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A high performance microfluidic analyser for phosphate measurements in marine waters using the vanadomolybdate method

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ABSTRACT

We report a high performance autonomous analytical system based on the vanadomolybdate method for the determination of soluble reactive phosphorus in seawater. The system combines a microfluidic chip manufactured from tinted poly (methyl methacrylate) (PMMA), a custom made syringe pump, embedded control electronics and on-board calibration standards. This “lab-on-a-chip” analytical system was successfully deployed and cross-compared with reference analytical methods in coastal (south west England) and open ocean waters (tropical North Atlantic). The results of the miniaturized system compared well with a reference bench-operated phosphate auto-analyser and showed no significant differences in the analytical results (student's *t*-test at 95% confidence level). The optical technology used, comprising of tinted PMMA and polished fluidic channels, has allowed an improvement of two orders of magnitude of the limit of detection (52 nM) compared to currently available portable systems based on this method. The system has a wide linear dynamic range 0.1–60 μM, and a good precision (13.6% at 0.4 μM, $n=4$). The analytical results were corrected for silicate interferences at 0.7 μM, and the measurement frequency was configurable with a sampling throughput of up to 20 samples per hour. This portable micro-analytical system has a low reagent requirement (340 μL per sample) and power consumption (756 J per sample), and has allowed accurate high resolution measurements of soluble reactive phosphorus in seawater.

1. Introduction

Phosphorus is a key nutrient in the marine environment and is involved in a range of biochemical processes [1,2]. Phosphate is the primary source of phosphorus for marine phytoplankton uptake and is essential for their growth and reproduction [3]. Although depleted levels of phosphate can limit primary production in marine ecosystems [4], an increase in phosphate concentrations due to anthropogenic inputs [5] can lead to excessive phytoplankton growth, with potentially adverse effect on water quality including nuisance blooms. Perturbations of phytoplankton communities have been directly linked to enhanced phosphate loadings [6]. Consequently, limits for phosphate concentrations in natural waters are being reduced by regulatory organisations [7]. This evolution of international water quality policy is increasing the need for the accurate determination of phosphate with sensitive automated analytical systems.

Various analytical techniques have been applied to measure soluble reactive phosphorus (SRP) in seawater, including ion chromatography [8,9] and electrochemistry [10]. However, flow analysis techniques [11] remain the first choice for seawater analysis [12] because of their selectivity, sensitivity and accuracy. The molybdenum blue method is the most commonly used analytical approach, involving a reaction between orthophosphate and a molybdate donor reagent, with subsequent reduction of the product to a blue coloured complex using ascorbic acid [13]. Alternative colorimetric techniques which are also based on the formation of heteropoly acids with molybdenum, can be used for SRP determination, including the vanadomolybdophosphoric method [14], as described here.

In situ analysis of phosphate is required to address the need for data at high spatial and temporal resolution [15–17]. This approach removes potential alterations of water samples during the sampling, sample handling and storage; these steps all constitute the main sources of contamination in standard analytical methodologies [18]. In the past, a range of autonomous analytical systems based on wet-chemistry have been developed for the determination of phosphate in natural waters [19–24]. One of the main challenges for the development of *in situ* chemical systems involves the reduction of their physical size. Large instruments are

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difficult to deploy, often expensive, and cannot be used on smaller marine platforms (*e.g.* buoys, remotely operated vehicles, CTD rosette frames). In addition, there are restrictions on the length of time that instruments can be deployed autonomously due to limitations on reagent and power availability, and often limited reagent stability.

To address these limitations we have applied "lab-on-a-chip" technology [25–27] to miniaturise already sensitive and accurate chemical methods. This approach addresses many of the technical constraints of *in situ* deployments and preserves the high performance of chemical analysis in natural waters. This study describes an automated low power and low reagent consuming microfluidic analyser to monitor SRP in marine waters. The microfluidic analyser uses the stable vanadomolybdate reagent and was field-tested and compared with bench-top analytical systems in coastal and open ocean waters. The combination of a stable reagent, low reagent use and low power consumption makes the micro-analyser a viable and economical option for implementation on various deployment platforms for monitoring phosphate in marine and freshwater systems.

