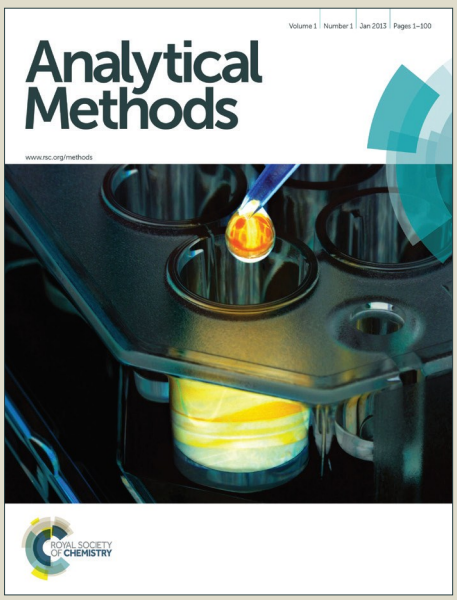
Analytical Methods

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Seasonal monitoring of inland bathing waters using a sequential injection method as a fast and effective tool for nutrients quantification (N:P)

Raquel B. R. Mesquita [1,2], Ana Machado[2], Inês C. Santos [1], Adriano A. Bordalo [2], António O. S. S. Rangel [1]*

[1] CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal

[2] Laboratory of Hydrobiology and Ecology, Institute of Biomedical Sciences Abel Salazar (ICBAS) Universidade do Porto, Rua de Jorge Viterbo Ferreira n.º 228, 4050-313 Porto, Portugal

[*] rmesquita@porto.ucp.pt; Phone:+351 225580001; Fax: +351 225090351.

Keywords: bathing waters quality; multiparametric determination; nutrients monitoring; flow analysis; nitrogen ions speciation; phosphate quantification.

Abstract

In this work, an expedite flow method for the combined determination of major nutrients, nitrogen base ions (nitrate, nitrite and ammonium) and phosphate, is described for seasonal monitoring of inland bathing waters. The individual determinations were adapted and comprised within the same manifold to attain a comprehensive assessment of the environmental status of natural waters. The multiparametric determination was obtained exploring the features of sequential injection analysis technique yielding a real-time assessment of various parameters and effective in-line sample handling. The sequential injection multiparametric method enabled the determination within the ranges: 15.0 – 150 µM nitrate; 0.15 – 5.00 µM nitrite; 1.00 – 60 µM ammonium; and 0.2 – 30 µM phosphate. The determination rates provided a full nutrient analysis within four hours, an overwhelming improvement in relation to the individual determination of each parameter. The described method was successfully applied to seasonal monitoring of six inland bathing waters dealing in-line with sample variability.

1. Introduction

The environmental assessment of nutrients in natural waters is essential to attain a comprehensive knowledge of the natural water quality. The determination of parameters like nitrate, nitrite, ammonium/ammonia and phosphate is crucial as it provides the establishment of the N: P relationship.[1] This key relationship, a consequence of the Redfield ratio described for ocean phytoplankton (106 C: 16 N: 1 P), has been widely used to predict and/or estimate the eutrophication phenomenon leading to algae blooms and therefore may emerge as an excellent bio indicator of the trophic status of the water body.[1,2] Recently, some aspects of other microbial interactions with the N:P relation have been explored [3,4] reinforcing the importance of major nutrient assessment. Overall, the accurate determination of major nutrient ions, nitrate, nitrite, ammonium and phosphate, is crucial and must therefore rely on robust, reliable, effective methods.

Therefore, the use of flow analysis methods, namely sequential injection, is highly advantageous as it combines characteristics as automation, miniaturization, robustness and versatility with real time, reliable determinations.[5] Nutrient determinations have been previously addressed using sequential injection methods [6-11] but it has never been assessed all parameters within the same methodology. By mastering the previous acquired experience in nutrient determination in natural wasters,[7-10] a comprehensive sequential injection method was developed. The determination of routine parameters, such as nitrate, nitrite, ammonium and phosphate was attained with expedite miniaturized automated method.

The determination of nitrate and nitrite was based on the well-known Griess reaction with in line nitrate reduction.[7] The ammonium determination was based in the colour change of the bromothymol blue (BTB) indicator, exploring the "no-consumption" approach that was previously used successfully.[9,11] The phosphate determination was based on the also well-known molybdenum blue reaction. The choice of these reactions was intentional as they are quite sensitive, reliable and provide comparable results.

The impact of weathering in the N:P relationship has been previously studied [12]reinforcing the pertinence of monitoring nutrient major ions as environmental quality bio-indicators. A reliable monitoring process can only be achieved using the automatic methods minimizing analyst and/or analysis interference, otherwise results may be questionable. An effective follow up of the weathering impact, seasonal variation, will anticipate potential problems and enable a fast intervention reducing risks and negative impact.

So, the aim of the developed work was to attain the effective determination of nitrogen and phosphate ions for environmental quality assessment of natural waters throughout seasonal and spatial variation. An effective analytical tool, based upon previously described individual methodologies,[7-10] was adapted to increase determination rate and resulted in a versatile multiparametric assessment. The developed procedure was effectively applied to the seasonal monitoring of nutrients in several recreational waters, namely inland beaches, proving to be quite robust. The described multiparametric method enabled real-time

assessment with the determination of nitrate, nitrite, ammonium and phosphate within 4 hours. The Redfield ratio (16 N: 1 P) was simulated for each selected location for evaluation of seasonal influence.

2. Experimental

2.1. Sample collection and preparation

Sub-surface water samples (30 cm depth) from six officially classified inland bathing waters, located in the North West of Portugal, were seasonally collected, in 500 mL sterile or acid-cleaned polyethylene bottles for microbial and chemical analysis, respectively. In order to minimize the risk of contamination by the operator, bottles were always emerged against the current. All samples were kept in the dark in refrigerated ice chests (about 4°C) until further processing.

