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Determination of traces of phosphate in sea water automated by programmable flow injection: Surfactant enhancement of the phosphomolybdenum blue response

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ABSTRACT

An assay protocol, based on programmable Flow Injection (pFI), is optimized by tailoring flowrates appropriately to the individual steps of an assay, thus allowing sample and reagent metering, mixing, incubation, monitoring and washout to be carried out more efficiently and in different time frames. This novel approach to flow based methods is applied here to optimize the determination of orthophosphate at nanomolar levels. Programmable Flow Injection was also used to facilitate an investigation of the properties of the phosphomolybdenum blue (PMoB) formed during this assay, by using the stop flow technique – an approach that revealed for the first time the influence of surfactants on the kinetics of formation of PMoB and its spectral characteristics. It was discovered that the two most frequently used surfactants (SDS and Brij) have profound and different influences on the spectra and formation of PMoB and this finding was used to enhance the sensitivity of the phosphate assay at nanomolar levels. The method was applied to the assay of trace levels of phosphate in sea water.

1. Introduction and principles

1.1. Continuous flow analysis

Continuous Flow Analysis (CFA), discovered by Skeggs seventy years ago [1,2], is based on sample processing, performed while a sample is being moved by a flowing stream through a manifold to a flow cell. The key feature of the CFA method is air segmentation that prevents intermingling of adjacent samples, an intervention that would be detrimental to the result of the assay. The instrument (Auto Analyzer®) uses a multichannel peristaltic pump (Fig. 1A) connected to a manifold, where appropriate reagents are mixed with the air segmented sample while it flows through a sequence of mixing coils on its way to flow cell. Interestingly, the phosphate assay based on phosphomolybdenum blue (PMoB) colorimetry was amongst the first reagent based assays, automated by CFA [3]. Invention of Flow Injection Analysis (FI) in 1974 revolutionized the concept of Flow Analysis (FA) by showing that air segmentation is not essential for successful performance of flow-based assays [4]. Flow Injection used simplified instrumentation (Fig. 1B) and provided a faster sampling rate. Interestingly, the first real life application of FI was also automation of PMoB colorimetry [5]. There are by now almost 300 publications on the phosphate assay by FIA listed in Hansen's Database [6]. The PMoB method automated by CFA or by FI became the essential tool for performing nutrient assays and is now widely used in environmental research and is the standard method in chemical oceanography.

There are, however, several drawbacks to processing samples by means of a *continuous* flow:

- Reagents are consumed continuously during sample processing and also during startup and shut down periods.
- Use of a constant flowrate hinders optimization of the assay protocol, because it's individual steps: sample and reagent metering, mixing, incubation, monitoring and efficient washout can be carried out more efficiently, that is at different flowrates.
- CFA manifolds have large internal volumes needed to accommodate the air segmented stream. Due to the compressibility of air the flow path cannot be miniaturized and therefore the instrumentation is unwieldy.
- Sample residence times in CFA manifold are too long (8 min) to allow exploitation of the benefits that come from the fast kinetics of reagent based assays.
- Continuous flow methods are not suitable for studying the kinetics of chemical reactions and their spectral characteristics and therefore

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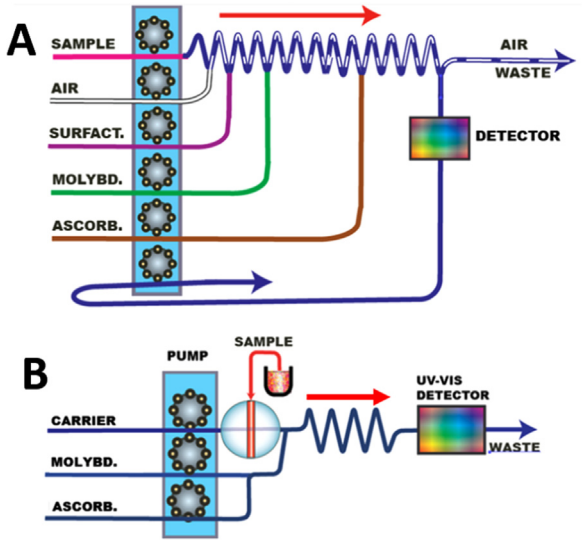

Fig. 1. A. Continuous flow air segmented analyzer flowchart configured for assay of phosphate. B. Flow Injection analyzer diagram configured for continuous Flow Injection analysis (cFI) of phosphate.

are difficult to optimize.

In spite of these disadvantages, the majority of flow based assays, are performed by continuous flow, the lasting legacy of Skeggs' Auto Analyzer®. This was evident on the recently organized voyage aboard the RV Investigator (CSIRO) from March 17th to 27th, 2017. This cruise, an Australian-run international nutrient inter-calibration cruise, was designed to compare advanced and current methodologies for nutrient determinations in seawater. The cruise brought together six international research teams from Australia, New Zealand, South Korea, China, Venezuela, and USA. The instruments and the methods used were, with a single exception, all based on Continuous Flow Air Segmented technology (CFA) and corresponding commercial instrumentation (SEAL Auto Analyzer AA3, SEAL QuAAtro39 Auto Analyzer, Technicon AutoAnalyzer II) [7].

1.2. Programmable Flow Injection

Flow programming, recently applied to Flow Injection (pFI), [8,9], avoids the shortcomings of CFA and conventional FI, by utilizing stopped, reversed and accelerated flowrates, tailored to the needs of the individual steps of the assay protocol. The key feature of the pFI instrument is a confluence point, situated at the center of the lab-on-valve (LOV) manifold, where sample and reagent inlets meet (Fig. 2, red circle). The essential components of the instrument are the two high precision bidirectional micro pumps, connected to the LOV manifold via two thermostated holding coils. Monitoring takes place in an externally mounted thermostated, long light path flow cell, interfaced to a spectrophotometer and light source by fiber optic cables. In contrast to all other flow-based techniques, sample and reagents are intermittently injected into a stream of deionized water, which serves as the carrier. In this way reagent consumption is minimized, the flow channels are thoroughly washed between each sample and the pumps, LOV and flow cell are filled with DI water when the instrument is idle.

