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**Development of a Low Cost Microfluidic Sensor for the Direct Determination of Nitrate Using Chromotropic Acid in Natural Waters**

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*Abstract:*
Progress towards the development of a miniaturised microfluidic instrument for the direct measurement of nitrate in natural waters and wastewater using chromotropic acid is presented. For the first time, the chromotropic method for nitrate analysis has been transferred to a microfluidic chip configuration that can withstand the extremely acidic nature of the reagent within a field deployable platform. This simple method employs one reagent mixed in a 1:1 ratio with the sample to produce a yellow colour absorbing strongly at 430 nm. A stopped flow approach is used which, together with the very rapid kinetics and simple reagent stream, enables an uncomplicated microfluidic design and field deployable platform with a sample throughput of 9 samples h$^{-1}$, limits of detection of 0.70 mg/L $NO_3^-$ and 0.31 mg/L $NO_3^-$ for seawater samples, with a dynamic linear range from 0 – 80 mg/L $NO_3^-$ and long-term reagent stability of up to 6 months. Validation was achieved by analysing split water samples by the analyser and ion chromatography, resulting in an excellent correlation co-efficient of 0.9969. The fully integrated sensing platform consists of a sample inlet with filter, storage units for chromotropic reagent and standards for self-calibration, pumping system which controls the transport and mixing of the sample, a microfluidic mixing and detection chip, and waste storage, all contained within a ruggedized, waterproof housing. The optical detection system consists of a LED light source with a photodiode detector, which enables sensitive detection of the coloured complex formed. The low cost of the platform coupled with integrated wireless communication makes it an ideal platform for *in-situ* environmental monitoring.

**Keywords:** Environmental monitoring, microfluidics, COP, nitrate, chromotropic acid, colorimetry

### 1. Introduction

Nitrate is an important nutrient to promote plant growth, but excess of nutrients like nitrate in water bodies promotes excessive growth of algae, *i.e.* eutrophication. Nutrient pollution is considered one of the most difficult environmental challenges as it is difficult to implement effective mitigation remedies.[1] Nitrate levels in various types of water are therefore regulated through the European Commission's Groundwater Directive (2006/118/EC) and the Drinking Water Directive (98/ 83/EC) which both state an upper nitrate limit of 50 mg/L.[2, 3] Reliable measurements of nitrate are therefore essential for effective assessment and management of environmental water quality. However, despite this growing demand for real-time, in-situ monitoring of nitrate, penetration of autonomous chemical sensing technologies into commercial markets remains disappointingly low. The cost and the technical drawbacks of commercially available wet chemical sensors for the determination of nitrate can be

prohibitive and often entail regular maintenance over time. This may be due to the difficulties associated with determining nitrate such as the relatively intricate procedures involved, probability of interferences present and the limited detection ranges associated with various methods. Many techniques have been developed and studied for the determination of nitrate *via* on-site measurements, including direct ultraviolet spectrophotometric screening, nitrate-responsive electrodes, and colorimetric techniques. Nonetheless, these methods can involve a number of multistage or complex procedures and can result in a high component cost. A field deployable platform for automated *in-situ* nitrate monitoring developed by Beaton *et al.* employed a reduction step through the use of a cadmium column followed by nitrite analysis using the Griess method.[4] Although this method is sensitive and has been employed for field measurements successfully, it is an indirect method, and requires measurement of background nitrite followed by a second measurement of total nitrate (after reduction to nitrite) and nitrite.  The nitrate concentration is then obtained by subtraction.  The reduction step adds complexity to the fluidic system; e.g. valves for diverting flow through the reduction column as well as the addition of buffers and column washing. Issues may also arise in the reproducibility of a cadmium column and the unstable nature of the Griess reagent and nitrite standards over time (few weeks).

In the realisation of a fully reliable low-cost platform, it is obviously an advantage to keep the fluidic handling requirement as simple as possible as complex, multistage approaches are correspondingly challenging and expensive to implement due to significant additional component costs, and higher maintenance costs. Therefore in this research we focussed on developing a simple, single stage direct colorimetric method for the determination of nitrate. Direct UV absorbance is an attractive option for *in-situ* monitoring of nitrate, as there is no need for fluidics and reagents and was the chosen detection method for 50 platforms in the Argo project, which was established in 2000 to provide distributed sensor information related to the global marine environment.[5]  This approach was also explored by Frank *et al.* for *in-situ* nitrate monitoring in the North Sea.[6] Although good correlations were achieved when compared to wet chemical analysis, discrepancies were found between reference data and *in-situ* data derived from the UV spectra due to the technical limits of the sensor. The conclusion was that direct UV may provide a practical solution where wet chemical analysers cannot due to technical difficulties or insufficient temporal resolution. However, interferences arising from dissolved organic matter and surfactants can be problematic and the high cost of optical components suitable for multiwavelength UV-spectroscopy are significant issues.[7]

Recently, direct detection of nitrate using a simplified chromotropic acid reagent method was reported by Cogan *et al.*[8] wherein chromotropic acid, in a sulphuric acid medium, was reacted with nitrate ions to produce a characteristic yellow colour associated with an absorbance band in the visible region ($\lambda_{max}$ = 430 nm). This modified method had a linear range of 0.9 – 80 mg/L nitrate, and a limit of detection of 0.73 mg/L nitrate. An issue with this method is the use of highly concentrated sulphuric acid (98%) in the chromotropic acid reagent. This strongly acidic medium drastically constrains the types of materials and components that can be used to implement the method in a microfluidic analytical fluidic system.  However, despite the highly reactive and corrosive nature of the reagent, we have successfully transferred the method from a conventional flow analysis platform into a microfluidic sensing platform through which we have implemented a fully automated, rapid and sensitive method, with a high sample throughput. The method can be applied to the analysis of nitrate in freshwater and wastewater, and also in marine waters following a system calibration of relevant seawater samples. The analyser shows excellent correlation with ion chromatography and good repeatability as well as a low limit of detection. In this paper, we describe the analytical performance of the method in this configuration, and

demonstrate that, through careful choice of key materials and components, a reliable and robust field deployable platform can be realised.

