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# Determination of nitrate plus nitrite in small volume marine water samples using vanadium(III)chloride as a reduction agent

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ABSTRACT

In this contribution a method to analyse nitrate in marine pore water, seawater and freshwater is presented. The method serves to replace the well-known cadmium column method for the reduction of nitrate to nitrite. Instead, acidic vanadium(III)-solution is used for the reduction avoiding the toxic Cd metal. Both, the already present and the newly produced nitrite are quantified by the established Griess–Ilosvay reaction. Sample preparation is easy because only one reagent solution has to be added to the sample. Efficiency of the nitrate reduction is 100 ± 3% (n = 12) using either a reaction time of 60 min at 45 °C or 10–20 h at room temperature. Measurements can either be done by conventional UV–VIS spectrophotometry using 1–5 cm cuvettes, by discrete sequential analysers based on the loop flow technique, by continuous flow technique or, when only small sample volumes are available, by microtiter plate readers (MR) in absorbance mode. The latter method requires only 0.5 mL of a sample for nitrite and nitrate quantification and has a precision of 2%. The limit of detection of the MR technique is comparable to conventional methods using a 1 cm cuvette (0.4 μM for nitrate, 0.07 μM for nitrite). The method requires only a spectrophotometer, and is simple and cost-effective; sample preparation is rapid and a salt error or an interference by dissolved organic carbon is not evident. Interferences induced by naturally coloured samples, or hydrogen sulphide can be corrected or eliminated. A procedure for direct nitrate determination is given as well as a procedure to compensate for the small loss of nitrite by nitric oxide formation for the nitrite present prior to the addition of a reductant.



## 1. Introduction

One of the most common methods applied to analyse nitrate in water samples is based on the reduction of nitrate to nitrite using a copperised cadmium column in a buffered solution. The reduced nitrate plus the nitrite originally present in the sample (in the following ascribed to as NOx) is then measured by the Griess–Ilosvay procedure (e.g. Hansen and Koroleff, 1999). Under acidic conditions nitrite reacts with sulphanilamide to form a diazo compound, which couples with N-1-naphthylethylenediamine di-hydrochloride (NEDD) to form a reddish-purple azo dye measured at 550 nm. Using this method, samples have to be measured twice; NOx is measured after reduction using a cadmium column (in some cases also copperised) and nitrite is measured without the reduction step; the nitrate concentration is calculated as the difference between both measurements.

Disadvantages of using Cd columns are the potential metal toxicity and efficiency variations with time depending on the number and nature of samples. Difficulties arise especially during analysis of marine pore waters due to contact of the reduction column with air, $H_2S$, Hg-poisoned samples and/or humic acids leading to a lower efficiency and representing serious sources of error (e.g. Hansen and Koroleff, 1999). Furthermore, marine pore water samples are often only available in small volumes. This low sample volume is very often not sufficient to analyse NOx by the traditional Cd column technique, which hampers a complete biogeochemical characterisation of the water samples.

An alternative reduction agent for nitrate in natural waters makes use of hydrazine in the presence of copper ions as a catalyst (Mullin and Riley, 1955). Disadvantages of this method are the time-consuming reaction time, the incomplete reduction of nitrate, a salt error and the high pH dependence (e.g. Henricksen, 1965; Hansen and Koroleff, 1999).

A third method to determine NOx is based on vanadium(III) ($VCl_3$) as an agent to reduce nitrite and nitrate to nitric oxide at temperatures of 80–90 °C followed by chemoluminescence detection (Braman and Hendrix, 1989). At room temperature, nitrite is rapidly reduced to NO by $VCl_3$ (while nitrate is not) and can be quantified with the same equipment. Analysis time for both, NOx or nitrite is about 4–5 min per sample and a sample volume of 2–4 mL is needed. This method provides the most sensitive measurement of nitrogen species compared to so far applied methods but requires more sophisticated laboratory equipment. A comprehensive review about methods for the detection and determination of nitrate, nitrite and NOx was published by Moorcroft et al. (2001).

Up to now $VCl_3$ was used for the chemical reduction of nitrate to nitrite at lower reaction temperatures of about 35–45 °C in medical applications like buffer, media, serum (Miranda et al., 2001) and in soil

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extractions (Hood-Novotny et al., 2010) using 96 well microplate spectrophotometry for detection. The method was also tested for a wide range of matrices including milk, river water, various agriculture extracts and urine and by using one single reagent ($VCl_3$ reduction solution and the Griess solution) (Doane and Horwath, 2003). For samples where nitrite is in excess to nitrate the method was modified by removing nitrite using sulfamic acid prior to nitrate measurement (Beda and Nedospasov, 2005). In the marine environment this method was applied to aquaculture systems using separate reduction solution, separate Giess reagents and a removal of nitrite by sulfamic acid prior to nitrate measurement (Cecchini and Caputo, 2012). These authors also investigated the influence of salt on NOx measurements and found no salt effects up to a salinity of 35. With detection limits of about 360 μM nitrate for the 96 well microplate technique and 36 μM nitrate for a 5 cm spectrophotometer cell (Cecchini and Caputo, 2012) the method is only useful for applications in the marine environment where nitrate concentrations are medium to high.