The assay protocol for a two reagent assay, such as PMoB method has the following steps (Fig. 3):

1) The molybdate reagent (MO, blue), is aspirated by flow reversal into a holding coil (HC1).
2) The MO reagent is then transferred into the second holding coil (HC2) through the confluence point in the LOV where it simultaneously mixes with ascorbic acid (ASC yellow). The mixing ratio of Mo with ASC is controlled by combining the delivery rate from HC1 with the aspiration rate into HC2. The flowrates shown result in a Mo/ASC mixing ratio of 1/1.
3) The combined reagents (green) are then aspirated into the first holding coil (HC 1) through the confluence point, simultaneously mixing with the sample (SAMPLE, red). The mixing ratio of the combined reagent with sample is again controlled by the delivery rate from HC2 and aspiration rate into HC1. The flowrates shown result in a SAMPLE/ reagents ratio 4/1.
4) The reaction product (PMoB, orange) is then moved from HC 1 into the flow cell for monitoring by a forward flow generated by P1.
5) The flow channels, including the flow cell, are then flushed with DI water.

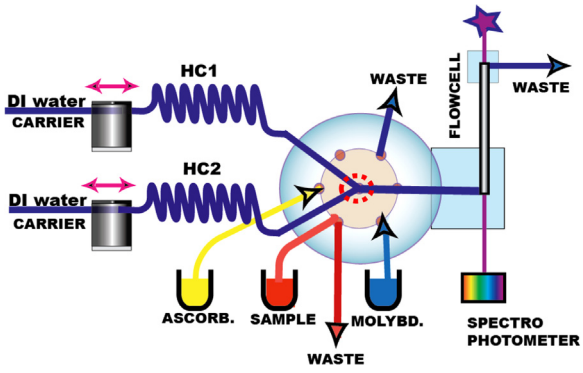

Fig. 2. Flow Injection analyzer based on programmable flow (pFI), configured for automated analysis of phosphate. The confluence point is marked by the red circle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

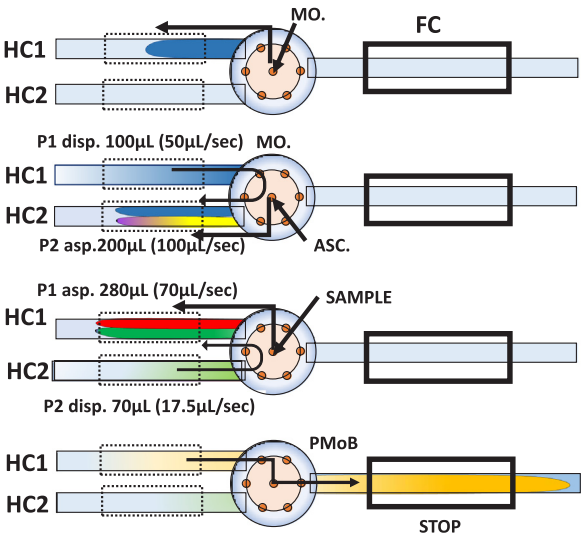

Fig. 3. Graphical representation of the assay protocol for phosphate determination. MO. molybdenum reagent, ASC. Ascorbic acid reagent, P1 and P2 pumps, HC1, HC2 holding coils. FC flow cell. For details see text.

Incubation of the sample with the reagents can take place either in the holding coil or within the flow cell. By stopping the flow in the holding coil (SHC mode) the reaction product will, as it moves through the flow cell, yield a response in the form of a peak. Alternatively, by stopping the flow when reaction mixture is within the flow cell (SFC mode, Fig. 3), the response is in the form of a reaction rate curve (Fig. 5) [8,9]. The SFC mode has been used throughout this work because it offers important insights into the reaction rates and the spectra of the reacting species, it offers higher sensitivity than SHC mode, and its results are not affected by changes in the refractive index at the

Table 1
(A) Reagent composition and (B) comparison of performance of CFA and pFI method.

(A)

Reagents	Autoanalyzer (SEAL Instruments)			pFI-PO_4 (this study)		
	Prepared conc.	Flowrates (mL/min)	Final conc. in FC*	Prepared conc.	Volumes (mL)	Final conc. in FC
MO (mM)	0.0153	0.23	0.002	7.653	0.100	0.957
Sb (mM)	0.379		0.042	0.359		0.045
H_2SO_4 (mM)	0.326		0.036	407.8		51
ASC. (%)	0.2	0.23	0.022	3	0.100	0.375
SDS (%)	1.18	0.42		1.5		0.19

(B)

	Autoanalyzer (SEAL Instruments)	pFI-PO_4 (this study)
Ratio of Mo/H^+	21.32	53.29
Incubation time (mins/assay)	8	1.5
Total volume (mL) of consumed reagents/assay	7.04	0.2
Sample/hour	30 samples	24 samples
Detection limit (μM)	0.030	0.032

* Final concentration has been calculated based on the flowrate.

interfaces of the various solutions [10].