2. Material and methods

2.1. Chemical method

The determination of SRP reported here is based on the rapid direct reaction of orthophosphate with an acidified vanadomolybdate reagent which produces a yellow coloured complex [28–31]. This "yellow" method was chosen for the stability of the reagent mixture over time, reported to be over one year, compared with four to six weeks for the classical "blue" method [32]. The inorganic phosphorus species in seawater include orthophosphoric acid, dihydrogen phosphate, and hydrogen phosphate, and their abundance at equilibrium depends on the pH. Under acidic conditions, orthophosphoric acid is dominant and reacts with molybdate ions to form molybdophosphoric acid:

$$H_3PO_4 + 12MoO_3 \rightarrow H_3PMo_{12}O_{40}.$$

In the presence of vanadate ions, this complex will form vanadomolybdophosphoric acid, $H_4PVMo_{11}O_{40}$, with a molar absorptivity of 3.6×10^3 mol^{-1} cm^{-1} at 385 nm [14].

The vanadomolybdate stock solution used in this study was prepared with 7.2 g ammonium molybdate (A-7302, Sigma, Dorset, England) and 0.36 g ammonium metavanadate (20555-9, Aldrich, Dorset, England) dissolved in 95 mL concentrated hydrochloric acid (Sigma-Aldrich, ACS reagent 37%) and made up to 1 L with high purity water (MilliQ, Millipore, $> 18.2\ \Omega\ cm^{-1}$). Phosphate working standards were prepared from a stock solution of 3 mM phosphate (0.40827 g L^{-1} of potassium dihydrogen phosphate, Sigma-Aldrich), and prepared using low nutrient sea-water (LNSW), collected from North Atlantic surface waters and aged in the laboratory for at least one year. A silicate stock standard of 1 mM (0.188 g L^{-1} of sodium hexafluorosilicate, Sigma-Aldrich) was used to spike solutions for studies into silicate interference on the phosphate measurements. The error generated by the silicate interference was investigated by preparing a 50 nM phosphate standard in LNSW with additions of silicate that ranged from 1 μM to 100 μM, corresponding to levels found in natural waters (World Ocean Atlas 2009, NOAA).

2.2. Microfluidic analyser description

The optical absorption measurement of the yellow complex provides a simple measurement of SRP. High power UV-LEDs (XRL-375-5E, 375 nm, 19–26 mW, Roithner) were used as a light source, and photodiodes (TSL257-LF, Texas Advanced Optoelectronic Solutions Inc.) for the absorbance detection [33]. Both LEDs and