In general, the advantage of using the low temperature $VCl_3$ reduction method is that the reduction agent has a lower toxicity than cadmium and needs no laborious preparation of Cd coils or columns.

To date, the low temperature $VCl_3$ reduction method has, however, not been tested for interferences occurring in marine samples from the coastal marine waters and in porewater samples. Therefore, this work focuses on the application of the $VCl_3$ reduction method to measure marine samples. The following topics are addressed:

a) The reduction of nitrate to nitrite was optimised by varying reaction times and sample to reagent ratios for nitrate and nitrite.
b) The detection limit for nitrite was improved to analyse small sample volumes using 96 well microplate readers (MR) technique in absorbance.
c) We tested to what extent nitrite is lost due to nitric oxide (NO) formation after addition of the combined Griess-$VCl_3$ reagent. A procedure is given to correct for this loss if NOx is analysed. Alternatively, a procedure is given for the direct nitrate determination after removal of the nitrite fraction present in the sample.
d) Procedures are proposed to correct or eliminate the major known potential interferences for samples from the marine environment (hydrogen sulphide, dissolved organic carbon, naturally coloured samples).
e) Validation procedures, including a comparison with the classical reduction method, and analytical precisions are given.

It is demonstrated, that our optimised reduction procedure leads to results comparable to those of the commonly used technique (Cd column reduction), but sample preparation is faster, less sample volume is required, the method is easy to use and less prone to interferences, and finally, lower amounts of toxic chemicals are involved.

## 2. Material, methods and procedures

### 2.1. Reagents

Nitrate and nitrite stock solutions were prepared by dissolving 85 mg $NaNO_3$ or 69 mg $NaNO_2$ (Merck, p.a. quality) in 1 L water resulting in 1000 mM, respectively. A second stock solution of nitrate and nitrite was commercially available (1000 mg $L^{-1}$; Merck, CertiPur®). To ensure accuracy of the calibration and dilutions both solutions were used for crosschecking during each run (except experiments). Low nutrient seawater (LNSW) was provided by OSIL (www.seawatersolutions.com) and artificial seawater (ASW) was prepared according to the procedure given in Doe (1994). DOC spiking was done by dilution of a stock solution of potassium hydrogen phthalate (1000 mg $L^{-1}$, Merck, CertiPur®). All dilutions were carried out with ultrapure water (>18 MΩ $cm^{-1}$, Sartorius).

The reagent solutions used for the analysis of NOx, nitrate and nitrite were prepared as follows (chosen procedure):

A) $VCl_3$ reduction solution (saturated): 800 mg $VCl_3$ (p.a., Merck) was partly dissolved in a small amount of ultrapure water (app. 20 mL), 8.4 mL HCl (37 wt.%) was added and diluted to 100 mL with ultrapure water. Using this procedure leads to a particle-free solution which is stable for at least a month.
B) NEDD solution (0.2%): 100 mg N-1-naphthylethylenediamine dihydrochloride (NEDD, p.a., Merck) was dissolved in 100 mL ultrapure water.
C) Sulfanilamide solution (2%): 2 g Sulfanilamide (p.a., Merck) was dissolved in 100 mL 10% (v/v) HCl.
D) NOx reagent (Griess + reduction reagent): 5 parts A + 1 part B + 1 part C.
E) Nitrite reagent (Griess reagent): 1 part B + 1 part C.
F) Sulfamic acid (60 mM): 583 mg Sulfamic acid (p.a., Merck) was dissolved in 100 mL ultrapure water.

The NOx reagent solution is stable for at least one month at room temperature, whereas the other solutions are stable for several weeks at 4 °C in the dark. Calibration ranges for the different instruments are given in Table. 2.

