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A miniature and field-applicable multipumping flow analyzer for ammonium monitoring in seawater with fluorescence detection

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ABSTRACT

In this article, a simple, economic, and miniature flow analyzer for ammonium in seawater based on the solenoid micropumps is presented. A single reagent of sodium tetraborate, ortho-phthaldialdehyde (OPA), and sodium sulfite was used and optimized applying the modified SIMPLEX method. A special-made detection cell for fluorescence detection of the reaction product isoindol-1-sulfonat was made and combined with a commercial photomultiplier tube, a long-pass optical filter, and an UV-LED as excitation light source. A LOD down to 13 nmol/L was achieved. The fabrication and application of a miniature reaction coil heating device for reaction rate enhancement is further described. The system featured an injection frequency of 32 h^{-1} at average standard deviation of 3%.

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1. Introduction

Ammonium is generated from the biodegradation of organic material and presents one of the important pools in the nitrogen metabolic cycle in estuarine, coastal, and open-ocean waters. Knowledge of ammonium concentration in seawater is of interest for characterization of the water masses for modeling nutrient fluxes and estimation of algae growth potential. Ammonium measurements are further accomplished in metabolic studies. Due to the substantially lower growth rate of ammonium-oxidizing bacteria compared to destruents and in consequence a delayed oxidation mechanism of ammonium to nitrite and nitrate, ammonium play also an important rule for the indication of metabolic activity in the water mass.

Spectrometric determination by the "Berthelot" or "indophenol-blue" reaction [1] is the classic and wide-spread applied analytical method for quantification of ammonium in seawater [2]. It is based on the reaction of ammonium with hypochlorite and further with a phenol compound to form the corresponding blue–green indophenol. Although there are manifold descriptions of automation of this reaction scheme on flow analysis platforms, the affection of the sample salinity [3], the required high reaction pH, which causes precipitation of earth-alkaline hydroxides, and hypochlorite degradation present important drawbacks [4]. The insufficient sensitivity for oligotrophic waters unless using long-path detection cells has to be named further [5,6].

Gas diffusion enrichment of ammonium and matrix separation is another common operation principle of analytical flow technique applications. Generally, the decrease of acidity of the acceptor solution, caused the dissolution of the gaseous ammonia, is quantified by monitoring the spectrum of an added pH indicator [19,20,23]. This method suffers from the required diffusion time to achieve an acceptable sensitivity for seawater monitoring [7] and required change or cleaning of the diffusion membrane to avoid failure due to biofouling or membrane blockage. Pervaporation or membrane-less gas diffusion have the potential to overcome the former drawbacks but did not achieve the required sensitivity for seawater analysis [8,9]

During the last two decades, quantification of ammonium by fluorescence spectrometry after its reaction with o-phthaldialdehyde (OPA) and reduction with sulfite has become commonly accepted. The reaction product isoindol-1-sulfonat shows an intense fluorescence at 425 nm using an excitation wavelength of 365 nm. The method shows less dependency on the sample salinity and higher sensitivity can be achieved. The method is further well-suited for trace analysis in seawater and estuarine waters. A more detailed

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comparison about mentioned and further methods for the analysis of ammonium is given elsewhere [10].

The method has been automated in former works using analytical flow techniques (FT) [4,11–17] using generally a commercial fluorescence spectrometer. FT are automation tools for preparative and analytical laboratory procedures carrying out the required steps in a tube assembly denoted manifold. Among the different techniques developed up-to-date differing in respect of flow conditions, operation scheme, manifold configuration, multipumping flow systems [18] have gained considerable interest due to the potential of miniaturization, individual control of each flow line, and simplicity. This technique is based in the use of solenoid micropumps as liquid drivers. They provide a semi-continuous flow of pronounced pulsed character, which enhance sample and reagent mixing. They allow the set-up of economic and multichannel flow systems and therefore present a powerful alternative to syringe pumps and peristaltic pumps as long as the backpressure in the system is low.

In this work we aimed the development of a simple, economic, and transportable analyzer system for ship-board monitoring. We adopted the OPA method on a solenoid micropump system applying a reverse FIA concept, i.e. the reagent was injected into the sample flow, which was consequently also used as carrier.

