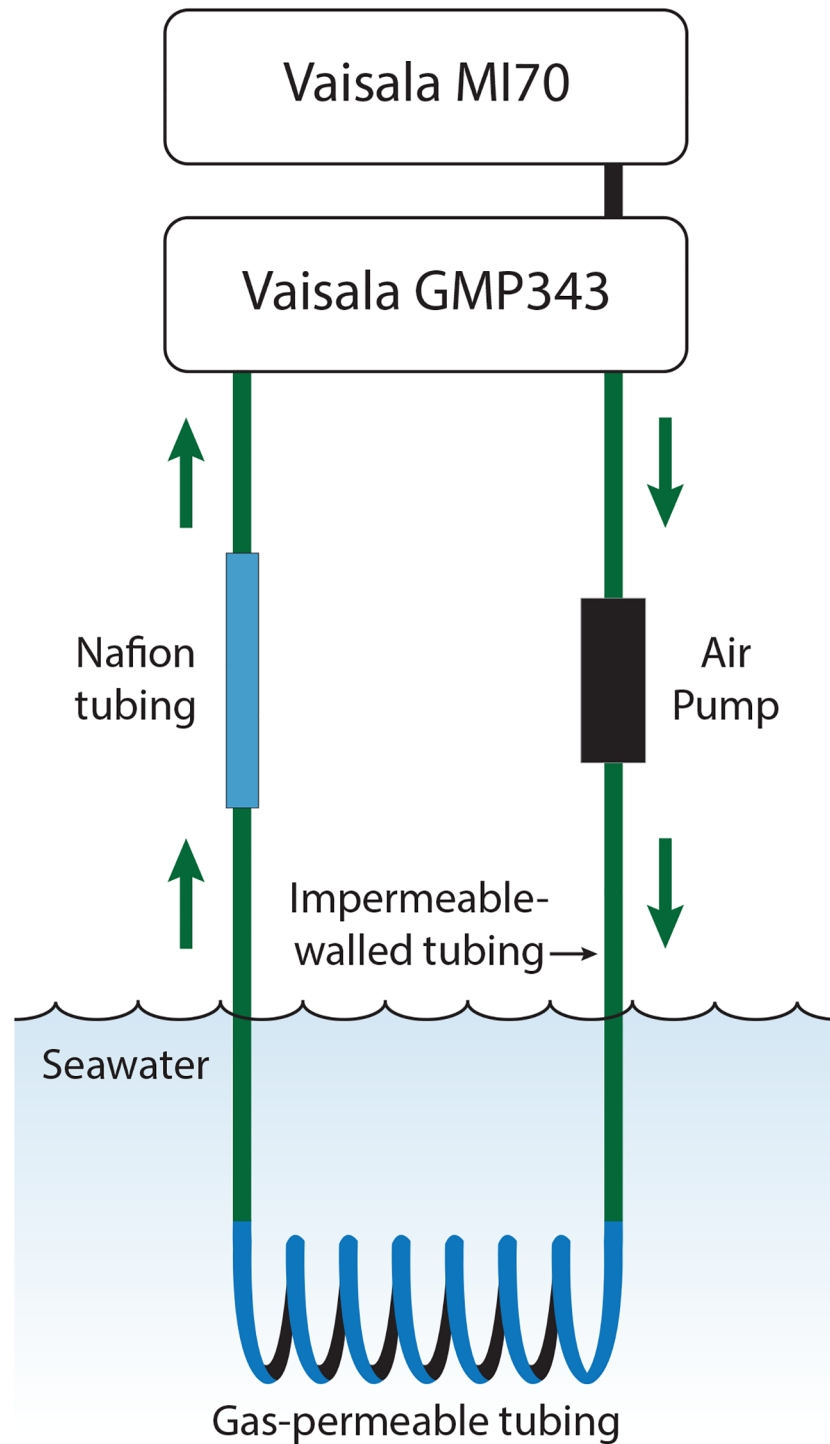
Fig 1. Diagram of the portable NDIR CO$_2$ equilibrator. The portable CO$_2$ equilibrator consists of a commercially available non-dispersive infrared (NDIR) CO$_2$ gas analyser and data logger display interface, in-line air pump, impermeable-walled tubing and a section of gas-permeable membrane that is submerged in water. Air is pumped in a closed loop around the system and equilibrates with CO$_2$ in seawater.

