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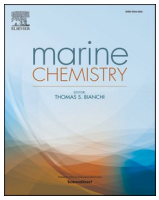

Consistency and stability of purified meta-cresol purple for spectrophotometric pH measurements in seawater

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ABSTRACT

Analysis of a global hydrographic data product showed a clear pH-dependent discrepancy between pH on the total scale measured spectrophotometrically (pH_{spec}) using purified meta-Cresol Purple (mCP) and pH calculated from total alkalinity and dissolved inorganic carbon. However, this was based mainly on US cruises, and three recent Japanese cruises do not show this pH-dependent discrepancy. One potential explanation is that purified mCP batches obtained from different institutions lead to significantly different pH_{spec}. Here, we tested this hypothesis by comparing the performance of purified mCP obtained from four different institutions. We demonstrate that consistent pH of ±0.0012 (95% C.I.) can be achieved regardless of the institution when impurities are properly removed. However, there was at least one batch from three of the four institutions that had significant pH-dependent errors that were as large as −0.008. The presence of impurities that led to pH-dependent errors was identified using HPLC and, for 8 out of the 9 cases, by spectrophotometry (although issues still remain for the latter). We conclude that pH-dependent errors due to impurities that remain after the purification process are, by themselves, too small to account for the differences observed between the recent set of cruises. Identifying the source of this difference should be a top priority. This study also highlights the importance of establishing robust quality assurance and quality control protocols to ensure consistent behavior with previously published equations to compute pH. We recommend a centralized system where one or a handful of institutions distribute purified mCP for the community, as this distribution approach will lead to lower prices and simplify quality assurance.

1. Introduction

Measurements of seawater pH saw a resurgence in the early 1990's due to advancements in spectrophotometric pH methodology using sulfonephthalein indicator dyes (Byrne and Breland, 1989; Clayton and Byrne, 1993). The most common indicator dye used in oceanography is meta-Cresol Purple (mCP), as the pK_A is in the nominal seawater pH range. Spectrophotometric pH measurements (pH_{spec}) were embraced by chemical oceanographers and the wider oceanographic community due to their excellent precision, reproducibility, and relatively quick sample analysis, and they are now considered the standard method for measuring seawater pH (Dickson et al., 2007). Seawater pH_{spec} is routinely utilized in a wide variety of oceanographic research areas, such as upwelling in eastern boundary current systems (Feely et al., 2008; Harris et al., 2013; Takeshita et al., 2015; Nam et al., 2015), time-series studies (Dore et al., 2009; Byrne et al., 2010; Bates et al., 2014), and ocean acidification experiments (Riebesell et al., 2010). Spectrophotometric pH has also been adapted for in situ sensing technology (Martz et al., 2003; Liu et al., 2006; Seidel et al., 2008; Rérolle et al., 2013; Wang et al., 2015). Furthermore, it is the primary method for calibrating ISFET-based pH sensors (Martz et al., 2010; Bresnahan et al., 2014; Takeshita et al., 2018) for global observational networks (Johnson

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et al., 2016; Bushinsky et al., 2019; Claustre et al., 2020). Thus, achieving consistent measurements of pH_{spec} has widespread implications.

Significant improvement in pH_{spec} measurements was achieved when impurities that altered the optical properties of the mCP dye were removed (Yao et al., 2007; Liu et al., 2011; Patsavas et al., 2013; DeGrandpre et al., 2014). These impurities typically caused the measured pH to be lower by an amount that followed a quadratic relationship with solution pH, and that approached −0.02 at pH greater than 8.1 (Liu et al., 2011). The biases are dye-lot specific, such that the levels and types of impurities that result from the manufacturing process are inconsistent from lot to lot. Purification seemed to solve this problem, however, producing pH measurements consistent to ±0.0005 when purified mCP from multiple commercial dye manufacturers were compared (Liu et al., 2011). In response to these significant improvements in the spectrophotometric pH metrology, multiple international institutions are now using purified mCP (Álvarez et al., 2020).

pH_{spec} measurements have become more routine on global repeat hydrography programs such as the Global Ocean Ship-based Hydrographic Investigations Program (GO-SHIP; Talley et al., 2016), and purified mCP dyes are increasingly commonly used for these measurements (Álvarez et al., 2020). However, while purified mCP improved the variability and repeatability of pH_{spec} between and among laboratories that used different lots of mCP over different cruises (Carter et al., 2018), it revealed a pH-dependent discrepancy between pH_{spec} and pH calculated from Total Alkalinity (TA) and Dissolved Inorganic Carbon (DIC; $pH_{TA,DIC}$) (Fig. 1, updated from Carter et al., 2018). Using a comparison between Ion Sensitive Field Effect Transistor (ISFET) pH sensors and pH_{spec}, pH-dependent biases in the spectrophotometric pH method were ruled out as a possible cause of this discrepancy (Takeshita et al., 2020), as Honeywell ISFET pH sensors have a Nernstian response (Takeshita et al., 2014). Thus the pH-dependent discrepancy was hypothesized to result from a combination of uncertainties that include an unaccounted source of alkalinity such as organic bases (Fong and Dickson, 2019).

The analysis in Carter et al., 2018 relied on pH measurements from U.S. institutions, where purified dyes were primarily (though not exclusively) sourced from R. Byrne's laboratory at the University of South Florida (USF). An updated analysis introducing hydrographic data in the 2019 release of the Global Data Analysis Project V2 (GLODAPv2) data product (Olsen et al., 2019) shows that data from 3 recent Japan Agency for Marine-Earth Science and Technology (JAMSTEC) cruises that utilized purified mCP from another source do not show this pH-dependent discrepancy, although a small offset still remains (Fig. 1). These data include GLODAPv2 secondary QC adjustments, but the slopes are significantly different ($p << 0.01$), with or without the pH adjustments applied. This raises the question: are the different slopes observed in Fig. 1 due to variable performance of 'purified' mCP between different institutions that purified them? While consistent pH_{spec} was demonstrated from mCP from a variety of commercial manufacturers and purified by a single laboratory (Liu et al., 2011), the comparability of mCP dye that has been purified by different institutions has not yet been assessed.

Here, we aim to characterize the variability of pH measured on the total scale using different batches of mCP that have gone through purification. We obtained purified mCP from 4 different institutions and compared measurements from these dyes to measurements with a reference dye to assess pH-dependent offsets. These offsets are sources of systematic errors when they differ from the behavior of the purified dye used to derive the spectral characteristics and pK_A of the mCP. Furthermore, the presence of light-absorbing impurities was assessed using spectrophotometry (Douglas and Byrne, 2017a) and HPLC (Liu et al., 2011; Patsavas et al., 2013). We compared the dye performance across multiple laboratories by making pH_{spec} measurements of equimolar Tris (2-amino-2- hydroxymethyl-1,3-propanediol) buffer in artificial seawater (pH = 8.0935 at 25 °C). Finally, we present mCP solution stability results over 2 years when the solution was stored as recommended, and an 'aggregated' dye perturbation correction approach for non-estuarine seawater samples as a best practice.