2. Material and methods

2.1. Reagents

All reagents were of analytical grade as not otherwise stated and Millipore quality water (>18 MΩ/cm) was used throughout. Aqueous stock solutions of 30 g/L sodium tetraborate, 8 g/L Na_2SO_3, and 0.5351 g NH_4Cl were prepared, the last two stored at 4 °C in the dark. A 40 g/L solution of OPA (Acros Organics, Geel, Belgium, Ref.: 131080050) in methanol was prepared and stored likewise at 4 °C in the dark.

The reagent working solution was prepared by mixing adequate quantities of the Na_2SO_3 solution about 1 h before usage. A 35% (w/v) solution of Brij-35 surfactant was prepared further and added to the reagent solution to achieve a final concentration of 0.01% (v/v).

Artificial seawater was used for the preparation of standard solutions applying a standard composition given elsewhere [22]. A saturated solution of poly(vinyl alcohol) of an average molar weight of 16000 g/mol (Across Organics) in 20% v/v ethanol, 0.5 mol/L NaOH was used for chemical modification of the PMMA detection cell flow channel.

For the comparison, a manual protocol based on OPA-sulfite was used. The used reagent was composed of 100 mL of sodium tetraborate stock, 2 mL of OPA stock, and 0.2 mL of sodium sulfite stock.

A solution of tri-sodium-citrate, adjusted to pH 9.0, was used as additive for the stabilization of calcium and magnesium carbonate.

2.2. Flow system

The flow system is shown schematically in Fig. 1. Two solenoid micropumps (SMP 1 and SMP2) from BIO-CHEM FLUIDICS (Boston, NJ, USA) of nominal 25 μL and 8 μL (types P/N120SP-12-25 and P/N090SP-12-8) were used for driving of sample and reagent, respectively. In-situ (in the flow system) calibration of the SMP as former recommended [23] showed effective pulse volumes of 36 μL and 9 μL, respectively. The SMP were controlled by an 8-channel relay card (SERDIO8R) from EasyDAC (Glasgow, United Kingdom) as described elsewhere [23].

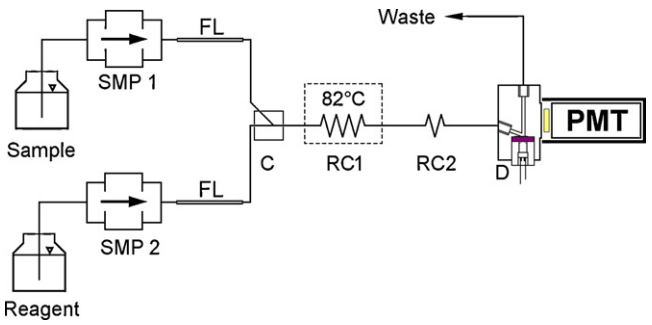

Fig. 1. Schematic drawing of the used flow system for the determination of ammonium. Two solenoid micropumps (SMP) are used for driving of sample and o-phthalaldehyde-sulfite reagent. Both solutions are mixed at a confluence (C) and stopped in a stainless steal heated reaction coil (RC1, 100 cm, 0.7 mm id). Afterwards they are pushed through a second reaction coil of PEEK (RC2, 30 cm, 0.7 mm id), in which the mixture cools down, towards the detection flow cell (D) with mounted photomultiplier tube (PMT) and optical filters. Flexible pumping tubes (FL) were inserted between each SMP and C to decrease pump pulsations.

The SMP outflows merge at a Y-shaped polymethylmetacyrlate (PMMA) confluence into the reaction coil. In between, pieces of elastic Tygon® peristaltic pumping tube (red–red, 4 cm effective length) were inserted in order to decrease flow pulsation of the SMP and smoother outflow.