CO$_2$ data from the sensor were generated every 2 seconds and mean values recorded every minute by the data logger. Values were logged until CO$_2$ readings stabilised. The graph plot on the MI70 was used to visualise data to ensure an equilibrium state was reached (stable plateau of the graph, S1 Fig). The seawater CO$_2$ value was recorded when the system was at equilibrium. Data files stored on the data logger were downloaded using the software MI70 Link (version 1.06, Vaisala, 2002). Reported accuracy of the GMP-343 sensor configuration used is ±13 ppm at 400 ppm CO$_2$, ±25 ppm at 1000 ppm CO$_2$, and ±33 ppm CO$_2$ at 1400 ppm CO$_2$.

Carbonate chemistry calculations

Carbonate chemistry parameters were calculated in CO2SYS [22] using the constants K1, K2 from Mehrbach *et al.* 1973 refit by Dickson & Millero 1987, and Dickson for K(HSO$_4^-$). The pH$_{NBS}$ scale was used for calculations in CO2SYS using pH$_{NBS}$ electrode data and the pH$_T$ scale was used for calculations using data from spectrophotometric pH$_T$. For each of the four methods, raw data are presented, and have not been adjusted for any offset compared with expected values from certified reference materials (CRMs). Seawater temperature was measured with a temperature probe (C26, Comark, Norwich, U.K.). Temperature during the experiment in this open system was 26.9 ± 0.7˚C (mean ± s.d.). Salinity data were obtained from moorings around Lizard Island, which form part of the Australian National Mooring Network Integrated Marine Observing System (IMOS) operated by the Australian Institute of Marine Science [23]. During the study, salinity was 35.4 ± 0.0 (mean ± s.d.) and A$_T$ was 2291.8 ± 5.6 µmol.kg^{-1} (mean ± s.d.). Levels of total P and Si in seawater were below detection limits (total phosphorus <3.2 µmol.kg^{-1} SW as P, silica <8.1 µmol.kg^{-1} SW), and thus set to 0 for calculations in CO2SYS.

Data analysis

Estimates of pCO$_2$ were compared among methods using generalised linear models (GLM) with the statistical software R [24]. A Gaussian distribution was used to assess the relationship between pCO$_2$ estimates derived from the three different methods against those derived from C$_T$ and A$_T$, while the log-link function and quasipoisson distribution were used to compare estimated aragonite saturation state against the estimated pCO$_2$ values. Estimated measurement uncertainties were calculated for each method by determining the relative difference in each carbonate chemistry parameter from values derived from C$_T$ and A$_T$ as a reference. The root mean square error (RMSE) (= root mean square deviation, RMSD) [25] was then determined for each method for pCO$_2$, the saturation state of seawater with respect to aragonite (Ω$_{ar}$) and [H$^+$] (Table 2). Absolute differences were also calculated by taking the mean of the deviations (as positive numbers) for each measurement and are reported in the text for pCO$_2$, Ω$_{ar}$ and pH.