### 2.2. Microphotometry of NOx and nitrite with MR (chosen procedure)

The MR technique has the advantage to be comparable in sensitivity to a 1 cm cuvette system but requires smaller volumes of the sample and chemicals. In addition, the MR technique is simple in operation and offers rapid measurement of a large number of samples (up to 96 wells). The latter is of advantage if alteration of labile chemical compounds (e.g. present in marine pore waters) has to be avoided. The MR technique has been applied for a large number of applications. For instance, pore waters of freshwater sediments were analysed for phosphate, ammonium and ferrous iron (Laskov et al., 2007), pore waters of soils for inorganic, organic and total dissolved nitrogen (Hood-Novotny et al., 2010), waters of marine aquacultures for nitrite, nitrate, ammonia and phosphate (Hernandes-Lopez and Vargas-Albores, 2003; Cecchini and Caputo, 2012).

For microphotometry of NOx, 180 μL sample or calibration solution and the 150 μL NOx reagent (D) were transferred into 96 well plates (flat bottom-type, polystyrene microtiter plates, Greiner). The transfer of sample and reagent solution is critical and should be done very carefully. We use a hand dispenser system (Multipette, Eppendorf) for loading the reagent solution and a variable pipette for sample solutions. If pre-dilution of samples is necessary, this is done in the wells by diluting an aliquot of sample with ultrapure water to the final sample volume. Precision highly depends on the pipetting error and is generally <2% if well serviced pipettes and low-retention or Teflon (PFA) pipette tips for small volumes of sample solution (<50 μL) are used. Samples were incubated in a cabinet dryer, an incubation oven or in the reader at 45 °C ± 5 °C for 60 min with a cover plate placed over the wells. Measurements were done on different microtiter plate readers (MR) in absorbance mode (FLUOstar OPTIMA, BMG Labtech; Multiscan Go, Thermo-Fisher; Spectra Rainbow Thermo, Tecan) using a wavelength of 540 nm. Prior to measurement, the samples were shaken for 10 s to remove small bubbles which might have formed in the wells during heating. The measurement of 96 samples only takes about 20 s in the most precise measurement mode of modern MR instruments.

Microphotometry of nitrite was done using a 300 μL sample/calibration solution and adding 30 μL of Griess reagent (E) to the wells. We used either an incubation temperature of 45 °C for 30 min or room temperature for 60 min.

Microphotometry for direct measurement of nitrate was done using 180 μL sample/calibration solution and adding 30 μL of sulfamic acid (F) to the wells. After 15 min reaction time to remove the nitrite present in the sample, the microtriter plate was shaken for 5 min in the reader to

**Table 1**
$NO_2$ absorbance and concentration for naturally coloured samples from small coastal tributaries (NW-Germany).

| Sample | Absorbance sample + reagent (sa + r) | Absorbance sample without reagent (sa w/o r) | Absorbance (sa + r) − (sa w/o r) | Concentration μM $NO_2$ w/o correction | Concentration μM $NO_2$ corrected | Concentration μM $NO_2$ difference |
|---|---|---|---|---|---|---|
| Mariensiel + HgCl2 | 0.0562 | 0.0568 | −0.0006 | 2.4 | 0.0 | −2.4 |
| Mariensiel | 0.1347 | 0.0626 | 0.0721 | 5.7 | 3.1 | −2.7 |
| Jade + HgCl2 | 0.0478 | 0.0482 | −0.0004 | 2.0 | 0.0 | −2.0 |
| Jade | 0.1914 | 0.0625 | 0.1289 | 8.1 | 5.5 | −2.6 |
| Schweiburg + HgCl2 | 0.0483 | 0.0418 | 0.0065 | 2.0 | 0.3 | −1.8 |
| Schweiburg | 0.1416 | 0.0466 | 0.095 | 6.0 | 4.0 | −2.0 |

remove bubbles, then 120 μL NOx reagent (D) was added. The subsequent incubation and measurement were done in the same way as for NOx.

### 2.3. Conventional photometry

A UV/VIS spectrometer (Lamda 12, Perkin Elmer) was used with either a 2 or 5 cm cell. Solution chemistry was done in the same way as for MR technique except for preparing a larger amount of sample and reagent solution.

### 2.4. Continuous flow analyser

For comparison, an automated continuous flow analyser (CFA, Quaatro, Seal Analytical) was used for the determination of NOx and nitrite. Nitrate was reduced to nitrite at pH 8 in a copperised cadmium reduction coil (method No. Q-035-04 Rev. 7, multitest MT3B on a Quattro; Seal Analytical, 2010), NOx and nitrite was measured using the Griess reaction and nitrate was calculated by difference (NOx − nitrite). ASW was used as wash liquid and LNSW was used to prepare calibration solutions if seawater samples were measured.

## 3. Results and discussion

All optimisation experiments were carried out using the MR technique. Each experiment was done in triplicate and all plates were measured three times. All error bars are given as 1 sigma relative standard deviation.