2. Methods

2.1. mCP purification

Purified meta-Cresol Purple dye in powder form was obtained from 4 different sources for a total of 9 dye batches: USF (n = 2), Sunburst Sensors (n = 2), JAMSTEC (n = 2), and Wako (n = 3); Wako purified mCP as a service for JAMSTEC. Two different lots of dye were obtained

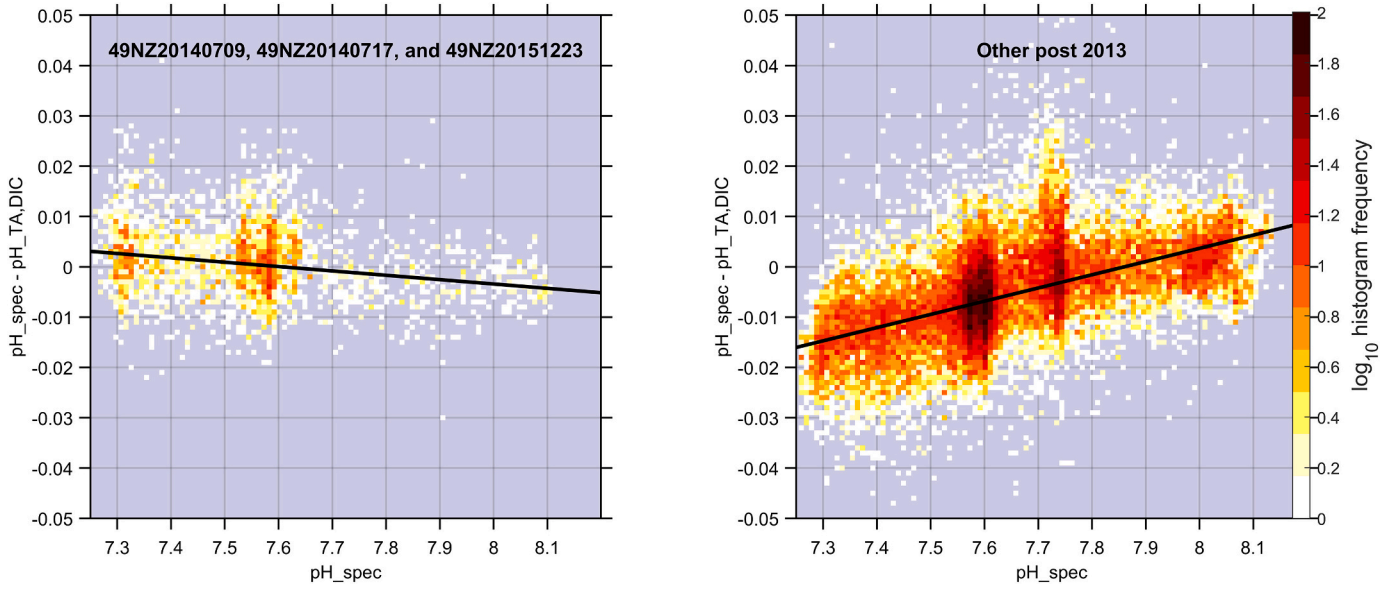

Fig. 1. Two-dimensional histograms showing the $\log_{10}$ frequency of the difference between measured pH and pH calculated from TA and DIC measurements versus seawater pH at 25 °C and 0 dbar. Data are limited to measurements after 2013 and from either Japanese led cruises (left, defined as cruises with expocodes beginning with "49") or from US cruises (right). Orange and red colors indicate 10's or 100's of measurements. Black lines are linear trendlines fit to the data shown. These data include GLODAPv2.2019 adjustments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from USF, Sunburst, and JAMSTEC, and dye was collected at three different elution times during the purification process from a single lot from Wako. JAMSTEC provided mCP for the cruise data shown in Fig. 1. The mCP dye was purified at USF and Wako using protocols outlined in Patsavas et al., 2013, and at Sunburst following protocols outlined in DeGrandpre et al., 2014. The mCP dye was purified at JAMSTEC following a procedure similar to the method of Patsavas et al. (2013), except that the gradient step was not used, and the pump flow rate was ~20 mL min^{-1}.

All dye solutions for pH measurements were prepared at the Monterey Bay Aquarium Research Institute (MBARI), and subsamples were sent to different laboratories for analysis as described below. Dye solutions were prepared to be ~2 mM, in a 0.7 M NaCl ionic background. The pH values of the dye solutions were adjusted to 7.8 ± 0.03 (range) by adding HCl or NaOH as needed. The absorbance ratio (R) of the dye solution was measured using a 0.5 mm pathlength quartz cell.

2.2. Assessment of pH dependent differences

Solutions ranging in pH from ~7.2 to ~8.2 were prepared at ~0.2 pH intervals by preparing Tris buffer with different Tris/$TrisH^+$ ratios in 0.45-μm filtered natural seawater, with a total Tris concentration of 0.08 mol kg-sol^{-1}. The objective was to prepare well-buffered solutions that relatively evenly spanned the target pH range of 7.2 to 8.2, thus final solution pH was not very important. The high buffer concentration ensured that solution pH did not change during the experiment, and allowed us to neglect the impacts of dye additions on the solution pH during analysis. Different ratios of Tris/$TrisH^+$ were prepared by adding 1 M HCl (Fisher SA48-1 or Acros 12421-0010) after adding Tris (Fisher T395-500) to solution. A new Tris/$TrisH^+$ solution was prepared directly before every experiment. Seawater was obtained from the flow-through seawater line at MBARI, pumped from 17 m depth. Seawater salinity was measured using a density meter (Mettler Toledo DM45), and ranged between 32.7 and 33.3 over different batches throughout the experiment. To ensure no carry over effects, the cell was manually emptied and refilled, then flushed with 50 mL of the new solution when switching solutions.

The pH of the Tris/seawater solution measured using mCP purified by the four institutions was compared to pH measured with a reference dye, chosen to be a lot obtained from USF, to ensure the purification process was consistent with that presented in Liu et al. (2011). During the measurements, the solution bottle was kept in a water-filled container that was controlled at the same temperature as the spectrophotometric cell. The solution was slowly stirred during the experiment (~1 h), and a soda lime trap was put in line with the vent hole to minimize effects of CO_2 on solution pH during the experiment. Spectrophotometric pH measurements were conducted sequentially with the experiment's dye and the reference dye, and repeated 3 times for each solution, resulting in 3 ΔpH measurements (pH_{exp_dye} - pH_{ref_dye}) per solution. The dyes were alternated using a multi-port syringe pump. The average precision for the pH measurements was ±0.0005 (1 σ), and 85% of the replicates had 1 σ lower than ±0.0008. Using this estimate of ±0.0008 as the precision, the average |ΔpH| must be greater than 0.0012 to be significant at the 95% confidence interval ($n = 3$). This experiment was conducted at 20 and 25 °C for all dyes, and recorded temperature was stable to 0.01 °C for all experiments, which equates to an uncertainty of ±0.0003 in solution pH. This experiment was conducted at MBARI.

