



Measurement of nano molar ammonium with a cyclic olefin copolymer microchip and low-power LED

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ABSTRACT

In oligotrophic regions, ammonium (NH_4^+) concentrations can be below 50 nM, however, few existing instruments can measure below this level with high confidence. This work, based on the o-phthalaldehyde (OPA) fluorescence assay, is applied to measure nanomolar NH_4^+ in a novel optofluidic Cyclic Olefin Copolymer (COC) microchip using a low-power (20 mW) Light Emitting Diode (LED) as the excitation source. The optical arrangement was first modeled using ray tracing software to determine the initial detection volume size. Ammonium standards made with artificial seawater of 5 nM to 1000 nM, were run in triplicates. The limit of detection (LOD) obtained was 1.5 nM ($3 \times \sigma$ of the blank) or a LOD of 15 nM when the y-intercept and the vertical variation of each measured concentration on the calibration curve were taken into consideration (y -intercept $+3. S_{y/x}$). Precision at 5 nM and 1000 nM was 3.3% and 0.5% respectively. The optofluidic system was also compared to an off-the-shelf fluorometer (Jasco FP2020) and an existing high-resolution shipboard analyser using five different standard concentrations. The LOD and the ammonium concentrations uncertainty for the Jasco FP2020, shipboard analyser, and current microsystem were 217 nM, 39 nM, and 15 nM and ± 232 nM, ± 48 nM, and ± 16 nM respectively. The optical setup was also validated using real samples from the Atlantic. This optical design, without optical fibres, makes the system simple and suitable for use with other fluorescent assays when compact, rugged, low-cost, and low-power consumption instrumentation is required.

1. Introduction

Ammonium (NH_4^+) is a key nutrient in the biogeochemical cycle [1]. Measuring ammonium contributes to a better understanding of the nitrogen cycle. The seawater NH_4^+ is formed due to the degradation of organic matter or nitrogen fixation from the water surface. NH_4^+ serves as a nutrition source for phytoplankton. The measuring of NH_4^+ in natural waters has also been associated with the exchange of ammonia in the atmosphere and the oceans, which has recently been one of the focus nutrients due to climate awareness. Uncertainty in measuring ammonium leads to incorrect conclusions about ecosystem processes and invalid comparisons across sites. In natural waters, the supply of nitrogen to phytoplankton is dependent on the uptake of nitrate and NH_4^+ [2]. Phytoplankton preferentially takes up nitrogen from NH_4^+ because this is more energy efficient given its low oxidation state [3]. Thus, NH_4^+ is found well below the micromolar concentrations and typically $<1 \mu\text{mol/L}$ in most marine environments. In oligotrophic regions, NH_4^+ can be

found below 50 nM concentrations [4].

Methods for measuring ammonium are numerous [5]. Commonly used methodologies and techniques for measuring NH_4^+ in natural waters include ion-selective electrodes [6,7], spectrophotometric [8], fluorometric [9], and methods based on matrix separation [10]. The ion-selective method is the most suitable for rapid monitoring but is rarely applied to the determination of low ammonium due to low sensitivity and interference from other ions. This is mainly due to the complexity and heterogeneity of the natural samples [11]. On the other hand, bench-top chemical analysers based on the matrix separation and spectrophotometric methods have achieved acceptable precisions of 3.8% (at 100 nM) [12] and 4.4% (at 50 nM) [13] respectively, whilst using long-path liquid core waveguide cell. The assay used for high-performing ammonium shipboard or benchtop analysers is mainly fluorescence-based using the product of o-phthalaldehyde (OPA) and NH_4^+ in the presence of sulphite. The reaction of OPA and NH_4^+ produces an intensely fluorescent isoindole derivative. The excitation (λ_{ex}) and

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Table 1
Performance comparison of ammonium microfluidic measurement systems.