A heated reaction coil was used to enhance the reaction rate, shown schematically in Fig. 2. It was especially-made of stainless steel capillary tube of 0.7 mm id (Scharlab SL, Barcelona, Spain). About 100 cm of the capillary plus 7 cm for connections on both sides were wounded and thoroughly tight around a 3 cm × 2.5 cm bronze cylinder. The bronze cylinder showed three drilling for the insertion of a digital thermometer, TPP1-C1 from BEHA-Amprobe GmbH (Glottertal, Germany), a 10 kΩ NTC thermistor probe, and a commercial halogen bulb (12 V, 20 W) used as heating source. A thermostat control circuit from CEBEK – Fadisel SL (Barcelona, Spain Ref. I-81) was used to control the switching-on time of the halogen bulb; the NTC thermistor was used as temperature sensor. The hysteresis of the thermostat control circuit was lowered to about 1 °C by soldering a higher resistor (2 MΩ, amplification factor 2000) as feed-back of the operational amplifier.

The reaction coil was connected to the detection cell by a 30 cm, 0.7 mm id PEEK tube. For the detection cell outlet, a 20 cm long, 0.7 mm id PEEK tube was used.

2.3. Detection cell

The used and specially-made detection cell of PMMA is shown in Fig. 3. The flow channel is illuminated with a UV-LED of 365 nm maximum emission for fluorescence excitation. The LED was powered by a constant current source at a supply voltage of about 3.5 V and was operated at about 2/3 of its maximal intensity. A photomultiplier tube (PMT) from Hamamatsu Phototonics K.K. (Hamamatsu,

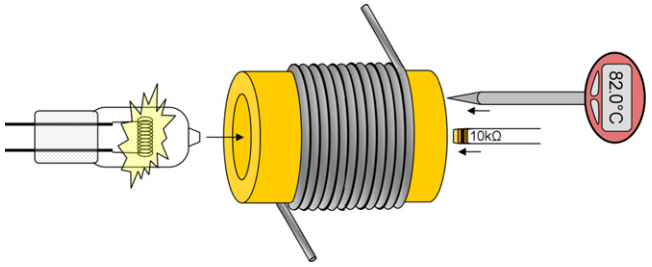

Fig. 2. Schematic drawing of the used heated reaction coil. The reaction coil is made of a stainless steal HPLC capillary (0.7 mm id.) wounded around a brass tube, in which the thermistor (right) and a halogen bulb (left) used as heating source are inserted.

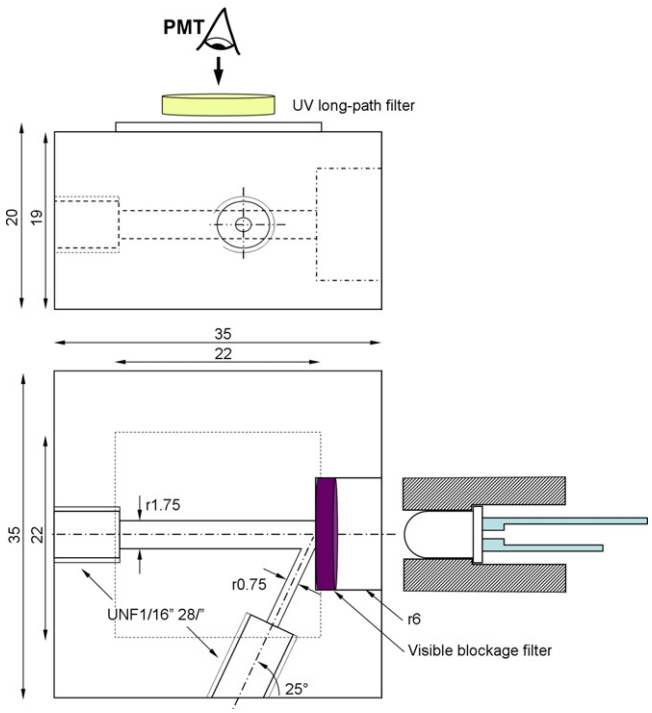

Fig. 3. Detection flow cell made of PMMA for fluorescence quantification of the reaction product isoindol-1-sulfonat. PMT: position of the photomultiplier tube.

Japan, Ref.: HS5784-04) was mounted onside the cell, perpendicular to the flow channel. A control unit from Sciware SL (Palma de Mallorca, Spain) was used for PMT supply and data readout.