The miniaturisation of analytical devices through the implementation of microfluidics is an important development for automated monitoring platforms, providing many advantages such as;
• The small volumes of sample and reagent required.
• Calibration procedures based on standard buffer solutions can be automated.
• Optical detection takes place within a microfluidic chip which can be protected from fouling effects by a combination of sample filtration and reagent-based fouling deterrents.
• Power efficiency and robustness (e.g. the sensor is not directly exposed to the sample as all detection takes place within the chip).

Polydimethylsiloxane (PDMS) and poly(methyl methacrylate) (PMMA) are often used for forming microfluidic chips as finely featured fluidic structures such as channels, mixing regions and detector flow cells can be easily produced.[9] However, as colorimetric reagents used for environmental analysis often tend to be of a strongly acidic/alkaline or redox active nature (in order to drive the reactions required to generate the analytical chromophore in the presence of the sample), in some cases, PDMS does not offer sufficient chemical resistance to the drastic nature of these reagents, and this can result in swelling and irreversible chemical alteration of the polymer. PMMA is suitable for use with dilute acid/alkali solutions but is not resistant to more concentrated acids. Methods based on very reactive chemical reagents are therefore usually implemented in fluidic systems based on highly inert materials such as glass or quartz. While these can be successful in small batches, they are difficult and costly to produce, requiring highly toxic etchants to produce the channels, and are not suitable for mass-production. This is also the case for robust fluoropolymers such as Teflons like polytetrafluoethylene (PTFE) and fluorinated ethylene-propylene (FEP). While the physical and chemical properties of these materials offer many advantages such as chemical inertness, thermal stability, and excellent anti-bio-fouling properties, sufficient adhesion and bond strength is hard to accomplish due to its low frictional resistance and therefore aggressive surface treatments are required for surface functionalization for sealing and bonding.[10] Focus has therefore turned to alternative thermoplastics such as cyclic olefin co-polymers (COC) and cyclic olefin polymers (COP). COP is a useful substrate material for the fabrication of microfluidic devices due to its excellent optical properties, low oxygen permeability and resistance to many chemical agents including acids, bases and polar solvents.[11] These surfaces have low surface reactivity and tend to be hydrophobic which can lead to difficulties in sealing and bonding these substrates together.[12] However, their surface reactivity can be enhanced by mechanical interlocking and interdiffusion of chains using techniques such as thermal cycling, solvent vapour treatment, acid and plasma surface activation. It has been well documented that energetic ions, electrons and UV photons achieve sufficient energy to break bonds on the surface of the substrate which results in the production of highly reactive free radicals which assists in forming the charged surface groups responsible for increasing the overall surface energy in order to improve the wettability between mating surfaces, and improving bond strengths.[13]

In this paper, we present a low-cost, robust sensing platform with a microfluidic reaction manifold and light emitting diode (LED) based optical detection system that can withstand the acidic nature of the chromotropic method in a cost-effective, simple and robust manner by using reliable materials that opens the possibility of long-term in-situ deployments. We also demonstrate the effectiveness of UV surface treatment and thermal fusion of COP surfaces and microchannels, to produce the first microfluidic chip capable of detecting nitrate directly

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using chromotropic acid. Considering the highly corrosive nature of the reagent, and the success of the implementation, we also discuss the crucial role that materials and design play in the realisation of reliable, robust long-term deployments of environmental sensors.

## 2. Experimental

### 2.1 Colorimetric reagent

The chromotropic acid reagent was prepared by dissolving 0.0735 g of chromotropic acid ($C_{10}H_6Na_2O_8S_2 \cdot 2H_2O$, Sigma-Aldrich, Ireland) in 250 mL concentrated (98% v/v) sulphuric acid ($H_2SO_4$, BDH Laboratory Supplies, UK). When protected from light, the reagent is stable for at least 6 months as shown in Fig. S1.

### 2.2 Deionised water and standards

All solutions were prepared using analytical grade chemicals. Deionised water ($< 18.2 \ \Omega \ cm^{-1}$) from a Millipore Milli-Q water purification system was used throughout the analysis. Nitrate stock standard solution (500 mg/L $NO_3^-$) was prepared from potassium nitrate ($KNO_3$, Sigma-Aldrich, Ireland) that was pre-dried for 1 hour at 110 °C. Nitrate standards are stable for several months due to the addition of 0.1% chloroform.[4]

### 2.3 Material Compatibility Study Generation 1 Prototype Platform

The chemical compatibility of various elastomers and polymers with sulphuric acid was investigated, by fully immersing the material in the acid (98% $H_2SO_4$, BDH Laboratory Supplies, UK) contained in a glass vial. Subsequently, a prototype bench-top system was developed to investigate the chemical compatibility of various components and materials with the nitrate-chromotropic acid complex. The prototype consisted of a peristaltic micro pump (Series 100, Williamson Manufacturing Company Ltd.) which incorporated various tubing within the pump head. Reagent and sample were pumped through a Kynar (PVDF) 1.6 mm Y-connecting hose barbed union (11806643, Cole-Parmer, Ireland) and delivered to a modified glass flow cell (Brand Ltd Cat. No.7477 15) at a flow rate of 600 µL/min over a period of 7 months. Initially, the coloured complex formed passed through a paired emitter-detector diode (PEDD) detection system similar to that described in detail by O'Toole *et al.* consisting of two LEDs, one operating as a light source/emitter and the other as a light detector, with $\lambda_{max}$ of 430 nm and 623 nm respectively.[8,16] Subsequently, an LED/PD detector was used to measure the coloured complex formed. The detector signal was transmitted wirelessly to a laptop via a Wixel controller (Pololu Corporation, USA) and saved as a text file which was subsequently analysed using Microsoft Excel.