### 3.1. Sample to reagent ratio for NOx and nitrite determination

For NOx analyses, sample and reagent varied between 150–250 μL (sample) and 80–180 μL (reagent D). We used an incubation temperature of 45 °C and a reaction time of 60 min. A mixture of 180 μL sample + 150 μL reagent D was found to give the best compromise between sensitivity (calculated from the slope, see equation in Fig. 1) and linearity (with a linear regression fit of $R^2 > 0.9996$) for a concentration range up to 100 μM (Fig. 1). The calibration curve was as well linear with 200 μL sample + 130 μL reagent or 150 μL sample + 180 μL reagent, respectively, but this resulted in a slightly lower sensitivity. When using an even higher or lower sample to reagent ratio a second order polynomial has to be applied for getting the best fit ($R^2 > 0.999$, not shown in Fig. 1). For this reason a sample to reagent ratio of 1.5 to 1.8 is recommended and the following experiments were carried out using this ratio (except were indicated). In contrast, variations of the Griess reagent, its volume or its concentration only have a minor effect on absorbance variation (<10%) (this work and Miranda et al., 2001).

As nitrite concentrations in environmental samples are mostly low (below 1 μM), we used a sample to reagent ratio of 10 (300 μL to 30 μL) to improve the detection limit. Tests have shown that water samples can be analysed correctly even with a sample to reagent ratio of 32, but the results are less precise possibly because the pipette error for the reagent may become too high.

### 3.2. Reaction time for 20 °C and 45 °C

To investigate the time necessary for the complete reduction of nitrate at room temperature (23 °C) a 50 μM solution was mixed with NOx reagent D according to the chosen procedure. To minimize evaporation the plate was covered with a lid. Measurements were done after 30, 60, 90 min, 2 h, 6 h, 7.5 h and 23 h. At room temperature the complete reduction of nitrate to nitrite is very slow and requires at least 10 h (Fig. 2). After approx. 15 h absorbance decreases slowly most possibly due to the destruction of the diazonium complex.

The same experiment was done using a standard drying oven at 45 °C. Every 10 min the measurement was repeated in the reader and the plate was placed back into the oven for further colour development. The time course shows that within 40 min the absorbance plateau is achieved and decreases only slightly until the end of the experiment (Fig. 3). The addition of the reagent solution to the samples as well as the measurement is almost simultaneously. Therefore a complete reduction of nitrate to nitrite and thus colour development is not critical for quantification of samples containing exclusively nitrate (but note nitrite behaviour during NOx measurement below).

### 3.3. Spectral interferences by coloured samples

Spectral interference effects on nitrite analyses (MR method) were investigated by using filtered aliquots of intensely yellow and brown coloured freshwater to brackish water samples from small tributaries along the NW-German coastline, draining a peat- and marsh-dominating hinterland. Nitrite analyses were corrected by subtracting the absorbance of the coloured sample without reagent E from that of the sample with reagent E (Table 1). The sample without reagent E was prepared by transferring an aliquot of the sample into a separate well and adding ultrapure water instead of the reagent solution E to compensate for the dilution effect. Table. 1 shows that absorbance of naturally coloured samples has to be taken into consideration for correct quantification. Without correction, nitrite concentrations are 2–3 μM higher in this specific matrix. The slight over-correction for two samples is related to the fact that the absorbance of the samples with and without reagent is very similar. The resulting difference between the measurements is small and can either result in a small negative or positive absorbance.

This effect could not be found for NOx as the blank absorbance of this method (containing only ultrapure water and the reagent D) is in excess of any naturally coloured sample due to the blue colour of the $VCl_3$ solution. In most environmental samples nitrite is present at much lower concentrations than nitrate and thus the effect of naturally coloured samples on nitrite analyses is more significant. To conclude, nitrite analysis should always be corrected for the additional absorbance caused by naturally coloured samples.

Note that this experiment includes other aspects. During sampling, the samples were split into two fractions: one without any preservation and one poisoned with $HgCl_2$ (final concentration of 100 mg $L^{-1}$ $HgCl_2$, Kattner, 1999). Comparison of the fresh poisoned and fresh non-poisoned sample set led to results within the standard deviation of the method indicating that $HgCl_2$ as a poisoning agent does not affect NOx