1.3. Phosphomolybdenum blue method

Determination of phosphate by the molybdenum blue method is one of the most frequently performed reagent based assays. The PMoB is produced in two steps; the first one results in formation of a polymolybdate structure around the phosphate anion, while during the second step, the yellow phosphomolybdate is reduced to a compound that appears to be blue [11]:

$$PO_4^{3-} + 12MoO_4^{2-} + 27H^+ \rightarrow H_3PO_4(MoO_3)_{12} + 12H_2O \quad (1)$$

$$H_3PMo(VI)_{12}O_{40} + Reductant \rightarrow [H_4PMo(VI)_8Mo(V)_4O_{40}]^{3-} \quad (2)$$

Both reactions are best performed in strongly acid solution (pH 0–1) in order to prevent interference from molybdenum blue (MoB) as well as from SiMoB which is formed in presence of silicic acid, albeit more slowly and under more mildly acid conditions.

A variety of reducing agents has been proposed in the perpetual quest for optimization of the PMoB method, resulting in bewildering variety of suggestions and descriptions of the properties of the phosphomolybdenum blue species. Briefly, nonmetallic reductants form PMoB with a single peak spectrum and molar extinction coefficient (ε = 7000 at λ 700 nm), and this spectrum changes upon heating (ε = 26,000 at λ 880 nm). Metallic reductants (such as $SnCl_2$) form PMoB with a twin peak spectrum (ε = 19,000 at λ 700 nm and ε = 16,000 at λ 620 nm). In the presence of Sb and a nonmetallic reductant (such as ascorbic acid) SbPMoB is formed, with a twin peak spectrum (ε = 20,000 at λ 880 nm and ε = 15,000 at λ 710 nm). Papers dealing with the properties of the various forms of PMoB, published between 1925 and 2012 list molar extinction coefficients anywhere between ε = 1000 and ε = 32,000 depending on the wavelength and reducing agent used (Fig. 6. and Table 3. in [Ref. 11].) These data and the wealth of additional information gathered in the recently published comprehensive review of PMoB chemistry [11], document the complexity of chemical reactions on which PMoB assay is based. As the authors of this outstanding work observed: “These significant differences (in spectral characteristics) are noteworthy, since there is, of course, only one actual 'molar absorptivity' for a particular compound at a given wavelength”. The question is therefore, to what extent are we dealing with several PMoB species (of different oxidation states or composition), or whether differences in reported spectral characteristics are due to uncorrected baseline, or assumptions about apparent absorptivity, versus actual molar absorptivity, or are due to the presence of different forms of PMoB, or are they caused by reactants that have not been considered to participate?

In the confusing multitude of proposed PMoB based assays, developed during last 70 years, the seminal work of Murphy and Riley published in 1962 [12], provides the foundation on which the majority of presently used, flow based assays, are based. This is because of the use of ascorbic acid in the presence of Sb as the reductant, which enhances the rate of formation of molybdenum blue, makes the method suitable for automation by CFA and by Flow Injection. Yet another lasting impact on the present concept of PMoB chemistry was Murphy and Riley's finding, that antimony is incorporated into the complex as $PSb_2Mo_{12}O_{40}$, which exhibits a twin peak spectrum at **λ** 710 nm and **λ** 910 nm, while the PMoB spectrum obtained without antimony has a single peak at **λ** = 850 nm. (Fig. 1. in ref. [12]). This finding was later confirmed by Going and Eisenach [13] and it is now accepted (Fig. 9. ref. [11]), although at present, the majority of methods that use ascorbic acid with SbK tartrate employ **λ** 880 nm for measurement of phosphate content. Since the purpose of this work is to develop an assay suitable for trace analysis of phosphate in sea water, we decided to investigate the kinetics of formation and spectral characteristics of phosphomolybdenum blue by using the modification of the Murphy-Riley method because it is commonly used in oceanography in CFA or Flow Injection format. Therefore, the composition of reagents (Section 2.2) in this work is identical or close to the ones most frequently used in oceanography [14–16] (Table 1).

Automation of the PMoB based assay by CFA necessitates addition of a surfactant to the air segmented stream (Fig. 1A) in order to maintain a regular pattern of air bubbles that prevents intermingling of neighboring samples. Yet another purpose of adding surfactant is to assist in the washout of PMoB and of reagent residues from the manifold and flow cell, since such contamination causes baseline drift and carryover. AutoAnalyzer® technology, predominantly used Brij 35, a nonionic surfactant and SDS [3], a negatively charged surfactant. Since then use of various surfactants has continued in both CFA based commercial systems (Seal Analytical, Astoria Pacific) as well as in

commercial FIA applications (Lachat Instruments, FIAlab Instruments.). For automation of the PMoB method, both research and commercial instruments mostly use SDS. While universally used, surfactants and their role is in all publications mentioned almost as an afterthought, limited to a scant description in the reagent section. Yet, interference of surfactants on the PMoB assay was observed as early as in 1976 [17], when soaps and surfactants were found to interfere in the determination of phosphate in household and industrial effluents by the manual PMoB method. Also, in the recently published comprehensive review of PMoB chemistry [11] surfactants are only viewed either as an interference (Section 4.3.3 in REF [11]), or as a means to prevent the coating of the channels of flow systems.

1.4. Trace analysis of soluble phosphate in sea water

The quest for increasing sensitivity and improving the limit of detection of the phosphate assay in sea water by spectrophotometry has recently advanced by employing two different strategies: extending the light path of the flow cell, by using up to 1 m long liquid core waveguide (LCW) [18,19], or by preconcentrating the PMoB from a large volume of sample by co-precipitation (MAGIC) [20] or by Sorbent Extraction. However, implementation of LCW and MAGIC present difficulties, since LCW is affected by the coating of the large area of the flow cell walls by PMoB, while the MAGIC method is labor intensive. Therefore, automated preconcentration by Sorbent Extraction (SE) using FI or SI is gaining in popularity, also because polymer based sorbents are efficient and readily available [21–24].

These advances have improved the limit of detection down to 10 nM P level, when sorbent extraction was automated in the Sequential Injection format [22] or by using LCW combined with Flow Injection [19].