2.3. Assessment of impurities

Douglas and Byrne (2017a) showed that the pH-dependent errors when using impure dyes are primarily due to the presence of other compounds that absorb light at 434 nm. The light absorbance due to impurities at 434 nm (A_{434imp}) can be estimated from the absorbance spectrum of mCP in a high-pH solution where only the basic (I^{2-}) form is present:

$$A_{434imp} = \left(1 - \frac{e_2}{e_3} R_{obs}\right) \times A_{434obs} \quad (1)$$

where e_3 and e_2 are the ratio of molar absorptivities and can be calculated from temperature and salinity (Liu et al., 2011; DeGrandpre et al., 2014), R_{obs} is the ratio of the measured absorbances at 578 and 434 nm, and A_{434obs} is the measured absorbance at 434 nm. Assuming that A_{434imp} is pH-independent and the contaminant compounds only absorb at 434 nm, A_{434imp} can be used to correct pH measurements made by an impure dye. Furthermore, A_{434imp} should be proportional to the amount of impurities in the mCP solution that absorb at 434 nm, and thus could potentially be used to assess the purity of a particular dye batch. Absorbance measurements were therefore made in 0.1 M NaOH in a 0.6 M NaCl background at 25 °C to obtain A_{434imp}, as recommended (Douglas and Byrne, 2017a). However, e_2/e_3 was calculated using formulations from DeGrandpre et al., 2014 because they characterized the molar absorptivity ratios in a 0.7 M NaCl solution, compared to artificial seawater used by Liu et al., 2011. These measurements were made at MBARI, USF, and Sunburst using the same dye solution prepared at MBARI. The reported A_{434imp} corresponds to absorbance at the isosbestic wavelength (487 nm; A_{iso}) of ~0.2–0.25. All nine dye solutions were measured at MBARI, eight at USF, and five at Sunburst.

2.4. HPLC

The different mCP dye solutions were also characterized on a Waters PrepLC HPLC system at USF. This system includes a Prep LC controller, two HPLC pumps capable of flow rates between 1 and 150 mL min^{-1}, a fraction collector, and a model 2998 Photodiode Array Detector. The Primesep B2 column from SIELC Technologies (Part B2-46-250.0510, 4.6 × 250 mm, particle size 5 μm) was used. The Primesep columns were housed in a Shimadzu column oven (model CTO-10A) at 30 °C. The HPLC mobile phase composition was 70% acetonitrile plus 30% H_2O; 0.05% trifluoroacetic acid (TFA) was present as a mobile phase modifier. The pump rate was 1.5 mL min^{-1}, and the injection loop volume was 20 μL. The Masslynx software allowed examination of the absorbance spectrum of the eluent between 200 and 600 nm as components eluted from the column. It is noted that the TFA in the mobile phase drops the solution pH to around 2, thus the mCP exists exclusively in the fully protonated form (H_2I), with absorbance peaks at 410 and 510 nm (strong reddish colour), and not at 434 and 578 nm, which are the absorbance peaks for the HI^- and I^{2-} forms, respectively.

2.5. Inter-laboratory consistency

In order to assess the consistency of pH measurements made from different laboratories using various lots of purified dye, the pH of equimolar Tris in artificial seawater (DelValls and Dickson, 1998) was measured at MBARI (9 dyes), USF (8 dyes), and Sunburst (4 dyes) at 25 °C using the same dye solution. Equimolar Tris buffer solutions (batch 34) were obtained from A. Dickson's laboratory at UC San Diego. Furthermore, spectrophotometric pH values of the same batch of equimolar Tris measured in the Dickson lab using the system described by Carter et al. (2013), and with purified mCP obtained from USF ($n = 12$; different batch than those tested in this study), were also included in this comparison.

2.6. Dye stability and perturbation corrections

The stability of mCP dye solution was assessed using the same protocol outlined in Section 3.2. Seven different dye solutions from the same dye batch obtained from USF (Dye 1) were prepared at different times over 23 months, and aged in solution form. Specifically, at the time of the experiment, the aged dye solutions tested here were 8, 11,

16, 18, 19, 22, and 23 months old. These aged dye solutions were compared to a freshly prepared dye solution from a separate dye lot obtained from USF (Dye 2); a separate dye lot had to be used for the control because we ran out of the original dye. The purities of both dye lots were verified using the HPLC analysis described above, and had indistinguishable pH measurements (Fig. 2). Dye solutions were prepared using the protocol in Section 2.1, with a mCP concentration of ~2 mM in a 0.7 M NaCl ionic background. Dye solutions were stored in borosilicate media bottles that were wrapped in aluminum foil and kept in a dark cabinet inside of a climate-controlled laboratory (18–22 °C).

The consistency of seawater pH dye perturbation corrections across seven dye batches, corresponding to dyes 1 and 3–8, was assessed. Dye perturbation corrections were assessed in filtered natural seawater sampled in 500 mL borosilicate bottles, with salinity between 33.4 and 33.5, and TA between 2240 and 2270 μmol kg^{-1}. Solution pH was adjusted by mixing ambient seawater with high-CO_2 seawater that was created by slowly bubbling 100% CO_2, and then waiting for at least 30 min before analysis. The pH of the solution was sequentially measured per bottle using different amounts of dye in a jacketed 10-cm cell. In order to keep absorbance measurements within the linear range of the spectrometer, dye additions targeted A_{iso} values of ~0.15, and ~ 0.3. Samples were drawn from the bottom of the bottle to minimize the impacts of gas exchange. Furthermore, each measurement requires 100 mL of sample, thus a total of 200 mL were used per bottle, leaving >60% of sample in the bottle which also reduced the effects of gas exchange. Dye perturbations at three different pH values (triplicate at each, $n = 9$ total) were assessed for each dye batch. Outliers, defined as greater than 2σ, were removed (3 out of 63). Reported dye perturbation is normalized to A_{iso} ($\Delta pH/\Delta A_{iso}$).

2.7. Spectrophotometric setup

At MBARI, absorbance measurements were made using an Agilent 8453 spectrophotometer using a semi-automated system based on the design described by Carter et al. (2013). A 10-cm jacketed cell was used to temperature control the blank and sample with a recirculating water bath. Temperature was measured in the jacketed waterflow directly after the cell using a thermometer (QTI DTU-6001-002) which was calibrated against a NIST-traceable thermometer (QTI DTU6028–002; ± 0.02 °C) between 1 and 40 °C. The temperature accuracy is estimated to be ±0.02 °C. Both the blank and sample/dye solution were kept in the cell for 5 min prior to making spectral measurements to ensure thermal equilibrium, as it is critical to accurately constrain temperature for solutions containing Tris (DeGrandpre et al., 2014). This long wait time for