Temperature, conductivity, turbidity, dissolved oxygen and pH were measured *in situ* using a probe YSI 6920 CTD.

Upon arrival at the laboratory, the water samples were directly introduced in the developed system after reaching room temperature. For microbial assessment samples were processed no later than 4 h after collection.

2.2. Analytical procedure

2.2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals and boiled deionised water (specific conductance less than 0.1 µS/cm).

The colour reagent (CR) for NOx determination was made of 20 g/L sulphanilamide (Merck, Darmstadt, Germany) with 2 g/L N-(1-naphthyl)-ethylenediamine dihydrochloride (N1NED, Merck, Darmstadt, Germany) in 0.5 M of *ortho*-phosphoric acid (Merck, Darmstadt, Germany). The cadmium granules (Merck, Darmstadt, Germany) were prepared and activated as described in the Standard Methods of Examination of water and wastewater,[13] and the column was assembled and conditioned as previously described by Mesquita *et al.*[7] The conditioning buffer consisted in 0.4 g/L EDTA (Merck, Darmstadt, Germany) and 20 g/L ammonium chloride (Merck, Darmstadt, Germany) solution at a final pH of 9.2, adjusted with commercial ammonia (d=0.91; 25% Merck, Darmstadt, Germany).

The colourimetric indicator bromothymol blue (BTB, Merck, Darmstadt, Germany), with 18 µM of BTB, was prepared by dilution of a 3 mM stock solution of BTB in ethanol. The sodium hydroxide solution (25 mM) was daily prepared by dilution of a 1 M stock solution, obtained from the dissolution of sodium hydroxide pellets (Merck, Darmstadt, Germany).

The molybdate reagent (MR) was prepared to final concentrations of 16 g/L ammonium heptamolybdate-tetra-hydrate (Merck, Darmstadt, Germany), 0.1 g/L of potassium antimony(III) oxide tartrate hemihydrate (Merck, Darmstadt, Germany) and 7.5 g/L of tartaric acid (Merck, Darmstadt, Germany) in 0.78 mol/L sulfuric acid (Merck, Darmstadt, Germany). The ascorbic acid solution, from Normapur (VWR, Leuven, Belgium), 30 g/L, was daily prepared.

Stock solutions for nitrite, nitrate, ammonium and phosphate were prepared from dissolution of the previously dried solids: sodium nitrite, sodium nitrate, ammonium chloride and sodium dihydrogen phosphate monohydrate, respectively. All of these reagents were obtained from Merck (Darmstadt, Germany). The working standards for nitrite, nitrate and ammonium were weekly prepared but the phosphate working standards were daily prepared. The used stock solutions and the dynamic working ranges are shown in Table 1.

Table 1. Standard solutions preparation for the respective working dynamic range of the assessed nutrients.

Analyte	**$[NO_2^-]$**	**$[NO_3^-]$**	**$[NH_4^+]$**	**$[PO_4^{3-}]$**
Solid	$NaNO_2$	$NaNO_3$	NH_4Cl	$NaH_2PO_4.H_2O$
Stock solution 1 (mM)	2.03	10.0	2.62	0.735
Stock solution 2 (µM)	20.0	$1.00x10^3$	100	110
Dynamic working range (µM)	0.150 – 5.00	15.0 – 150	5.00 – 150	0.2 – 30

If high salinity samples are aimed the standards should be prepared in the same level of salinity, namely simulated sea water.

2.2.2. Sequential injection manifold and sequence protocol

The described method for the multiparametric determination of nutrients combines the determination of nitrate, nitrite, ammonium and phosphate in a single manifold. In Fig. 1 is depicted the designed manifold and the corresponding photograph.

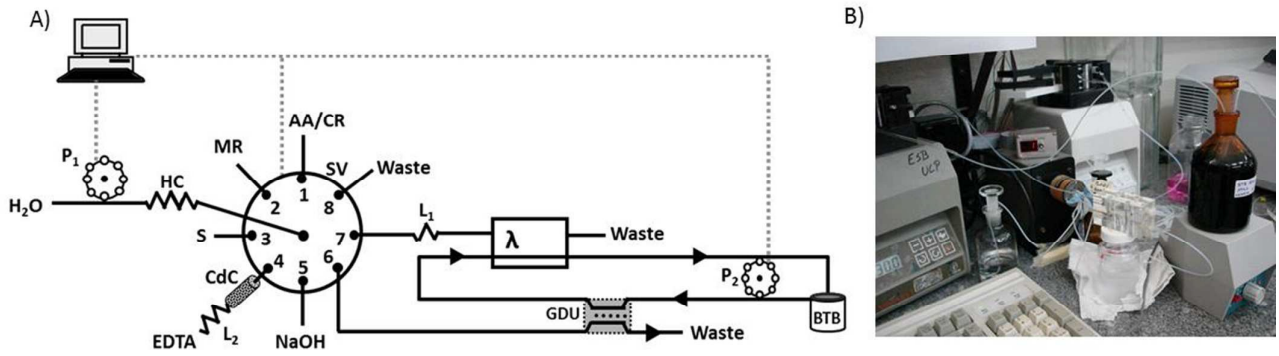


Fig. 1. Sequential injection manifold for the multiparametric nutrients determination. A) Schematic representation of the SIA manifold; SV, eight port selection valve, P_i, peristaltic pumps; HC, holding coil with 300 cm; L_1, reaction coil with 30 cm length for nitrite/nitrate determination and 1.5 m length for phosphate determination; L_2, 25 cm tube length; GDU, gas diffusion unit assembled with an hydrophobic membrane; λ, Spectrophotometer at 543, 620 or 800 nm for nitrite/nitrate, ammonium and phosphate determination, respectively; AA/CR, Ascorbic acid (30 g/L) for the phosphate determination or colour reagent (20 g/L sulphanilamide with 2 g/L N1NED in 0.5 M of H_3PO_4) for nitrite and nitrate determination; MR, molybdate reagent (16 g/L ammonium heptamolybdate, 0.1 g/L potassium antimony(III) oxide tartrate, 7.5 g/L tartaric acid in 0.78 M of H_2SO_4); S, sample or standard; CdC, copperised cadmium column; EDTA, conditioning buffer solution (0.4 g/L EDTA and 20 g/L NH_4Cl) at pH 9.2; BTB, bromothymol blue (180 μM) reagent at pH 6.6. B) Photograph of the SIA manifold.