Results and discussion
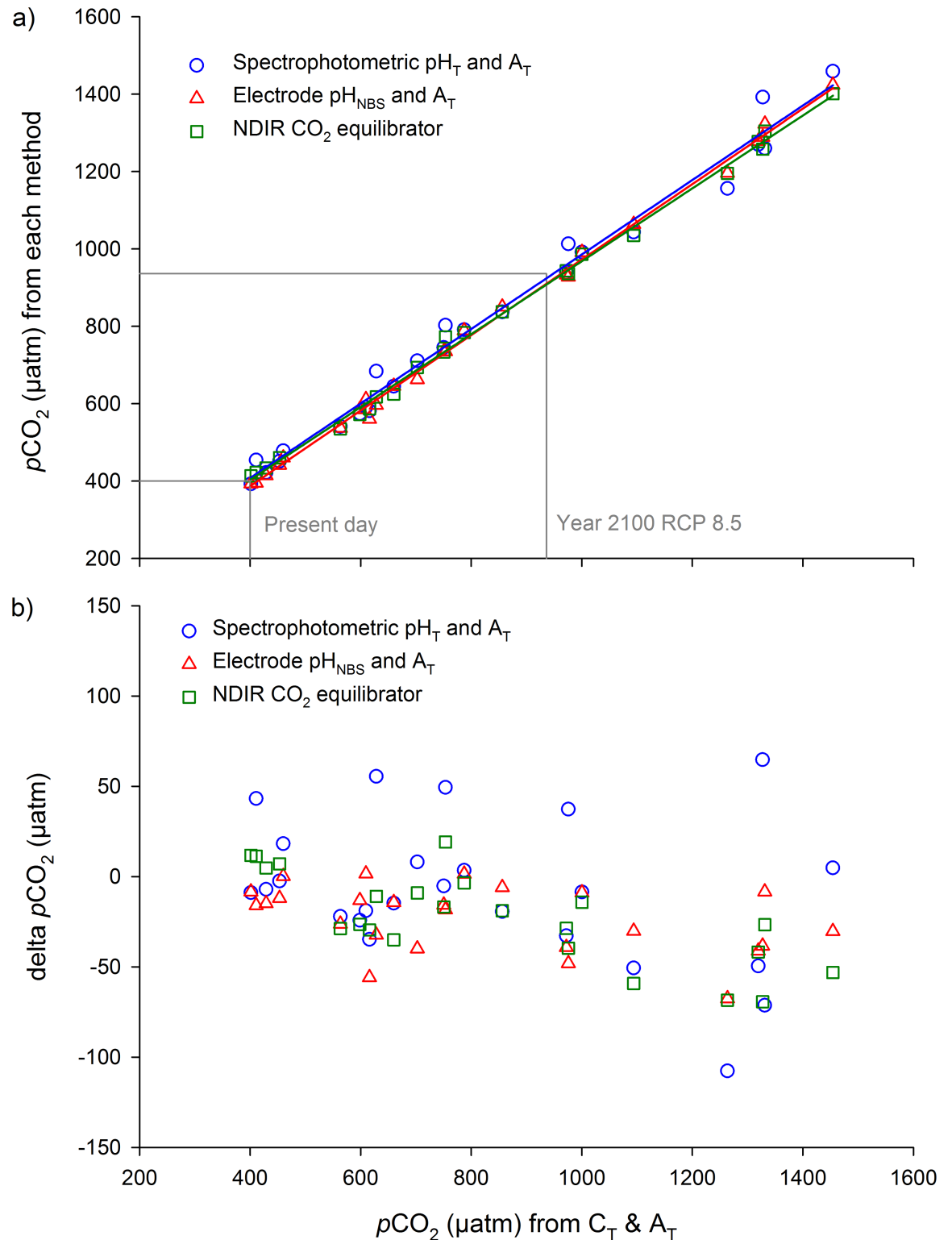
All four methods: 1) C$_T$, 2) spectrophotometric pH$_T$, 3) electrode pH$_{NBS}$, and 4) the CO$_2$ equilibrator, were compared across the pCO$_2$ range tested in this study: 370 to 1460 µatm (Fig 2).

Table 2. Estimated measurement uncertainties associated with each method determined from C$_T$ and A$_T$-derived reference values.

Parameter	Spectrophotometric pH$_T$ and A$_T$ uncertainty (%)	Electrode pH$_{NBS}$ and A$_T$ uncertainty (%)	NDIR CO$_2$ equilibrator uncertainty (%)
pCO$_2$	4.6	3.6	3.5
Ω$_{ar}$	3.3	2.7	2.7
[H$^+$]	3.9	3.1	3.0

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Fig 2. Seawater pCO$_2$ calculated from C_T and A_T, compared with three other methods: 1) spectrophotometric pH_T and A_T (n = 25), 2) electrode pH_{NBS} and A_T (n = 25), and 3) the direct measurement of seawater CO$_2$ with a NDIR CO$_2$ equilibrator (n = 23); a) for pCO$_2$ data and b) for the difference in pCO$_2$ compared to pCO$_2$ derived from C_T and A_T (delta pCO$_2$).

Estimated measurement uncertainties for pCO$_2$ from spectrophotometric pH$_T$, electrode pH$_{NBS}$ and the CO$_2$ equilibrator were ≤4.6% (Table 2). Overall, there was no effect of method on pCO$_2$ data when compared with pCO$_2$ data derived from C$_T$ and A$_T$ (GLM analysis after exclusion of the non-significant interaction terms between pCO$_2$ and method: F$_{2,69}$ = 2.60, p = 0.082, Fig 2).