Method/ Technique	L.O.D	Excitation Source Power	Tested range Precision	Detection Material	Detection Volume	Environment	Ref Year
Electrochemical/ISE	1.7 μM	-	4.4 μM – 55 mM	COC	2 μL	Standard solutions, fresh & wastewater	[7] 2023
Electrochemical/ISE	5.5 nM	-	5.5 nM- 55 mM	PDMS/Glass	-	Standard solutions	[6] 2014
Electrochemical	40 μM	-	-	PDMS	-	Standard solutions, wastewater & tap water	[41] 2019
Colorimetric	97 μM	-	55 μM – 5.5 mM	Paper	-	Standard solutions	[42] 2023
Colorimetric/gas diffusion	-	LED/Fibre Optics	11 μM - 2.8 mM	PDMS/ 3 layers	-	Standard solutions and ash deposition	[24] 2019
Colorimetric	< 0.56 mM	-	0.56–57.1 mM	Paper	1.2 mL	Standard solutions	[43] 2018
Colorimetric/ FIA	8.4 μM	LED -	28–280 μM	D-ABS/ Graphene PLA	100 μL	Standard solutions & two river samples	[44] 2020
Colorimetric	2.5 μM	-	3–70 μM	SU-8	395 nL	Ammonia in cell culture media	[45] 2020
Colorimetric	17.9 μM	-	28–1680 μM	Paper: BTB- based	8 μL	Standard solutions, freshwater samples and certified reference material	[46] 2019
Colorimetric	26.3 μM	-	112–560 μM	Paper: NY-based	8 μL	Standard solutions, freshwater samples and certified reference material	[46] 2019
Colorimetric	187 μM	-	Upto 280 μM	Paper	15 μL	Standard solutions & wastewater samples	[47] 2020
Spectrophotometric	111 μM	LED 20 mW	112–840 μM	PDMS/ Ninhydrin	-	Standard solutions	[48] 2018
Spectrophotometric	0.02 μM	LED -	1–100 μM	PDMS	0.5 μL	Standard solutions, tap, rain & lake samples	[31] 2012
Spectrophotometric	1.5 nM	LED 20 mW	5 nM - 1 μM < 6%	COC	25 μL	Standard solutions with artificial sea water & low nutrient seawater	Current work

emission (λ_{em}) are located around 360nm and 420nm respectively [14,15]. Some groups added formaldehyde to form a stable α -hydroxy methane sulfonate complex [16], which resulted in an increased reaction response of OPA to ammonium, improved selectivity for ammonium, and hence increased the fluorescence signal. To our knowledge, the highest resolution ammonium measuring system using the OPA, sulphite and formaldehyde assay is a shipboard-type system with a precision of 6.7% (at 1 nM) [17]. Our previous work utilized the OPA and sulphite reagent without formaldehyde (due to its toxicity to humans and the environment) achieving a precision range of 1–4% (at 5 nM to 25 μM) in oligotrophic seawaters [18]. The selectivity of the fluorescence assay to ammonium and potential interferences with salinity, amino acids, amines, and algae were also discussed [16,18,19]. The fluorescence method without the use of formaldehyde is acceptable in terms of selectivity when the target ammonium concentrations are low.

On the other hand, microfluidic technologies provide an excellent platform for measuring nutrients in seawater [20] but have been underutilized for in-situ ammonium applications and field measurements in the nanomolar range. This is mainly due to the availability of multiple ammonium methods, each associated with specific electronics and/or optical requirements producing varied performance. Usually, the ammonium measuring methods that produce high precision results are relatively complex and more challenging to integrate on a microfluidic chip such as the long 1 m or 2.5 m liquid core waveguides used by Kodama et al. [12] or Zhu et al. [13]. In addition, when it comes to remote in-situ deployments using microfluidics, optical sources used for excitation such as lasers [21,22] could become a challenge to integrate ruggedly, not to mention the limited power budget available. Hence, recent analytical instruments have successfully demonstrated the use of light emitting diodes (LED) as the excitation source instead of lasers. A more in-depth review of LEDs for analytical chemistry applications is studied in detail [23]. LEDs coupled to optical fibres using a