Solutions were propelled by two Gilson Minipuls 3 peristaltic pumps with a PVC pumping tube (i.d. 1.02 mm). One of the pumps, P1, was connected to the central channel of an eight port selection valve (Valco VICI 51652-E8). The other pump, P2, was connected to the acceptor channel of the gas diffusion unit (GDU), which in turn was connected to the spectrophotometer and back to the reagent container. All tubing connecting the different components was made of Teflon from Omnifit, with 0.8 mm i.d.

A Thermo Spectronic Helios γ UV-Vis spectrophotometer set at selected wavelength, 543 nm for nitrite and nitrate, 620 nm for ammonium and 800 nm for phosphate, was used as detection system. The flow-cell was a Hellma 178.711-QS flow-cell (10 mm light path, 30 μL inner volume). Analytical signals were recorded in a Kipp & Zonen BD 111 (Delft, The Netherlands) chart recorder. A personal computer (Samsung SD 700) equipped with a PCL818L interface card, running with a homemade software written in QuickBasic 4.5, controlled the selection valve (SV) position, the pump 1 sense and speed and the pump 2 activation. Four different speeds were used, 10, 20 30, 40 rpm corresponding to different flow rates, 15.1, 30.2, 47.6, 60.8 μL/s, respectively. These flow rates were calculated by establishing a linear relationship between aspirated/propelled volumes versus time. Pump 2 speed was manually set at 30 rpm, flow rate of 48 μL/s. An acrylic gas diffusion unit (GDU) as described in Mesquita and Rangel,[14] with a straight flow channel, was used as separation device. A hydrophobic Millipore Durapore® membrane filter (ref. HVHP09050) was used between the two channels. The membrane was replaced every two months of daily use.

The sequence of steps with the respective time and volumes for the determination of nutrients is shown in Table 2.

Table 2. Protocol sequences for the determination of nutrients.

Parameter	Step	SV position	Pump 1 flow rate (µL/s)	Volume (µL)	Time (s)	Pump 2	Description
Phosphate (P)	A_P	1	61	180	3.0	off	Aspiration of ascorbic acid
	B_P	2	61	120	2.0	off	Aspiration of molybdenum blue reagent
	C_P	3	61	490	8.0	off	Aspiration of sample/standard
	D_P	7	61	4260	69.8	off	Propelling to detector, mixture of reagents and sample and absorbance measurement
Nitrite (N2)	A_{N2}	1	61	550	9.0	off	Aspiration of colour reagent
	B_{N2}	3	61	560	9.2	off	Aspiration of sample/standard
	C_{N2}	7	61	3350	54.9	off	Propelling to detector, mixture of reagent and sample and measurement (nitrite determination)
Nitrate (N3)	A_{N3}	4	48	215	4.5	off	Aspiration of conditioner (EDTA in NH_3/NH_4^+ buffer)
	B_{N3}	2	48	120	2.5	off	Aspiration of sample/standard
	C_{N3}	4	15	150	10.0	off	Propelling to column for reduction
	D_{N3}	4	0	0	45.0	off	Stop period (45 s) for promoting the reduction reaction
	E_{N3}	4	15	45	3.0	off	Aspiration for preparation of the port
	F_{N3}	8	61	250	4.1	off	Propelling to waste to wash holding coil
	G_{N3}	1	61	300	4.9	off	Aspiration of colour reagent
	H_{N3}	4	30	100	3.3	off	Aspiration of reduced sample/standard
	I_{N3}	5	61	2740	44.9	off	Propelling to detector, mixture of reagent and sample and measurement (nitrite + nitrate determination)
	J_{N3}	4	48	240	5.0	off	Aspiration of conditioner (EDTA) to wash the column
	L_{N3}	8	61	300	4.9	off	Propelling to waste to wash holding coil
Ammonium (N4)	A_{N4}	1	61	200	3.3	off	Aspiration of hydroxide (NaOH)
	B_{N4}	2	61	900	14.8	off	Aspiration of sample/standard
	C_{N4}	3	61	200	3.3	off	Aspiration of hydroxide (NaOH)
	D_{N4}	7	48	1800	37.5	off	Propelling to the donor channel of the GDU the mixture of hydroxide and sample, gaseous ammonia
	E_{N4}	4	30	1350	45.0	on	Propelling to detector, the BTB in the acceptor channel to measure the colour change

For the phosphate determination, the first to be performed, ascorbic acid, molybdenum reagent and sample/standard (steps A_P-C_P) were sequentially aspirated into the holding coil. Then, mixing was promoted by reversing the flow while propelling towards the detector for absorbance measurement of the colour product (steps D_P).

In the nitrite determination, the colour reagent for the Griess reaction was aspirated followed by the sample/standard (steps A_{N2}-B_{N2}) and sent to the detector for measurement of the coloured product obtained from the reaction of sulphanilamide and nitrite in the presence of N1NED (step C_{N2}).

For the nitrate determination the sample/standard was aspirated after a portion of conditioning buffer (EDTA in NH_4^+/NH_3 buffer) and sent to the cadmium column for nitrate reduction (steps A_{N3}-C_{N3}). Prior to the detection, there was a stop period of 45 s to enhance the nitrate reduction followed by the preparation of the port and washing of the holding coil (steps D_{N3}-F_{N3}). After the reduction of nitrate to nitrite, a similar procedure to the nitrite determination was employed: the colour reagent was aspirated followed by the sample/standard, from the cadmium column, (steps G_{N3}-H_{N3}). The reaction was promoted by flow reversal by propelling to the detector for absorbance measurement of the formed coloured product (step I_{N3}). The coloured product formed corresponds to the sum of nitrite and nitrate in the sample and the concentration of nitrate was obtained by the difference between both determinations. In the end, the cadmium column was washed and reconditioned to prepare for the next cycle (steps J_{N3}) followed by the washing of the holding coil (steps L_{N3}).