It was found that the UV absorbance characteristic of PMMA lead to a cut-off below wavelengths of 380 nm. However, low intensity emission beyond 380 nm still caused saturation of the PMT. Therefore, a long-pass glass filter of 420 nm cutoff wavelength from Edmund Optics (Barrington, NJ, USA, references NT46-425) was placed between the detection flow cell and the PMT. Later modifications on the detection cell included further the insertion of an UV-band pass filter from the same company between flow channel and LED to block emission > 390 nm (reference NT46-081), similar to a former reported approach [13]. The final design of the detection flow cell is given in Fig. 3 while transmission spectra and LED emission spectra are given in Fig. 4.

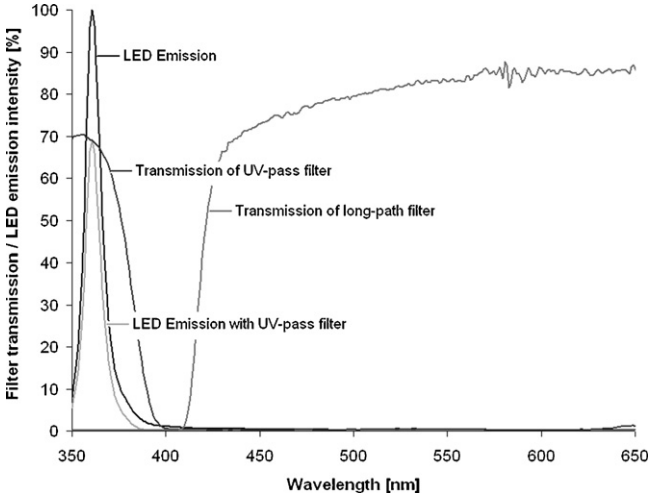

Fig. 4. Transmission spectra from the used filters and emission spectra of the UV-LED and after passing both filters.

2.4. Software control

Control of the relay card and detector readout was accomplished via RS232 interfaces using the software AutoAnalysis 5.0 from Sciware SL (Palma de Mallorca, Spain). Instrumental control is done via specific dynamic link libraries establishing the communication to the individual instrumental assembly, here the relay card and the PMT controller.

The development of the analytical procedure is done by the assembly of an instruction list. Module-wise programming is possible by the use of global procedures such as for cleaning steps or repeated measurements.

2.5. Analytical protocol

For analysis, 360 μL of sample and 90 μL of reagent were injected by simultaneous pulses (10 each) at operation frequency of 2 Hz, respectively. By two further pulses from SMP1, the mixture was completely driven into the heated reaction coil. After 80 s, the mixture was driven through the detection cell for fluorescence quantification by the expulsion of final 2.2 mL of sample. After each sample change, 1.5 mL of the new sample were required in addition to fill the system and to expulse the old sample.

For all measurements, averaging over 5 data points with and a data acquisition time of 100 ms were applied. Quadratic polynomial smoothing over 7 values following the Savitsky–Golay algorithm was further applied.

2.6. Manual protocol

To 10 mL of sample, 4 mL of reagent were mixed in polyethylene tubes and incubated in a water bath for 1 h at 37 °C. Measurements were made using a LS50B Fluorescence spectrometer from Perkin Elmer (Waltham, Massachusetts, USA) applying 365 nm for excitation and 425 nm for emission with 2.5 nm slit width for both and an average time of 5 s.

3. Results and discussion

3.1. Detection cell

High care had to be taken to achieve smooth and clear surfaces during the manufacturing of the detection cell. In order to increase the method's sensitivity, the back and lateral sides of the cell were covered with a plastic mirror. Shielding the PMT form ambient light was achieved with black isolation tape and aluminum foil. In- and out-flow tubes were of the nontransparent polymer PEEK.

In spite of the of the commercial 420 nm cut-off long-pass filter (see Section 2.3), rest emission of the UV-LED at longer wavelengths still caused saturation of the PMT at the highest gain. Therefore, optimization experiments were performed at 2.3% of the maximum gain.

