A fast and direct spectrophotometric method for the sequential determination of nitrate and nitrite at low concentrations in small volumes

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Abstract

The use of vanadium (III) has been proposed recently as a suitable alternative to cadmium for the reduction of NO_3^- to NO_2^- during spectrophotometric analysis. However, the methods proposed suffer from decreased sensitivity and additional steps for the measurements of nitrite and nitrate. We have developed an improved fast and sequential protocol that permits the determination of low concentrations of nitrite and nitrate in marine and freshwater samples using small volumes. NO_2^- concentration is firstly determined using the common Griess reaction. The subsequent addition of a 2% VCl_3 solution in 6N HCl in the same sample and the reaction at 60ºC for 25 minutes results in an efficient reduction of the NO_3^- to NO_2^- (> 95%), which is also detected by the already added Griess reagents. The method has a detection limit <0.05 μM, a high precision (ranging from 0.2 to 11%) and accuracy (0.07 μM) for the determination of $NO_3^- + NO_2^-$ concentrations lower than 30 μM. Comparison of the proposed method with the established Cd column method using samples from a variety of environments (fresh water reservoir, sediment freeze lysable pore water, estuarine water samples and samples from an acid mine drainage impacted reservoir) showed good agreement between the two methods, with a difference between methods of 0.073 ± 0.099 μM. The analysis can be performed in large batches (~60 samples) using small sample volumes (≤1 mL) for the determination of both NO_3^- and NO_2^- in less than one hour.

Introduction

Nitrate is a key compound in the N cycle of natural ecosystems and artificial environments, being substrate or product of several microbial, plant, and animal metabolic processes. In aquatic environments, nitrate is produced by nitrification in a two-step reaction: ammonium oxidation to nitrite and nitrite oxidation to nitrate. Nitrate can then be assimilated by photosynthetic organisms, thus being an important nutrient for primary production. Nitrate is also consumed in a variety of bacterial processes such as the anaerobic denitrification or dissimilatory nitrate reduction to ammonium (DNRA). Denitrification reduces nitrate to nitrous oxide, a potent greenhouse gas (Lashof and Ahuja, 1990), or molecular nitrogen gas, reducing the nitrogen load of the system, whereas DNRA reduces nitrate to biologically available ammonium that remains in the system (Megonigal et al., 2003). Therefore, the measurement of nitrate and nitrite concentrations in aquatic systems is an important aspect of most studies related to the N cycle in order to determine its production and consumption rates.

Numerous methods for the determination of nitrate are available in the literature. Highly sensitive methods are based on the reduction of nitrate to nitric oxide, which is quantified by chemiluminiscence (Aoki et al., 1997; Braman and Hendrix, 1989), or to nitrous oxide, later quantified by gas chromatography (Christensen and Tiedje, 1988). However, both these techniques require expensive and specialized equipment. Other methods involve the use of strong acids often at elevated temperatures (Mir, 2008; Zhang and Fischer, 2006), which complicates handling and analyses of the samples. In contrast, the simplest and most regularly applied method involves the reduction of nitrate to nitrite and its subsequent measurement by colorimetry using the Griess reaction (Grasshoff et al., 1983; Marzinzig et al., 1997). This method has low detection limit, high accuracy and high specificity without using expensive instruments or complex procedures.

The critical step for the accurate determination of nitrate is its efficient reduction to nitrite. Nitrate reduction to nitrite can be accomplished by specific nitrate reductases (Guevara et al., 1998; Marzinzig et al., 1997) or by the use of different reducing metals, with cadmium being the most commonly used one (Grasshoff et al., 1983; Wood et al., 1967). Although various adaptation of the cadmium reduction method have been proposed in order to increase sample throughput and decrease sample volume required (Harris and Mortimer, 2002; Jones, 1984), the method suffers from various shortcomings; it is time consuming, efficiency of the column varies, a continuous activation of the Cd-column is required, and cadmium is highly toxic (European Chemical Agency: www.echa.europa.eu and Occupational Safety and Health Administration, United States Department of Labor: www.osha.gov) making handling of samples and waste hazardous.

Miranda et al. (2001) described a spectrophotometric method using a vanadium solution (VCl$_3$) for the reduction of nitrate. V(III), which is less toxic than cadmium (European Chemical Agency and Occupational Safety and Health Administration, United States Department of Labor), has been commonly used for the reduction of both nitrate and nitrite at high temperatures (80-90ºC) to nitric oxide, then measured by chemiluminiscence (Braman and Hendrix, 1989). Miranda et al. (2001) showed that at room temperatures nitrate is reduced to nitrite, which can react with Griess reagents and be measured in a spectrophotometer. However, the proposed protocol resulted in a low molar absorptivity for nitrate, indicating low reaction efficiency in the reduction of NO$_3^-$ to NO$_2^-$. As a result, nitrite highly interferes with the determination of nitrate. Beda and Nedospasov (2005) included an initial step in the method for the elimination of nitrite by the reaction with sulfamic acid, reducing thus the high NO$_2^-$ interference in the NO$_3^-$ determination. However, as the subsequent steps involving the reduction of NO$_3^-$ to NO$_2^-$ were not modified, the overall efficiency of the reaction was not improved. As a result the precision of the method was lower than that using the classic Cd-columns for nitrate reduction.