A comparison of the four methods (C$_T$, spectrophotometric pH$_T$, electrode pH$_{NBS}$ and CO$_2$ equilibrator), showed there was no difference in their estimates of pCO$_2$ and aragonite saturation (GLM analysis after exclusion of the non-significant interaction terms between pCO$_2$ and method: F$_{3,160}$ = 0.148, p = 0.931, Fig 3). Each of the four methods is discussed in more detail below.

1) Determination of seawater dissolved inorganic carbon (C$_T$) and total alkalinity (A$_T$)

The combination of C$_T$ and A$_T$ is currently the preferred method for the characterisation of open ocean carbonate chemistry, and certified reference materials (CRMs) (sterilized natural seawater) for C$_T$ and A$_T$ are readily available [17] to ensure the accuracy and reliability of C$_T$ and A$_T$ determination. In this study, measurement standard deviations of C$_T$ and A$_T$ were within 3 and 2 μmol.kg^{-1} of CRMs, respectively, determined from repeat analysis of CRMs, run in conjunction with study samples. The sample processing time (approx. 23 min per sample) for C$_T$ and A$_T$ on the VINDTA allowed 7–8 samples to be processed per standard working
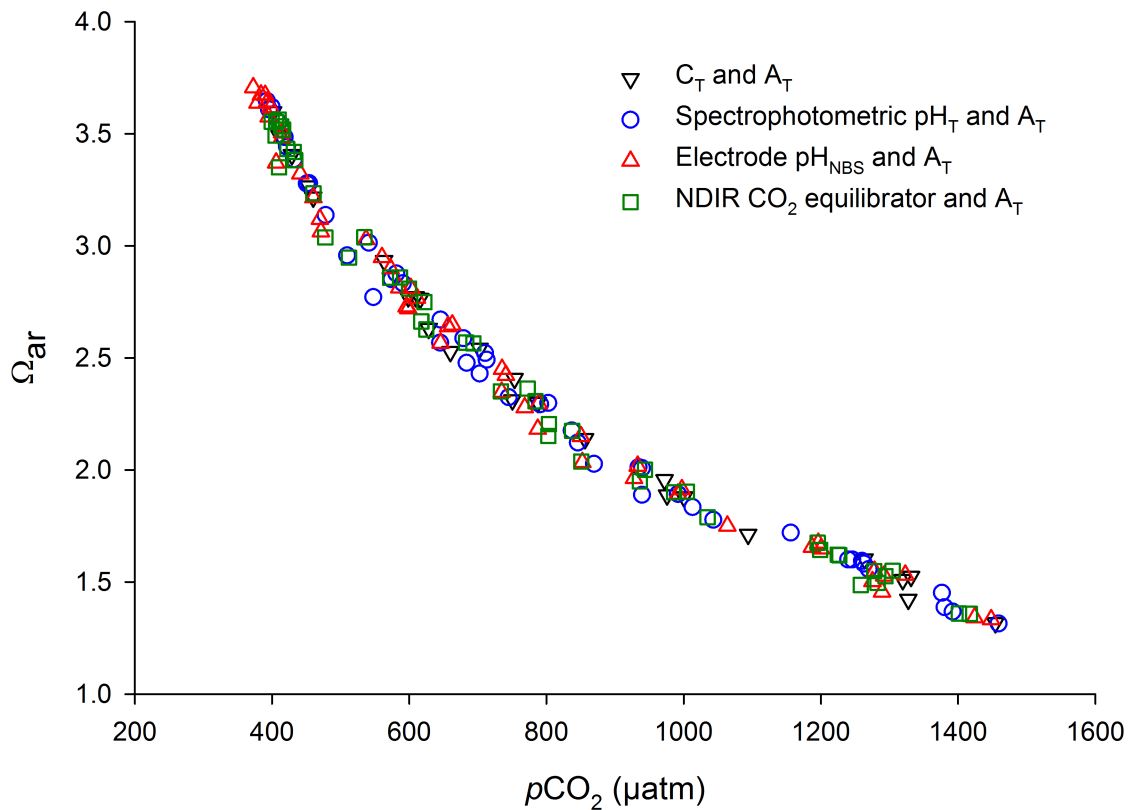
Fig 3. Relationship of seawater *p*CO₂ and aragonite saturation state (Ω$_{ar}$) determined by four different methods: 1) C$_T$ and A$_T$ (n = 25), 2) spectrophotometric pH$_T$ and A$_T$ (n = 45), 3) electrode pH$_{NBS}$ and A$_T$ (n = 49), and 4) the direct measurement of seawater CO$_2$ with a NDIR CO$_2$ equilibrator (n = 46).

day after appropriate machine warm-up (*c.* 2 hr) and control with CRMs, or 16–20 samples during an extended 11–12 hr shift.