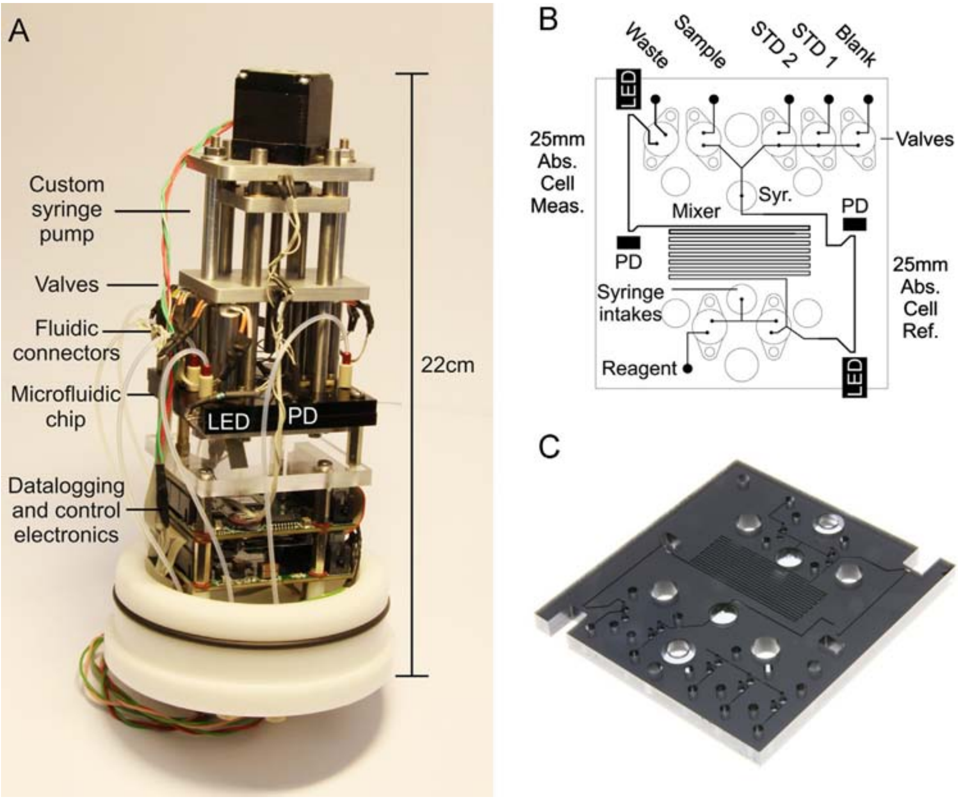

Fig. 1. (a) Photograph of the micro-analyser, including a custom syringe pump, valves, microfluidic chip and electronics. The system measures 22 cm high and 10 cm in diameter. (b) Microfluidic chip schematic. (c) Photograph of the chip prior to bonding (micro-milled in tinted-PMMA).

photodiodes were directly glued onto the microfluidic device with an optical adhesive (Norland 63). Many other microfluidic devices use a short optical path length [24] or complex solutions for coupling the optical components to the chip [34], however these may negatively affect the sensitivity and the limit of detection (LOD) of the analysis.

The microfluidic device was produced by micro-milling (LPKF Protomat S100 Micromill) of 5.0 mm thick tinted poly (methyl methacrylate (PMMA, Plexiglas GS 7F60, Röhm). The use of tinted PMMA reduces the amount of light reaching the detector coming from ambient sources, and also any stray light from the LEDs that did not pass through the analyte [35]. A solvent vapour bonding procedure was used to bond the two halves of the chip: the microfluidic manifold and the lid [36]. This technique produces smooth channel surfaces and allows for longer optical cells. The specific optical set-up of the micro analytical system was characterized using varying concentrations of a food colouring dye (Yellow food colouring, Super Cook). The fluidic manifold of the chip included two absorbance cells of 25 mm length: a reference and a measurement cell (Fig. 1). All channels were 150 μm wide and 300 μm deep, except the two optical absorption cells which were 300 μm wide and 300 μm deep. Fluid handling was performed using seven micro-inert valves (LFNA1250125H, Lee Products Ltd.) which permitted switching from the sample inlet to blank or on-board standards stored in 500 mL fluid bags (Sartorius Stedim). A stepper motor was used to drive two 200 μL liquid delivery syringes, which were controlled by two Hall-effect sensors. The pre-calibrated distance between these Hall-effect sensors and a magnet placed on the sliding plate of the pump formed a linear displacement transducer for the syringe pump. The dead volume of the microfluidic device was 500 μL. The dead volume was minimised as all fluidic connectors, optical alignment grooves, valve mounts, and syringe pump mounts were directly milled into the microfluidic device. Seawater was filtered at the sampling inlet line using a 0.45 μm pore size polyethersulfone MILLEX-HP filter unit (SLHP033RS, Millipore) with a dead volume of 100 μL.

The outputs of the photodiodes and the final data were stored on a 2 GB flash memory card (filtered to 1 Hz). A fault tolerant file system ensured data was not lost or corrupted in the event of a power loss. The micro-analyser was automated using a custom-designed embedded electronics package (5 cm × 6.2 cm × 4 cm) (Fig. 1). The entire system (Fig. 1) was housed in a water-tight acrylic tube (not shown) with a 10 cm outer-diameter and a height of 22 cm.

2.3. Analytical procedure

The analytical protocol involved a series of four main steps: (1) withdrawal of the water sample from the environment; (2) injection of the sample, mixing of sample and reagent into the on-chip mixer; (3) colour formation and (4) absorbance measurement. The absorbance of the sample, blank or standard was determined in the reference optical cell prior to the addition of the reagent. This step was necessary to correct for any background absorbance. Mixing is achieved on the chip by passing blank, standard or sample together with reagent through a 250 mm long serpentine mixer. Both the reagent and the sample were pumped through the chip at a flow rate of 200 μL min^{-1}, at a ratio of 1:1. The flow was stopped to allow colour formation and the associated absorbance was measured in the second optical cell. This reaction time was between 180 and 300 s, so as to allow sufficient time for the reaction to develop colour. Depending on the sample SRP concentration; shorter reaction times will allow faster analysis but result in a reduced sensitivity. The same