**Table 2**
Analytical parameters of NOx and $NO_2$ using MR, CFA or conventional spectrophotometry.

| Method | MR | MR | CFA | CFA | 1 cm | 1 cm | 2 cm | 2 cm | 5 cm | 5 cm |
|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | NOx | $NO_2$ | NOx | $NO_2$ | NOx | $NO_2$ | NOx | $NO_2$ | NOx | $NO_2$ |
| | VC13 | | Cd/Cu | | VCb | | VCl3 | | VCl3 | |
| Detection limit (μM)$^+$ | 0.4 | 0.07 | 0.03 | 0.01 | 0.31 | 0.09 | 0.18 | 0.04 | 0.16 | 0.003 |
| Sensitivity (slope) | 0.02 | 0.04 | 0.47 | 0.81 | 0.016 | 0.04 | 0.032 | 0.08 | 0.086 | 0.20 |
| Range (μM) | 0–80 | 0–1 | 0–80 | 0–2 | 0–80 | 0–10 | 0–50 | 0–10 | 0–10 | 0–1 |
| Typical blank absorbance (OD) | 0.12 | 0.04 | 0.01 | 0.01 | 0.15 | 0.00 | 0.31 | 0.0006 | 0.78 | 0.02 |
| Typical precision (RSD) | 0.8 | 1.6 | <2 | <2 | <2 | n.d. | <2 | <2 | n.d. | n.d. |

$^+$: detection limit based on 30 blank samples, n.d.: not determined, OD: optical density, RSD: relative standard deviation (1 s).

determination. Directly after sampling no nitrite could be detected in both solutions (below detection limit, data not shown), whereas the results presented in Table. 1 are from measurements performed after nine weeks. An increase in nitrite is observed in samples without preservation, most probably due to bacterial oxidation of ammonia in the samples (ammonia level is up to 40 μM) as solely chemical oxidation should have affected both sample sets.

### 3.4. Interference by dissolved organic material (DOC)

One of the problems related to the use of a cadmium column for reduction of nitrate to nitrite is the observation that DOC present in samples leads to degradation of the reduction column. If not corrected, this results in an underestimation of NOx and thus nitrate. Porewaters in terrestrial soils and marine sediments often are highly enriched in DOC. For this reason we investigated this effect for the $VCl_3$ reduction solution. A 20 μM calibration solution was spiked with DOC concentrations up to 100 mg $L^{-1}$ and measured against a nitrate calibration. All results are within the pipetting error (<2%) indicating no influence on the efficiency of the reduction.

### 3.5. Interference by $H_2S$

According to the sequence of diagenetic reactions in marine sediments NOx is not present in redox regimes where $H_2S$ is a stable component, as all oxidised nitrogen compounds should be reduced to $NH_4$ (Froelich et al., 1979). In some environments sampling resolution is not sufficient to clearly separate zones where nitrate or nitrite occurs from zones with $H_2S$. Also, in areas characterised by advection an overlap of species may occur and is even indicative for such environments. Sulphide interference is evident at a level exceeding 2 μg $L^{-1}$ (Timmer-Ten Hoor, 1974). Therefore, we investigated this effect by spiking separate nitrate or nitrite calibration solutions with $H_2S$ concentrations up to 1 mM (Fig. 4A) and quantified these samples based on a nitrate calibration. The results clearly show that the presence of $H_2S$ leads to a de-colourisation and thus lower recoveries for both species. In addition, nitrate is more sensitive to the presence of $H_2S$ than nitrite. When purging water samples with $N_2$ or Ar gas it may take hours to get rid of for $H_2S$ in some marine porewaters. Therefore we tested other procedures.

The procedure given by Hansen and Koroleff (1999) for nitrite analysis in sample solutions containing $H_2S$ by adding first the acidified sulfanilamide solution (C, see above), purging with $N_2$ before adding the NEDD solution (B) was not successful (Fig. 4B). Most probably nitrite ions are almost quantitatively lost (but not completely) by formation of nitrous gases according to the reaction:

$$2NO^-_2 + 2H^+ \rightarrow NO\Uparrow + NO^-_2\Uparrow + H_2O.$$

This finding is consistent with the investigation of Granger et al. (2006). Addition of ascorbic acid with a pH of 3.5 to water samples containing nitrite leads to the formation of nitrous gases, which can be quantitatively removed by bubbling with inert gas. The method was used to remove nitrite from water samples for the N and O isotope analysis of nitrate but requires about 3 to 5 h for quantitative removal.

For samples containing only nitrate the procedure given by Hansen and Koroleff (1999) works (Fig. 4B). The reason simply is that HCl addition will not lead to the reduction of nitrate to nitrous gases. Thus, for NOx determination this procedure to remove $H_2S$ can only be applied to samples with negligible concentrations of nitrite.