### 2.4 COP Microfluidic chip and detector design

Bonding of COP layers together has mainly involved solvent bonding, adhesive printing bonding, surface treatment bonding and thermal fusion bonding.[14] A study by Tsao *et al.* showed that UV/ozone surface treatments could be used for achieving strong low temperature bonding of PMMA and COP microfluidic substrates. When applying pressure to PMMA and COC substrates at a temperature of 90°C, the UV/Ozone treated surfaces displayed bond strengths orders of magnitude more than control units not subjected to this treatment.[12] A recent report by Jackson *et* al. characterised UV activation of microstructures in (COC) followed by thermal fusion bonding for circulating tumour cells analysis.[15] In this study, direct UV exposure to COP was demonstrated as an alternative approach to achieving high bond strengths between the microfluidic system layers.

The function of the microfluidic chip shown in Fig. 1, is to mix the sample, blank or nitrate standard with the reagent and to present the resulting mixture to the detector which consists

of an LED light source with a photodiode (PD) detector. The seven layers were fabricated using a Roland CNC micro-mill (Modela MDX40A) from 2.0 mm thick COP (Zeonex mcs-COP-04, Microfluidic Chip Shop, Netherlands). The chip with dimensions 60 mm x 30 mm x 14 mm contained 6 inlets in total, 3 for reagent followed by a sample, high and low inlet all of 1.7 mm diameter and a 109.73 mm serpentine mixer succeeded by a micro-cuvette with a pathlength of 8 mm. To ensure mixing of the reagent and sample was in 1:1 ratio (which is ideally suited for an automated platform)[17], channels were of equal length and cross-sectional area of 500 μm x 500 μm. This ensures that the fluidic resistance is equal for all channels leading to the serpentine channel, provided that the solutions to be mixed are injected at equal pressure.

The milled layers were cleaned and degreased using isopropyl alcohol and deionised water. To assemble the chip the mating surfaces were irradiated with UV light (Dymax 5000-PC) (λ < 180 nm) for approximately 15 min. This causes the normally hydrophobic COP surfaces to become hydrophilic which allows for bonding below the glass transition temperature of COP which is 135°C (Fig.S2). The chip is then assembled using 1.5 mm steel dowel pins at the chip corners to ensure correct alignment. The assembled chip is clamped between two brass plates to apply pressure. The alignment of the plates is checked at the four corners using a vernier callipers to ensure even pressure is applied across the chip faces. The chip is then heated to 110°C for 2 hr.
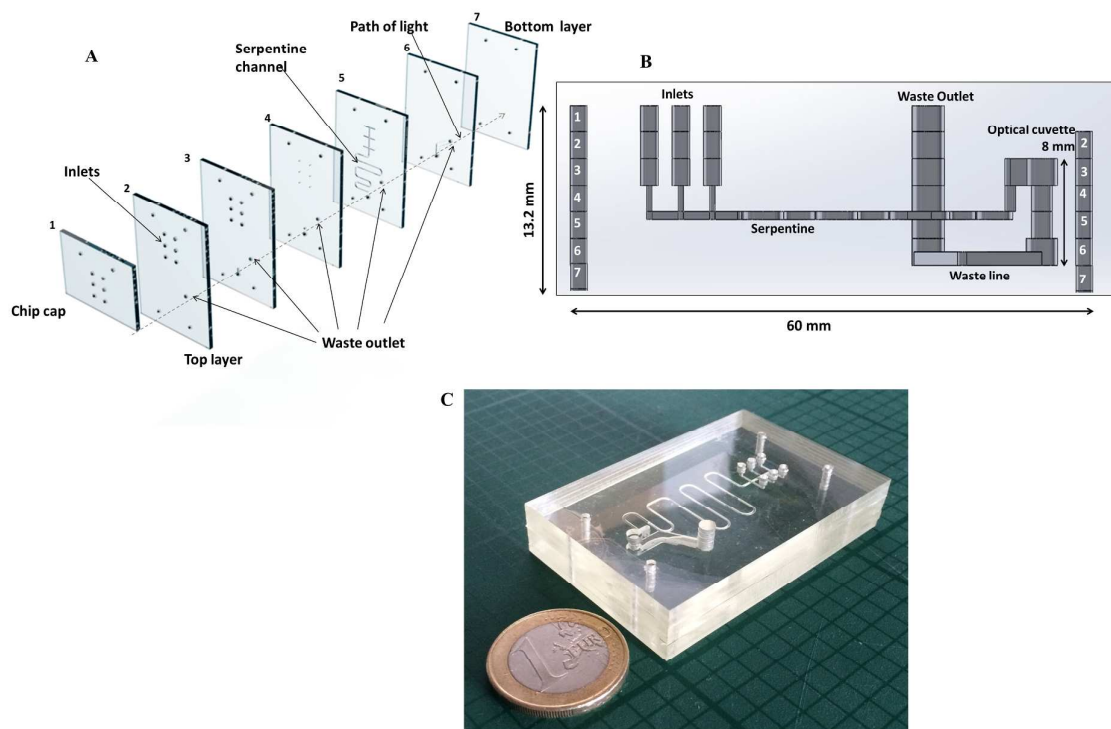


**Figure 1.** (A) Expanded view of microfluidic chip layers (B) Side view of fully assembled chip layers showing the micro-cuvette (C) fully assembled microfluidic chip.

Once the chip layers were bonded, Teflon® tubing connected to the peristaltic pumps was inserted into the chip input through holes by feeding through a chip cap which holds the