The detection flow cell was modified after accomplishing the optimization of the reagent composition. To achieve a higher sensitivity, the detection cell flow channel was slightly widened from 3 mm id to 3.5 mm id.

Stacked air-bubbles in the flow channel caused light scattering and variation of the flow channel volume. In consequence, increase of baseline, decrease of sensitivity and poor reproducibility was found.

Thorough polishing of the flow channel surface was insufficient to decrease air-bubble stacking. So, water affinity of the flow channel inner surface was increased applying a protocol reported earlier [21]. The cell was left filled with first 3 mol/L nitric acid for about 10 min and second with a saturated solution of poly(vinyl alcohol), emptied, and let dry overnight. This modification finally proved to

Table 1
Simplex optimization of OPA and sodium sulfite concentration of reagent. Conditions: 2.3%, 82 °C reaction temperature, 8 μmol/L ammonium chloride.

No	OPA (g/L)	Sulfite (g/L)	Signal after 1 s[a]	Signal after 120 s[a]	Difference
1	0.80	0.016	11 ± 5	446 ± 19	435
2	1.57	0.020	22 ± 8	878 ± 46	856
3	1.01	0.032	30 ± 9	1048 ± 39	1018
12	8.02	0.200	39 ± 1	1883 ± 62	1844
13	9.76	0.255	40 ± 5	1892 ± 58	1852
15	9.65	0.248	30 ± 2	1939 ± 49	1909

[a] $n = 3$.

be highly efficient in decreasing air-bubble stacking and increasing the methods robustness, stability, and reproducibility.

The emission spectrum of the UV-LED reached down to 600 nm with relative intensities in respect of the LED maximum of 1.1% at 400 nm, 0.23% at 500 nm, and 0.25% at 600 nm. The use of an additional UV band-pass filter between LED and flow channel decreased the baseline signal to <3% of the working range and permitted PMT gain values of >12% of the maximum gain. The given modifications of the detection cell resulted in about 2.5-fold higher sensitivity at the same PMT gain.

3.2. Mixing conditions

The reaction coil showed an inner volume of about 400 μL. Simultaneous pump pulses for sample (SMP1) and reagent (SMP2) were found to yield higher response and better peak height reproducibility than alternating pulses. A lower sample to reagent volume, i.e. higher dilution of the sample by the reagent gave lower peak heights. With 10 pulses of sample and reagent each, a dispersion coefficient of 1.4 was obtained, representing a good compromise between sensitivity and time of analysis.

3.3. Reagent composition

Weighted centroide simplex optimization of the both reagent components OPA and sodium sulfite was performed, starting from elsewhere recommended concentrations. Initial vertices and final vertices are given in Table 1. Doubling of the method's sensitivity was achieved within 15 cycles.

The influence of both reagent components was evaluated by univariant studies with the results depicted in the Figs. 5 and 6. Three different reaction times were applied; the waiting time of 1 s applied for the univariant study of OPA concentration allows the evaluation of the reagent blank. The univariant studies showed that the concentration optimum of the OPA was lower than estimated by the simplex optimization, while the sodium sulfite concentration was found to coincide well.

Table 2
Results form the study of the Brij-35 content of the reagent. Conditions: 120 s reaction time, Gain 2.3%, 6 g/L OPA, 0.27 g/L sodium sulfite, 82 °**C** reaction temperature, 8 μmol/L ammonium chloride.

Brij final (%, V/V)	Signal after 100 s[a]
0.000	1977 ± 92
0.004	1991 ± 41
0.007	2004 ± 25
0.014	1958 ± 28
0.028	1898 ± 25
0.042	1887 ± 20
0.070	1871 ± 21
0.140	1814 ± 25

[a] $n = 3$.