In samples containing both species, $H_2S$ was removed by adding a $HgCl_2$ solution to a sample aliquot and centrifuging the HgS precipitation according to Timmer-Ten Hoor (1974). This procedure leads to a 100 ± 2% recovery (Fig. 4C) of both species. The amount of added $HgCl_2$ depends on the concentration of $H_2S$ present: adding 10 μL of a 0.2 M $HgCl_2$ to a sample volume of 1 mL results in a quantitative precipitation of 1 mM $H_2S$ in this sample. Insufficient addition of $HgCl_2$ solution is recognised by the formation of a brown residue and turbid solution, even after centrifuging for 30 min, instead of a blackish-brown residue and a clear solution. Dilution effects by the added chemicals can be avoided by adding these to all solutions (including calibration standards) in the same run or by applying dilution factors.

### 3.6. Quantification of nitrate in the presence of nitrite using $VCl_3$ as reduction agent

Miranda et al. (2001) have shown that accuracy of samples with low nitrate concentrations and high nitrite to nitrate ratio is lower as for

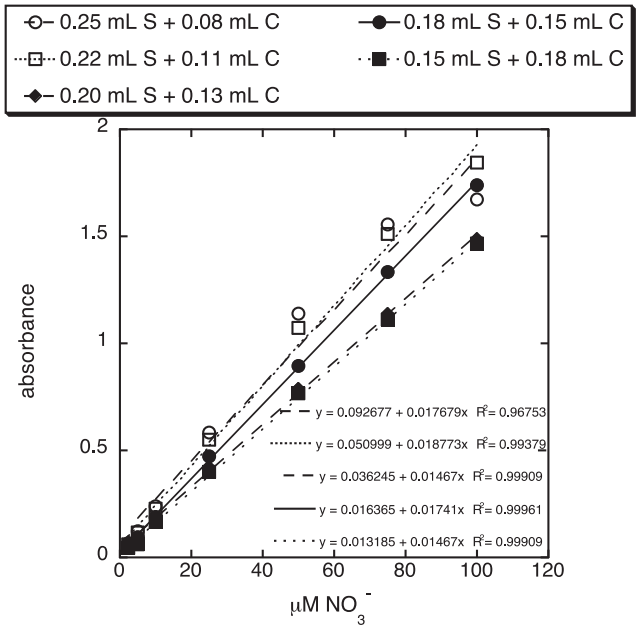


**Fig. 1.** Absorbance versus $NO_3^-$ concentrations for different sample to reagent ratios, a fixed incubation temperature (45 °C) and a fixed reaction time (60 min). The best compromise between linearity and sensitivity is achieved using a 0.18 mL sample (S) and 0.15 mL reagent solution (C).

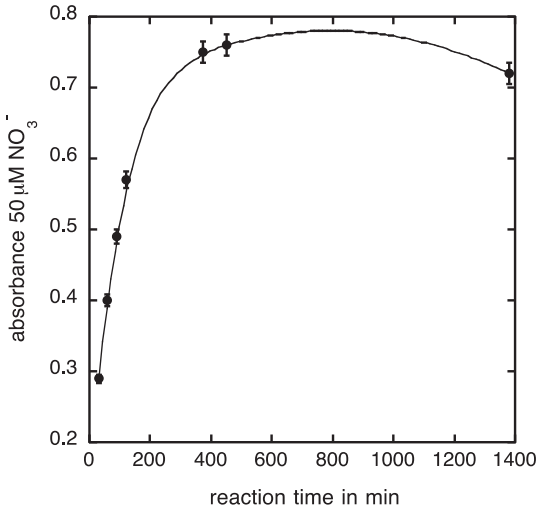


**Fig. 2.** Time course in absorbance for a 50 µM $NO_3^-$ solution using a 0.18 mL sample and 0.15 mL reagent solution and an incubation temperature at ambient conditions (approx. 23 °C). A reaction time of at least 600 min (10 h) is necessary for a complete $NO_3^-$ reduction.

samples with a low ratio. One reason for this is the fact that nitrate is calculated by difference (NOx minus nitrite). If nitrite and NOx concentrations are close together the resulting difference is small and can result in slightly negative or positive values for nitrate. This is true for all methods based on the difference between two measurements.

A second reason for the less precise quantification of nitrate in samples with significant amounts of nitrite is possibly related to NO production. It is known from investigations of Braman and Hendrix (1989) and Yang et al. (1997) that at high temperatures (80–85 °C) $VCl_3$ reduces nitrate and nitrite to NO, which then is detected by a chemoluminescence technique (without use of the Griess reagent). At room temperature only nitrite is significantly reduced by acidified $VCl_3$ solution to NO. The loss of nitrite by adding HCl is also large and demonstrated in the experiment shown in Fig. 4B using the Hansen and Koroleff (1999) procedure for $H_2S$ interference elimination. In addition, also nitrite produced by the reduction of nitrate by $VCl_3$ suffers from NO loss.