polydimethylsiloxane (PDMS) gas-diffusion membrane, for ammonium colorimetric detection was reported for concentrations above 11 μM [24]. Also, the use of LEDs and optical fibres in fluorescence-based microfluidic systems was not limited to ammonium analysis [25]. A wide range of nutrients were measured using similar optical arrangements. For example, a fluorescence-based analytical system targeting phosphate using an ultra-bright LED coupled to an optical fibre demonstrated a limit of detection (LOD) of 2.1 μM [26]. A PDMS microchip with two low-power LEDs coupled with optical fibres have been used for Lead (Pb) measurements with a LOD of 24 nM [27]. Also, using the PDMS microchip, a single low-power blue LED was used to measure amino acid derivatives with a reported LOD of 120 nM, using an optical fibre at the detection end [28]. A summary of the microfluidic sensor systems applied for ammonium measurements is shown in Table 1.

From the reliability point of view and previous underwater in-situ experience [29], we believe the use of LEDs, without any optical fibres or lasers, in chemical sensing is becoming our preferred option [30]. The use of optical fibres can lead to loss of optical power due to light alignment challenges [31], and reduce the ruggedness of the system.

However, in microfluidic chips, the use of LEDs instead of lasers as the excitation source without optical fibres comes at a price. For minimum power consumption, the preferred use of relatively low power LEDs (20 mW) means less focused excitation, consequently less fluorescence output and, hence, less signal-to-noise ratio making the use of cheap detection (such as the TSL257) a challenge and limiting the measurements to only the high ammonium concentrations. This is evident as shown in previous work [31] utilizing a PDMS chip with a LED as the excitation source and a photon multiplier tube (PMT) as the detector (in an orthogonal alignment). The measured ammonium fluorescence was carried out over the range of 1.2–128 μM with a further repeatability analysis conducted at 12.8 μM [31].

In this work, we aim to design a new and simple optofluidic design

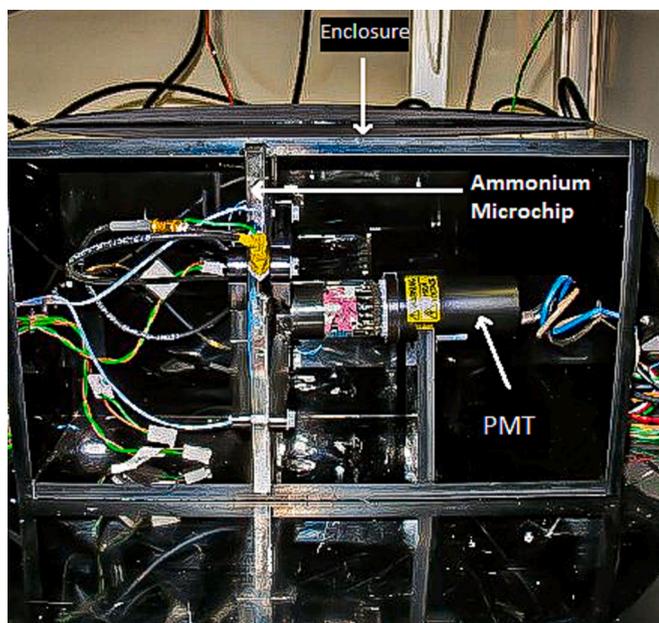


Fig. 1. Ammonium microsystem tested on the bench.