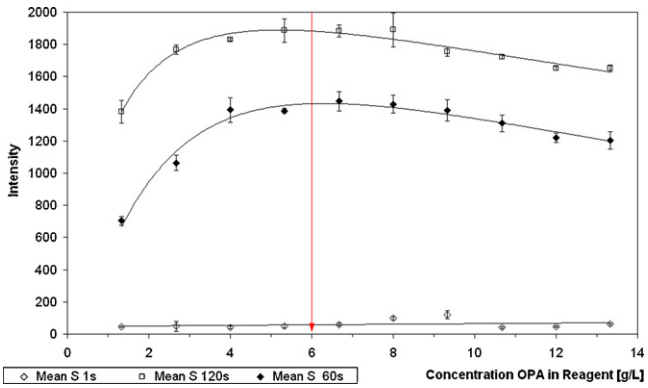

Fig. 5. Univariant study of the reagent concentration of ortho-phthaldialdehyde for incubation times 1 s, 60 s, and 120 s. Conditions: Gain 2.3%, 0.27 g/L sodium sulfite, 82 °C reaction temperature, 8 μmol/L ammonium chloride.

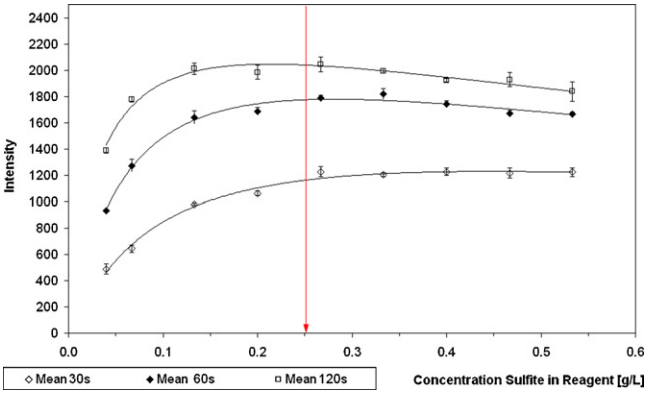

Fig. 6. Univariant study of the reagent concentration of sodium sulfite for incubation times 30 s, 60 s, and 120 s. Conditions: Gain 2.3%, 6 g/L OPA, 82 °C reaction temperature, 8 μmol/L ammonium chloride.

Concentrations of 6 g/L OPA and 0.25 g/L sodium sulfite were finally chosen. It was found that the optimum concentrations were higher for shorter incubation times; therefore, concentrations between the optimal values for 60 s and 120 s were chosen for both reagent components. During the application of the proposed analyzer to seawater samples, calcite formation on the inner walls of the heated reaction coil. This effect was finally minimized by the addition of 2.5 g/L tri-sodium-citrate.

3.4. Wetting surfactant

In former works, Brij-35 was used as wetting surfactant [4,16]. In the first optimization experiments, day-to-day variations of sensitivity and poor baseline stability were observed. Stacked air bubbles in the detection cell flow channel were identified was the main reason for these defections, so likewise, the use of Brij-35 was studied with results and conditions given in Table 2.

Table 3
Results from the study of the reaction time and temperature. Conditions: gain 2.3%, 6 g/L OPA, 0.27 g/L sodium sulfite, 8 μmol/L ammonium chloride.

Time [s]	Signal at 63.5 °C[a]	Signal at 71 °C[a]	Signal at 82 °C[a]	Signal at 86.5 °C[a]
40	216 ± 3	498 ± 21	932 ± 22	1054 ± 39
60	434 ± 16	840 ± 14	1143 ± 23	1184 ± 33
80	625 ± 31	1002 ± 41	1258 ± 59	1286 ± 53
100	792 ± 9	1143 ± 69	1335 ± 18	1355 ± 41
120	970 ± 39	1227 ± 17	1397 ± 45	1347 ± 47

[a] $n = 3$.