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tubing securely in place. Epoxy resin adhesive (Loctite 3421 A&B Hysol, Henkel) was applied to the chip cap and the cap pushed into place. The surface around the tube inlet of the chip was previously roughened using sand paper to increase the bond strength. The detection components and chip were placed into the detection cell holder generated by a 3D printer (Dimension SST 768), which incorporated custom designed features for housing the photodiode (epd-440-0/2.5, UV, Roithner) and the 430 nm LED (1045418, Blued, 5 mm, emission bandwidth of 40 nm, Farnell, Ireland)[18] which overlaps the spectrum of the nitrate-chromotropic acid complex as shown in Fig. S3. The photodiode detector and LED were aligned on opposite sides of the chip's optical cuvette allowing for absorbance measurements of the nitrate-chromotropic acid complex (Fig. 2(A)).
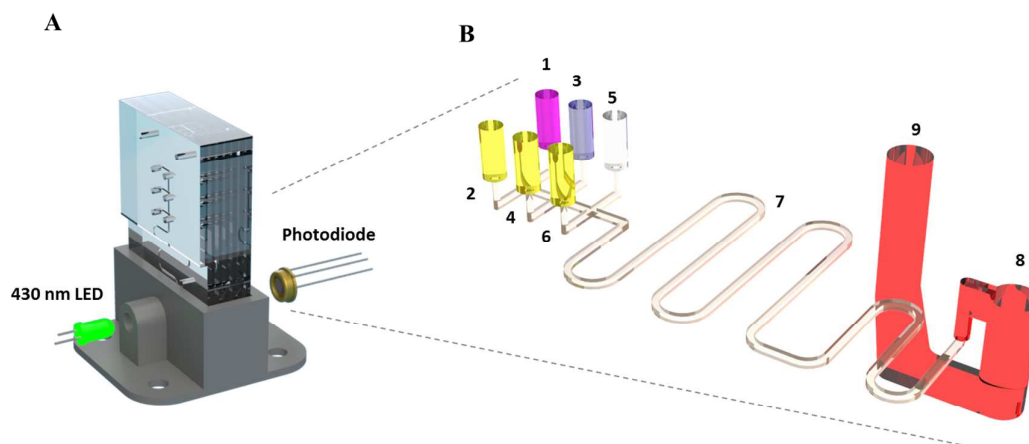


**Figure 2.** (A) Rendered representation of the flow system and LED/PD detection cell with LED and photodiode (B) Details of the fluidic system design [1] High standard inlet (1.7 mm diameter) [2] Reagent inlet (1.7 mm diameter) [3] Low standard inlet (1.7 mm diameter) [4] Reagent inlet (1.7 mm diameter) [5] Sample inlet (1.7 mm diameter) [6] Reagent inlet (1.7 mm diameter) [7] Serpentine/mixing channel (500 μm x 500 μm x 109. 74 mm) [8] micro cuvette (3 mm x 8 mm) [9] Waste Outlet (3.1 mm diameter).

### 2.5    Autonomous nitrate analyser

The nitrate analyser shown in Fig. 3 is a fully integrated system consisting of sampling, detection and communication components. Components included storage units for the chromotropic acid reagent, waste solution and calibration standards, pumping system to control the transport and mixing of the sample, and a microfluidic mixing and detection chip resulting in a low cost, rapid and simple instrument for the measurement of nitrate.

Three miniature double headed peristaltic pumps (102-005-012-016/4, Williamson Ltd.) allowed for the pumping of reagent and sample into the microfluidic chip at a flow rate of 600 μl/min using the same motor to ensure a 1:1 v/v reagent to sample ratio. Viton® tubing of 1.6 mm internal diameter and 5 mm outer diameter (Williamson Pumps Ltd.) was used within the peristaltic pump. To prevent back-flow within the system, polyvinylidene fluoride (PVDF) miniature check valves with a Viton® diaphragm (11873843, Cole-Parmer, Ireland) were used. Teflon tubing of 0.3 mm inner diameter and 1/16" outer diameter (008T16-30-20, Kinesis Ltd.) was used to introduce the sample and reagent to the chip and Teflon tubing of 1/16" inner diameter, 1/8" outer diameter was used for the waste line. Epoxy resin adhesive (Loctite 3421 A&B Hysol, Henkel) provided leak-free connections between the Viton tubing,

the Teflon tubing and the microfluidic chip. By using the epoxy resin adhesive additional components such as Teflon unions, tees or adapters were avoided, effectively lowering the component cost of the platform.

For each sample assay, the instrument measured a 'blank' solution (0 mg/L $NO_3^-$, deionised water as per section 2.2) and a 'high' nitrate standard (80 mg/L $NO_3^-$). The high standard measurement was performed initially. The reagent and high standard were pumped into the serpentine/mixing channel *via* inlets 1 and 2 as per Fig. 2.(B). The resultant mixture was delivered to the optical cuvette within the chip for measurement, followed by transfer to the waste storage container *via* outlet 7. The procedure was then followed for the blank (low standard, 0 mg/L $NO_3^-$) and sample measurements respectively.
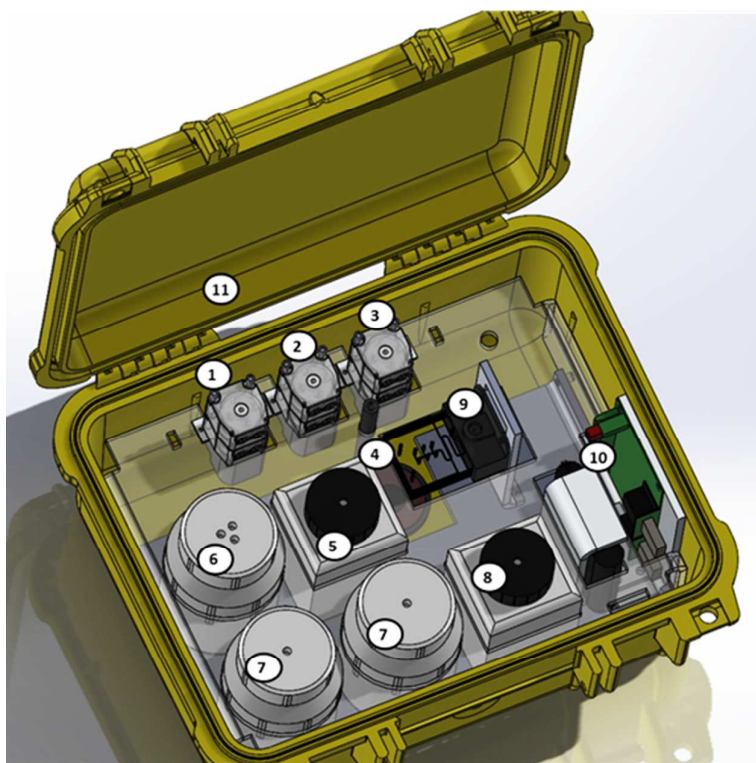


**Figure 3.** Integrated nitrate analyser (Tubing removed for easy viewing); [1] sample pump [2] high standard pump [3] low standard pump [4] sample inlet [5] high standard container [6] reagent container [7] waste containers [8] low standard container [9] microfluidic chip with LED/PD [10] control and communications board with 14 V lithium-ion polymer battery [11] waterproof housing.