In the ammonium determination, there is no reagent consumption due to the use of the close loop approach. The sample is aspirated between two plugs of hydroxide to ensure the conversion of ammonium to ammonia (steps A_{N4}-C_{N4}). The mixing is promoted by reversing the flow while sending the stacked plugs through the donor channel of the GDU where the molecular ammonia diffuses through the membrane to the acceptor channel (Step D_{N4}). The pH of the BTB on the acceptor channel changes, resulting in a colour change measured at the spectrophotometer when the pump 2 is activated (Step E_{N4}). During the propelling to the detector, pump 1 is also on, in order to wash the holding coil before the next cycle.

2.3. Microbiological assessment – Fecal indicators

In order to ascertain the microbiological quality of the waters, particularly in the case of the studied inland bathing waters, two indicators were studied: *Escherichia coli* (EC) and fecal enterococci (FE). Samples for enumerations were filtered onto sterile cellulose nitrate membranes (0.45 µm pore size, 47 mm diameter, Whatman, UK), placed in Chromocult agar (Merck) incubated at 37 ^{o}C for 24 h (EC) and Slanetz-Bartley agar (Oxoid) incubated at 44.5 ^{o}C for 48 h (FE).[15] Typical colonies were counted and results expressed as colony forming units CFU/100 mL.

3. Results and discussion

3.1. Sequential injection methodology for nutrients determination

The design of the sequential injection manifold was based on previously described methods,[7-10] aiming to combine all the different determinations in a single manifold. This approach is of great advantage, especially when time and preparation procedures are a concern. By adapting several methods,[7-10] the idea of time saving was imperative to attain a complete assessment in real-time. To achieve this purpose, a relevant change to the previously described methods was to use the same detection system for all the determinations, namely a conventional spectrophotometer with 1 cm flow cell.[7-10]

3.1.1. Phosphate determination

The phosphate quantification was the first to be performed (Table 3), giving the imperative need for minimal time elapse between collection and analysis. Phosphate determination was based in the molybdenum blue chemistry: the reduction of the molybdenum-phosphate complex to attain the coloured product. Thus, to guarantee that the molybdenum-phosphate was produced prior to reduction, the plugs were aspirated sequentially to ensure that the sample mixtures first with molybdate reagent to produce the phosphate-molybdenum complex and only afterwards with the ascorbic acid for the complex reduction and formation of the coloured product. This proved to be the better aspiration order.[8] The volumes of the reagents and samples were previously studied [10] accounting for the need of mixing between the three aspirated plugs, the molybdate reagent had a minimal value to attain reproducibility.[16] The diameters of the tubes for the holding and reaction coil were the same as in the previous work, to guarantee the same conditions.[10]

3.1.2. NOx determination

The nitrite and nitrate determination was based in the Griess reaction with in-line reduction of nitrate. The determination parameters, namely volumes and reagent concentrations, were carefully studied in the work of Mesquita *et al.* [7] which intended the simultaneous determinations of the targeted anions. The diameters of the tubes for the holding and reaction coil were the same as in the previous work, to guarantee the same conditions.[7]

In the proposed method, the two determinations were attained running separate protocols to improve determination rate. In fact, for the nitrite determination a 3 fold increase was obtained and for the nitrate determination the separate protocols approach resulted in over 1.5 fold increase. In the nitrate protocol sequence a stop period had to be included to ensure 100% reduction rate. A 45 s step was set, representing a reduction to the previous describe work.[7]

3.1.3. NHx determination

The ammonium determination uses the reagent in a close loop with the detector exploring the indicator capacity of the BTB, and enabling to avoid reagent consumption per determination. The sample is aspirated between two plugs of hydroxide solution, to ensure the conversion to ammonia, and propelled through the donor channel of a gas diffusion unit. The molecular ammonia diffuses through a hydrophobic membrane to the acceptor channel, where the BTB in acidic medium converts the ammonia in ammonium producing a colour change of the indicator. The volumes and concentrations had been previously studied,[9] so they were adopted. The diameters of the tubes for the holding and reaction coil were the same as in the previous work, to guarantee the same conditions.[9] However, in their work, Segundo *et al.* [9] reported the use of sample/standard double plug, a concept dropped to increase the determination rate. So, only one plug of sample was aspirated between two plugs of hydroxide solution and a 1.4 fold increase of the determination rate. Considering the recirculation approach, time was needed for reagent stabilization, so the determination of ammonium/ammonia should be the last one to be performed. The time for the determination of the other parameters, phosphate, nitrate and nitrite, ensures the reagent stabilization and avoids unnecessary waiting time.

3.1.4. Multiparametric determination

As mentioned above the aim was to comprise all the determinations within the same manifold, so the detection system chosen was spectrophotometry enabling the detection of the coloured products formed and the BTB colour change. The reagents were placed around the selection valve, in order to facilitate the protocol sequence minimizing the execution time. The order of the determinations was also carefully though to optimize the overall procedure: i) phosphate was first due to the previously mentioned imperative need for a determination within 4 hours of collection, with the spectrophotometer set at 800 nm; ii) then the ascorbic acid in port 1 was replaced by the Griess reagent, the wavelength change to 543 nm and the determination of nitrite carried out; iii) using the same conditions, the protocol sequence for nitrate determination was run, and the nitrate concentration calculated by subtracting the nitrite concentration; iv) finally for the ammonium/ammonia determination, the connections of the flow cell were change to include the detector in the BTB reagent loop, and the wavelength set at 620 nm.