In addition to the ease of availability of CRMs, the advantage of using C_T as a carbonate chemistry parameter is that water samples can be poisoned and analysed later at a convenient time. The disadvantages of C_T, however, are that 1) access to equipment to measure C_T (such as a VINDTA) can be limited and may be costly, 2) the often long time lag to obtain results with no immediate data for field or lab CO$_2$ perturbation experiments, and 3) the requirement to take and store many water samples. Additionally, water samples collected for C_T measurement must be air-tight as C_T values are affected by gas exchange. The disadvantages of preserved water samples include the fact they are heavy and freight can therefore be costly, that hazardous chemicals (mercuric chloride) are required to fix the samples, and that the shipment of seawater as corrosive and dangerous goods is controlled nationally and restricted internationally through customs. Hazardous chemicals also require specialist facilities for use (e.g. appropriate protective equipment) and proper disposal.

The advantages and disadvantages of A_T are the same as those for C_T, except that access to titration equipment, such as an automatic titrator or manual titration equipment, is more readily available and less costly, and A_T measurement is not prone to gas exchange. As such, A_T is routinely used as a second parameter in combination with other techniques.

2) Spectrophotometric determination of seawater pH$_T$

Seawater pH measured with a spectrophotometer using a procedure adapted from SOP 6b [19] for field station use produced values within a range of -0.0048 to 0.0087 (0.0012 ± 0.0045 mean ± s.d.) pH$_T$ units of certified Tris buffer in synthetic seawater. An accuracy within 0.01 pH$_T$ units of certified Tris buffer was achieved with the field system set-up used here, and replicate measures of the same seawater sample were within 0.005 pH$_T$ units.

Seawater chemistry calculated with spectrophotometric pH$_T$ and A_T produced pCO$_2$ estimates close to those calculated from C_T and A_T, with an average difference of 30.5 ± 26.1 µatm (mean ± s.d.). The estimated measurement uncertainty of pCO$_2$ using the spectrophotometric pH$_T$ and A_T technique was 4.6% (Table 2). Spectrophotometric pH$_T$ values were on average within 0.014 ± 0.010 (mean ± s.d.) of pH$_T$ values calculated from C_T and A_T, and Ω_{ar} calculated from pH$_T$ and A_T was on average within 0.06 ± 0.05 of Ω_{ar} values calculated from C_T and A_T.

The advantage of the spectrophotometric pH$_T$ method is that it produces pH values on the total scale (pH$_T$). Measurement of pH on the total scale is preferred [17] given the ionic strength of seawater. However, the disadvantages are that certified reference materials (CRMs) for spectrophotometric pH$_T$ (certified Tris buffer) are often limited [17], spectrophotometers may need custom modifications for seawater pH$_T$ measurement and are unlikely to be available 'off the shelf' (SOP 6b; [19]), and dye impurities can affect measurement accuracy [26]. Other disadvantages are that spectrophotometers can be large and bulky compared with pH electrodes and portable NDIR CO$_2$ sensors, and traditional spectrophotometers may not be suitable for transport to field stations. Smaller spectrophotometers have, however, recently become available and may be better suited to field use than traditional spectrophotometers.

Measurement of spectrophotometric pH$_T$ is an *in vitro* technique and requires more equipment and more time per sample than electrode pH measures. When working with seawater at temperatures >25˚C, such as in the tropics, samples must be first cooled to 25˚C. Consequently a standard heated water bath is not suitable, and a chiller bath or chilled room is required. We found achieving temperature precision (±0.1˚C) whilst chilling water samples to the specific temperature required was time consuming in a field setting. Temperature adjustment (cooling) of the sample to laboratory temperature (25˚C) required about 15–30 min.