In this work we will use programmable Flow Injection to gain a better understanding of PMoB chemistry and to develop an optimized assay protocol based on the investigation of:

- The kinetics of PMoB formation in the absence or presence of surfactants.
- The spectra of PMoB in the absence or presence of surfactants.
- The influence of surfactants on the sensitivity of the PMoB method.
- The influence of the salinity of the sea water matrix on the performance of PMoB method developed in this work.

2. Experimental

2.1. Instrumentation

The instrument, miniSIA-2 (GlobalFIA, Fox Island, WA, USA), fitted with a 13 cm long thermostatted flow cell of internal volume 65 microliters [25], was used without any modifications. The instrument comprises two high precision, synchronously refilling milliGAT pumps, two thermostatted holding coils, and a 6-port lab-on-valve module with an external flow cell (Fig. 2). All tubing connections, downstream from the milliGAT pumps including the holding coils (volume 1000 μL), were made with 0.8 mm I.D. polytetrafluoroethylene (PTFE). The conduits between the carrier stream reservoirs and the milliGAT pump were made from 1.6 mm I.D. PTFE tubing in order to minimize degassing under reduced pressure at higher aspiration flowrates. A spectrophotometer (USB4000, Ocean Optics, Dunedin, FL) and a tungsten light source within the miniSIA-2 instrument were connected to the flow cell using optical fibers. All assay steps were computer controlled using FloZF software supplied by Global FIA (Fox Island, WA, USA).

2.2. Reagents and materials

Deionized (DI) water was prepared using a Barnstead Water Purification System, Nano Pure Diamond (Thermo Fisher Scientific, www.thermofisher.com). When DI water is used as the carrier stream air dissolved within it can exsolve forming bubbles that compromise pumping accuracy and spectrophotometric measurement. To eliminate this problem, DI was either stored over night before use or was deaerated by stirring under a mild vacuum.

Potassium antimony tartrate stock solution was prepared by dissolving 0.45 g of Potassium antimony(III) oxide tartrate trihydrate ($C_8H_4K_2O_{12}Sb_2{\cdot}3H_2O$, Sigma-Aldrich, https://www.sigmaaldrich.com) in 45 mL of the DI water. The solution was stored in the refrigerator.

Mixed Molybdate Reagent (ammonium molybdate/Sb/acid solution) was prepared by dissolving 0.4 g of ammonium heptamolybdate tetrahydrate, ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, certified ACS. Fisher Scientific, https://www.fishersci.com) in ~100 mL of deionized water in a 250 mL bottle, to this was added 4 mL of potassium antimony tartrate stock solution and 8 mL of conc. H_2SO_4 (certified ACS. Fisher Scientific, https://www.fishersci.com), DI water was added to make a final volume of 200 mL.

3% Ascorbic acid solution was prepared by dissolving 1.2 g of L (+)-Ascorbic acid ($C_6H_8O_6$, Alfa Aesar, https://www.alfa.com/) in 40 mL of DI water and was prepared daily.

Brij L23 solution is a nonionic surfactant, (Sigma-Aldrich, https://www.sigmaaldrich.com) with Critical Micelle Concentration (CMC) = 0.091 mM, 0.01%

3% Brij in 3% Ascorbic acid solution was prepared by dissolving an appropriate amount of ascorbic acid in 3% Brij solution.

Sodium dodecyl sulfate is an anionic surfactant (SDS, $CH_3(CH_2)_{11}OSO_3Na$, Affymetrix, Thermo Fisher Scientific, www. thermofisher.com), an anionic surfactant mw. = 288.5 g, CMC = 6–8 mM, (0.1728–0.2304%, w/v)

6% SDS in 3% Ascorbic acid solution was prepared by adding an appropriate amount of solid of SDS into 3% Ascorbic acid solution.

Note that the concentration of surfactants listed in the following sections are the concentrations of surfactants in the final reaction mixture that is monitored within the flow cell. Since reagents are diluted eight times in the flow system (Fig. 3) the ascorbic acid-surfactant reagent concentrations described above will yield 0.001% Brij35 and 0.75% SDS in the flow cell.

A 100 μM stock standard phosphate solution was prepared by diluting a commercial PO_4 standard containing 1000 ppm Phosphate Standard solution (LabChem, https://www.labchem.com/) in DI water.

The stock solution was further diluted to obtain working standards in DI water and seawater.

" Phosphate free" sea water samples were collected from the Hawaii Ocean Time-series station ALOHA, in the North Pacific Ocean, and were filtered through a 0.45 μm filter (Supor 450 Membrane filter, Pall, https://shop.pall.com/), and were stored in the dark.

The carrier solution was DI water without any added surfactant.

2.3. Development and optimization of pFI protocol

For trace analysis work the priority is to achieve maximum sensitivity and the lowest limit of detection of the target analyte. The key factors in optimization of programmable Flow Injection (pFI) - spectrophotometry are;

- Length of the flow cell light path
- Temperature of the reaction
- Mixing ratio of reagents
- Mixing ratio of sample with mixed reagent (S/R)
- Positioning of the reaction mixture within the flow cell
- Incubation time (WAIT time)
- Injected sample volume (Sv)

The *configuration of the flow cell* (light path 13 cm, internal volume 65 μL) was maintained throughout the optimization procedure. *The temperature* of the reacting mixture was kept at 40 °C by thermostating