The study of possible interferences from the inland bathing water matrices in the determination of phosphate, nitrite, nitrate and phosphate has already been made in previous studies.[7-10] No significant interferences were observed from this type of matrix and therefore no further studies were performed in this work.

3.2. Features of the developed multiparametric method for nutrients assessment

The described sequential injection method, comprise the determination of nitrate, nitrite, ammonium and phosphate within the same sample in about 20 minutes. The features of the combined procedure, a single manifold for determination of nutrients in natural waters, are shown in Table 3.

Table 3. Features of the developed sequential injection method for the multiparametric determination of nutrients.

Parameter	Typical calibration curves A = slope ± SD (μM) +intercept ± SD	LOD[a] (μM)	Determination rate (h^{-1})	Consumption values per determination	
				Mass	Volumes (μL)
Phosphate	$A = 4.10x10^{-3} \pm 1.2x10^{-5} [PO_4^{3-}] + 8x10^{-3} \pm 1.1x10^{-2}$	0.17	40	2.0 mg $(NH_4)_6Mo_7O_{24}.4H_2O$, 12 μg $C_4H_4KO_7Sb$, 0.94 mg $C_4H_6O_6$, 9.2 mg H_2SO_4	120
				5.4 mg $C_6H_8O_6$	180
Nitrite	$A = 2.91x10^{-2} \pm 3.0x10^{-3} [NO_2^-] - 3x10^{-3} \pm 2x10^{-2}$	0.15	45	11 mg sulfanilamide; 1.1 mg N1NED; 27 mg H_3PO_4	550
Nitrate	$A = 3.07x10^{-3} \pm 2.2x10^{-4} [NO_3^-] + 4x10^{-3} \pm 1.3x10^{-2}$	12	26	6.0 mg sulfanilamide; 0.60 mg N1NED; 15 mg H_3PO_4	300
Ammonia	$A = 4.11x10^{-3} \pm 5.7x10^{-4} [NH_4^+] + 1.32x10^{-1} \pm 1.4x10^{-3}$	1.0	33	0.40 g NaOH	400

[a] detection limit as three times the standard deviation of the blank signal or the intercept

The presented calibration curves (Table 3) are a mean of consecutive days, for NOx n=3; for NHx and PO_4^{3-} n=2. The limits of detection were calculated according to IUPAC recommendations:[17,18] three times the standard deviation of the blank signal for nitrite and phosphate determinations, and three times the intercept for nitrate and ammonium determinations. The determination rate was calculated as the time spent per analytical cycle of each determination. An analytical cycle corresponds to the time needed to execute the protocol sequence instructions and the extra time that the equipment needs to respond. The consumption values were also calculated per analytical cycle.

The determination of nutrients in one single sample takes about 7 min if no replicas and no calibration curves are taken into account. However, a proper analysis requires calibration curves for all the parameters and replicas for the sample and the standards. So, the multiparametric analysis of 6 samples with 3 replicas and four calibration curves, one for each determination, with 5 standards and 3 replicas each, could be attained in 3.8 hours.

In the ammonia quantification, there is no reagent consumption per determination as the reagent is in recirculation. Daily regeneration of the BTB reagent was attained by pH adjusting.

3.3. Bathing waters monitoring using the sequential injection methodology

The collected samples were introduced directly in the sequential injection system without need for sample pre-treatment. The assembled sequential injection method, with manifold depicted in Fig. 1 and the protocol sequences in Table 2, enabled to attain the nutrients quantification for the different seasons. So, the determination was directly performed in the collected samples (Fig. 2).