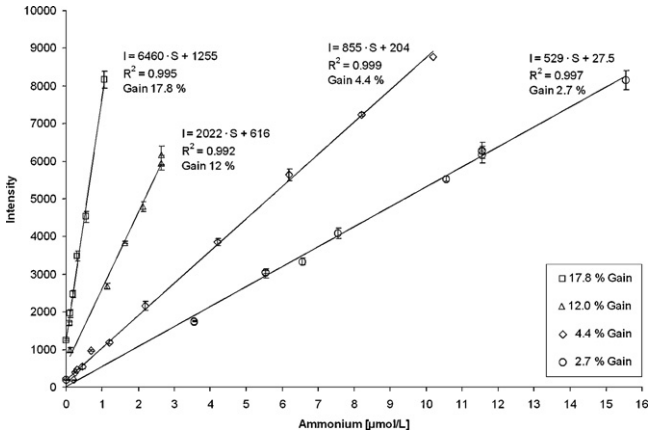

Fig. 7. Examples for calibration curves obtained with PMT gains of 2.7%, 4.4%, 12%, and 17.8% performed with Milli-Q-water. Peak examples are from calibration with gain 2.7%. Reagent: 6 g/L OPA, 0.26 g/L sodium sulfite, 2.5 g/L sodium citrate and 19 g/L borax.

It was observed that addition of Brij increased significantly signal repeatability and in concentrations between 0.005 and 0.015% (V/V) also slightly the methods sensitivity, while at higher concentrations, sensitivity decreased slightly. A concentration of 0.01% (V/V) was therefore chosen.

3.5. Reaction temperature and time

The reaction time and temperature in the ranges of 40–120 s and 63.5–86.5 °C, respectively, were studied with the results and conditions given in Table 3. Higher temperatures were not applied to avoid salt precipitation for the sample and consequent blocking of the reaction coil. On the other side, lower temperatures would have required to unacceptable long reaction time. The time response followed in approximation a PT2 lag behavior. Calculated final values were lower for higher temperatures indicating degradation of the reaction product while the reaction was considerably enhanced by higher temperature. As a compromise between sensitivity and reaction time, 100 s at 82 °C were chosen corresponding to 92% of the final value estimated for this temperature. After modification of the detection cell, decreasing of the reaction time to 80 s was done corresponding to 85% of the final value.

3.6. Characterization

3.6.1. Methods performance

The method's performance was evaluated by repeated calibrations with seawater standards. A sample throughput of 32 h^{-1} was achieved in monitoring mode, i.e. without sample change. Cleaning of the sample channel required a time of 30 s, since a relatively slow flow rate had to be applied to avoid cooling down of the flow cell. At a higher flow rate as 3 mL/min, cooling by the fresh and not sufficiently heated sample lead to an about 30% lower peak height in the first measurement of the sample.

The method's repeatability, evaluated by subsequent injections was 3% RSD on average. LOD and LOQ values were calculated from the concentration yielding the triple and ten-fold standard deviation of the blank standard, respectively.

As shown in Fig. 7, the method can be adapted to different ammonium concentrations by the increase of the PMT gain or the LED intensity but on the costs of a higher reagent blank and baseline level due to the detectable (visible) rest emission of the LED. For the highest tested PMT gain of 17.8%, a LOD of 13 nmol/L and a LOQ of 44 nmol/L with a linear working range of up to 1 μmol/L ammonium were found. For the lowest tested PMT gain of 2.7%, a LOD of 210 nmol/L and a LOQ of 700 nmol/L were found with a linear working range of up to 16 μmol/L ammonium at least. In consequence, the method showed to be applicable to oligotrophic samples as well as for coastal waters or be applied for respiratory experiments.

Table 4
Results of interference study.

Compound	Tested concentration (μmol/L)	Signal yield	Relative sensitivity to ammonium
EDTA	8	0.01 (<LOD)	−0.1%
Glycine	4	0.14	3.6%
Hydrazinesulfate	4	0.15	3.7%
Trimethylamine	4	0.13	3.3%
Urea	4	(<LOD) 0.09	2.2%
Uric acid	4	0.29	7.2%

Former analyzer system based on the same methodology used mainly air-segmented flow analysis [24] or classical flow injection analysis [25]. Here, the sample was injected into the reagent-containing carrier or continuous confluence of different reagent component solutions was carried out, which increases considerably reagent consumption and waste generation. In this work, we constructed a simpler, smaller, economic and transportable analyzer system with the objective of future ship-board application.

3.6.2. Sample influence and interferences

Comparing the methods performance with standard at ambient temperature (25 °C) and cooled standards (15 °C, 0 °C), the sensitivity and repeatability were not affected significantly. The only significant affection caused by a high air saturation of the standards was the observance of very narrow ghost-peaks from time to time.