Sampling processing time was around 2–3 min per sample; however, if sample temperature or absorbance at the non-absorbing wavelength changed, then the sample was re-run until the quality control criteria were met. Consequently, a custom-manufactured chiller unit with precision temperature control could be useful for spectrophotometric pH_T measurement for tropical ocean acidification experiments. Variation in carbonate chemistry data from spectro-photometric pH_T was likely due to the challenges of maintaining constant temperature (lower then ambient in the tropics) during sample analysis, even in a temperature controlled room. A more recent study describes a formula to use m-cresol purple over a range of temperatures [26] which may circumvent the requirement to measure the samples at 25.0˚C.

3) Electrode measurement of seawater pH_{NBS}

Electrode pH_{NBS} measurement produced pCO_2 estimates with an average difference of 23.5 ± 18.1 µatm (mean ± s.d.) compared with pCO_2 estimates derived from C_T and A_T. The estimated measurement uncertainty of pCO_2 using the electrode pH_{NBS} and A_T technique was 3.6% (Table 2). Electrode pH_{NBS} values were on average within 0.011 ± 0.008 (mean ± s.d.) of pH_{NBS} values calculated from C_T and A_T, and Ω_{ar} calculated from pH_{NBS} and A_T was on average within 0.05 ± 0.04 of Ω_{ar} values calculated from C_T and A_T.

There are some advantages of electrode pH_{NBS} measurement. Electrodes produced the most rapid measurement of seawater chemistry of all techniques assessed in this study, stabilising initially in a few minutes or less, and then typically in one minute or less for subsequent measures. Measurements can be taken over a range of seawater temperatures (although in much cooler waters, electrodes can take longer to stabilise), and 2 or 3 point (or more) calibrations are possible using readily available reference materials. Thus using an electrode to measure pH can allow the measurement of many tanks (e.g. 50+) per day, which can be useful for large ecological experiments with many replicates and field-based studies. With careful electrode calibration with certified reference materials (CRMs) and further cross-checks of electrode pH_{NBS} measures against pH_{NBS} calculated from NDIR CO_2 in combination with approximate expected or actual A_T, we found that it is possible to achieve pH accuracy comparable to estimated measurement uncertainties reported from non-state-of-the-art techniques that use reference materials (0.01–0.03 pH units) [17]. The benefit of recording immediate carbonate chemistry data for multiple tanks, and thus enhanced tank data resolution, is significant because any tank differences can be detected rapidly during the experiment and appropriate action taken during the experiment or in the analyses.

The disadvantages of pH_{NBS} electrodes is that the uncertainty in measuring can be up to 0.05 pH_{NBS} units for seawater measurements [17]. However, with careful use our results indicate that improved accuracy within ≤0.02 pH_{NBS} units can be achieved. In general, and to achieve the greatest measurement certainty, we recommend electrode pH_{NBS} measurements are validated by cross-checking data with another method, such as one of the three other methods evaluated here, to ensure accurate results. This is important because undetected, the potential uncertainty (of up to 0.05 pH units) [18] from pH_{NBS} electrodes may create uncertainty in estimated pCO_2 of around 50–150 µatm over the 375–1250 µatm pCO_2 range often used in biological ocean acidification studies.

4) Measurement of seawater CO_2 with a non-dispersive infrared (NDIR) gas analyser

The NDIR CO_2 equilibrator system gave very similar pCO_2 estimates to those derived from seawater chemistry using C_T and A_T, with an average difference in CO_2 values of 27.6 ± 19.8 µatm (mean ± s.d.). The estimated measurement uncertainty of pCO_2 using the

CO$_2$ equilibrator was 3.5% (Table 2). Ω_{ar} calculated from equilibrator CO$_2$ values and A$_T$ was on average within 0.05 ± 0.03 (mean ± s.d.) of Ω_{ar} values calculated from C$_T$ and A$_T$.

The ability to measure pCO$_2$ directly in seawater is particularly beneficial, firstly because pCO$_2$ is the key experimental target condition in many biological ocean acidification perturbation experiments, and secondly, because direct pCO$_2$ measurement allows for appropriate pCO$_2$ dosing in manipulation experiments when analysis of other carbonate chemistry parameters, such as A$_T$, is not immediately available. Recording pCO$_2$ directly could also save some of the time and cost required to process other seawater carbonate chemistry parameters (e.g. pH, A$_T$ or C$_T$, and the associated equipment) if pCO$_2$ is the principal carbonate chemistry parameter of interest in a study. Improved confidence in seawater carbonate chemistry can be achieved if A$_T$ is confirmed as a common unchanging value in experiments.