Step	Sequences	Device Name	Input Format	Input Paremeters
Flushing system	FLOWCELL	COV	Port#	2
	Pump1 dispense	Pump1	volume (µL), flow rate (µ/sec)	1000,100,1
	Pump2 dispense	Pump2	volume (µL), flow rate (µ/sec)	1000,100,1
	WAIT	System	times (s)	2
Set up Spectrometer	Spectrometer get reference spectrum	Spectrometer	N/A	N/A
	WAIT	System	times (s)	2
	Spectrometer start acquire	Spectrometer	request period (seconds)	0.5
	WAIT	System	times (s)	2
MO.	MO.	COV	Port#	3
	Pump1 aspirate	Pump1	volume (µL), flow rate (µ/sec)	250,125,1
MO. + ASC.	ASC.	COV	Port#	5
	Pump2 aspirate	Pump2	volume (µL), flow rate (µ/sec)	200,100,0
	Pump1 dispense	Pump1	volume (µL), flow rate (µ/sec)	100,50,1
SAMP. + REAG.	SAMP.	COV	Port#	4
	Pump1 aspirate	Pump1	volume (µL), flow rate (µ/sec)	280,70,0
	Pump2 dispense	Pump2	volume (µL), flow rate (µ/sec)	70,17.5,1
	FLOWCELL	COV	Port#	2
INCUBATE MONITOR	Pump1 dispense	Pump1	volume (µL), flow rate (µ/sec)	190,20,1
	WASTE	COV	Port#	1
	WAIT	System	times (s)	120
	FLOWCELL	COV	Port#	2
	Pump1 dispense	Pump1	volume (µL), flow rate (µ/sec)	500,20,1
	save data to file	Data	N/A	N/A
	Pump1 dispense	Pump1	volume (µL), flow rate (µ/sec)	1000,100,1
	Pump2 dispense	Pump2	volume (µL), flow rate (µ/sec)	1000,100,1
DATA	subtract baseline	Data	at times (s)	15
	set data window	Data	min time (s), max time (s)	(see text)
	cal peak height	Data	data index	1
	save data to file	Data	N/A	N/A

Fig. 4. Assay protocol for determination of phosphate by programmable flow injection.

the holding coil and the flow cell, in order to eliminate any influence from ambient temperature fluctuations. *The mixing ratio of reagents* is controlled by the difference between the volume dispensed from a holding coil HC1 and the volume aspirated into holding coil HC2, resulting in 1/1 mixture of molybdate and ascorbic acid reagents (Fig. 3 step two). *The mixing ratio of the sample with the combined reagents* (S/R) is similarly controlled by the difference in the volume dispensed from a holding coil HC2 and the volume aspirated into holding coil HC1 when the confluence point is connected to the sample port. (Fig. 3.step three). Note also that the ratio of the flow rates must be the same as the ratio of the volumes dispensed and aspirated. By applying the flow volumes and flowrates expressed in microliters and depicted in Fig. 3, a mixing ratio of S/R = 4/1 was achieved and used for all subsequent experiments.

The position of the reaction mixture within the flow cell is a key parameter in optimizing the sensitivity of the SFC method and to avoid the Schlieren effect. This is achieved by placing the middle of the reacting sample zone into the middle of the light path of the flow cell, because such positioning ensures maximum sensitivity and reproducibility of the measurement. The volume of the sample/reagent mix that is pumped into the flow cell is selected to be much larger (280 µL, see Fig. 4) than the internal volume of the tubular flow cell (65 µL). This ensures that the interfaces between the carrier solution and the sample/reagent mixture (the cause of the Schlieren effect) are outside the light path of the flow cell while the middle of the sample/reagent mixture completely fills the light path of the flow cell during the stop flow period. The positioning is achieved by moving the reaction mixture from the holding coil (HC1) into the flow cell by delivering a defined volume of carrier (the return volume Rv) [8,9] using P1. For the current setup and sample and reagent volumes Rv = 190 µL delivered at a rate of 20 µL/s (Fig. 4. INCUBATE and MONITOR).

The incubation time is the duration of the stopped flow period within which the PMoB is being formed. The SFC protocol (Fig. 4), allows exploration of the kinetics of the formation of PMoB by arresting the reaction mixture in the flow cell for selected periods of time (WAIT) while the flow cell is physically isolated from flow system by turning the valve to the waste position.

Increasing the volume of the injected sample increases the sensitivity of all FI measurements in a linear fashion until the $S_{1/2}$ value is reached, above which the increase in peak height continues in an asymptotic

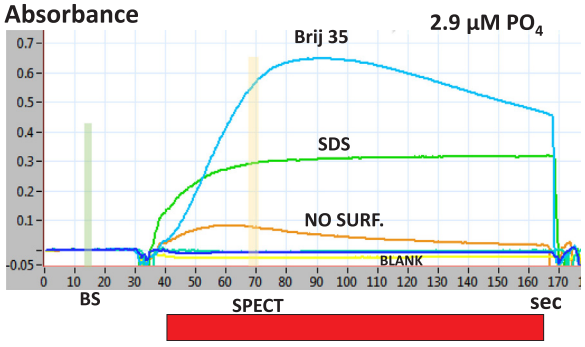

Fig. 5. Influence of surfactants on the change of absorbance during the formation of PMoB while reaction mixture is held within the flow cell. Brij35 absorbance was monitored at 775 nm, while SDS and no surfactant were monitored at 880 nm. The 510 nm wavelength was used as reference absorbance for all measurements. The spectra shown in Fig. 6. were collected at the incubation time marked SPECT. The red line along the X axis shows the period when the fluid in the flow cell was stopped. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

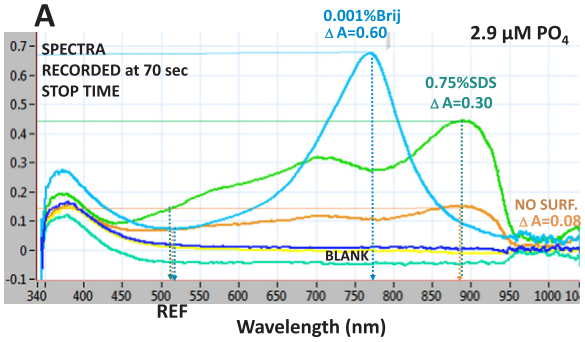

Fig. 6. Spectra of PMoB in absence and presence surfactants were recorded at 70 s time interval (SPECT in Fig. 5) during stop flow incubation period.

fashion. For the following experiments, carried out in the SFC mode the sample volume was kept unchanged (Sv = 210 µL Fig. 3).