We describe here an optimised protocol for the sequential measurement of nitrite and nitrate in small volumes (< 1mL) of the same sample by adjusting factors such as vanadium chloride and HCl concentrations, temperature and time of reaction that improved the efficiency of the nitrate reduction to nitrite and the signal measured. As a result, efficiencies higher than 95% were obtained, resulting in a simple, fast and accurate method for the determination of nitrate and nitrite. The proposed methodology was also compared with the most widely used method for NO$_3^-$ analysis, the Cd column method) by analysing in parallel samples from different environments (fresh water reservoir, sediment freeze lysable pore water, estuarine water samples and samples from an acid mine drainage impacted reservoir).

Materials and methods

Instrumentation

Spectrophotometric measurements were performed on Unicam UV/Vis UV2-200 spectrophotometer (Unicam, U.K.) using 1-cm path length quartz cuvettes. The spectrophotometer was equipped with a thermostatized cuvette holder regulated by an external water bath. The UV-probe software (Unicam, U.K.) was used to control measurements and record absorbance spectra. Spectrophotometric measurements in 96-well mi-

croplates were performed on a microplate reader Powerwave 340 (Bio-Tek Instruments Inc., USA), using the control program KC JuniorTM Data Analysis software. Incubations of samples were performed in a temperature controlled water bath Unitronic OR (JP Selecta, Spain).

Reagents and standards

Reagents were of analytical purity grade. All solutions and dilutions were prepared with pure water (Milli -Q). Griess reagents for the analysis of NO_2^- were prepared as described in Grasshoff. et al. (1983). Sulphanilamide reagent was prepared by dissolving 5.0 g of sulphanilamide in 50 mL of concentrated (12N) hydrochloric acid (HCl) diluted in about 300 mL of pure water and after cooling made up to 500 mL with pure water. N-(1-naphthyl)-ethylenediamine dihydrochloride (NED) reagent was prepared by dissolving 0.5 g NED in 500 mL of pure water (MiliQ). Both reagents were mixed in equal proportions just prior to performing the analysis (hereafter referred as Griess-reagent). Reagents were stored in glass dark bottles and maintained at 4ºC. The reagents are stable for at least one month. NED reagent can be used until a brown discoloration occurs.

Vanadium (III) chloride (VCl_3) reagent 2% w/v was prepared in a 6N HCl solution (VCl_3-reagent). The time needed for the complete dissolution varies depending on the concentration used but was generally about 1 hour. The complete dissolution was evidenced by the shift from a turbid to a transparent solution. The solution was finally filtered through a 0.7 µm nominal pore size glass fibre filter in order to eliminate any impurities of the reagent. VCl_3-reagent was prepared weekly and stored in a dark glass bottle at 4ºC. The VCl_3-reagent is stable for several months.

Artificial seawater was prepared following the complete salt composition described by Grasshoff et al. (1983).

Stock nitrate standard solutions (10 mM) were prepared by dissolving 1.011 g oven dried (100ºC, 1 h) KNO_3 in 1 L pure water. Stock nitrite solution (10 mM) was prepared by dissolving 0.690 g $NaNO_2$ to 1 L of pure water. Working solutions were prepared from stock solutions as required by dilution with pure water for all the initial optimization experiments. Standard solutions were prepared in artificial seawater of the appropriate salinity for the salinity effect experiment.

Proposed procedure for the sequential determination of NO_2^- and NO_3^-

A 2-step protocol was tested and validated, in order to allow the sequential determination of both NO_2^- and NO_3^- in the same sample as follows:

Step 1: Nitrite determination

One mL of sample was transferred into 1.5 mL eppendorf vials followed by the addition of Griessreagent (50 µL) and gently mixed. Vials were incubated at ambient temperature (~25ºC) for 20 min. Then, 350 µL of this solution (sample + reagents) were transferred into 96-well flat bottom polysterene microplates and absorbance was measured at 540 nm. NO_2^- concentrations was determined by parallel analysis of a set of NO_2^- standards.

Step 2: Nitrite plus Nitrate determination

A volume (70 µL) of VCl_3-reagent was added to the remaining sample volume (700 µL) in the eppendorf vials. The vials were closed to prevent evaporation, gently mixed and incubated in a temperature-controlled bath at 60ºC for 25 min. Then, the vials were cooled down to room temperature in a water bath and 350 µL of each sample was transferred into 96-well microplates and the absorbance was measured at 540 nm. Parallel analysis of a set of NO_2^- and NO_3^- standards were performed simultaneously for the determination of NO_3^- concentration as described below.

Calibration curves
- Nitrite determination (Step 1)

During *step 1* a set of NO_2^- standards is used to determine the concentrations of NO_2^- in the standard/ sample using the equation:

$$ABS^1_{NO2} = S^1_{NO2} \times [NO_2^-] + ABS^1_{reagents} \quad (1)$$

Where ABS^1_{NO2} is the absorbance of the NO_2^- standards; S^1_{NO2} is the slope of the calibration curve (ABS µM^{-1}) for NO_2^-; $[NO_2^-]$ is the NO_2^- concentration, and $ABS^1_{reagents}$ is the absorbance of the reagents, i.e. the intercept of the calibration curve

- Nitrate determination (Step 2)