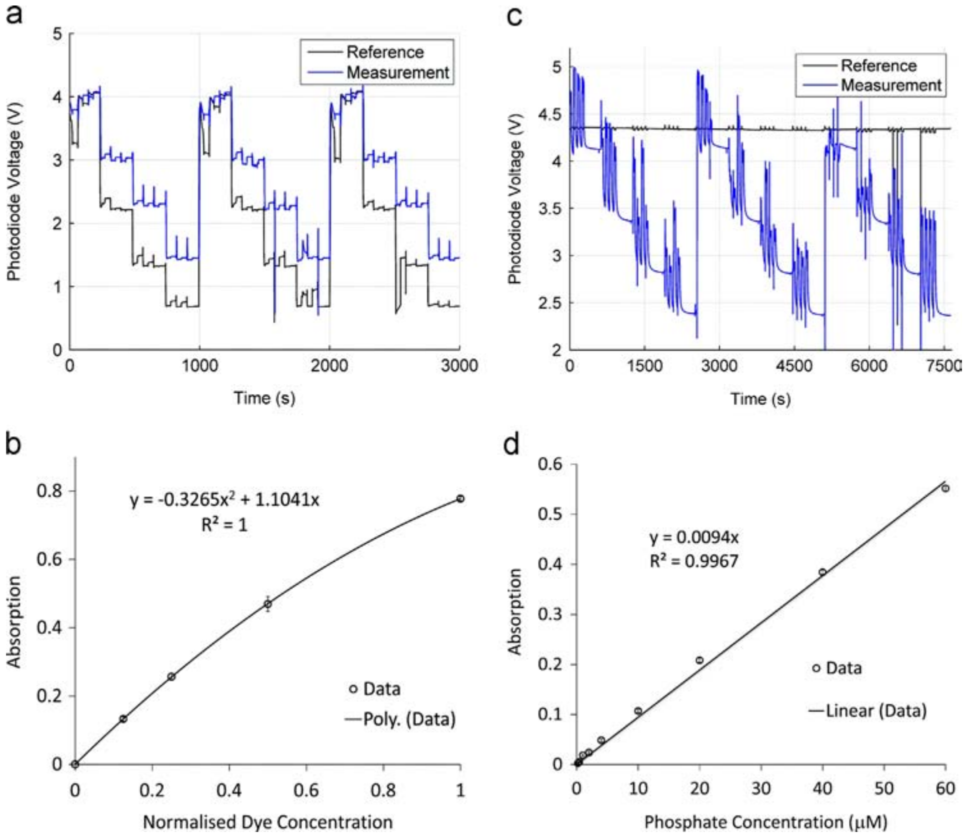

Fig. 2. (a) Signal from dye dilutions blank, 0.025%, 0.05% and 0.1% v/v., passing through the microfluidic device. (b) Absorption values *versus* normalised dye concentration. (c) Output voltage for a sequence of phosphate standards. (d) Calibration curve for phosphate standards *versus* phosphate concentration.

analytical protocol and reaction times were applied to blanks, standards and samples. To minimise any potential contamination or carry-over between samples, an additional 320 μL sample volume was used to flush the channels between samples. The flushing stage is equivalent to four withdrawal and injection cycles, and is observed as the ripple effects in Fig. 2a and c (due to the noise of the pump running).

3. Results and discussion

3.1. Calibration and limit of detection

The photodiode output voltage was proportional to the incident light intensity and therefore the voltage decreased as solution absorbance increased. The light intensity was measured to characterize the response of the specific optical set-up to varying solution concentrations (Fig. 2). At higher absorption values (> 0.5 AU), the polynomial nature of the calibration curve (Fig. 2b) was the result of mismatched spectra for the LED output and dye absorption [26]. An example of the change in photodiode output voltage for the reference and measurement absorption cells for a sequence of phosphate standards is presented in Fig. 2c. The calibration curve of the analytical system is shown in Fig. 2d for phosphate standards between 0.2 and 60 μM concentrations. The system offers a dynamic range with a linear response up to 60 μM and a precision of13.6% at 0.4 μM, $n=4$. The limit of detection (LOD), defined as three times the standard deviation of the blank baseline, was based on the measurements of 16 LNSW blanks. A LOD of 52 nM ± 6.2 nM ($n=16$, $k=3$) was determined using the recommended approach by the International Union of Pure and Applied Chemistry (IUPAC), considering that the analyte measurement is statistically different from the analytical blank value with a factor $k=3$ [37]. This LOD determination includes system error and various external sources of noise, including electrical interference, background light, fluidic stability: flow rate fluctuations and bubbles.