The CO$_2$ equilibrator itself too has several advantages. It is simple, portable, relatively low cost and reasonably rugged. Conveniently, CO$_2$ data are available in close to real-time. The closed-loop takes some time to equilibrate which makes the time lag to obtain results longer than electrode pH$_{NBS}$, but on a par with spectrophotometric pH$_T$. The time taken to reach equilibrium with a 12.2 m length of gas-permeable tubing was up to approximately 1 hour for each measurement (S1 Fig). Faster equilibrium times can be achieved if the starting CO$_2$ level is closer to the final CO$_2$, or if a longer length of gas-permeable tubing and/or shorter length of impermeable tubing or smaller diameter tube was used to reduce total system:permeable tubing air volume ratio. Potentially separate coils could be used and connected in turn to one NDIR CO$_2$ sensor close to stabilisation time to accelerate the process of obtaining measurements from multiple tanks.

The CO$_2$ equilibrator described here can be used in small bodies of water c. 10–20 litres in volume, and smaller versions can be easily made for even smaller water bodies (<5–10 litres). Alternatively, the equilibrator can be modified to use a 'shower head' device to spray seawater into a closed loop of air for use with small volumes of water and to reduce equilibration time. This shower head method is, however, more bulky in size than the membrane coil and consequently less portable for field use. Notably, the CO$_2$ equilibrator tested here provides a portable system that is light-weight and compact suitable for measurement in field laboratory situations, and is not intended to be compared to underway CO$_2$ measuring systems such as that described by Bandstra et al. [27].

In summary, the CO$_2$ equilibrator tested here is a simple, small, lightweight, relatively low cost device that provides a method for the direct measurement of CO$_2$ in water and is suitable for laboratory and field-based experimental studies. It is robust enough for use at field locations where pH may be the only other parameter of seawater carbonate chemistry that is immediately measurable. The CO$_2$ equilibrator can thus provide cost-effective, near real-time estimates of in-$situ$ seawater pCO$_2$ for biological experiments, providing a major advantage to biological perturbation experiments where achieving a desired pCO$_2$ is key.

Evaluation

In combination with A$_T$ as the second carbonate chemistry parameter, all four methods produced very similar pCO$_2$ estimates, and the three field methods 1) spectrophotometric pH$_T$, 2) electrode pH$_{NBS}$, and 3) NDIR CO$_2$ equilibrator, performed comparably to C$_T$ with careful use. In this study, electrode pH$_{NBS}$ and the CO$_2$ equilibrator gave consistently close results to C$_T$-derived pCO$_2$ values, and had the smallest ranges. Spectrophotometric pH$_T$ produced pCO$_2$ values that were on average slightly further from C$_T$-derived pCO$_2$, compared with electrode pH$_{NBS}$ and the CO$_2$ equilibrator. All methods calculated Ω_{ar} within ≤0.06, which is within the recommended <0.2 units [28].

When choosing a technique to use, consideration should be given to the required precision and accuracy, and the number and frequency of measures required. For example, rapid methods such as electrode pH$_{NBS}$ can provide the scope to measure many tanks requiring daily or more frequent assessment, whereas lower frequency techniques including methods that require water samples may be more suitable for experiments with fewer replicates. Due consideration should be given to the potential uncertainty inherent in all techniques, which can be larger for some methods, such as pH$_{NBS}$, without careful use. Consequently, the use of reference materials, and cross-validation wherever possible, is strongly recommended for all methods used.

Although sample measurement time is not necessarily long for C$_T$ and A$_T$ (i.e. 10–25 min once the system is calibrated and running), the limited availability of instruments to measure these at field sites often means such water samples are batch processed at a later time, often after the end of the experiment. The time lag to obtain results therefore becomes an important consideration. Spectrophotometric pH$_T$, electrode pH$_{NBS}$ and the CO$_2$ equilibrator provide data in real-time or near real-time (Table 1).