3. Results and discussion

3.1. Kinetics of formation and spectra of PMoB in the presence of surfactants

The change of absorbance with time during the stopped flow period was recorded by monitoring 2.9 µM PO_4 standard solutions prepared in deionized water using the protocol summarized in Fig. 4. The influence of surfactants was investigated by adding surfactants to the ascorbic acid reagent. It was discovered that the rate of absorbance change in the absence and presence of surfactants differ considerably (Fig. 5). In the absence of surfactants the initial rise in absorbance starts to *decline* after 1 min of incubation, although production of PMoB continues. In contrast, in the presence of SDS, the absorbance increases rapidly, reaching a steady-state plateau after 70 s. The presence of Brij35 enhances absorbance even more, reaching a maximum at 90 s after which absorbance begins to decline.

Surfactants also influence the spectral characteristics of PMoB. Thus absorbance values of PMoB in presence of SDS and Brij35 are elevated over the entire spectral range (Fig. 6). Addition of SDS elevates absorbance values at 880 nm almost four fold compared to the spectrum of PMoB without a surfactant, while the form of the spectral response remains unchanged with maxima at 720 nm and at 880 nm. Surprisingly, the spectrum in the presence of Brij35, exhibits a well-defined single peak at 760 nm with an absorbance almost 8 times higher compared to spectrum of PMoB without surfactant measured at 880 nm. This along with a well-defined peak and low absorbance at 510 nm which is suitable for placing the reference wavelength, appears to make the Brij35 enhanced assay the most favorable alternative.

The spectra of PMoB were collected after 70 s incubation time (Fig. 5. SPEC), yet they also undergo a change with time. Thus the single peak spectrum in the presence of Brij35, starts to change after 5 min of incubation, converting into a double peak spectrum similar to the one encountered with SDS. However, since the purpose of this work is to optimize a fast assay protocol, we limited this exploratory work to incubation periods to 3 min.

3.2. Selecting a surfactant for sea water determinations

Determination of phosphate in sea water presents a challenge if samples are collected in an estuary, or any area where the salinity of the water varies, because the slope and position of the calibration lines depends on composition of the sea water matrix. Because the SFC variant of pFI technique was shown to be independent of salinity variations when trace Fe(II) were determined [10], the SFC method is also used here because monitoring the reaction rate while the reaction mixture is arrested in the flow cell eliminates the influence of the refractive index. By comparing calibration lines obtained in deionized water (DI) with those in sea water (SW), the influence of sea water matrix can be evaluated.

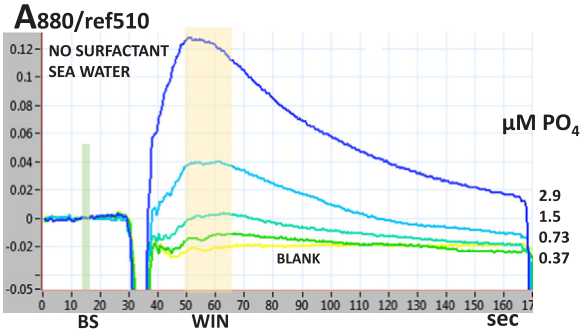

Fig. 7. Change of absorbance recorded during incubation stopped flow period obtained with reaction mixture with no surfactant and phosphate samples prepared in sea water. For details see text.

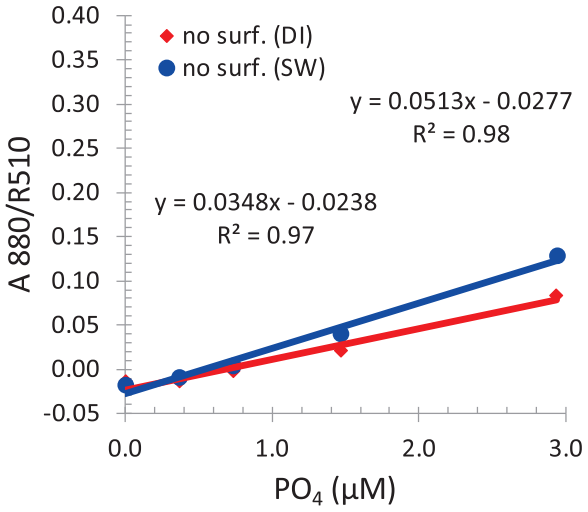

Fig. 8. Calibration graphs obtained from experiments in the absence of surfactant in the deionized water or seawater.

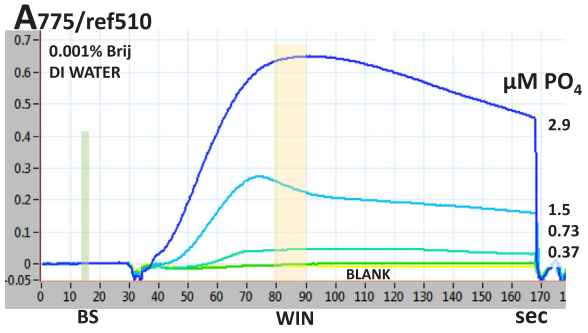

Fig. 9. Change of absorbance recorded during incubation stopped flow period obtained with reaction mixture with Brij35 and phosphate samples prepared in deionized water. For details see text.