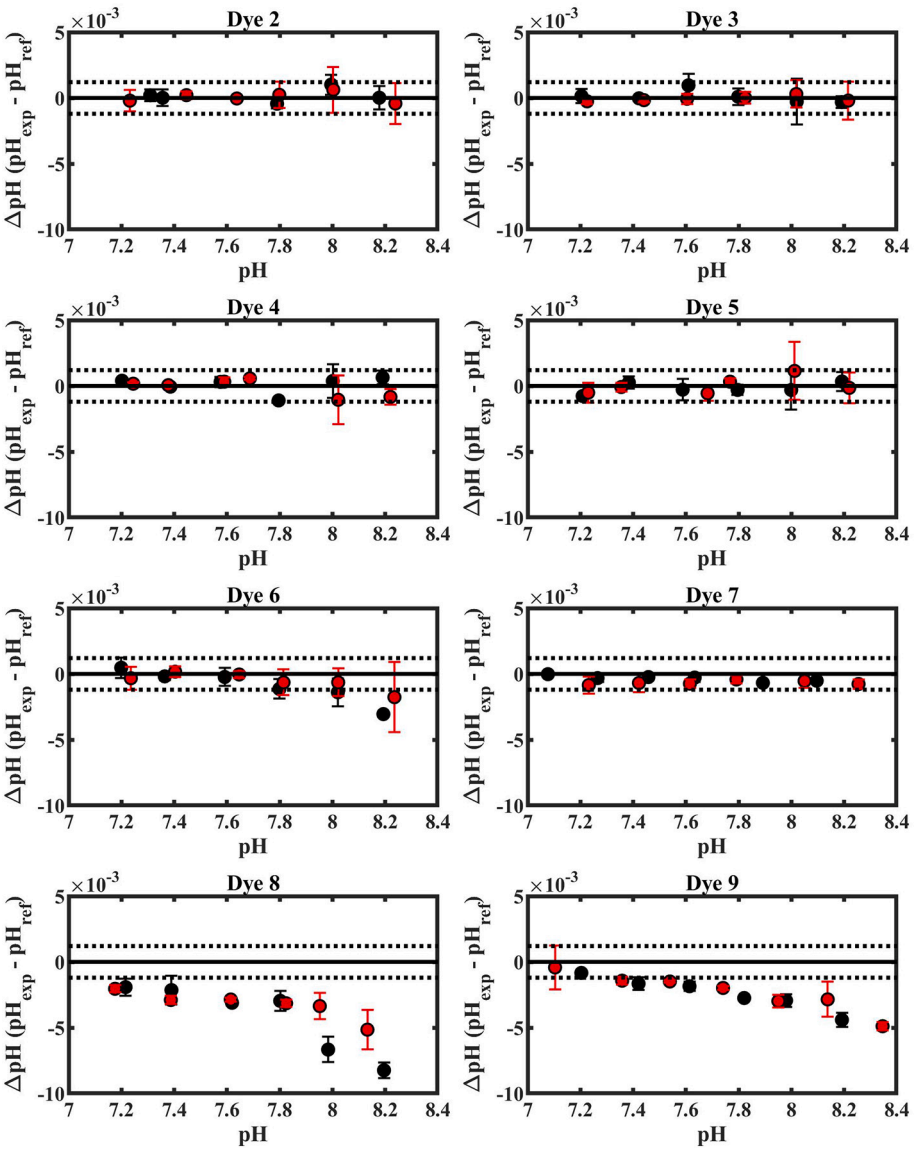

Fig. 2. ΔpH ($pH_{exp\_dye} - pH_{ref\_dye}$) as a function of solution pH obtained from the reference dye at 25 °C (black) and 20 °C (red) for the dye batches that were tested. Dye 1 is not shown because that is the reference. Error bars represent one standard deviation ($n = 3$). The dashed lines are ±0.0012, which is the 95% C.I. of the propagated uncertainty based on measurement precision. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

thermal equilibrium is essential to obtain accurate measurements for solutions containing Tris because of its large temperature coefficient. Shorter wait times led to more variable results. Three spectra each were taken for the blank and sample, then averaged. Dye additions were adjusted so A_{iso} was near 0.25. The same protocol was used for pH and A_{434imp} measurements. This protocol was also used for dye perturbation measurements, except that the wait time for thermal equilibration was 2 min. A shorter thermal equilibration time is sufficient for seawater measurements because the pH change with temperature is smaller than that of Tris solutions.

At USF, absorbance measurements were made on a Varian Cary 400 UV–Vis spectrophotometer with a custom cell holder. Ten-cm cylindrical cells (with an internal volume of about 27 mL) were used for the absorbance measurements. The cells containing the samples were pre-equilibrated to 25 °C in a temperature-controlled bath prior to the absorbance measurements to ensure samples were at the target temperature. Five absorbance measurements at 434, 578, and 730 nm were taken each for both the blank and sample, then averaged. An aliquot of 50 μL of dye solution was added to the cell, resulting in A_{iso} of about 0.2–0.25. Temperature of the internal solution was measured after the absorbance measurements using a thermometer that was calibrated to a NIST traceable thermometer with an accuracy ±0.03 °C, and this value was used for subsequent calculations. The same protocol was used for pH and A_{434imp} measurements.

At Sunburst, absorbance measurements were made using a Varian Cary 100 UV–Vis spectrophotometer. Ten-cm cylindrical cells (with an internal volume of about 27 mL) were used for the pH measurements. Samples were pre-equilibrated to the target temperature in a circulating water bath prior to being transferred to the measurement cells. Cells were filled with no head space to avoid gas exchange. Temperature of the cell was controlled using a water-jacketed cell holder, and the cells were kept in the holder for 10 min prior to making the blank absorbance measurements to allow the solution to thermally equilibrate. Three additions of dye were conducted, and absorbances at 434, 578, and 740 nm were measured after each addition. The volume of dye added depended on the pH of the solution, and was adjusted to keep the absorbances at 434 and 578 nm between 0.1 and 1.0. Temperature of the internal solution was measured using an Omega DP25-TH digital thermometer with a thermistor that penetrates through the stopper on the cell. This thermometer was calibrated to a NIST traceable thermometer, and we estimate the accuracy to be better than ±0.04 °C. The pH is calculated at the measured temperature, and corrected to 25 °C, and is the average of the pH measurements after each addition of dye. A slightly different protocol was used for A_{434imp} measurements. A 1-cm cylindrical cell (internal volume ~ 3 mL) was used, and only a single addition of dye was made, where A_{iso} was ~0.25. Temperature of the internal solution was measured using an Omega HH41 digital thermometer after absorbance measurements.

All solution pH values were calculated using formulations in Liu et al. (2011). All labs routinely verified wavelength and absorbance accuracy using NIST traceable standards.

3. Results

3.1. Assessment of pH dependent differences

In general, all batches of mCP dye showed good agreement with one another over a pH range of 7.2 to 8.2, and pH was consistent to 0.005 and 0.01 at 20 and 25 °C, respectively (Fig. 2). In fact, no significant difference was observed for the majority (six out of nine) of the dyes and, for these six dyes, agreed to better than ±0.0012 with the reference dye at all solution pH. At least one batch of dye from every institution showed consistent behavior with the reference dye. Dye 6 was only significantly different at solution pH > 8, and |ΔpH| was <0.003. Two batches of dye (Dye 8 and 9), however, showed significantly different behavior across most of the solution pH range, with a larger magnitude of ΔpH at higher solution pH. At 25 °C, the maximum discrepancy was 0.003 at pH 7.2, whereas the maximum discrepancy increased to 0.008 at pH 8.2. At 20 °C, the maximum discrepancy was 0.005 at pH 8.2. The ΔpH had a quadratic relationship with solution pH for these two batches of dye, similar to that observed in Liu et al., 2011 for dyes that contained impurities.

3.2. Impurity in mCP

A_{434imp} was significantly higher ($p < 0.05$) for the two batches of dye that had significantly different behavior than the reference dye in Fig. 2 (dye batches 8 and 9: 0.0014 ± 0.0005, $n = 17$) compared to those that did not (−0.0002 ± 0.0008, $n = 41$). In theory, A_{434imp} should be 0 for dye containing no impurities (dye batches 1–7), and the average across the three laboratories was not significantly different from 0 at the 95% confidence interval. Dye batch 7 however, had relatively high A_{434imp} at one laboratory, but did not show significantly different performance (Fig. 2). The impact of A_{434imp} on the calculated pH increases for higher solution pH, as absorbance at 434 nm is lower. Approximately, A_{434imp} of 0.002 can affect the calculated pH by roughly 0.002 and 0.004, at solution pH of 7.2 and 8.2, respectively.