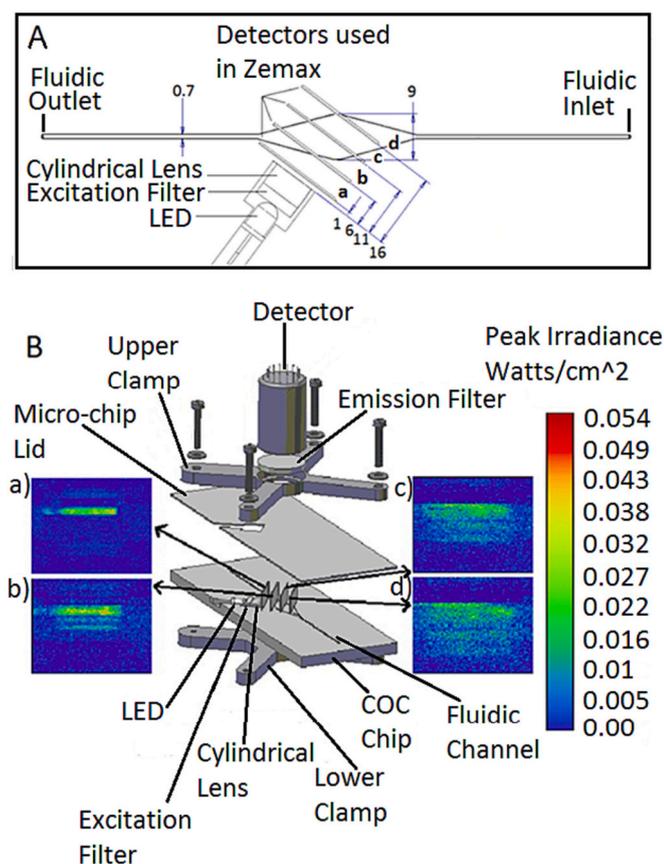


Fig. 2. A) 2D schematic of the chip detailing flow cell, excitation optics, and location of virtual detectors 'a' to 'd' (200×200 pixels) used in Zemax simulation (dimensions are in millimetres) B) 3D CAD used in Zemax simulation together with simulated irradiance results at each of the virtual detectors labeled 'a' to 'd'.

machined on a cyclic olefin copolymer (COC) chip with the use of one low-powered ultra violet (UV)-LED as the excitation source whilst targeting and measuring ammonium using artificial seawater in the nano-

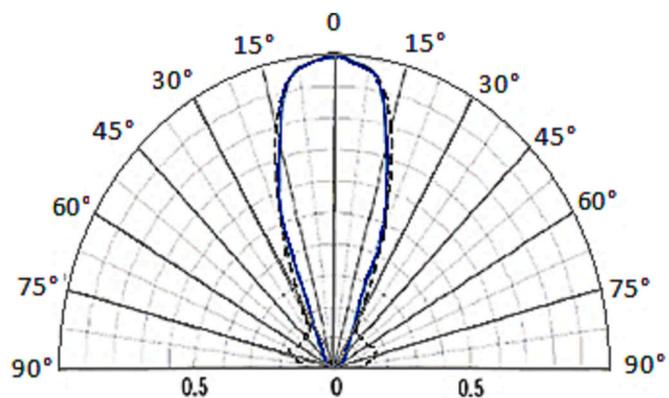


Fig. 3. Polar radiant intensity for the LED used (solid —) and LED created in Zemax (dotted - - -).

molar range.

In this work, we plan to use a PMT as the fluorescence detector to assess the microsystem design performance against other shipboard and microfluidic systems reported in the literature. The possibility of using a cheap photodiode instead of the PMT will be also discussed.

2. Materials and methods

2.1. Chip material and optical design

Microchip materials reported in the literature were compared for both compatibility with the assay used and optical requirements. For example, PDMS is relatively porous, and may lead to carryover between samples [32], has a less long-term performance, and is incompatible with flushing fluids used for high NH_4^+ concentration measurements [33]. A potential active ingredient for flushing high ammonium concentrations is isopropyl alcohol [18]. One challenge for developing NH_4^+ measurement on a chip is that the fluorescence product has absorption and emission spectra centered at 370 nm and 425 nm respectively. This prevents the use of many materials like polycarbonate (PC) and poly methyl methacrylate (PMMA) due to material yellowing or low UV transmission [34]. On the other hand, the advantages of COC material are well explained [35]. The material optical properties combined with the need to use low-power LEDs without couplings to optical fibres, results in further low optical efficiencies [36]. Although quartz glass has a high transmission for the desired wavelengths, micro-fabrication methods (e.g. deep hydrofluoric acid etching) are demanding and not suitable for laboratory prototyping. In contrast, the COC material can be machined, has good inherent optical properties at the required range, is chemically inert, with no or limited autofluorescence [37], and is suitable for cyclic heating making them ideally suited to NH_4^+ measurement. The COC material has the potential for full integration of the ammonium fluorescence method in the future.