3.2. Dissolved silicon interference study

Dissolved silicon is a known interference for the molybdenum yellow method for phosphate analysis [38,39]. The similarity in ionic structure of silicate compared to phosphate, makes it a competitor in the reaction with the molybdate ions; both silicon and phosphorus atoms are covalently bonded to four oxygen atoms. The tetrahedral unit of the molybdophosphoric ion formed has a formal charge of −IV since each oxygen atom presents a formal negative charge. The analytical signal can therefore be affected by the formation of similar molybdate heteropolyacid compounds. The influence of silicate additions on the phosphate signal is described in Table 1. The interference of silicate concentration on the apparent phosphate signal can be characterised with different relations, depending on the phosphate concentration. The SRP measurements were corrected for the silicate interference using these relations. The dissolved silicon concentrations along the offshore transect described below (Section 3.3.2.) were low and near constant: (0.71 ± 0.06 μM). The micro-analyser SRP measurements were then corrected for the silicon interference with an average value equivalent to 0.007 μM of the SRP signal. River and estuarine environments typically have high silicate concentrations and in these waters the determination of SRP can be particularly affected by silicate. A new design of the system could tackle the silicate interference directly with the addition of a tartaric acid stream to mask the silicate interference [38].

Table 1
Response of apparent phosphate output signal with silicate additions in different matrices: no phosphate, low phosphate and high phosphate conditions.

[P] (μmol L^{-1})	[Si] (μmol L^{-1})	$[P]_{apparent}$ (μmol L^{-1})
0	1	0.0 ± 0.01
0	10	0.3 ± 0.08
0	50	0.5 ± 0.07
0	100	0.5 ± 0.12
0	150	0.6 ± 0.11
1	1	1.02 ± 0.02
1	10	1.09 ± 0.07
1	50	1.12 ± 0.07
1	100	1.18 ± 0.11
1	150	1.22 ± 0.09
10	1	10.0 ± 0.04
10	10	10.1 ± 0.09
10	50	11.5 ± 0.07
10	100	11.5 ± 0.05
10	150	11.6 ± 0.07

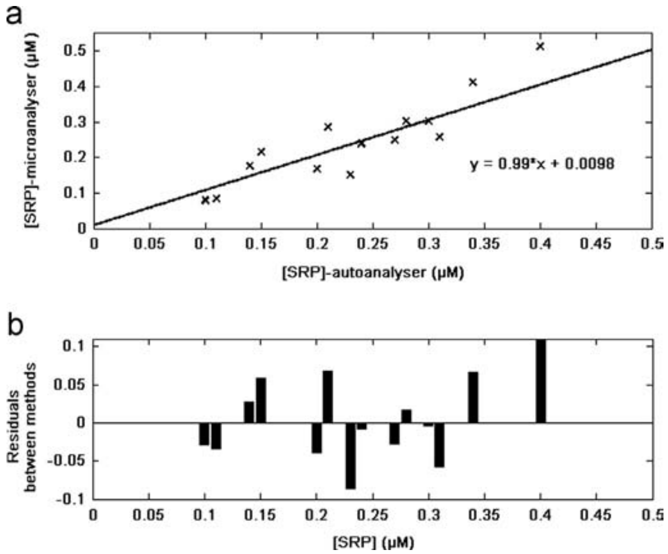

Fig. 3. (a) Scatter plot of the micro-system phosphate measurements off Plymouth coast compared with a segmented flow colorimetric auto-analyser, (b) Plot of residuals between both methods in function of the phosphate concentration.

3.3. Field evaluations in marine waters

3.3.1. Coastal waters

The microfluidic analyser was tested off the south-west coast of England near Plymouth, UK, on-board the RV *Plymouth Quest* as part of the Western Channel Observatory programme (www.westernchannelobservatory.org.uk). A sampling transect was carried out from inshore waters to the L4 buoy (50°15.00′N, 4°13.02′ W) in September 2010. We analysed 15 samples on the transect, and the concentrations encountered ranged between 0.1 and 0.5 μM. The mean value is comparable with the records of the L4 station at this time of year (http://www.westernchannelobservatory.org.uk/l4_nutrients.php). However the high spatial resolution of the transect highlights the high variability in phosphate concentration with deep water entrainment after the summer blooms. The measurements were compared with a bench-operated segmented flow colorimetric phosphate analyser [40](Fig. 3). Good agreement was found between the measurements performed using the micro-analyser and the reference auto-analyser (Fig. 4). The relationship between both systems can be expressed using the following linear relationship: $SRP_{Micro\text{-}analyser}=0.99\times SRP_{Auto\text{-}analyser}+0.0098$, with SRP concentrations in μM. A paired