Other considerations in method choice include the availability of equipment and reference materials, and cost. For example, some techniques require sophisticated equipment, such as a thermostated spectrophotometer cell (e.g. SOP 6b; [19]) or VINDTA, which may not be available 'off-the-shelf' and require further custom manufacturing. Certified reference materials for techniques such as spectrophotometric pH$_T$ may also be difficult to acquire [17]; certified Tris buffers (from Prof. A.G. Dickson, Scripps Institution of Oceanography, U.S.A.), for example, are often in short supply. One option may be to collaborate with research groups who have access to the required equipment to ensure that carbonate chemistry quality is not compromised.

Conclusions and recommendations

Our results indicate that the portable CO$_2$ equilibrator used in conjunction with one of the other methods described here (C$_T$, spectrophotometric pH$_T$, or electrode pH$_{NBS}$) provides a suitable combination for estimating and maintaining pCO$_2$ levels in biological ocean acidification experiments. The other three methods (C$_T$, spectrophotometric pH$_T$, or electrode pH$_{NBS}$) all require a second carbonate chemistry parameter in order to determine pCO$_2$, and all four methods require a second carbonate chemistry parameter to calculate other parameters of the seawater carbonate chemistry system. A$_T$ is well suited for this purpose, and the measurement or calculation of A$_T$ is also useful to characterise the seawater used in the experiment. For perturbation experiments that manipulate C$_T$ (such as CO$_2$ injection), where A$_T$ remains constant, limited numbers of A$_T$ samples can be taken and analysed later (e.g. after the experiment). During the experiment, a NDIR sensor coupled with a CO$_2$ equilibrator can be used to ensure seawater pCO$_2$ in manipulation experiments is correct.

For all techniques, we recommend the used of certified reference materials (CRMs) to ensure high quality control for seawater carbonate chemistry and we recommend cross-checking measurements with another technique to further ensure quality control wherever possible. For example, cross-checking electrode pH$_{NBS}$ measures against pH$_{NBS}$ calculated from NDIR CO$_2$ in combination with expected A$_T$ can reduce uncertainty associated with electrode pH$_{NBS}$ measures whilst still allowing for high frequency sampling, such as in studies with high tank replication.

Importantly, we are not advocating the replacement of established methods to measure open ocean chemistry and constrain the ocean CO$_2$ system for real-time measures of ocean change, nor for the measurement of small changes in seawater pCO$_2$. However, for biological

perturbation experiments measuring differences of over 100 μatm pCO_2 among treatments, we find the four methods described here can be adequate and with careful use they can all produce similar results. Although methods such as the portable CO_2 equilibrator and pH_{NBS} electrodes do not replace standard methods, such as C_T and spectrophotometric pH_T, for high-accuracy quantification of carbonate parameters in seawater; they can, provide a cost-effective means to determine pCO_2 in large ecological experiments investigating the effects of ocean acidification on marine organisms providing options for greater tank and temporal resolution.

In summary, we show that all four combinations of methods tested here 1) C_T and A_T, 2) spectrophotometric pH_T and A_T, 3) electrode pH_{NBS} and A_T, and 4) the NDIR CO_2 equilibrator, can achieve pCO_2 values accurate enough for biological ocean acidification manipulation experiments with careful use. In addition, we find the portable NDIR CO_2 equilibrator tested provides a cost-effective system for near real-time measures of CO_2. For all methods, we recommend the used of certified reference materials (CRMs) and cross-checking data with another method to ensure quality control in biological ocean acidification experiments.

Supporting information

S1 Fig. CO_2 measurements recorded by the portable CO_2 equilibrator over time from the start of a test period until equilibrium is reached (boxed area). Stabilisation time was 1 hour. This time period is a conservative estimate since equilibration time is shorter if the pCO_2 difference between two samples is less.
(PDF)

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Author Contributions

Conceptualization: Sue-Ann Watson, Katharina E. Fabricius, Philip L. Munday.

Data curation: Sue-Ann Watson.

Formal analysis: Sue-Ann Watson, Katharina E. Fabricius.

Funding acquisition: Sue-Ann Watson, Philip L. Munday.

Investigation: Sue-Ann Watson.

Methodology: Sue-Ann Watson, Philip L. Munday.

Project administration: Sue-Ann Watson.

Resources: Katharina E. Fabricius, Philip L. Munday.

Validation: Sue-Ann Watson.

Visualization: Sue-Ann Watson, Katharina E. Fabricius.

Writing – original draft: Sue-Ann Watson.

Writing – review & editing: Sue-Ann Watson, Katharina E. Fabricius, Philip L. Munday.

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