Hence, we have implemented the proposed optical design using the COC for the measurement of NH_4^+ . The experimental setup is shown in Fig. 1 and the optical design adopted is shown in Fig. 2A. The fluorescence was induced using a 20 mW LED (375 nm, XRL-375-5E, Roithner Lasers) modulated at 80 Hz. A narrow (FWHM of 5 Hz) analogue band pass filter (BPF) at 80 Hz, with -40 dB at 50 Hz was used to demodulate the signal. An excitation filter (Semrock FF01-370/10) and an uncoated borosilicate glass cylindrical lens (5 mm diameter) were used to filter the light and focus it into a sheet through the middle of the channel respectively. The fluorescence signal was filtered (Semrock FF01-427/10) and measured at a 90° angle using a PMT (Hamamatsu 5784-20). Fig. 2A shows the LED and lenses securely seated into the COC chip. This ensures good alignment of light from the LED to the detection channel. Fig. 2B shows an expanded CAD model of the chip and optical setup used as the

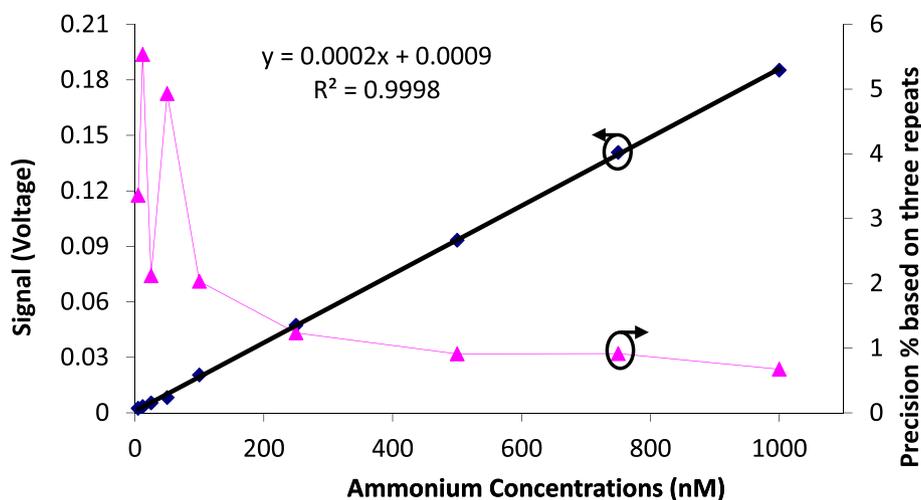


Fig. 4. Average optical signal response when running triplicates of ammonium standards 5, 12, 25, 50, 100, 250, 500, 750 and 1000 nM. Errors were minute for display and thus were not shown in this figure.

basis for the optical modelling. The software used is Zemax (Radiant Zemax, USA) with the default non-sequential ray-tracing mode. The optical properties of the COC material were chosen from the Zemax library. The LED used in Zemax was developed as a custom representation to match the polar irradiance intensity of the off-the-shelf LED used on the ammonium chip as shown in Fig. 3.

The detection volume was analysed and determined in Zemax based on the Milli-Q water absorption as shown in Fig. 2B. The absorption of the UV-LED was analysed in Zemax using four virtual 200×200 pixel detectors (2x2cm each) placed at 1, 6, 11, and 16 mm away from the cylindrical lens (results are shown in Fig. 2B). The LED irradiance at the virtual detector (d, 16 mm), was absorbed by >80%. Hence, the detection volume was chosen as a rhombus around $450 \mu\text{m}$ deep (25 μL) to aid fluidic flushing, reducing carry-over between samples, whilst maximizing the fluorescence capture.