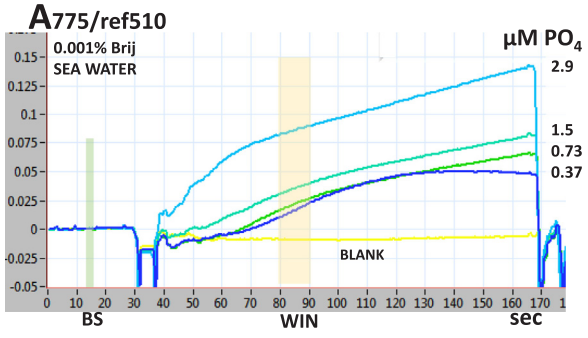

Fig. 10. Change of absorbance recorded during incubation stopped flow period obtained with reaction mixture with Brij35 and phosphate samples prepared in seawater water. For details see text.

In the *absence of surfactants*, standards prepared in DI and in seawater were analyzed by means of the assay protocol (Fig. 4) while using an incubation interval of 120 s and a data collection window (WIN) and baseline subtraction (BS) set as shown in Fig. 7. The baseline subtraction (BS) is the reference point to set the zero absorbance point of the spectrophotometer. We chose the position of BS to obtain an absorbance value that is reproducible, because it is recorded at the time

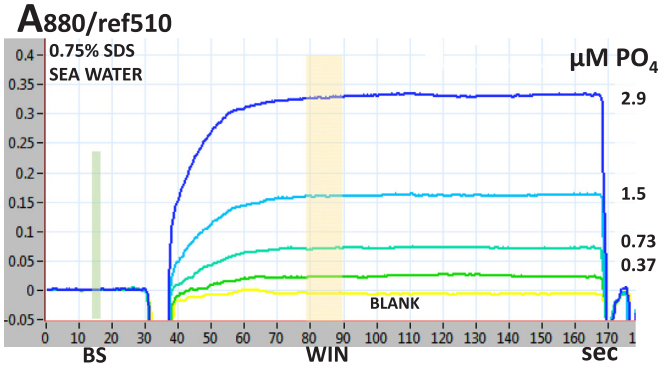

Fig. 11. Change of absorbance recorded during incubation stopped flow period obtained with reaction mixture with SDS and phosphate samples prepared in sea water. For details see text.

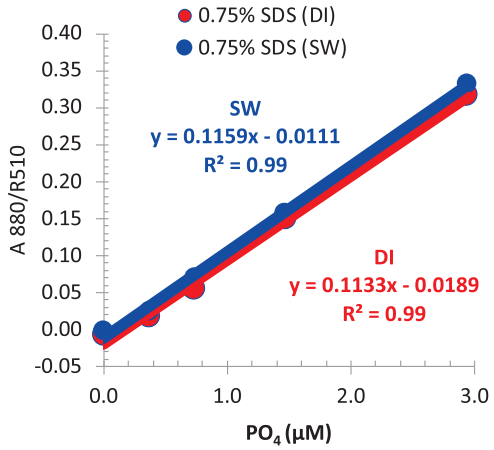

Fig. 12. Calibration graphs obtained from experiments in the presence of SDS in the deionized water or seawater.

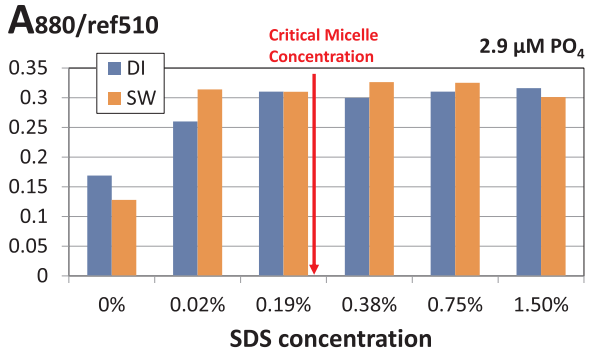

Fig. 13. Influence of the concentration of SDS on absorbance of 2.9 µM phosphate standards prepared in deionized (DI) and sea water (SW). SDS concentration refers to surfactant concentration in reaction mixture.

when the flow cell is filled with deionized water, the carrier. The absorbance values determined this way were used to construct calibration lines (Fig. 8) that share an intercept, but differ considerably in slope. This difference in slopes makes this approach unsuitable for the assay of sea water.

In the *presence of Brij 35* the reaction rate in DI water is slower (Fig. 9) which necessitated setting the data collection window (WIN) after an incubation time of 90 s. The absorbance at the peak top was eight times higher compared to no surfactant experiment. Unfortunately, the reaction rates observed with sea water standards (Fig. 10) were much lower, making the method unsuitable for determination of phosphate in seawater. Increasing the concentration of Brij35 did not solve the problem as it slowed the reaction rate even further, for both DI and sea water standards.

Table 2
The results of the "phosphate free" seawater spiked with o-phosphate analyzed by this study (pFI) and S-Lab.

Standard solution (µM)	This study	S-Lab	S-Lab adjusted
SW + 0 µM	− 0.014 ± 0.013 (n = 3)	0.11	0.00
SW + 0.351 µM	0.268 ± 0.005 (n = 3)	0.44	0.33
SW + 0.695 µM	0.668 ± 0.006 (n = 3)	0.79	0.68
SW + 1.371 µM	1.387 ± 0.013 (n = 3)	1.47	1.36
SW + 2.804 µM	2.835 ± 0.018 (n = 3)	2.88	2.77

In the *presence of SDS* the reaction rate curves recorded for seawater (Fig. 11) were identical with those obtained with DI water, and in contrast to experiments without a surfactant or with Brij35, absorbance values reached a steady state plateau. By setting the data collection window at 80 s, the calibration lines obtained with DI water and sea water standards were identical (Fig. 12). High reaction rates and reproducible absorbance values were obtained for both DI and sea water samples at a wide range of SDS concentrations (Fig. 13). It is significant that enhancement of absorbance and stability of PMoB measurement is achieved at, and above the critical micelle concentration of SDS.