In the samples, where both NO_2^- and NO_3^- are present, it is necessary to discriminate between the contributions from the two compounds. After the reaction with VCl_3-reagent, the measured absorbance (ABS^V_{NOX}) is a combination of the individual contribution of each compound (NO_2^- and NO_3^-) plus the absorbance of the reagents, i.e.:

$$ABS^V_{NOX} = ABS^V_{NO2} + ABS^V_{NO3} + ABS^V_{reagents} \quad (2)$$

The use of NO_2^- standards and NO_3^- standards allow performing a calibration of the complete reaction in the presence of VCl_3-reagent, being:

$$ABS^V_{NO2} = S^V_{NO2} \times [NO_2^-] + ABS^V_{reagents} \quad (3)$$

$$ABS^V_{NO3} = S^V_{NO3} \times [NO_3^-] + ABS^V_{reagents} \qquad (4)$$

where, ABS^V_{NO2} and ABS^V_{NO3} are the absorbance of the NO_2^- and NO_3^- standard, respectively; S^V_{NO2} and S^V_{NO3} are the slope of the calibration curves (ABS µM^{-1}) for NO_2^- and NO_3^-, respectively; $[NO_2^-]$ and $[NO_3^-]$ are the NO_2^- or NO_3^- concentration of the standards, respectively and $ABS^V_{reagents}$ is the absorbance of the reagents without NO_2^- or NO_3^-, i.e. the intercept of the calibration curve.

The measured absorbance is a combination of the individual contribution of each compound plus the absorbance of the reagents, i.e. the combination of Equations 3 and 4:

$$ABS^V_{NOX} = S^V_{NO3} \times [NO_3^-] + S^V_{NO2} \\ \times [NO_2^-] + ABS^V_{reagents} \quad (5)$$

The actual NO_3^- concentration of the sample is calculated as:

$$[NO_3^-] = (ABS^V_{NOX} - ABS^V_{reagents} - S^V_{NO2} \\ \times [NO_2^-]) / S^V_{NO3} \quad (6)$$

Where: $[NO_2^-]$ is the NO_2^- concentration determined in the step 1 and $[NO_3^-]$ is the NO_3^- concentration of the sample.

Environmental samples

A set of environmental samples were collected from different environments and analysed both with the traditional cadmium reduction method on a TRAACS 800 Technicon autoanalyser using standard protocols (Grasshoff et al 1983) and with the protocol described here. Estuarine water column surface samples were collected along a salinity gradient (salinity range 6-26) in the inner Gulf of Nicoya, Costa Rica in July 2011 (Seguro et al. in prep). Freshwater water column samples (0-16 m depth, salinity 0) were collected from the Bornos water reservoir, SW Spain, in August 2010 (Romero-Martínez et al., 2013). Water column samples from an experiment using sediment cores from an acid mine drainage affected water reservoir, Sancho reservoir, SW Spain, were collected in December 2011 (Torres et al. 2013) to test for the effect of high metal concentrations. Water samples were filtered by 0.7 µm glass fibre filters and stored frozen at -20ºC until analysis. Pore water nutrient samples extracted from frozen sediment cores (Freeze Lysable Inorganic Nutrients, FLIN) (0-3.5 cm depth, salinity 32-40) were collected from the Rio San Pedro tidal creek, SW Spain, in March 2013, using the procedure described in Garcia-Robledo et al (2010). FLIN samples were stored frozen until analysis. Samples were aliquoted in triplicate and analysed with each method.

Results & Discussion

Effect of temperature, VCl₃ and HCl concentration

Maximum absorbance and the corresponding minimum time needed to reach it as a function of varying VCl_3 and HCl concentrations are represented in Figure 1. For VCl_3, maximum absorbance was obtained when concentrations were equal or higher than 1%, being maximum at 2% (Fig. 1A). In order to follow the effect of temperature on colour development, the cuvette holder of the spectrophotometer was maintained at a constant temperature while the absorbance was measured every 5 minutes.

Temperature had a significant effect, with absorbance being highest at 40ºC and lowest at 80ºC (Fig. 1A). However, the combined effects of temperature and VCl_3 concentration strongly influenced the reaction time (Fig. 1C). Although the maximum colour development was measured at 40ºC, reaction times exceeded 2 hours, reducing its application for daily routine analysis. We also tested lower temperatures such as the ambient temperature (25 ºC) which was used in the protocols by Beda and Nedospasov (2005) and Miranda et al. (2001); however, reaction times exceeded 5 hours (data not shown), and therefore temperatures lower than 40ºC were not considered during subsequent tests. In contrast, temperatures of 50-70ºC combined with 2% VCl_3 solution had high absorbances with reaction times <1 hour. The increase in temperature from 60 to 70ºC did not affect the performance of the reaction substantially, so further comparisons were done between 50 and 60ºC.

We also tested for the effect of HCl concentration on the reaction. Maximum absorbance was measured when the concentration was higher than 4 N (maximum at 6-8 N) (Fig. 1B). Reaction time was also influenced by HCl concentration, decreasing exponentially to reaction time less than 30 min at concentrations =6N HCl (Fig. 1D). Therefore, a concentration of 6N HCl was determined as the minimum concentration producing the maximum signal with the minimum reaction time. As a consequence, the conditions selected based on the current setup that resulted in the most efficient reaction overall was a solution of 2% VCl_3 in 6N HCl and a reaction temperature of 60ºC.