2.2. Material and fabrication

The COC substrates (Topas, type 5013S) comprising the microchip were pre-annealed at 115°C . The upper and lower substrates used were 1 mm and 7 mm thick respectively. Solvent bonding using the cyclohexane vapour was used to bond the substrates and to optimise the optical efficiency. Substrates were exposed to cyclohexane vapour for four minutes at 25°C and then pressed at 55 N/cm^2 for 40 min [38].

2.3. Chemicals and reagents

The reagents were made from sodium sulphite (Na_2SO_3), disodium tetraborate decahydrate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$), and OPA with a minimum purity of 97% (Sigma Aldrich). Initially, three stock solutions 10 mL of 0.06 M sodium sulphite, 50 mL of 1.25 M OPA and 1000 mL of 0.079 M of disodium tetraborate decahydrate were prepared as described in [18]. From the above stock solutions, two working reagents for our experiment were made. One reagent (R) with OPA and the other without OPA each with a pH of 9.4. The use of the two reagents is mainly to minimise organic matter fluorescence, OPA autofluorescence, and any density change in the mixture. This was taken into consideration in the subtraction process between the (standard + R + OPA) and (standard + R - OPA) signals at two different stages with and without a heater. The procedure is explained in more detail in [18] with the main aim of maximizing the system's sensitivity to the signal of interest. Artificial NH_4^+ standards were prepared using artificial seawater (ASW). The ASW contains 24.54 gL^{-1} of NaCl (0.42 M) and 0.2 gL^{-1} of NaHCO_3 (2.38×10^{-3} M) dissolved in Milli-Q water to give a salinity of 35 and pH of 8.4.

Detailed preparation steps are described in detail [18]. Reagents and standards were made in acid-washed laboratory glassware. The concentration of the reactive OPA, described above, was determined empirically to achieve the maximum fluorescence across the desired concentration range of NH_4^+ . Contamination from handling and laboratory air becomes a critical factor when dealing with nano-molar concentrations. Samples are prepared in relatively larger volumes using glassware to minimise atmospheric contaminations. In addition, the newly prepared samples are transferred immediately to hermetically sealed polyvinyl chloride (PVC) bags. The bags are connected to flexible tubings using $1/4''$ -29/8 UNF male / Luer-lock adaptors. The tubing then couples to the 3-way Biochem valves using polyether ether ketone (PEEK) fittings. Since the main reagent is sensitive to light, associated bags, and tubing are shielded from light. The samples are run immediately after preparation and are not re-refrigerated. Ammonium adsorption is also reduced when standards are made with 0.2 g L^{-1} NaCl solution instead of pure water [14]. According to EN ISO 5667-3, samples can be stored in plastic bottles for up to one month in freezer at below -18°C . Some reported stable samples for up to 4 months when stored between -18°C and -20°C .

3. Results and discussions

The 95% confidence calibration curve when running triplicates of ammonium standards of 5, 12, 25, 50, 100, 250, 500, 750, and 1000 nM, in the proposed optofluidic design, is linear in the form of:

$$Y = (0.000185 \pm 2.14\text{E} - 06) x + (0.000918 \pm 0.000981)$$

A typical calibration curve of the resultant device is shown in Fig. 4, where error bars were minute and not shown in the figure. The precision (%) is shown on the right axis (Fig. 4). The measurements were conducted sequentially and the blank baseline was re-established after running either high or low ammonium concentrations with the same amount of flushing in all cases. Flushing the chip with Milli-Q has an estimated Reynold number below 0.01. This laminar flow ensures consistent flushing. In this experiment, off-the-shelf peristaltic pumps (Instech- model P625) and solenoid valves (Biochem-075 T series isolation valves-3 way) controlled by Labview were used to deliver the samples automatically.