3.3. Application of pFI-SFC method to determination of phosphate in sea water, and verification by comparison with CFA method

Correlation of results obtained by established methodology with a newly introduced technique, is the critical test to which any new approach must be submitted. Since in oceanographic community the air segmented continuous flow analysis (CFA, originally introduced as AutoAnalyzer®), is still most frequently used [7] we obtained assistance from S-LAB at University of Hawaii, where determination of phosphate in sea water has been routinely determined by means of SEAL AA3 Nutrient Auto Analyzer. As mentioned earlier, the pFI method developed by us, uses the same reagents as the CFA technique, albeit the concentrations of the principle reagents used for pFI are higher, because DI water is used in pFI as a carrier (Table 1), while in CFA reagents serve as carrier stream.

Samples were prepared by spiking "phosphate free" sea water with o-phosphate standards, and they divided to two batches. These batches were analyzed by pFI by us and by CFA in S-lab (Table 2). For pFI method, the assay protocol used was as shown in Fig. 4. while the calibration graph shown in Fig. 12, was used to calculate results displayed in Table 2. Results of analysis of the spiked samples obtained by CFA are consistently higher than the expected value, however, when they are adjusted by the difference between the nominal zero value and found value (0.11 µM of PO_4,), the expected and determined concentrations obtained by pFI and by CFA correlate very well (r^2 = 0.9995). Overall this agreement of results obtained by pFI and CFA confirms viability of pFI method, and reveals that reliability of the phosphate assay is founded on the underlying chemistry, but it is independent on the mode of fluidic manipulations, as long as they are performed in strictly reproducible way.

3.4. Surfactant influence

Surfactants have been used to optimize a wide variety of reagent based assays, with the aim of enhancing spectrophotometric, fluorescence, chemiluminescence or turbidimetric detection. In the context of this work, determination of sulfate by $BaCl_2$ is an interesting example, because this turbidity measurement was enhanced by stabilizing the particles of *insoluble* $BaSO_4$ using various surfactants [26, Table 2] Similarly, the assay of phosphate by the phosphomolybdenum- malachite green method was also enhanced by using surfactants, including SDS [27,28]. Furthermore, it is known that PMoB is sparingly soluble in

acids as particulate PMoB has been filtered from the acidic solutions and subsequently dissolved in an organic solvent with aim to improve sensitivity of phosphate determination [29]. Also, PMoB has been adsorbed on Sepharose and eluted by methanol [30] or adsorbed on a hydrophobic sorbent (Oasis HLB) and eluted with sodium hydroxide [21,32]. Alkaline solutions are commonly used to remove PMoB deposits from flowcells, because PMoB becomes negatively charged and soluble at high pH (Eq. (2)). It therefore follows that in acid solution and in the absence of a surfactant the PMoB is initially *colloidal* and then starts to aggregate as seen from the decrease in the monitored absorbance (Fig. 7). In contrast, when PMoB is stabilized within the micelles of SDS, the absorbance continues to rise until it reaches steady state (Fig. 11). Support for the colloidal nature of PMoB also comes from its blue color. True solutions such as the alkaline form of bromothymol blue adsorb at 620 nm, however the spectrum of PMoB does not exhibit a peak at that wavelength. PMoB *appears* to be blue, due to light scattering from the 40–900 nm sized nanoparticles due to the Tyndall effect [31]. This is the same effect that makes sky appear to be blue due to light scattering on aerosols. This also explains the wide range of "molar extinction coefficients" that have been reported in the literature [11], which are due to the fact that the measurement is really a blend of absorbance and turbidity, the latter being affected by the size of PMoB particles which scatter the light. Therefore, PMoB prepared under different reaction conditions, such as by using metallic or non-metallic reductants results in different particle sizes, which aggregate at different rates and under the various conditions. The spectra (Fig. 6) of PMoB prepared in this work, by precisely controlled (automated) procedure, are of the same compound (presumably $PSb_2Mo_{12}O_{40}$), but differ due to a difference in particle size and degree of aggregation, caused by interaction with the surfactants. The long linear chains of nonpolar Brij35 act differently than short negatively charged molecules of SDS that form micelles that stabilize PMoB (Fig. 13). While tempting, it is beyond the scope of this work to speculate more on this topic, which is intriguing and exciting. Yet, undeniably, this initial work opens further the Black Box – or rather Pandora Box - of PMoB chemistry to further exploration.

4. Conclusions

What this all means for trace analysis of phosphate in sea water can be briefly summarized as follows.

- PMoB analysis, based on Murphy-Riley method [12] has to be carried in a strictly reproduced time frame, automated by flow based technique.
- Long Incubation times (beyond 120 s) do not contribute to an increase of sensitivity. Therefore, CFA technique which operates at incubation time of 8 min is not optimized. Actually, FI methods designed for analysis of phosphate at micro molar level use incubation time as short as 20 s to obtain sampling frequency over 120 s/h [5].
- The choice of surfactants and their concentration has to be carefully considered. Batches from different manufacturers may differ in concentration of active component.
- Optimization or modification of PMoB based assay should always include a study of kinetics and spectra of PMoB species produced.
- Beyond the scope of flow based assays, use of surfactants, such as SDS should be considered for manual and automated batch analysis of phosphate by PMoB method.

Observations made in the course of this work, and the method developed for trace determination of phosphate in sea water, were made possible only through availability of a novel tool, the programmable Flow Injection method, and of associated instrumentation.

We hope that this paper will inspire further research in optimization of existing reagent based techniques, and in the design of new methods based on the study of the kinetics of reagent based assays.

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