As mentioned above, the nitrate analyser implemented a two-point measurement protocol using a 'low' blank solution (0 mg/L $NO_3^-$) and a 'high' standard solution (80 mg/L $NO_3^-$), the concentration of which can be varied depending on the particular site/sample in question. The reagent was stored in a Teflon® 250 mL bottle and the waste was stored in 2 x 250 mL Teflon® bottles to facilitate a larger volume of waste due to high and low standard measurements (VWR International Ltd., Ireland). The reagent storage capacity allowed for ca. 200 nitrate measurements. Teflon bottles are not necessary for the high and low standards and therefore standard 250 mL polyethylene bottles (HS25ON, Richmond CTP Ltd.) were

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used. The reagent bottle was attached to the tubing of one channel of each peristaltic pump; the tubing of the second channel of the pump was attached to the water sample, low standard or high standard as required.

Development of a field-deployable sensing platform requires a rugged enclosure to ensure the device is fully protected from the environment while still allowing for reliable sampling and communication. Therefore the system was installed into a waterproof and crushproof polycarbonate box (Peli™ Case 1400 Case). The interior dimensions (30 x 22.5 x 13.2 cm) allowed for housing of the peristaltic pumps, microfluidic chip and detection system, reagent and waste storage containers. The sampling inlet consisted of a sampling port with filter (Supor® 25 mm membrane filters, pore size 0.45 mm) holder. The filter holder (Fig.4) was designed and manufactured to minimise inlet dead volume while utilising a large portion of the filter surface. The filter membrane support unit shown in Fig.4(A), was fabricated from milled 1.5 mm thick PMMA using a Roland CNC micro-mill (Modela MDX40A) and placed onto a brass fitting using epoxy (Loctite 3421 A&B Hysol, Henkel). A knot was tied in the inlet tube and placed in the centre of the mould. Silicone sealant (Silcoset 158, ACC Silicones Ltd.) was poured into the brass fitting and allowed to dry for 48 hours. The filter membrane was placed on the mould and secured in place using a 25 mm diameter o-ring, 25 mm diameter washer and the brass fitting cap shown in Fig.4(B). To ensure all the containers, electronics and chip were secure; a holder was designed and fabricated from 3.5 mm thick PMMA using a laser cutter (Epilog Zing Laser), hot wire strip heater (CR Clarke 600) and folding jig (CR Clarke F0600). The transparency of the PMMA allowed easy visual checking for blockage or damage of the fluidics, and the system was designed so that components like bottles/batteries could be simply and safely removed and replaced when necessary.

A microcontroller (MSP430, F449, Texas Instruments) was used to control the operation of the pumping system and optical detector. The data was stored on a flash memory chip. For wireless communications, a GSM module interface powered by a rechargeable 14 V lithium-ion polymer battery (Olimex Ltd.) was used. Data files were in text format and communications employed RS232 data handling protocols.
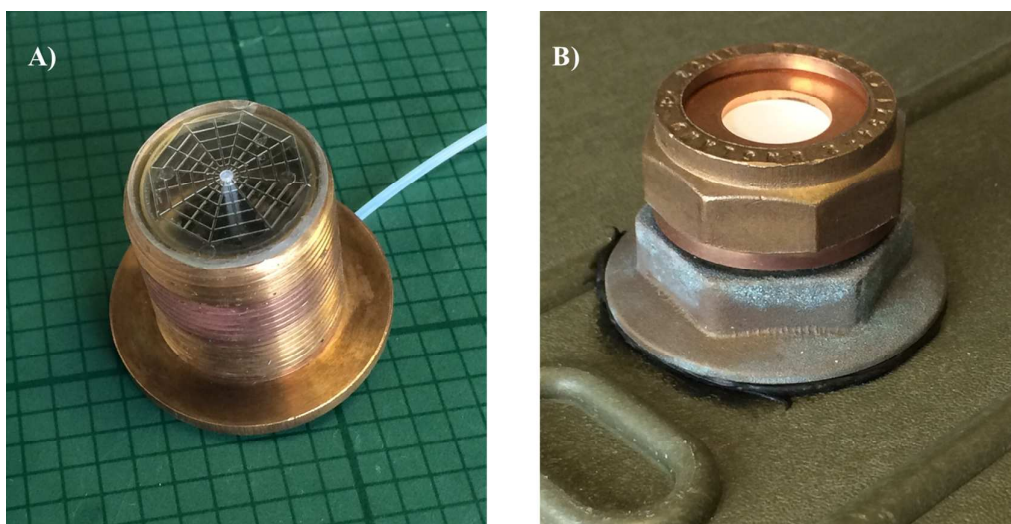


**Figure 4.** (A) Filter holder membrane support unit (B) membrane installation.

*2.6    Analytical procedure*

The analytical protocol involves four main steps:

1) Introduction of the water sample into the analyser for analysis.
2) Pumping of sample and reagent into the microfluidic chip, and on-chip mixing of sample and reagent.
3) Colour formation.
4) LED/PD absorbance measurement.
5) Storage of waste.