All solutions used were equilibrated at room temperature before conducting the experiments. To further assess the current optical design performance, NH_4^+ standards of $\sim 5 \text{ nM}$, 25 nM , 50 nM , 500 nM , and 1000 nM were tested in triplicate using an off-the-shelf Jasco FP2020 fluorometer and a high-resolution shipboard ammonium analyser. The

Table 2

Comparison of calibration curves of the form $y = bx + a$, of the COC micro chip optical design (current work) against off-the-shelf fluorometer (Jasco FP-2020) and a high resolution shipboard analyser. The slope is b and the y-intercept is a . The number of calibration points is n , r^2 is the correlation coefficient, S_x is the standard deviation (uncertainty) of the calculated NH_4^+ concentrations. The statistic t -values were chosen based on 95% confidence level.

Platform used	n, t, r^2	$a \pm t.Sa$ $b \pm t.Sb$	S_x (nM)	LOD nM \pm (t. S_x)
Jasco FP-2020	5, 3.18, 0.97933	0.0052 ± 0.0092 0.000069 ± 0.000018	73	$217 \pm (232)$
Shipboard	5, 3.18, 0.99931	0.025 ± 0.0060 0.00025 ± 0.000012	15	$39 \pm (48)$
Current work	5, 0.18, 0.99988	0.00042 ± 0.0017 0.00018 ± 0.0000035	5	$15 \pm (16)$

shipboard ammonium analyser used, as one of the benchmarks, is tested and characterized in the field and its performance is cross-compared with manual sampling. In addition, background fluorescence, contamination, and sample interaction with matrixes are all discussed in detail [18]. The relative standard deviation results for the Jasco FP2020, shipboard analyser, and current microanalyser at 5 nM concentration were 6%, 1.3%, and 0.2% respectively.

For a fair comparison with the work reported in the literature, the LOD was calculated in two ways. First, the LOD was considered as $3 \times \sigma$ (σ is the blank standard deviation) calculated as 1.5 nM, which compares to the 26 nM [31] and 5 nM [18]. The second preferable method [39], used for the LOD calculation, considers the residual of each point on the calibration plot (standard y-deviations $S_{y/x}$) together with the intercept 'a' of the regression line as shown in Table 2. i.e. the LOD (in volts) is considered as $(a + 3 \times S_{y/x})$. Then, the equivalent concentrations' confidence limits were obtained using the calculated standard deviation of the concentration S_x (nM) with $(n-2)$ degrees of freedom yielding a confidence limit of $\pm (t \times S_x)$ nM. The uncertainty in

calculating any ammonium concentration using the obtained calibration curves (Table 2) for the Jasco FP2020, shipboard analyser, and current work was ± 232 nM, ± 48 nM, and ± 16 nM respectively. Hence, the proposed optical design compared favorably to the off-the-shelf Jasco FP2020 fluorometer and the high-resolution shipboard ammonium analyser in terms of both the LOD and concentrations uncertainty with 95% confidence. The system has good performance over the concentration range demonstrated by the characterisation shown in Fig. 4 and Table 2. In addition, real samples using low nutrient seawater (LNSW) spiked with 10, 20, 30, 40, and 50 nM were used. Results are shown in Fig. 5. The calculated LOD, using the y-intercept $+3 \cdot S_{y/x}$ method, is 4 nM. The limit of quantitation, using the y-intercept $+10 \cdot S_{y/x}$ method, is 16 nM. The current optical design is a one-step forward toward investigating the reaction efficiency at the micro scale as one of the potential challenges facing microfluidic sensor systems [40].

To evaluate the suitability of using low-cost detectors, the fluorescence irradiance at low ammonium concentration was estimated from the PMT output, and the likely performance, using the TSL257 detector, was estimated by calculation. The PMT output signal corresponding to the blank standard deviation σ value is around 0.1 mV (equivalent to 0.5 nM NH_4^+ concentration). Comparing the PMT datasheet information in terms of sensitivity and gain at 425 nm wavelength, then for a standard deviation σ (0.5 nM), the estimated fluorescence irradiance is around 1 pW/cm². For a TSL257 (cost \$2) detector, the published sensitivity is 1.18 V/ μ W/cm² at 425 nm (i.e. the signal output will be around 1 μ V for a 0.5 nM concentration). Considering the area detection ratio, this can be improved by 40 folds or more if proper focusing optical lenses are used. The TSL257 noise (shot and thermal) is typically around 6 μ V/sqrt (Hz). Thus, with a 10 Hz bandwidth filter used in the demodulation system, the output noise is around 19 μ V. Thus, it is anticipated when using the TSL257 as the detector, the LOD will be around 60 nM ($3 \times \sigma$ of the blank). It is important to highlight that these calculations exclude the circuit or external optical noise.