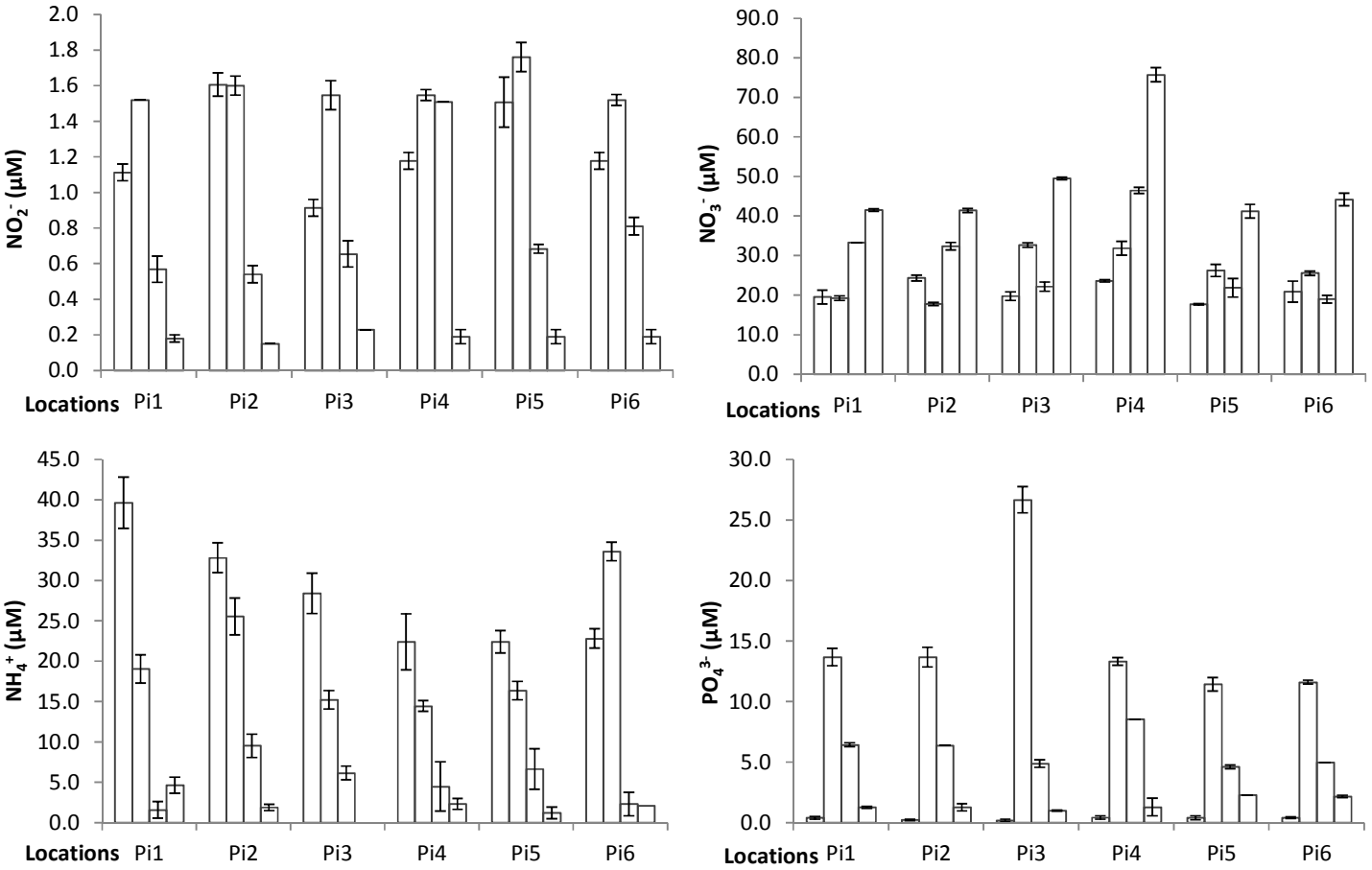


Fig. 2. Seasonal variability of nutrients in the monitored inland bathing waters; spring, diagonal stripes; summer, horizontal waves; autumn, grid; winter, small dots; Error bars correspond to the standard deviation (n=3).

The nitrite concentration value showed grossly a 10 fold variation between the values determined in spring/summer when compared to those found in winter. A similar trend was observed for ammonium while nitrate showed more consistent values throughout the seasons. The phosphate had a different temporal behaviour. In spring the values tended to be close to the detection limit, but in the summer survey reached a 20 fold increase in some locations (e.g. Pi3). The presence of nutrients in inland bathing waters such as N and P originated from both sewage discharges and non-point agriculture run-off can promote aa temporal

scale, albeit not instantly, the eutrophication of the water, and in the end, frequent or massive algal blooms. The direct consequences are well known: water discoloration, unpleasant smell, and upon contact, allergies or even toxicity. So, despite the expected seasonal variability (Fig.2), the water samples could always be directly introduced in the system and the determination of N and P was possible in all samples. Also, the obtained results proved that the sequential injection methodology was applicable regardless of the physical chemical parameters of the sample, namely pH, salinity and conductance (Table 4).

Table 4. Values obtained with the sequential injection methodology for nutrient assessment of inland bathing waters; G, conductance, D.O., dissolved oxygen; TUR, turbidity; n.d., non-detectable; SD, standard deviation (n=3).

		In situ					Sequential injection analysis methodology							
							Phosphate		Nitrate		Nitrite		Ammonium	
Season	Sample ID	T (°C)	pH	G (µS/cm)	D.O. (mg/L)	TUR (NTU)	PO_4^{3-} (µM)	SD	NO_3^- (µM)	SD	NO_2^- (µM)	SD	NH_4^+ (µM)	SD
Spring	Pi1	11.38	8.00	45	11.70	0.8	0.398 ±	0.113	19.5 ±	1.7	1.11 ±	0.05	39.6 ±	3.2
	Pi2	13.48	7.00	51	10.98	9.6	0.235 ±	0.063	24.3 ±	0.7	1.61 ±	0.07	32.8 ±	1.8
	Pi3	14.38	7.17	44	10.74	0.4	0.180 ±	0.113	19.7 ±	1.1	0.91 ±	0.05	28.4 ±	2.5
	Pi4	11.48	6.82	39	11.78	0.4	0.435 ±	0.137	23.6 ±	0.3	1.18 ±	0.05	22.4 ±	3.5
	Pi5	10.56	6.89	35	11.92	0.0	0.398 ±	0.166	17.7 ±	0.2	1.51 ±	0.14	22.4 ±	1.4
	Pi6	9.34	6.87	33	12.22	0.0	0.416 ±	0.054	20.9 ±	2.7	1.18 ±	0.05	22.8 ±	1.2
Summer	Pi1	19.80	7.09	28	9.48	0.0	13.7 ±	0.7	19.3 ±	0.6	1.52 ±	0.00	19.0 ±	1.8
	Pi2	26.70	7.20	35	9.50	10.5	13.7 ±	0.8	17.8 ±	0.4	1.60 ±	0.05	25.5 ±	2.3
	Pi3	23.30	6.95	36	8.70	0.3	26.7 ±	1.1	32.6 ±	0.6	1.55 ±	0.08	15.2 ±	1.1
	Pi4	19.41	6.50	28	9.15	0.7	13.3 ±	0.3	31.8 ±	1.7	1.55 ±	0.03	14.4 ±	0.7
	Pi5	18.70	7.16	30	9.52	0.4	11.4 ±	0.6	26.2 ±	1.5	1.76 ±	0.08	16.4 ±	1.1
	Pi6	18.88	7.38	27	10.05	0.0	11.6 ±	0.2	25.5 ±	0.6	1.52 ±	0.03	33.6 ±	1.1
Autumn	Pi1	15.95	6.25	36	8.17	0.8	6.44 ±	0.16	33.3 ±	0.0	0.569 ±	0.074	1.6 ±	1.0
	Pi2	16.03	6.26	36	8.33	1.1	6.35 ±	0.00	32.4 ±	1.0	0.541 ±	0.049	9.5 ±	1.4
	Pi3	17.53	6.94	36	9.17	2.5	4.89 ±	0.32	22.1 ±	1.2	0.655 ±	0.074	6.2 ±	0.8
	Pi4	18.03	6.88	41	9.16	12.8	8.54 ±	0.00	46.4 ±	0.8	1.51 ±	0.00	4.5 ±	3.1
	Pi5	17.43	6.59	29	8.95	1.2	4.61 ±	0.16	21.9 ±	2.3	0.683 ±	0.025	6.6 ±	2.5
	Pi6	17.17	6.45	27	8.85	1.9	4.98 ±	0.00	19.0 ±	1.0	0.811 ±	0.049	2.3 ±	1.4
Winter	Pi1	9.24	6.80	27	11.94	0.9	1.28 ±	0.08	41.5 ±	0.3	0.180 ±	0.020	4.7 ±	1.0
	Pi2	9.26	6.70	27	11.93	1.0	1.28 ±	0.29	41.4 ±	0.5	0.150 ±	0.000	1.9 ±	0.4
	Pi3	11.60	6.93	27	11.08	0.4	1.01 ±	0.05	49.5 ±	0.3	0.230 ±	0.000	n.d.	
	Pi4	11.02	7.06	33	11.28	1.2	1.30 ±	0.73	75.7 ±	1.8	0.190 ±	0.040	2.3 ±	0.7
	Pi5	10.29	7.11	23	11.5	1.8	2.29 ±	0.00	41.2 ±	1.7	0.190 ±	0.040	1.2 ±	0.7
	Pi6	10.37	7.30	22	11.59	1.2	2.18 ±	0.10	44.2 ±	1.6	0.190 ±	0.040	2.1 ±	4.9