HPLC analysis of the dye batches verified that impurities remained in some of the mCP dye batches (Fig. 4). The peaks that appear at ~24 and ~1.7 min represent mCP and the solvent, respectively, and are observed in all of the chromatograms; other peaks represent impurities. Impurities were clearly evident for dyes 8 and 9, which showed significantly different pH-dependent behavior relative to the reference dye (Fig. 2), as well as higher A_{434imp} values compared to the other dye batches (Fig. 3). Examination of the impurity absorbance spectra showed that the spectral peak centered around 400 nm for dye 8 and, for dye 9, spectra that were very similar to that of mCP in acid media. These impurities would affect pH measurements, as they absorb at 434 nm, 578 nm, or both. Chromatograms for individual dyes can be found in the supplementary materials.

3.3. Inter-laboratory consistency

Compared to the pH of 7 batches of dye measured at all 3 laboratories (Fig. 5), the pH of equimolar Tris buffer solution was lower for dye

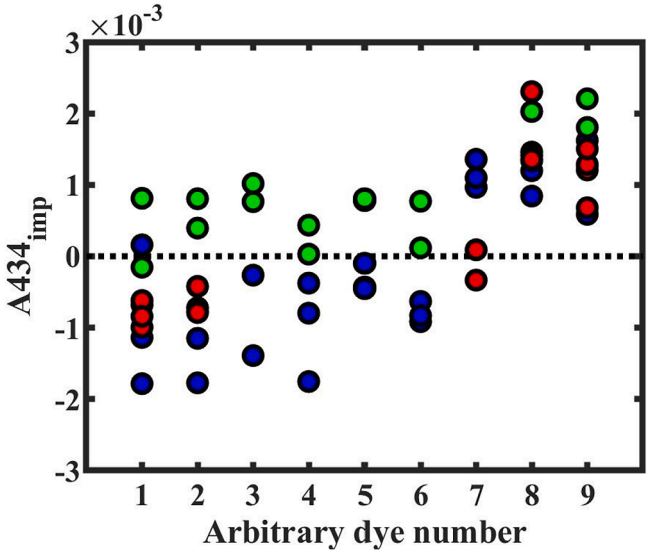

Fig. 3. A_{434imp} measured at the various laboratories (blue = MBARI, green = USF, and red = Sunburst). Dyes number 8 and 9 were the dyes that showed significantly different behavior from the reference dye in Fig. 2. Dye numbers correspond to those in Fig. 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

batches 8–9 that, based on HPLC analysis (Fig. 4), contained impurities. Measurements made at USF were consistent with the calculated pH values from DelValls and Dickson, 1998; the average difference across dye batches 1–7 was 0.0005 ± 0.0008 (1 σ). On the other hand, measurements from the other three laboratories were slightly lower, and were on average − 0.0032 ± 0.0004 (1 σ) for dye batch 1–7. The two batches of dye containing impurities were on average 0.005 lower than the other batches respective to the measurements made at each laboratory. Excellent agreement of better than 0.001 was observed between MBARI, SIO, and Sunburst (dye batch 1, 7, and 9). It is unclear why pH measurements at USF were approximately 0.003 higher than the other laboratories. It is noted that a temperature bias of 0.1 °C could account for this discrepancy, which could be caused by difficulties associated with accurately measuring temperature of a small volume of solution such as the thermistor touching the cell wall, or the thermal mass of the thermistor changing the temperature of the solution itself.

3.4. Dye stability and perturbation

There was no clear evidence that the performance of purified mCP solutions changed over the course of 23 months. Reproducibility of <0.001 pH was obtained over a period of almost 2 years over a pH range of 7.3 to 8.2 (Fig. 6). All stored dye solutions showed consistent behavior to better than 0.001 relative to the freshly prepared dye solution, except for one measurement at pH 8.2 from the oldest dye (23 months). However, the second oldest dye solution was 22 months old, and was not significantly different than the new dye solution. Therefore, this could have been caused by random error.

Addition of mCP dye to seawater perturbs the pH of the seawater (Dickson et al., 2007). The objective of this section was to determine whether different batches of purified mCP would perturb seawater pH differently. The effects of dye perturbation on the measured pH of seawater were repeatable from dye batch to dye batch, and the perturbation was zero near the dye pH of 7.8 (Fig. 7). The slope for individual dye batches were not significantly different based on a one-way ANOVA test ($p = 0.35$), although we note that with $n = 9$ for each dye batch, the statistical power to resolve a difference (0.084) is low. This suggests that dye perturbation effects do not have to be characterized for each batch of dye solution that is produced, but rather, a single 'aggregated' dye perturbation correction can be utilized, as long as the pH of the stock dye solution is adjusted accurately and verified (Section 2.1). These results are in general agreement with the magnitude of dye perturbation predicted from a chemical speciation model (Chierici et al., 1999). For example, model output predicted a dye perturbation of 0.006 when pH of seawater is 0.7 lower than the dye pH for a 5-cm cell, which is consistent with our results assuming the magnitude of dye perturbation for a 10-cm cell is half that of a 5-cm cell. In order to assess the error that can be expected if dye perturbation is characterized for each batch, the magnitude of the dye perturbation correction was calculated for pH 7.3 and 8.3 for all dye batches assuming A_{iso} of 0.25. The maximum difference between dye batches was 0.0015 and 0.0018 at pH 7.3 and 8.3, respectively. This error would increase proportionally with more dye added.

4. Discussion

The majority of the dye batches tested in this study (six out of nine), including at least one batch from each institution, produced consistent seawater pH measurements with the reference dye to better than ±0.0012 between solution pH 7.2–8.2 (Fig. 2). Another batch of dye had consistent behavior for most of the pH range except at pH 8.2, where the discrepancy increased to −0.0025. This demonstrates that consistent pH measurements can be achieved when mCP is purified properly, regardless of the source of the original commercial mCP manufacturer and the institution that conducted the Flash purification. However, two mCP dyes that went through the purification process (Dyes 8 and 9) had significant pH-dependent biases throughout most of the pH range (Fig. 2), caused by impurities that absorb in the relevant wavelengths that were not fully removed (Fig. 3 and Fig. 4). In the worst case, an error in pH of −0.008 was observed at pH 8.2, which was similar to or worse than the performance of some mCP directly obtained from the commercial manufacturer without purification (Liu et al., 2011; Patsavas et al., 2013). Dyes 8 and 9 also had significantly lower measured pH for equimolar Tris at all laboratories, validating these results (Fig. 5). These results demonstrate that while consistent behavior of mCP can be achieved from multiple institutions if purified properly, it is extremely important to verify the success of the purification process through stringent Quality Assurance (QA) and Quality Control (QC) protocols for

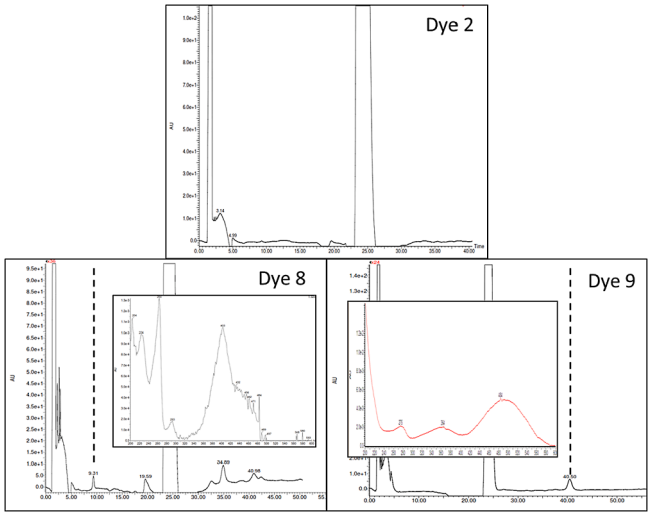

Fig. 4. HPLC results from 3 selected dyes. Dye 2 represents pure mCP with no impurities remaining, and dye 8 and 9 represent mCP that still contained impurities that absorb in relevant wavelengths. Insets for dye 8 and 9 show absorbance spectra (200–600 nm) corresponding to the peaks along the vertical dashed lines.