Optimization of the temperature and reaction time for laboratory use

The dependence of the maxima signal and reaction time on temperature was further tested using a temperature controlled water bath. This heating method is the most common procedure in laboratories and the heat transference efficiency is higher compared to that of the spectrophotometer's cuvette holder. At the highest tem-
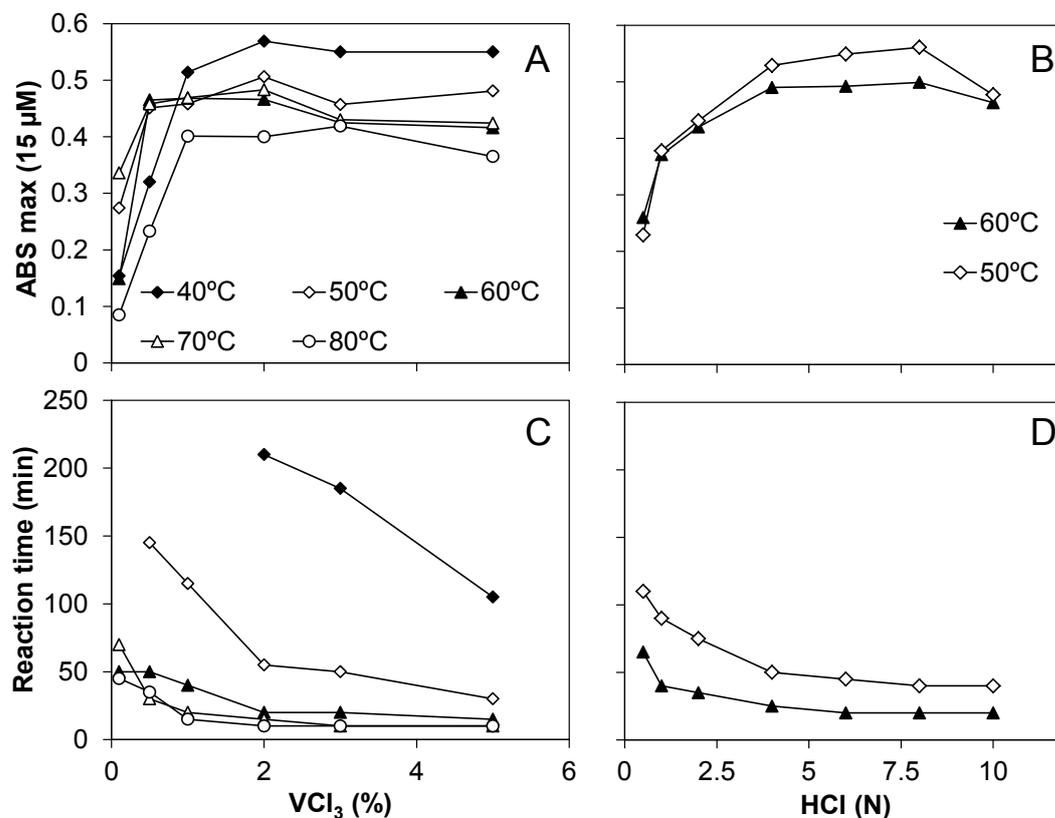
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Figure 1. (A) Effect of varied temperature and VCl$_3$ reagent concentration in 10N HCl on reduction and measurement of the maximum absorbance for a 15 μM NO$_3^-$ solution (2.5 mL standard + 250 μL Griess reagent +250 VCl$_3$-reagent); (B) Effect of varied temperature and HCl concentration (using 2% VCl$_3$) on reduction and measurement of the maximum absorbance for a 15 μM NO$_3^-$ solution (2.5 mL standard + 125 μL Griess reagent +250 μL VCl$_3$-reagent); (C) and (D) corresponding minimum time required to reach the maximum absorbances for (A) and (B), respectively.

peratures (70 and 80ºC), V(III) reduces nitrate in 5 minutes or less (Braman and Hendrix, 1989) and the produced nitrite reacts quickly with Griess reagent resulting in maximum signal in the first measurement (5 min incubation) (Fig. 2A). However, the addition of reducing compounds has been proved to interfere in complex manners with the Griess reaction (Lebaron et al., 2002), resulting in a progressive reduction of molar absorption with time. In contrast, at 40ºC the reaction was slow, resulting in reaction times exceeding 2 hours. At 50 and 60ºC, maximum responses were obtained after 40 and 15 min of incubation, respectively, and maintained constant for further 15 min of incubation (Fig. 2A). Considering that, the maximum molar absorptivity was obtained after 25 min of incubation at 60ºC, 5% higher than the value obtained at 50ºC, the optimal conditions chosen for the method in the previous tests were confirmed.

Optimization of the reagent volume

Reagent volumes were adjusted to produce the minimal dilution of the sample and obtain the maximum molar absorbance. The maximum efficiency was obtained using 100 μL 2% VCl$_3$-solution per mL of sample (Fig. 2B). With lower volumes, VCl$_3$ concentration was probably too little to reduce the entire nitrate in the sample within 25 min. In contrast, an excess of VCl$_3$ favoured the further reduction of nitrite to nitric oxide, resulting in a decrease of the measured signal. On the other hand, Griess-reagent volumes over 25 μL resulted in similar signal, being maxima with the addition of 50 μL per mL of sample (Fig. 2B).