With all the obtained determinations, possible relationships between the assessed values and the microbiological assessment were evaluated by construction of seasonal correlation table (Table 5).

Table 5. Correlation between all the assessed parameters for all season and all locations; G, conductance, D.O., dissolved oxygen; TUR, turbidity; EC, *Escherichia coli*; FE, fecal enterococci.

Parameters	*T ℃*	*pH*	*G (µS/cm)*	*D.O. (mg/L)*	*TUR (NTU)*	*PO_4^{3-} (µM)*	*NO_3^- (µM)*	*NO_2^- (µM)*	*NH_4^+ (µM)*	*EC*	*FE*
T °C	1.00										
pH	-0.07	1.00									
G (µS/cm)	0.08	0.17	1.00								
D.O. (mg/L)	-0.80	0.45	-0.04	1.00							
TUR (NTU)	0.34	0.05	0.43	-0.18	1.00						
PO_4^{3-} (µM)	0.85	-0.05	-0.12	-0.67	0.08	1.00					
NO_3^- (µM)	-0.34	-0.07	-0.28	0.19	0.03	-0.14	1.00				
NO_2^- (µM)	0.60	0.21	0.42	-0.28	0.30	0.53	-0.58	1.00			
NH_4^+ (µM)	0.18	0.56	0.58	0.18	0.07	0.07	-0.62	0.70	1.00		
EC	0.19	-0.20	0.05	-0.37	0.41	0.00	-0.01	-0.01	-0.34	1.00	
FE	0.10	-0.27	-0.18	-0.24	-0.01	-0.02	-0.19	-0.05	-0.20	0.59	1.00

The most of the significant correlations ($p < 0.05$) observed were expected: the increase of nitrite and ammonia with the decrease of nitrate as result of nitrate use by microorganisms, photosynthetic and chemosynthetic as well as aquatic plants; the decrease of dissolved oxygen with increase of phosphate promoting freshwater algae bloom; the physical impact of temperature rising promoting the oxygen release from the water due to increase of gas pressure.

There were also other correlations such as the increase of pH and conductivity with the increase of ammonium, the latter could be explained with the high mobility of the ion ammonium. Since ammonium concentration is based on the determination of NHx, which means both ammonium and ammonia are determined; it is less clear what was happening. The increase of both nitrite and phosphate concentration with the temperature increase and with each other's concentration, appeared to contradict itself. The increase of nitrite could be explained by higher biological activity with higher temperatures but that would result in a decrease of phosphate as well. Eventually, some external factors not taken into account caused this contradiction. Also some rather peculiar, non-expected relationships namely the increase of pH with dissolved oxygen, the conductivity with the turbidity were observed. The influence of a non-assessed parameter could be causing these apparently confusing results.

The obtained results, enabled to plot the seasonal N:P relationship for each location (Fig.3), enhancing the importance of these parameters as effective bio indicators.

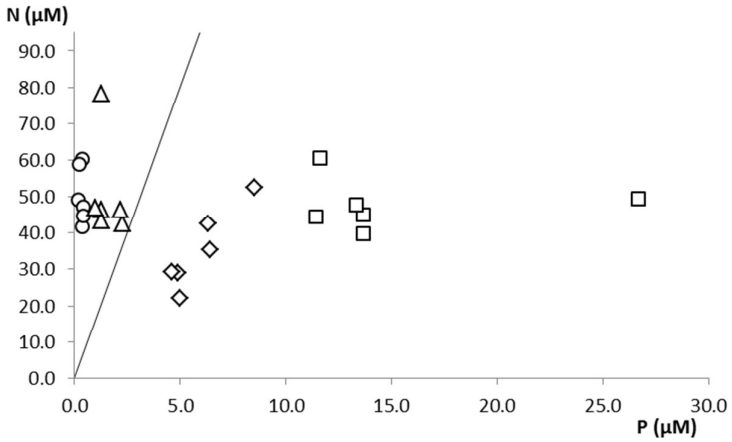


Fig. 3. Seasonal variability is reflected in the N:P relationship of the monitored inland bathing waters; ○, spring; □, summer; ◇, autumn; Δ, winter; the full line represents the Redfield ratio of 16:1 (N:P).

In fact, a seasonal signal for nutrients was detected, with winter and spring with N:P relationship above the Redfield ratio and summer and autumn clearly below (Fig.3). The clarity of the differences between seasons observed, strengthen the main purpose of designing a single manifold for the determination of nutrients.