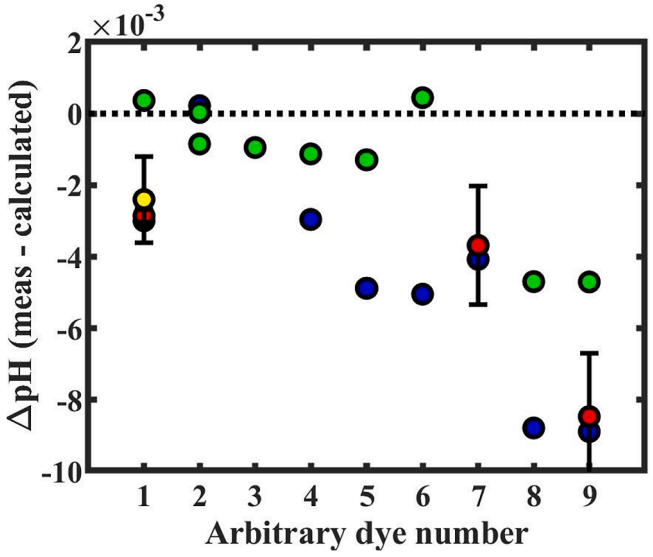

Fig. 5. The difference between pH of equimolar Tris in artificial seawater at 25 °C (B34 from A. Dickson; pH = 8.0935) and pH measured at 4 different laboratories (blue = MBARI, green = USF, red = Sunburst, yellow = SIO). Error bars represent 1 σ and are only shown when at least triplicates were measured, which were limited by Tris solution availability. The dye numbers are the same as Fig. 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

each batch of purified mCP.

HPLC analysis was able to identify the dye batches (Dye 8 and 9) that still contained impurities that led to pH-dependent errors (Fig. 4). For dyes 1–7, no peaks from impurities were observed in the chromatograms, whereas clear impurity peaks were observed for dyes 8 and 9. In general, the impurities absorbed more at 434 nm than at 578 nm. This leads to biases in spectrophotometric pH measurements, resulting in lower pH measurements at higher solution pH due to residual absorbance at 434 nm (Fig. 2). Our results indicate that HPLC is an effective technique to verify the molecular purity of the mCP as it provides the elution pattern of the components as the injection moves along the column, as well as the spectrum of each of the components.

The A_{434imp} results were consistent with HPLC and pH-dependent behaviors (Fig. 2), except for one dye batch at one laboratory. For example, Dyes 1–7 had an average A_{434imp} of -0.0002 ± 0.0008 (Fig. 3), were classified as pure based on HPLC, and showed pH measurement results that were consistent with the reference dye (Fig. 2). On the other hand, Dyes 8 and 9 had higher average A_{434imp} (0.0014) compared to the pure dyes. HPLC results verified the presence of impurities that absorbed at 434 nm (Fig. 4) in these two dyes, and both these dyes had pH-dependent biases in seawater (Fig. 3) and lower measured pH for equimolar Tris (Fig. 5). Dye 7 had similarly high A_{434imp} as Dye 8 and 9 at one laboratory, yet there were no impurity peaks in the HPLC analysis, and this dye had consistent behavior with the reference dye (Fig. 2). However, it is possible that this anomaly could be due to measurement error. Nonetheless, given that A_{434imp} for 8 of 9 batches of mCP reflected the observed pH-behavior of the dye, this demonstrates the potential for A_{434imp} measurements to be used as a simple QA measurement to identify optically impure mCP dye. This is attractive because measurements for A_{434imp} are easy to obtain relative to a complete HPLC extraction and analysis. However, before this can be implemented as a sole QA metric, further investigation is required to understand under what circumstances A_{434imp} can accurately identify optically impure dye, and what the limit of detection is for this technique.

It is important to note that A_{434imp} values are sensitive to which e_2/e_3 is used. For example, on average, A_{434imp} for dye batches 1–7 was -0.0022 when e_2/e_3 was calculated using formulations from Liu et al., 2011; negative values for A_{434imp} should not be possible, and in theory

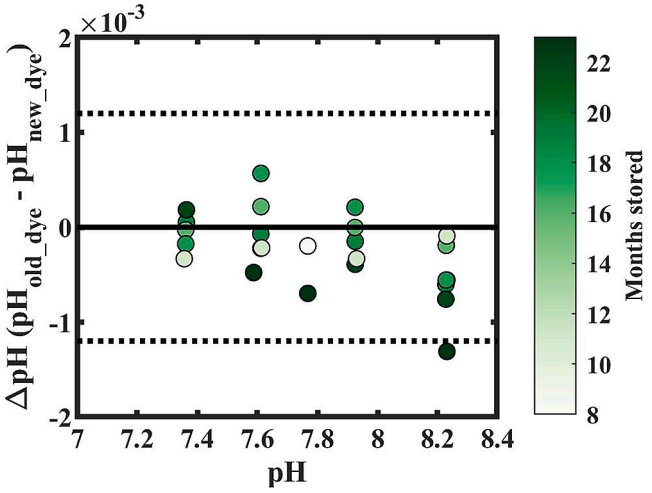

Fig. 6. Dye solution stability over 23 months, represented as the difference between mCP aged in solution form, and a freshly prepared mCP solution. Each point is the mean of 3 measurements, with an average standard deviation of ±0.0005; error bars are not shown for clarity. Darker green colors indicate older dyes, ranging from 8 months to 23 months. The dashed line is ±0.0012 and represents the 95% confidence interval. The one point that is significantly different is from the oldest dye solution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

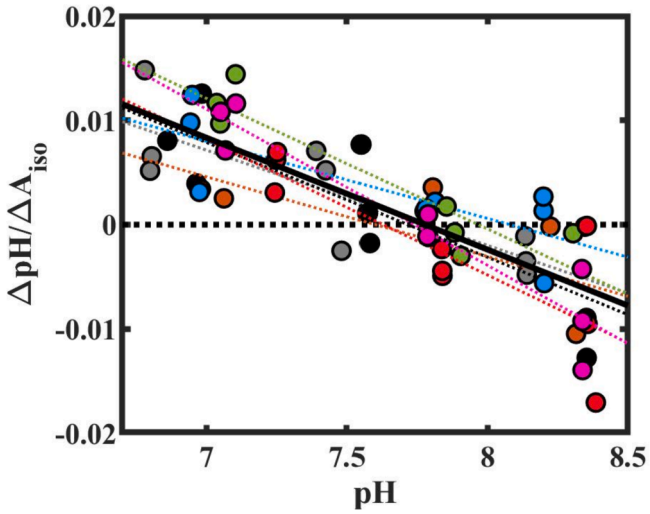

Fig. 7. Dye perturbation results from 7 batches of purified mCP. Different batches of dye are represented with different colors. Dashed lines represent linear regression made using individual dye batches, and the solid black line represents the linear regression when using all data. The horizontal black dashed line is zero.