Mixing was performed on the chip by pumping the sample, low standard or high standard together with reagent through the serpentine mixer. Both the reagent and sample were pumped through the chip at a flow rate of 600 µL/min. When the chip had been completely filled, the flow was stopped for 5 min (as per section 3.2) to allow colour formation to reach a steady-state, and the associated absorbance was measured in the micro cuvette detector. Performing analysis using a stopped flow procedure not only results in a simple flow design but also minimises the consumption of reagent and standards, and generates less waste. The colour of the fluid in the micro-cuvette was then measured using a LED-PD set up. Combining unknown measurements with simultaneous reference measurements using standards enabled automatic compensation for drift and temperature effects on the system detector over time. Each unknown measurement was therefore compared to a blank and an 80 mg/L $NO_3^-$ standard.  The unknown sample concentration was estimated using equation 1 via a calibration model created previously with standard nitrate solutions (0, 5, 20, 40, 80 mg/L $NO_3^-$).  This approach enabled the overall stability of the calibration model to be checked and adjusted for each sample measurement.

$$NO_{3\ conc} = h_c \frac{log_{10}\ (s_a/l_a)}{log_{10}\ (h_a/l_a)} \hspace{3cm} \textit{(Equation 1)}$$

Where $NO_{3conc}$ is the nitrate concentration in mg/L, $h_c$ is the high standard concentration in mg/L, $s_a$ is the sample ADC reading, $l_a$ is the low standard ADC reading and $h_a$ is the high standard ADC reading.

## 3.  Results and discussion

*3.1    Material Study*

Wet chemical analysers contain many components that are exposed to a range of hostile environments due to the highly reactive reagent components like hydrochloric acid, phosphoric acid or sodium hydroxide that are often used.  These can adversely affect the useful lifetime of autonomous analysers, which therefore require regular servicing and replacement of components due to degradation.[4, 17, 19, 20] For example, we found that Tygon tubing used within the previous flow analysis platform[8] had a shelf life of less than 1 month of continuous use with the chromotropic acid method, due to the poor chemical compatibility with sulphuric acid which resulted in softening and discolouration (Fig. S4). Sulphuric acid is a strong acid, and a highly corrosive and viscous liquid. It is a powerful protonating and oxidising agent and vigorous dehydrating agent. We therefore implemented an extensive

study of over one year into the chemical compatibility of the chromotropic acid reagent and sulphuric acid (as per section 2.1) with various materials (see table 1).

**Table 1**. Effect of prolonged exposure (ca. 12 months) of concentrated sulphuric acid on a range of materials commonly used in microfluidics.

| *Material* | *Compatibility* |
|---|---|
| Polypropylene | Severe effect |
| Polyurethane | Severe effect |
| PTFE (Teflon) | Excellent stability |
| Kynar (PVDF) | Excellent stability |
| Polyether ether ketone (PEEK) | Severe effect |
| PVC (Polyvinyl chloride) | Severe effect |
| PMMA (Poly(methyl methacrylate)) | Severe effect |
| Silicone | Severe effect |
| Tygon® | Severe effect |
| Tygon® Fuel (Lubricant) Tubing | Severe effect |
| Neoprene | Severe effect |
| Santoprene | Severe effect |
| Viton® | Excellent stability |
| COP (cyclic olefin polymer) | Excellent stability |

Degradation was observed with many of the materials with sulphuric acid almost instantly (Fig.S5), specifically with Polyether ether ketone (PEEK) as shown in Vid.S1, and in some cases, contrary to the manufacturer specifications. For example, polypropylene and polyethylene we screened had specifications that stated excellent or good resistance to 98% sulphuric acid according to the manufacturers.[21, 22] In contrast, Viton® (a brand of synthetic rubber and fluoropolymer elastomer from DuPont Performance Elastomers L.L.C.) showed excellent resistance with no observable degradation effects, corrosion or discoloration when used in the presence of 98% sulphuric acid even when the exposure period was extended to 18 months as shown in Fig. S5.G. As a result, peristaltic pumps were the preferred option over syringe pumps as Viton® tubing could be used with a peristaltic pump for the safe and reliable movement of fluid throughout the sensing platform. The prototype system described in section 2.3 was tested continuously over a period of 7 months in which the chemical compatibility of nitrate chromotropic acid complex with Viton® tubing and the peristaltic pumps were tested. Calibration plots were carried out in April 2014 and November 2014 and are comparable with a slope ratio of 1: 1.02 as shown in Fig.5. As a PEDD detection system was used in April and a LED/PD detection system was used in November, the limit of detection (LOD) was calculated for both April and November plots, by multiplying the average standard deviation (sd) of the baseline absorbance of the blank by 3 (repeated $n = 5$ times, each baseline standard deviation taken from 30 baseline data points, frequency of measurement: 1 Hz). When this was applied to the equation of the line, the LOD was found to be 1.48 mg/L $NO_3^-$ (April 2014 using a PEDD detection system) and 1.78 mg/L $NO_3^-$ (November 2014 using a LED/PD detection system), respectively. These results are significant as the high concentration and aggressive nature of the highly concentrated sulphuric acid does not affect the materials in the fluidic system, in particular the Viton® tubing and peristaltic pump making this an ideal set-up for a field deployable platform. Furthermore, the reagents and method (optimised as reported previously)[8] are suitable for use over an extended period of time.
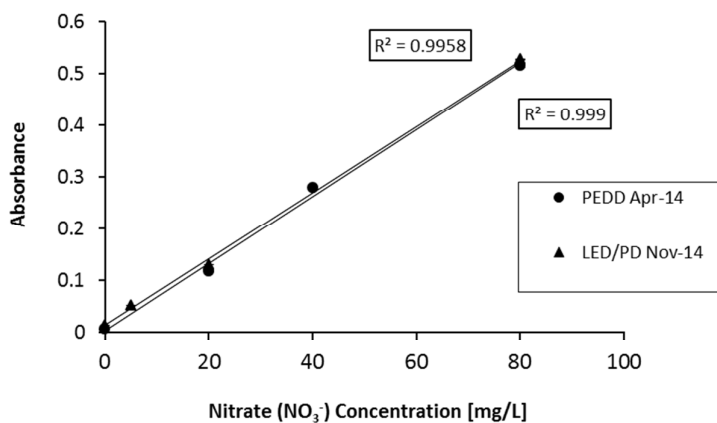
**Figure 5.** Calibration curves using the nitrate prototype analyser from 0 - 80 mg/L $NO_3^-$ and chromotropic acid complex from April 2014 – Nov 2014 (7 months). The standard deviations are represented as error bars ($n = 3$).