Limit of detection, measuring range and stability

The optimized conditions selected were tested in order to define the measuring range, detection limit and accuracy of the method (Fig. 3). When just NO$_3^-$ was present, reaction was linear between 0-40 μM NO$_3^-$ (Fig. 3A), similar to the one obtained by cadmium re-
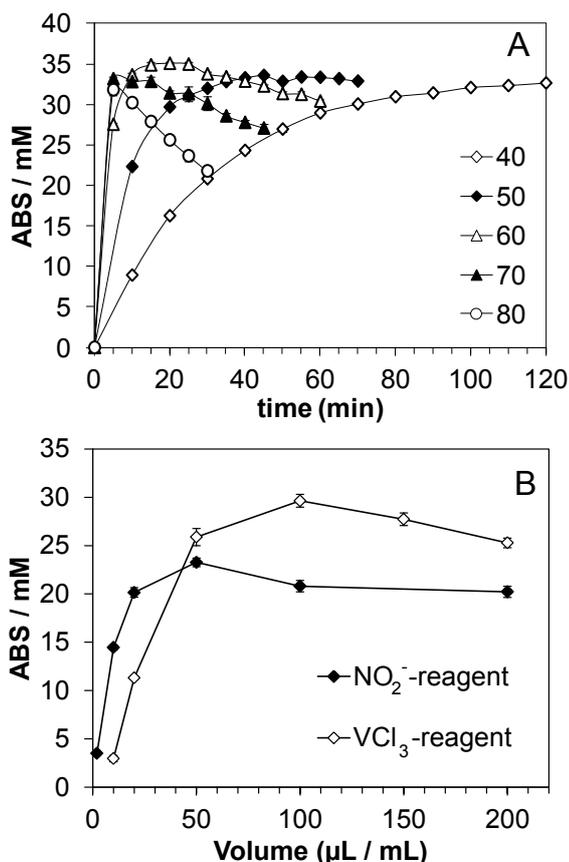
Figure 2. (A) Time evolution of the NO_3^- molar absorptivity at different temperatures following reaction (1 mL standard + 50 µL Griess-reagent + 100 µL 2% VCl_3 in 6N HCl) of NO_3^- calibration curves (0-15 µM range). Standards were measured every 5-10 minutes. **(B)** Effect of varied volumes of Griess-reagent (1 mL standard + 2, 10, 20, 50, 100 or 200 µL Griess reagent + 90 µL VCl_3-reagent) and VCl_3-reagent (1 mL standard + 50 µL Griess reagent + 10, 20, 50, 100, 150 or 200 µL

action columns (Wood et al., 1967), and within the range of nitrate concentrations commonly found in unpolluted aquatic environments.

The procedure showed a high precision. The standard deviation of the 6 standard replicates used for the calibration shown in Figure 3B ranged from 0.01 to 0.11 µM, which mean a coefficient of variation between 0.2 to 11%. The limit of detection (LOD) was calculated as 3 times the standard error of the intercept divided by the slope of the calibration as defined by Konieczka and Namiesnik (2009), resulting in a value as low as 0.04 µM NO_3^-.

As shown above, the reduction of NO_3^- and NO_2^- with V(III) progresses rapidly at high temperatures, resulting in gradual decrease of the signal after 30-35 min at 60ºC (Fib 2A). However, at slightly lower temperatures (e.g. 40ºC), the reaction slows down signifi-

cantly, requiring several hours to reach maximum absorbances (Fig. 2A). Therefore, it is suggested that the reaction time should be controlled carefully to be 20-30 min at 60ºC and then the samples to be cooled down to ambient temperature (25ºC). In this way, the signal remains stable for at least 1 hour, slowly decreasing with time thereafter (Fig. 3C). This procedure facilitates the analysis of a large number of samples simultaneously without the need to measure the absorbances of the samples immediately.

Salt effect

A slight decrease in the molar absorbance was observed with increasing salinities in the determination of NO_2^- using the common procedure of the Griess reaction (ABS = 94.163 + 5.822 * exp (-0.120 + salinity), r = 0.958) (Fig. 4), in contrast to previous suggestions (Grasshoff et al., 1983). Norwitz & Keliher (1985) also reported a salt effect but at much higher concentrations than the ones used here and for individual salts. The effect of salinity on the determination of NO_2^- was the same when VCl_3 was present in the reaction (ABS = 93.033 + 7.104 * exp (-0.088 + salinity), r = 0.984). The salt effect remained stable in salinities between 20 and 50, producing a molar absorbance about 94% of the value obtained in distilled water. A stronger effect was observed during NO_3^- determination in the presence of VCl_3, with the molar absorptivity decreasing to 85% of the value obtained in distilled water at salinities between 30 and 50 (ABS = 82.519 + 17.469 * exp (-0.055 + salinity), r = 0.998). Therefore, it is suggested that the calibration curves should be performed using standards at salinities similar to those found in the samples to be analyzed.

Nitrite interference

Environmental samples of marine and freshwater systems primarily contain NO_3^-, with NO_2^- being either absent or present at low concentrations. However, there are aquatic environments such as marine coastal areas and Oxygen Minimum Zones or fresh water hypolimnion where both NO_3^- and NO_2^- concentrations can increase and therefore both compounds should be measured accurately. The protocol described by Miranda. et al. (2001) described a fast and simple spectrophotometric protocol using VCl_3 for NO_3^- reduction. However, they reported a high interference from NO_2^-, with the error in the NO_3^- determination being proportional to the NO_2^- concentration. As a result, the error in a sample with higher concentrations of NO_2^- than NO_3^- could be superior than the actual NO_3^- concentration (Beda and Nedospasov, 2005).