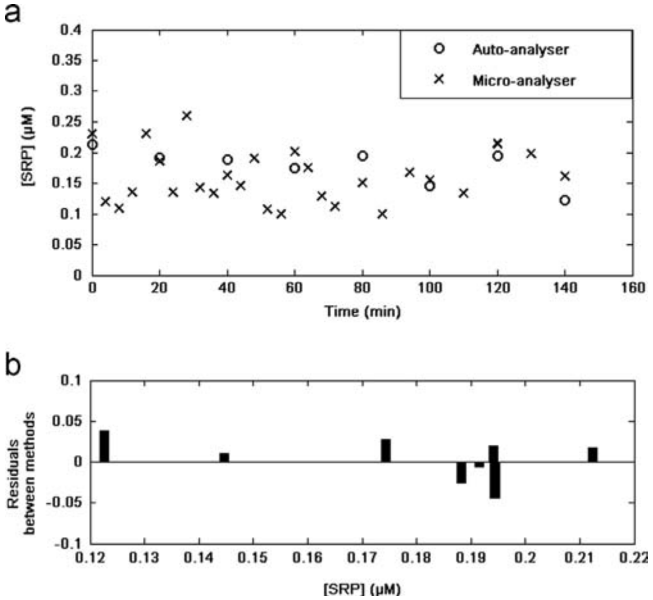

Fig. 4. (a) Scatter plot of the micro-analyser phosphate measurements off Senegalese waters compared with a segmented flow colorimetric analyser, (b) Plot of residuals between both methods in function of the phosphate concentration.

t-test was used to evaluate whether there was a significant difference between the methods over the range of concentrations encountered. The calculated *t*-value compared with Student's *t*-value at 95% confidence ($p=0.05$, $n=15$) showed no significant statistical difference. Thus the micro-analyser was applied successfully to natural samples and demonstrated a high analytical performance when compared with reference methods.

3.3.2. *Offshore waters*

The microfluidic analyser was also deployed in January and February 2011 on an oceanographic cruise in the tropical north-eastern Atlantic Ocean, as part of UK-GEOTRACES programme (http://www.ukgeotraces.com/). The microfluidic analyser was connected to the underway system of the ship (sample inlet at 4 m depth) and was programmed to measure a sample every 3 min (27 samples were successfully measured), whilst sailing from the Senegalese coast and through a deep water upwelling region, into more nutrient depleted offshore waters. Discrete samples (8 comparative samples) were taken every 20 min from a towed fish and analysed using a high precision segmented flow auto-analyser for phosphate [40] and dissolved silicon [41].

Despite the relatively low phosphate concentrations encountered, from 0.09 to 0.26 µM, and the high variability of the phosphate concentrations in this dynamic shelf sea environment, the results from the microfluidic analyser followed a similar trend to the bench-top auto-analyser (Fig. 4). A paired *t*-test showed no significant statistical difference between samples analysed on the microfluidic analyser and the conventional bench-top system at 95% confidence ($p=0.05$, $n=8$). Furthermore, our measurements obtained using the microfluidic analyser agreed well with reported phosphate concentrations for west African continental shelf-edge waters; between 0.35 and 0.74 µM during a strong upwelling event in April–May 2009 [42]. This deployment demonstrated the capacity of the micro-analyser to produce high quality fine scale measurements and its ability to observe spatial variations that were not noticeable with analysis of discrete samples using the conventional analyser.

4. Conclusions

A novel automated lab-on-a-chip colorimetric analyser for SRP was successfully deployed in coastal and open ocean environments. The system has a LOD of 52 nM and can analyse up to 10 samples per hour. The measurements undertaken by the novel micro-analyser agreed with those by a conventional bench-top SRP analyser. The technique used for the fabrication of the microfluidic device will permit an extension of the path length of the optical cell [35,36] which will further improve the LOD according to the Beer–Lambert law, and allow future use of the system in low nutrient oligotrophic waters. The reagent (340 µL per sample) and power consumption (756 J per sample) of the microfluidic system were orders of magnitude lower than for conventional systems and because of the enhanced stability of the reagent of the "yellow" method will allow long term monitoring of SRP in natural waters.

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