A_{434imp} should equal 0 for pure mCP. The negative values are likely caused by matrix effects on the molar absorptivity ratios, since Liu et al., 2011 characterized e_2/e_3 in artificial seawater and A_{434imp} measurements are made in NaCl media. Thus, we suggest that values from DeGrandpre et al., 2014 should be used for e_2/e_3 for A_{434imp} measurements (which are made in 0.7 M NaCl media) moving forwards, but further validation is warranted. Furthermore, these molar absorptivity ratios were measured in 0.7 mol kg^{-1} NaCl solution and were not quantified over a range of ionic strength. Therefore the solution to measure $A_{434,imp}$ (i.e. 0.1 M NaOH and 0.6 M NaCl) should be accurately prepared. Finally, when calculating pH for seawater samples, we recommend using e_2/e_3 from Liu et al., 2011, as these ratios were quantified in artificial seawater using an iterative approach, which accounts for all absorbing species in solution.

The results from this study suggest that it is possible that pH-dependent biases could be introduced into hydrographic data that utilized 'purified' mCP (Fig. 1), since some 'purified' mCP could still contain impurities. The use of optically impure mCP will lead to a less-positive/more-negative slope in the pH-discrepancy between pH_{spec} and $pH_{TA,DIC}$, because the measured pH is biased low at higher seawater pH (Fig. 2). However, the largest pH-dependent bias we observed had a slope of approximately −0.01, which is less than half of the slope (0.023) between the pH-dependent discrepancy between pH_{spec} and $pH_{TA,DIC}$, and measured pH in the right panel of Fig. 1 (or about a third of the 0.031 slope obtained without GLODAPv2 inter-cruise consistency adjustments applied). Therefore, dye impurities are unlikely to fully account for the difference in pH-dependent discrepancies. Furthermore, the impurities in mCP cannot account for the offsets of about ±0.0075 at low seawater pH (Fig. 1a), because errors caused by dye impurities are minimal at low pH. Additionally, Fig. 5 suggests that there are still unexplained sources of inter-laboratory bias for pH_{spec} for equimolar Tris buffers in artificial seawater on the order of 0.004, even between experienced laboratories. It is possible that these biases could be larger, as differences of 0.008 have been reported in the literature (Müller and Rehder, 2018). Whether these biases are constant offsets or are pH-dependent due, for example, sample handling is not clear and should be investigated further. However, it is noted that pH_{spec} measurements of Tris buffers are very sensitive to temperature (approximately 0.003 pH per 0.1 °C), thus, small biases in temperature measurements can account for these differences. Seawater pH_{spec} measurements are much less prone to errors due to temperature biases, thus it is possible that

interlaboratory consistency would be better than that for Tris buffers, but additional comparison experiments are required for this assessment. Nonetheless, while the use of 'pure' mCP that still contained some impurities could have reduced the slope a bit, it is not enough to fully explain all of the differences between the US and recent Japanese cruises, particularly the offsets at low pH. Further investigation is urgently needed to reconcile these differences, such as the Fong and Dickson (2019) study that investigated many other potential causes for variations in pH discrepancy vs. solution pH.

There are several considerations that could improve the purification process for mCP. For example, selecting mCP lots that contain fewer contaminants is important. In particular, it is important to select mCP lots that have a clear separation of the mCP and contaminant peaks during Flash chromatography, as this indicates the contaminants have different chemistry than mCP, and thus are easier to separate. Therefore, the chromatograph of each lot of mCP should be visually inspected, and dye lots that have a shoulder on the main mCP peak should be avoided, as this might cause difficulties in separating mCP and the contaminants. In our experience, as different lots of mCP from the same commercial manufacturer can have differing amounts and types of contaminants, it is important to conduct this screening process for every lot of mCP. Therefore, if routine purification of mCP is planned, it is advisable to purchase a large quantity of a single lot that passes the screening process. In addition, it is important to frequently verify the purity of the product using HPLC. This is particularly true if a large stock of purified mCP is being accumulated. To avoid potential contamination, the purity of each batch of purified mCP should be verified before adding to the main stock. These recommendations extend to other indicator dyes used for spectrophotometric pH measurements such as thymol blue, bromocresol purple, and cresol red. However, based on our experience, it is noted that some indicator dyes are more difficult to purify than others, especially the lower solubility and lower pK_a dyes like bromocresol purple.

The measurements of equimolar Tris from the different laboratories do not provide a concrete assessment of whether spectrophotometric pH measurements made at one laboratory were more accurate than measurements made at others (Fig. 5). This is because the batch of equimolar Tris solution used in this study was prepared using the same recipe described in DelValls and Dickson, 1998, but was not standardized using a Harned Cell. The pH of equimolar Tris solutions that are carefully prepared in the same laboratory can be different by 0.0034 (Nemzer and Dickson, 2005), which is the same magnitude as our observed variability between laboratories. To properly assess accuracy, spectrophotometric pH measurements should be made in solutions that are directly traceable to the Harned Cell (Müller and Rehder, 2018; Müller et al., 2018). Nonetheless, all laboratories measured significantly lower pH with Dyes 8 and 9 compared to the optically pure dyes, providing evidence that the biases caused by the impurities are consistent and repeatable.

It is extremely encouraging, however, that pH from four laboratories in this study using Dyes 1–7 produced pH measurements that were consistent to 0.004 pH in equimolar Tris, and in fact 3 laboratories agreed to 0.001 (Fig. 5). This level of agreement (0.001) is comparable to a difference of ~1 μmol kg^{-1} of DIC or TA in a seawater sample with 2300 μmol kg^{-1} TA and 1950 μmol kg^{-1} DIC. All four laboratories used high quality spectrophotometers, where errors from wavelength accuracy and bandwidth resolution are minimal (DeGrandpre et al., 2014). Thus, this experiment reveals the differences that can be expected between laboratories that routinely conduct spectrophotometric pH measurements with high levels of expertise. This was similar to the level of variability observed in the intercomparison experiment when only examining the results of laboratories that used purified mCP, which was 0.004 (Bockmon and Dickson, 2015). Therefore, it is likely that the large overall variability in the Bockmon and Dickson (2015) intercomparison experiment (up to ±0.04), particularly for low pH samples, arises from a combination of sources such as sample handling (loss or gain of CO_2), dye perturbation correction, or variable quality, consistency, and maintenance of the spectrophotometers used, rather than the spectrophotometric pH method itself. Consequently, addressing these methodological issues for the majority of laboratories, rather than dye purity, is of primary importance for obtaining accurate pH measurements.