A third reason for the difficulties in the quantification of nitrate at high nitrite concentrations is related to their different reaction kinetics.

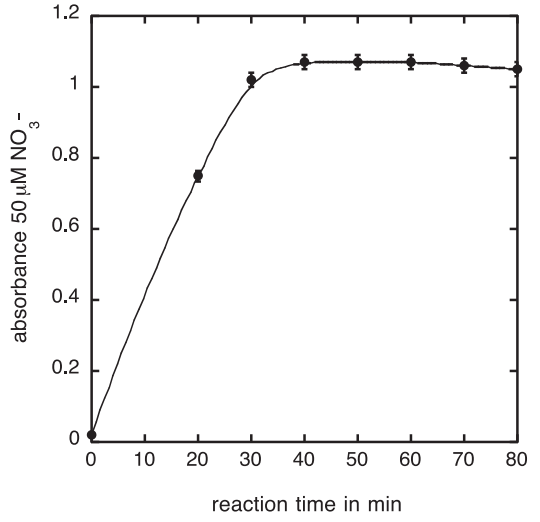


**Fig. 3.** Absorbance versus reaction time of a 50 µM $NO_3^-$ solution. Same experiment as in Fig. 2 except using an incubation temperature of 45 °C. A reaction time of 40 min is sufficient for reduction of nitrate.

Miranda et al. (2001) have shown that the reduction of nitrate by $VCl_3$ is slower than the reaction of nitrite with the Griess reagent. Thus, loss of the nitrite fraction already present in the sample by NO formation is larger than the nitrite fraction originating from $VCl_3$ reduction of nitrate. This effect is probably minimized if using a single reagent solution (Doane and Horwath, 2003). In this case any present and newly produced nitrite may react faster with the Griess reagent than nitrite is reduced to NO in the reagent solution.

To demonstrate the effects of NO production and the different kinetics on the quantification of nitrite with the NOx method a calibration curve based on the dilution of a nitrate stock solution (0–100 µM) and a sample solution with 20 µM nitrite was prepared. The plate was stored in a drying oven at 45 °C and measured by MR every 10 min. The time series (Fig. 5) show that the reaction kinetics of nitrate and nitrite are different in the beginning and have the same slope after 110 min. The different temporal development results from the slower reduction of the nitrate calibration solutions (and thus its colour development) compared to the faster colour development of the nitrite species with the Griess reagent. NO loss shown by the decline in absorbance for nitrite and for nitrate after full reduction to nitrite is present. The rate of NO loss from nitrite should be stable when all nitrate is converted to nitrite resulting in stable nitrite concentrations. In this experiment the nitrite loess is stabilized at 0.7 µM (19.3 instead of 20 µM nitrite) after 110 min whereas no loss apparently is present at ca. 65 min. We have performed this test several times and with different concentrations and did in most cases find a gain or loss in nitrite if reaction time or reaction temperature is too low or too high. Quantification of the nitrate species is not affected because the calibration curve is based on this species.

There are two options to minimize or to correct these three errors.

1. The following equation correct for the NOx concentration in marine samples where nitrite is present but not higher than nitrate. Correction should be done when a reference solution of known nitrite concentration deviates more than 4% (two times of the typical precision, see below) from the expected concentration using a nitrate calibration:

$$NOx_{corrected} = NOx_{measured} + [NO_2^-{}_{measured}(F-1)]$$

where: $NO_2^-{}_{measured}$ is the nitrite concentration measured separately in the sample using only the Griess reagent E. $F = NO_2^-ref_{expected}/NO_2^-ref_{found}$, i.e., the ratio between expected and found concentration of the nitrite reference solution measured within the same run as the NOx samples.

As shown above NO loss from nitrite occurs during the whole time course. The correction term in the square brackets is positive when reaction time or reaction temperature is to long. In this case nitrite concentration in the sample is underestimated and $NOx_{corrected}$ is higher than the measured NOx concentration. The correction term is negative if measurement is done without a sufficient long reaction time or reaction temperature. In this case higher than expected nitrite concentrations result and $NOx_{corrected}$ is lower than the measured NOx concentration. During routine measurements using the chosen method (see above) the nitrite reference solution deviates less than ±10%. As an example for the significance of this error we investigated marine waters of the coastal North Sea which have a nitrite concentration of at maximum 4 µM during winter. A 10% deviation of the reference solution would result in a correction of +0.44 µM or −0.36 µM for NOx. The typical level during winter is 40–80 µM NOx. Well-trained technicians are able to reduce the pipetting error to approx. 2%. This shows that the correction is within the range of the pipetting error and thus negligible for this environment. The correction becomes significant when nitrate concentrations are low and nitrite concentrations are high or nitrate and nitrite concentrations are on the same level, e.g. suboxic to anoxic porewaters, oxygen minimum zones off Namibia or redoxclines within the water column as present in the Baltic deeps or the Black Sea.