After the complete reaction with VCl_3, both the initial NO_2^- and NO_2^- from the reduction of NO_3^- will con-
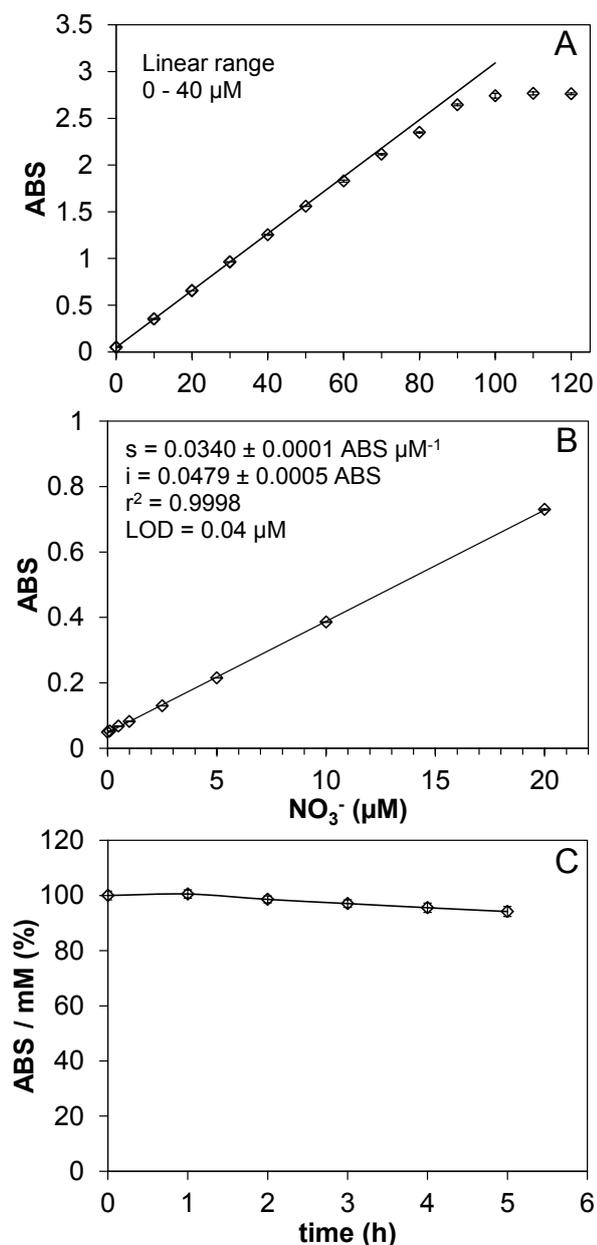
Figure 4. Effect of salinity on the molar absorptivity of NO_2^- in the normal Griess reaction and of NO_3^- and NO_2^- after the reaction with VCl_3 (1 mL + 50 µL Griess reagent + 100 µL VCl_3-reagent) expressed as percentage of the value obtained in pure water (MilliQ). Values are means ± SE.

Figure 3. (A) Linear range, **(B)** low concentration calibration with the calculation of the limit of detection (LOD) and **(C)** decrease of the molar absorptivity over time at ambient temperature (25ºC) expressed as percentage of the initial. Reactions were carried out for 25 minutes at 60ºC using 1mL NO_3^- standards (n = 3 - 6) + 50 µL Griess-reagent + 100 µL VCl_3-reagent and absorbances measured using 1 cm cuvettes. Values are means ± SE.

tribute to the measured absorbance. However, the contribution of each compound to the total absorbance depends on a number of factors. The addition of the VCl_3-reagent itself produces a slight dilution (1:1.1) of the
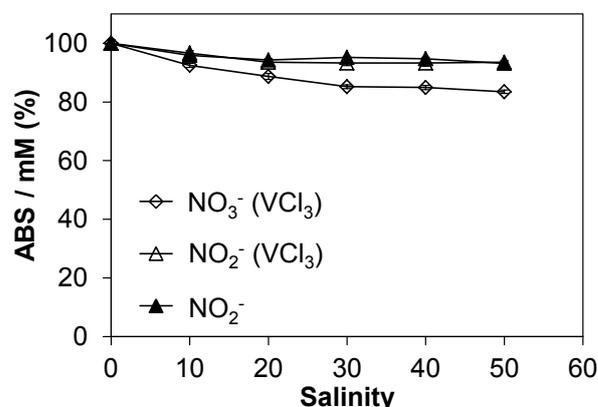
usual Griess reagent/sample volume ratio, resulting in a decrease of the expected NO_2^- molar absorptivity. In addition, the azo dye signal decreases with time likely caused by interference from the VCl_3 reagent (Lebaron et al., 2002), which also reduces the NO_2^- molar absorptivity with time (Fig. 2). Thus, NO_2^- molar absorptivity, as measured using solely NO_2^- standards, decreases from a value of 0.042 to 0.036 ABS μM^{-1} after the addition of VCl_3-reagent (Fig. 5A). This value was slightly lower than the theoretical decrease produced by dilution due to reagents addition (0.038 ABS μM^{-1}), emphasising the need to determine the effect of VCl_3-reagent addition to the azo dye signal obtained in step 1 using a set of NO_2^- standards in parallel to those of NO_3^-.