To our knowledge, the stability of mCP solution has not been reported in the literature. Our results indicate that there is likely no significant change in dye performance for approximately 2 years when dyes are stored in aqueous solution, and dyes are capable of producing consistent pH measurements to ±0.001 throughout this time when stored in the dark under climate controlled conditions. These are encouraging results, particularly for laboratories that run spectrophotometric pH measurements infrequently. However, it is emphasized that the results presented here represent one lot of dye stored under near-ideal conditions (i.e., climate controlled, dark environment, pH = 7.8), and further studies to verify these results are important. It is known that mCP degrades under UV light (Husheer, 2001), thus care should be taken to store these solutions in a completely dark location or otherwise the dye should be shielded with UV absorbing materials. Furthermore, plastic bottles and certain glasses are known to leach contaminants that can alter the pH of a solution (Huang et al., 2012). Therefore, storage in borosilicate bottles is suggested. Carbon dioxide uptake by the dye solution can also lead to changes in pH over time, thus, properly sealing the container is also recommended.

Currently, the best practices for pH_{spec} recommends that double dye additions are made to the samples to quantify the impacts of dye perturbation (Dickson et al., 2007). However, this significantly extends the time for sample analysis and also consumes more dye, which can be expensive for purified mCP. Furthermore, for spec pH measurements using long cell length such as 10 cm, the magnitude of the dye perturbation approaches that of the instrumental precision. Therefore, this low signal-to-noise could actually introduce errors into the corrections that rival the magnitudes of the dye perturbation corrections. Given these issues, some groups have been successfully using a dye perturbation relationship that is characterized for each batch of dye that is prepared, rather than conducting double dye additions for each sample (Clayton and Byrne, 1993; Carter et al., 2013). Our results presented in this study demonstrate that the effects of dye perturbation are repeatable between dye solutions and batches, as long as dye pH is adjusted properly and CO_2 absorption is minimized. Therefore, this indicates that dye perturbation effects do not have to be characterized for each batch, but rather, a single perturbation relationship can be utilized that has been determined using a large number of double dye additions. It is likely that the correction is system-dependent, thus every laboratory needs to conduct their own characterization. It is also advisable to check new solutions to verify that they are yielding adjustment relationships that agree with those determined for previous batches, as this will both validate the use of a bulk relationship and add additional constraints for the bulk adjustment. When conducting double dye additions, it is important to keep the absorbance within the linear range of the spectrometer, as adding too much dye could compound the effects of pH changes due to the addition of mCP and spectrophotometer errors, leading to erroneous corrections. Finally, since the magnitude of dye perturbation is dependent on the amount of dye added, and since the volume added can be precisely (though perhaps not accurately) quantified using A_{iso} as a proxy, it is advisable to determine dye perturbation corrections as a function of the change in A_{iso} rather than by assuming that identical dye volumes are delivered with each dye addition.

It is important to note that an 'aggregated' dye perturbation characterization is only applicable where the alkalinity of the solution of interest is similar to that used for the dye perturbation characterization (Li et al., 2020). This is because the magnitude of $\Delta pH/\Delta A_{iso}$ will increase at lower sample alkalinity because the solution is less buffered (Chierici et al., 1999; Li et al., 2020). Therefore laboratories that work with low salinity waters such as estuarine samples would need to characterize dye perturbation across a range of salinity (as a proxy for alkalinity) for the most accurate results (Mosley et al., 2004; Lai et al.,

2016). However, we note that the maximum dye perturbation difference using a stock solution of mCP in 0.7 M NaCl injected in samples with salinity ranging between 20 and 35, and pH ranging from 7 to 8.5, for a 10-cm cell (with $A_{iso} \sim 0.25$), is <0.001. These results are based on a dye perturbation model presented in Li et al., 2020, where the TA of the sample was adjusted in proportion with salinity. Therefore, unless work is done in an estuarine environment, the proposed 'aggregated' dye perturbation correction should be valid for all oceanic samples. For estuarine samples, dye perturbation effects may be large enough to obtain a robust signal-to-noise in low-salinity, weakly-buffered samples. Thus double dye additions may be a more practical approach for these samples.

5. Conclusions

In this paper, we assessed whether pH-dependent errors due to impurities remaining in 'purified mCP' could account for the different trend in $pH_{spec} - pH_{TA,DIC}$ observed in recent Japanese hydrography cruises relative to US cruises (Fig. 1). We tested the behavior of pH_{spec} using purified mCP from four different institutions, including the institution that supplied mCP for the Japanese hydrography cruises. We demonstrated that pH measurements using purified mCP from different institutions can be consistent to better than ±0.0012, when purified properly. However, there was at least one batch from three of the four institutions that had significant pH-dependent errors, that could be as large as −0.008. The magnitude of these errors increased with solution pH. However, we conclude that it is unlikely that the different behavior between pH_{spec} and $pH_{TA,DIC}$ for the recent Japanese cruises was solely due to remaining impurities in mCP, as the magnitude of this discrepancy was larger than any pH-dependent errors observed due to impurities in this study (Fig. 2). Therefore further research is needed to understand the cause of this discrepancy, including resolving inter-laboratory pH_{spec} measurement differences (Fig. 5), as it represents a considerable hurdle for creating a consistent global carbon dataset (Olsen et al., 2019). We suggest that future intercomparison experiments should consider use of buffers that are less temperature dependent than Tris.

The inconsistent behavior of purified mCP from the various institutions highlights the importance of establishing consistent and accurate QC protocols to verify that impurities have been sufficiently removed during the purification process. Such protocols will ensure that the purified mCP dyes will have consistent behavior with published equations to compute pH (Liu et al., 2011; DeGrandpre et al., 2014; Douglas and Byrne, 2017b; Müller and Rehder, 2018). Our results indicate that verifying the molecular purity of the purified mCP using HPLC analysis is an effective approach to detecting any impurities that may remain (Fig. 4). However, this requires expensive and specialized equipment and expertise, and will add cost and time for the institutions that purify the dye. Alternatively, measurements of A_{434imp} seems to be a promising QC tool, though there are outstanding issues that must be resolved before this approach should be implemented as the sole check on dye purity. It is imperative that the purity of mCP is verified prior to distribution and, if impurities are detected, they should refrain from distributing such batches of mCP. If a batch of impure mCP must be distributed due to logistical requirements, such as meeting a cruise deadline, coefficients for a 2nd order polynomial to correct the pH measurements (Liu et al., 2011) should be determined using protocols outlined in Section 3.2. We note, however, that it is not advisable for institutions to establish batch-specific coefficients to compute pH (e.g., pK_a and molar absorptivity ratios, $e_{x)}$, as this will greatly expand the complexity of inter-laboratory comparisons, and data synthesis and quality control efforts.

Given the challenges associated with establishing a consistent QC process across multiple institutions, we recommend a centralized approach where a small number of laboratories or institutions purify and distribute purified mCP for the oceanographic community. This will likely lead to lower cost due to economy of scale, as each facility will produce larger batches of purified mCP. Furthermore, it will be easier to conduct intercomparison exercises between a smaller number of centralized facilities at the appropriate frequency to ensure the quality of the purified mCP dyes. We have demonstrated that mCP dye solutions remain stable for up to 2 years when stored properly, but did not assess the stability of purified mCP in crystalline form. This would be an important assessment moving forward.

An alternate approach to addressing dye purity concerns would be to utilize a different indicator dye for spectrophotometric pH measurements of seawater, as some off-the-shelf indicator dyes such as thymol blue contain fewer impurities (Hudson-Heck and Byrne, 2019). This will likely lead to lower or potentially insignificant errors in pH stemming from dye impurities. Conducting spectrophotometric pH measurements using off-the-shelf indicator dyes would lead to substantial reduction in cost and potential mistakes in the future. However, further verification is required before such a recommendation can be made.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marchem.2021.104018.

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