COP discs of 2 mm thickness and 120 mm diameter (Zeonex mcs-COP-04, Microfluidic Chip Shop, Netherlands) were tested using 98% concentrated sulphuric acid over a period of 6 months. No degradation or colour formation appeared within this time as evident from Fig. S6, indicating that this would be a suitable material for producing microfluidic chips for this method.

### 3.2    Kinetics Study

In previous work, it was shown that the simplified chromotropic acid method followed first order kinetics, with the absorbance increasing rapidly until approximately 180 sec after reagent/sample mixing, at which point a steady state was reached.[8] For the same method implemented within the microfluidic chip, the signal stabilised after around 300 sec at room temperature, Fig.6. As, with the current fluidic design, it takes 1 min pumping to transport the sample through the fluidic system and 5 min to achieve the steady state under stopped flow conditions, the minimum measurement time for each measurement is ca. 6.5 min.
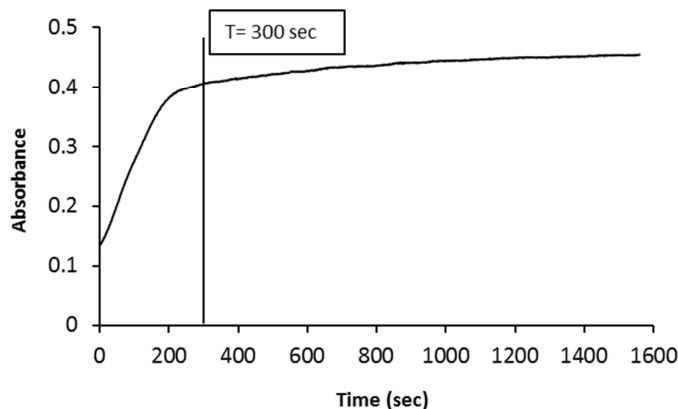


**Figure 6.** Time profile of the dynamics of colour formation obtained with 80 mg/L $NO_3^-$ standard and chromotropic acid reagent at room temperature.

*3.3 Calibration Study*

A calibration study was carried out with the microfluidic sensing platform using nitrate standards ranging from $0 - 80$ mg/L $NO_3^-$ at a wavelength of 430 nm. Results (Fig. 7) show a correlation coefficient of 0.9901 with an average RSD of 7.38% and an apparent extinction coefficient ($\varepsilon$) of $4.45 \times 10^2$ L mol$^{-1}$.cm$^{-1}$. The method gives a linear response to nitrate concentrations up to 80 mg/L $NO_3^-$ after which the absorbance plateaus.
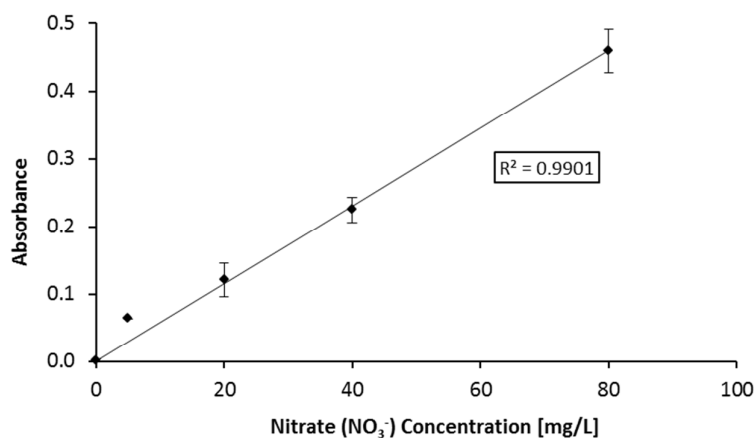


**Figure 7.** Chromotropic acid method calibration curve generated with the microfluidic nitrate analyser, using nitrate standards ranging from $0 - 80$ mg/L nitrate. The error bars represent standard deviations for *n* = 3 replicates.

*3.4 Repeatability and Limit of detection*

The repeatability of the measurement was determined by stop flow analysis of the blank (0 mg/L $NO_3^-$) and the high measurement (80 mg/L $NO_3^-$) seven times. The sample and reagent were passed through the chip simultaneously at a flow rate of 600 µl/min. When the flow stopped diffusional mixing took place and the two solutions then reacted to form the nitrate-chromotropic acid complex. Excellent signal stability and repeatability of the measurement technique is shown in Fig.8 with an average RSD of 0.28% for the blank and 0.19 % RSD for the high standard.
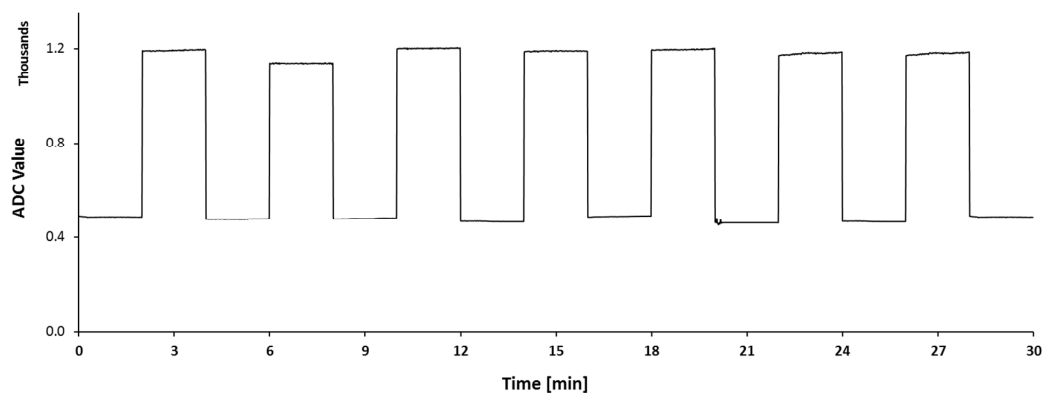
**Figure 8.** Multiple injection plugs of 0 mg/L $NO_3^-$ and 80 mg/L $NO_3^-$ with chromotropic acid reagent under stopped flow conditions.