The efficiency of the NO_3^- reduction to NO_2^- will also influence the measured signal; part of the NO_3^- may not react, the NO_2^- produced could further react with VCl_3 or the VCl_3 reagent could interfere with the azo dye production or signal, all resulting in efficiencies lower than 100%. Indeed, the molar absorbance obtained for NO_3^- was slightly lower than that for NO_2^-, but the efficiency of the reaction was always higher than 90% (94% in the example shown in Fig. 5A) during the tests of the method.

The method developed by Miranda. et al. (2001) achieved much lower molar absorptivities for NO_3^- that the ones observed in the present method due to 1) the larger volume of the reagents used by these authors, resulting in a 1:2 dilution of the sample and 2) a much lower conversion efficiency. The molar absorptivities obtained were 0.0089 ABS μM^{-1} which, after consider-
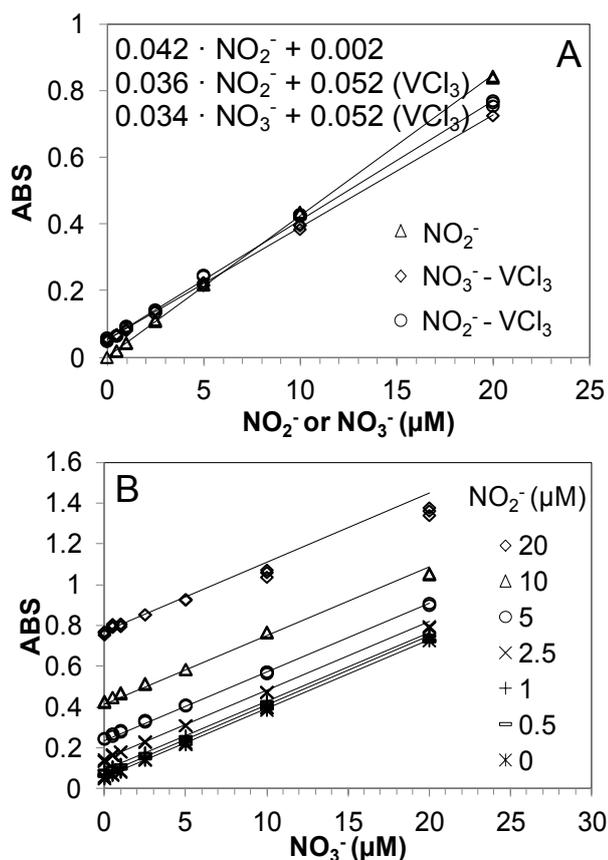
Figure 5. (A) Calibration curves obtained in the sequential measurement of NO_2^- and NO_3^- for NO_2^- after *step 1* (standard + Griess-reagent) and separately for NO_2^- and NO_3^- after *step 2* (sample + Griess-reagent + VCl_3-reagent, 25 minutes reaction at 60ºC). Standard error was <0.0003 for the slopes and <0.003 for the intercepts. **(B)** NO_3^- calibrations curves (0 to 20 μM) measured in the presence of varied NO_2^- concentrations (0 to 20 μM). Measured absorbance is represented as dots. Solid lines represent the calculated values using the calibration curves shown in (A) and equation 4 of the text.

ing the molar absorptivities obtained for NO_2^- with the Griess reaction (around 0.038 ABS μM^{-1}) and the dilution due to reagents as described their protocol, would signify a conversion efficiency of ~40%. As a result, the absorbance obtained per mol of NO_2^- with the method of Miranda. et al. (2001) is more than 2 times higher than that for NO_3^-. In addition, the differential signal obtained for NO_3^- and NO_2^- was not taken into account, resulting in lower accuracy and a large NO_2^- interference.

In contrast, the procedure proposed here achieves a similar absorbance signal for both compounds, resulting in minimal NO_2^- interference and high accuracy. In addition, the signal for each of the NO_3^- and NO_2^- is obtained separately during the calibration, and is constant during each analysis regardless of the NO_2^-:NO_3^-

ratio present in the sample. This was confirmed by the low error (= 0.5 μM) measured in the determination of NO_3^- in standards with a range of NO_2^- concentrations (Fig. 5B). Nitrate was, however, underestimated when the sum of NO_2^- and NO_3^- (NO_X^-) concentrations exceeded 30 μM, which is higher than the upper limit for the measurement of NO_2^- (Grasshoff et al., 1983). Therefore, for measurements of NO_X^- above 25 μM, especially with high NO_2^- concentrations (>30% NO_X^-), samples should be diluted in order to obtain an accurate NO_3^- measurement.

We have performed the complete procedure for large series of samples (>60 samples) for the determination of both NO_2^- and NO_3^- in the same water sample (=1 mL) in less than one hour. The method can also be adapted to be performed in its entirety on the same microplate by first taking the absorbance reading for NO_2^- after *step 1*, heating on a microplate and for NO_x^- after *step 2*. Thus, sample volumes <300μL (250 μL sample + 12.5 μL Griess reagent + 25 μL VCl_3-reagent) are utilised, making it especially suitable for analysis of sediment porewater samples.