The influence of the sample salinity on the method's sensitivity was studied with volumetric mixtures of Millipore water and artificial seawater between 0% and 35% of salinity and ammonium standard additions of 2 μmol/L and 4 μmol/L. A slight and in approximation linear increase of the method's sensitivity with the samples salinity of ca. 0.25% per 1% of salinity was observed. Calibration with standard artificial seawater is therefore reliable for quantification of ammonium in open ocean seawater. For alterations of more than ±4% of salinity such as in estuarine water, the adaptation of the standard's salinity or compensation would be recommended.

The influence of the sample's pH was studied over a range of 7.3 to 8.4. Between pH 7.6 and 8.3, no significant influence on the method's sensitivity was found.

The following possible interfering compounds were tested: EDTA, glycine, hydrazine, trimethylamine, urea, and uric acid. The results are given in Table 4. The sensitivities relative to ammonium were generally below 4% bearing in mind that none of the tested compounds is likely to be present in seawater in concentrations even ten-times lower than ammonium. The only exception was uric acid with 7% relative sensitivity. Being the carrier metabolite of nitrogen excretion of birds, interfering concentrations could be estimated only near to bird colonies. In conclusion, the system showed sufficient selectivity for application to seawater analysis.

The stability or time robustness of the method was tested over a period of 8 h. A standard of 5 μmol/L and PMT gain of 4.4% were used in order to exclude possible errors by contamination over the evaluation time. The signals were stable with a relative standard deviation of <2% as shown in Fig. 8.

3.6.3. Real sample analysis

The method was applied to surface seawater sampled at different coastal zones of the Bay of Palma de Mallorca. The samples

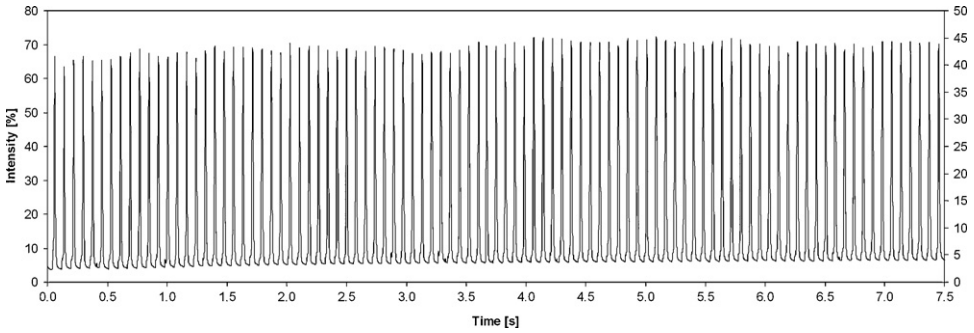

Fig. 8. Time stability of the method demonstrated by repeated measurement of a 5 μmol/L standard over 8 h applying a PTM gain of 4.4%.

were measured unfiltered but led time for the sedimentation of particles. For calibration, unfiltered seawater of low nutrient content was used and spiked with ammonium standards. The results were compared with the manual method (see Section 2.6). The comparison of both methods gave good correlation with mean difference between both measurement of 0.15 μmol/L. Four samples showing a difference of >20% between both methods were analyzed applying standard addition and recovery values of 96 ± 3% were obtained proving the reliability of the proposed analyzer.

Filtered seawater showed higher ammonium concentrations than the corresponding unfiltered sample. This might be due to contamination by laboratory air or due to elimination of algae, which would consume ammonium from bacterial activity. The best strategy to evaluate the reagent blank was the measurement of a low nutrient seawater or Milli-Q water with the reaction coil heating turned off.

4. Conclusion

A simple, miniaturized, and field applicable instrumentation for the fluorometric determination of ammonium using orthophthaldialdehyde based on solenoid pumps is described. The system showed to be reliable and robust in respect of pH, salinity, temperature, and gas content of the sample. The method's sensitivity is adjustable by the gain of the used photomultiplier tube allowing the application to both seawater analysis and metabolism studies.

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