4. Conclusions

The main aim of the developed work was to prove the pertinent need for improving nutrients analysis with an expedite, efficient, real-time and automatic methodology. As routine parameters, nutrients are effective bio indicators and enable to infer about the evaluation of the environmental status of the bathing waters. The sequential injection methodology developed provided:

- Reliable, reproducible data all obtained with spectrophotometric determinations, reinforcing the consistency;
- Direct introduction of the collected samples, with no need for sample pre-treatment and/or clean up processes;
- A single manifold, and consequent a unique set of equipments for all the assessed parameters, minimizing analysis cost;

- Independency from the analytical performer as result of automation.

Acknowledgements

I. C. Santos thanks Fundação para a Ciência e a Tecnologia (FCT, Portugal) and Fundo Social Europeu (FSE) through the program POPH – QREN for the grant SFRH/BD/76012/2011. This work was also supported by National Funds from Fundação para a Ciência e a Tecnologia (FCT), Portugal, through project PEst-OE/EQB/LA0016/2013.

References

1. P. M. Glibert, *Curr. Opin. Environ. Sustain.,* 2012, **4**, 272-277.

2. T. M. Lenton and A. J. Watson, *Global Biogeochem. CY.*, 2000, **14**, 225-248.

3. P. Carrillo, M. Villar-Argaiz and J. M. Medina-Sánchez, *J. Plankton. Res.,* 2001, **23**, 537-547.

4. Y. Li, A. M. Waite, G. Gal and M. R. Hipsey, *Ecol. Model.,* 2013, **25**, 196-213.

5. R. B. R. Mesquita and A. O. S. S. Rangel, *Anal. Chim. Acta*, 2009, **648**, 7-22.

6. M. Miro, E.H. Hansen and D. Buanuam, *Environ. Chem.,* 2006, **3**, 26-30.

7. R. B. R. Mesquita, M. T. S. O. B. Ferreira, R. L. A. Segundo, C. F. C. P. Teixeira, A. A. Bordalo and A. O. S. S. Rangel, *Anal. Methods*, 2009, **1**, 195-202.

8. R. B. R. Mesquita, M. T. S. O. B. Ferreira, I. V. Tóth, A. A. Bordalo, I. D. McKelvie and A. O. S. S. Rangel, *Anal. Chim. Acta,* 2011, **701**, 15-22.

9. R. L. A. Segundo, R. B. R. Mesquita, M. T. S. O. B. Ferreira, C. F. C. P. Teixeira, A. A. Bordalo and A. O. S. S. Rangel, *Anal. Methods,* 2011, **3**, 2049-2055.

10. R. B. R. Mesquita, I. C. Santos, A. A. Bordalo and A. O. S. S. Rangel, *Anal. Methods,* 2012, **4**, 1452-1457.

11. S. M. Oliveira, T. I. M. S. Lopes, I. V. Tóth and A. O. S. S. Rangel, *J. Environ. Monit.*, 2009, **11**, 228-234.

12. T. M. Lenton and A. J. Watson, *Global Biogeochem. Cy.*, 2000, **14**, 225-248.

13. APHA-AWWA-WPCF, Standard Methods for the Examination of Water and Wastewater, American Public Health Association, Washington DC, 20th edn, 1998

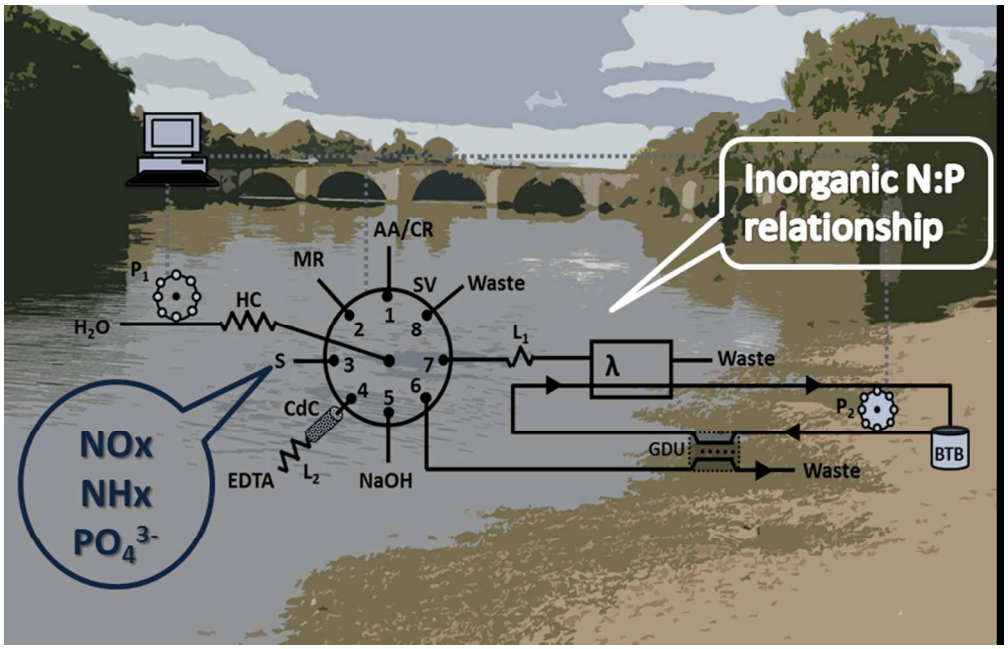
14. R. B. R. Mesquita and A. O. S. S. Rangel, *Talanta*, 2005, **68**, 268-273.

15. A. A. Bordalo, *Water Res.*, 2003, **37**, 3233-3241.

16. R. B. R. Mesquita and A. O. S. S. Rangel, *Anal. Sci.*, 2004, **20**, 1205-1210.

17. International Union of Pure and Applied Chemistry, *Pure Appl. Chem.,* 1995, **67**, 1699-1723.

18. International Union of Pure and Applied Chemistry, *Anal. Chem.,* 1976, **45**, 99-103.



156x99mm (150 x 150 DPI)