Comparison of cadmium column and vanadium methods

Contribution of NO_2^- to NO_x^- in the measured samples ranged from 0.2- 32.1% (median 5.1%). Samples were diluted appropriately (dilution factor range 1-12) to obtain NO_x^- concentrations lower than 30 μM. Our diluted samples with a high NO_2^- contribution showed NO_x^- concentrations lower 20μM. Comparison of the classical cadmium column reduction method and the protocol proposed here over a range of salinities (0-40) and type of samples (i.e. freshwater reservoir, acid mine drainage affected reservoir, estuarine, sediment freeze lysable) gave an excellent agreement between the two methods (Fig. 6). Paired t-test showed that the results from the two methods are homogenous (t = 0.736, p = 0.472, n = 17). Using the Cd column method as the reference methodology, the accuracy of the proposed method with VCl_3 could be calculated as the difference between the measured samples (Konieczka and Namiesnik, 2009), resulting in an accuracy of 0.073 ± 0.099 μM. These independent determinations show that the vanadium reduction method using spectrophotometry does not bias the results. Furthermore, 60 samples were analyzed for both NO_2^- and NO_3^- in less than 1 h by the vanadium method without the need of a specially trained technician and an expensive autoanalyser system by the automated cadmium column reduction method.
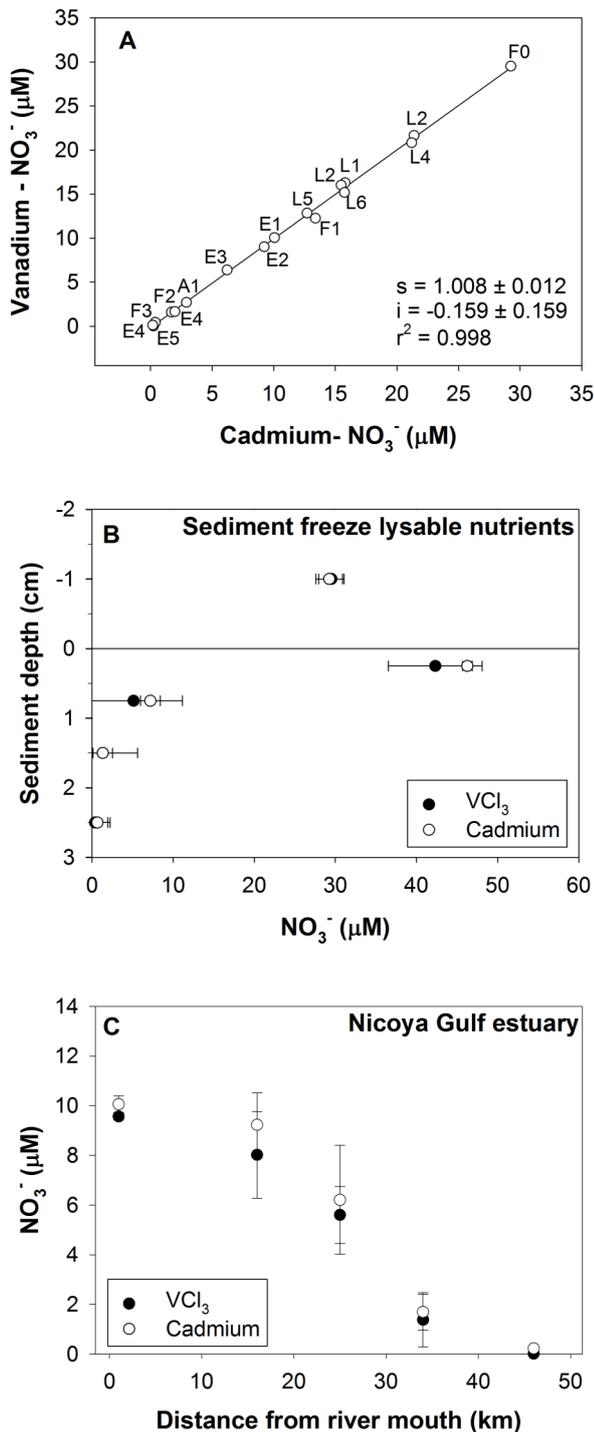
Figure 6. (A) Linear regression comparing the cadmium column and vanadium methods. L 1-6: Bornos water reservoir, A1: Sancho acid mine drainage impacted reservoir, E 1-5: Nicoya Gulf estuary, F 0-4: overlying water and sediment freeze lysable nutrient samples. Regression coefficients and standard error are shown (n = 17). (B) Vertical profile of overlying water and sediment freeze lysable nitrate concentration in sediment from the inner Bay of Cadiz, Spain determined with the two methods. (C) Horizontal profile of nitrate concentrations measured with the two methods along the Nicoya Gulf estuary. Error bars on graphs (B) and (C) represent 95% confidence intervals.

Conclusion

We present a protocol for the sequential measurement of low NO_2^- and NO_3^- concentrations in both freshwater and marine samples based on an initial detection of NO_2^- with Griess reagents followed by the reduction of the NO_3^- to NO_2^- with VCl_3 and subsequent detection with the excess Griess reagent present. The method has a detection limit < 0.05 μM and a high accuracy and precision in the determination of both NO_2^- and NO_3^- for combined concentrations lower than 30 μM. The procedure does not require specialized equipment, expensive reagents or tedious procedures. The method allows the fast analysis of larges series of samples using low volumes, such as those of sediment porewater profiles.

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