The limit of detection (LOD) was found by multiplying the standard deviation (sd) of the baseline absorbance by 3 (*n* = 7, 10 data points per each blank measurement, frequency of measurement: 1 Hz). Using the equation of the line, a LOD of 0.70 mg/L $NO_3^-$ chromotropic acid reagent complex was obtained, which is similar to an equivalent bench set up (0.73 mg/L $NO_3^-$).[8]


### 3.5    Application to Real Samples

The analyser was applied to determine the nitrate concentration in nine water samples from various environmental sources including surface water, effluent, seawater and standards. The samples were split, and parallel assays independently performed at the T.E. Laboratory site (Carlow, Ireland). The samples were filtered prior to analysis using the membrane and sample inlet described in section 2.5. The results obtained for the nine samples with the autonomous analyser, and a Hach® handheld portable colorimeter using NitraVer® 5 reagent powder pillows (detection range: 1.32 – 133 mg/L $NO_3^-$), were compared to reference measurements obtained using ion chromatography (IC), see table 2. Overall, the results for IC and the nitrate analyser are in good agreement while the Hach® handheld colorimeter results are generally higher than both IC and the analyser. Samples #8 and #9 were measured with reference to nitrate standards (0, 10, 40, 80 mg/L $NO_3^-$) made using artificial seawater free from nitrates (Instant Ocean® Aquarium Systems, Inc.) due to the highly different background matrix which shifts the calibration of the chromotropic acid method slightly (Fig.S7). The LOD using seawater standards was found by multiplying the standard deviation (sd) of the baseline absorbance by 3 (*n* = 7, 10 data points per each blank measurement, frequency of measurement: 1 Hz). Using the equation of the line, a LOD of 0.31 mg/L $NO_3^-$ chromotropic acid regent complex was obtained. Samples #8 and #9 were calculated as <0.31 mg/L $NO_3^-$ and 24.44 mg/L $NO_3^-$, respectively. These results are in good agreement with those obtained by ion chromatography, indicating that the chromotropic method can be successfully applied to the determination of nitrate in marine waters. Overall, an excellent correlation coefficient of 0.9969 was obtained between the analyser and ion chromatography for samples #1 to #9 (Fig.S8).

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**Table 2**. Determination of nitrate in nine water samples using the microfluidic nitrate analyser (*n*= 3) and comparison with ion chromatography reference method and Hach® colorimeter.

| *Sample reference* | *Sample characteristics* | *Ion chromatography ($NO_3^-$ mg/L)* | *Microfluidic Nitrate analyser ($NO_3^-$ mg/L)* | *Hach® colorimeter ($NO_3^-$ mg/L)* |
|---|---|---|---|---|
| 1 | Drinking water | 10.6 | 12.29 ± 0.82 | 14.7 |
| 2 | Surface water | 20.3 | 19.86 ± 0.73 | 27.8 |
| 3 | Effluent | 11.1 | 12.93 ± 0.50 | 20.6 |
| 4 | Effluent | <0.5 | <0.70 ± 0.95 | 1.3 |
| 5 | Surface water | <0.5 | <0.70 ± 1.25 | 5.7 |
| 6 | Deionised water (Blank) | <0.5 | <0.70 ± 1.89 | 5.5 |
| 7 | Deionised water (50 mg/L $NO_3^-$ standard) | 50 | 50.47 ± 0.81 | 63.7 |
| 8 | Seawater (Blank) | 0 | <0.31 ± 0.61* | 3.07 |
| 9 | Seawater (25 mg/L $NO_3^-$ standard) | 25 | 24.44 ±0.62* | 31.5 |

**\***These results were obtained using the nitrate analyser calibrated with nitrate standards prepared from nitrate-free artificial seawater (Instant Ocean® Aquarium Systems, Inc.).

## 4. Conclusion

A low-cost, novel portable system for long-term monitoring of nitrate has been developed. This completely autonomous device incorporates sampling, reagent and waste storage, colorimetric detection, wireless communication and a power supply into a complete, miniaturized system. For nitrate detection, the method is simpler than the popular Griess method due to the elimination of the necessity for a reduction step. We have successfully identified materials and components that are chemically resistant to the acidic nature of the reagent, enabling a robust microfluidic chip based platform to be produced. The analyser was applied to real samples including drinking water, freshwater, wastewater and seawater. The results obtained were in excellent agreement with ion chromatography. Table 3 summarises the overall analytical figures of merit and system specifications for the microfluidic analyser platform.

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**Table 3.** System Specifications

| | Matrix | |
|---|---|---|
| | *Deionised water* | *Artificial Seawater* |
| *LOD (NO$_3^-$ mg/L)* | 0.70 | 0.31 |
| *Linearity (R$^2$)* | 0.9901 | 0.9887 |
| *Repeatability RSD % (n =3)* | 7.38 | 1.62 |
| *Sample throughput* | 9 sample h$^{-1}$ | |
| *Reagent stability* | At least 6 months | |
| *Reagent consumption per sample* | 1.2 ml | |
| *Component cost* | €600 | |
| *Size and weight* | 30 x 22.5 x 13.2 cm, 3.5 kg | |

The prototype system has a component cost of ca. €600 as shown in Fig. S9, potentially delivering a highly competitive cost compared to commercially available autonomous sensing platforms for nitrate (YSI$^{TM}$/YSI96000 autonomous nitrate analyser retails at an indicative cost of ca. €25000 per unit; similar instruments from Ecotech$^{TM}$/FIA NUT1000, EnviroTech$^{TM}$/AutoLab and S:CAN spectrolyser typically retail in the range €15000 - €70000).[23, 24] The advantages of this device include its simplicity, reduced costs, efficient energy use, low consumption of reagents and less waste production, compact design, acceptable analytical performance and high sample throughput.

## 5. Acknowledgments

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**Graphical abstract for TOC only**

This study has demonstrated, for the first time, a microfluidic autonomous analyser for the direct determination of nitrate, incorporating a modified version of the chromotopic method resulting in a direct, quick, inexpensive and simple procedure to measure nitrate *in situ*.