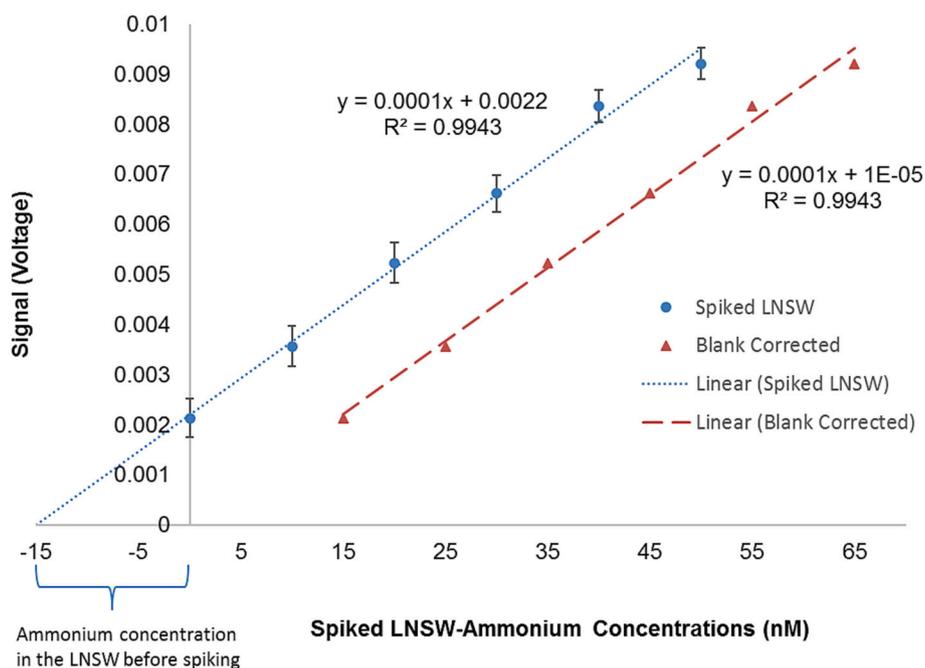


Fig. 5. The above graph shows the fluorescence signal detected by a PMT using a 20 mW LED as the excitation source. The dotted regression line (.....) is the calibration line of spiked low nutrient seawater (LNSW) with 10, 20, 30, 40, and 50 nM ammonium concentrations. Error bars resemble $\pm \sigma$. By extrapolation, the ammonium concentration in the LNSW was found to be around 15 nM. The dashed regression line (- - -) resembles the corrected samples after removing the background concentration of the LNSW.

4. Conclusions

In this work, we have demonstrated a high-performance proof of concept instrument that has a simple optical design with the potential for measuring NH_4^+ in the nanomolar range. In addition, the system was also demonstrated at relatively higher concentrations showing the system's potential for in-situ measurements over a wider dynamic range. The COC material used has allowed for high-efficiency fluorescence detection, whilst using low-cost and power LEDs as the excitation source without using optical fibres. Hence, the reduction of the detection volume is worth investigating. Future work could also look into the COC chip's long-term physical and chemical stability together with incorporating low-cost detection units. Fluorescence assay improvement is also a need to reduce the complexity of the method.

Authors' contributions

First Author: Samer Abi Kaed Bey (S. AKB).

Second Author: Matthew Mowlem (M. M).

S. AKB and M. M conceived of the presented idea. S. AKB developed the theory and performed the computations. S. AKB performed the experiments and measurements. M. M verified the analytical methods. M. M supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

Declaration of Competing Interest

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

Data availability

No data was used for the research described in the article.

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