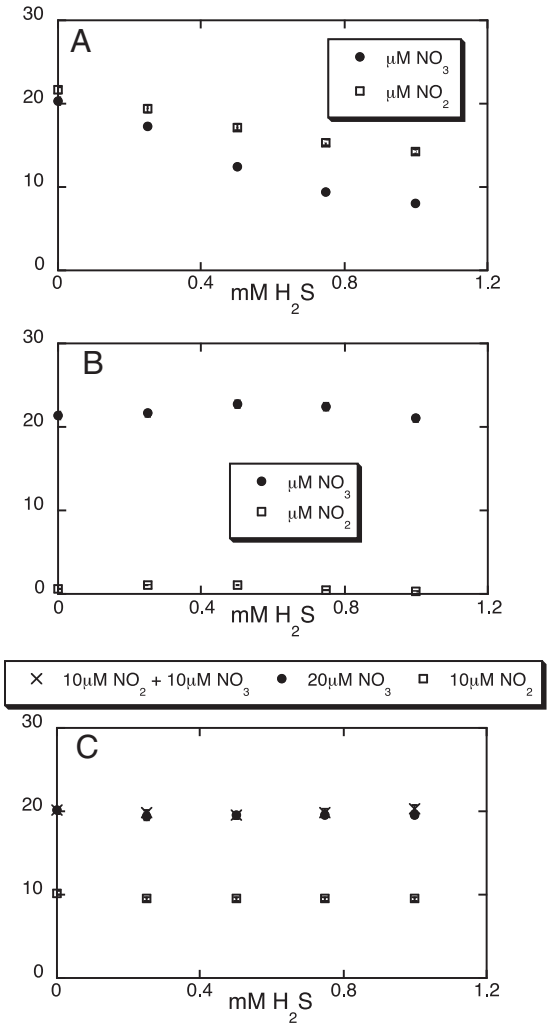


**Fig. 4.** A) Quantification of $NO_3^-$ and $NO_2^-$ (20 μM) using a $NO_3^-$ calibration for samples with increasing $H_2S$ concentration; B) same experiment as in A) but with sample preparations to remove $H_2S$: for $NO_2^-$ according to the procedure given by Hansen and Koroleff (1999) and for $NO_3^-$ by adding 50 μL HCl (2 M) to 1 mL sample and purging for 5 min with $N_2$; C) same experiment as in A) but with sample preparation to remove $H_2S$: 20 μL $HgCl_2$ solution (2 mM) was added to 1 mL sample and centrifugation was done for 30 min at 3500 rpm; the clear supernatant was used for measurement.

2. For samples where nitrite is higher than nitrate (industrial wastewater, intense fish farming aquaculture, sewage treatment plants) a direct quantification of nitrate is the better choice. Removal of nitrite can be done using sulfamic acid to convert nitrite to NO prior nitrate measurement (Beda and Nedospasov, 2005). The authors state that the disadvantage of using sulfamic acid is that any additional sulfamic acid in the sample solution after removal of nitrite affects the yield of the azo-dye and thus lower the sensitivity. In practice, at least two calibration curves of nitrate including the lowest and the highest concentration of nitrite in the samples must be prepared. From these curves and the known concentration of nitrite, the nitrate concentration can be extrapolated. We tested the procedure for nitrite concentrations from zero to 50 μM, by using a fixed sulfamic acid concentration (excess concentration) and different reaction times for nitrite removal. Preparation and measurement was done as given in the methods (direct measurement of nitrate). Table 3 shows that after adding sulfamic acid solution a reaction time of 10 min is sufficient to remove nitrite (see column: 0 μM $NO_3^-$ + 25 μM $NO_2^-$), thus all nitrite was converted to NO. If no nitrite is present in the samples (see column: 10 μM $NO_3^-$ + 0 μM $NO_2^-$) a relatively high concentration of residual sulfamic acid is present and this may lead to a 5% lower sensitivity. This effect is not present any more if the reaction time is increased to 15–40 min. If samples contain nitrite even after 10 min a correct quantification of 10 μM nitrate is possible in the presence of 10 to 50 μM nitrate. The procedure to eliminate nitrite at different levels can easily be modified by adjusting sulfamic acid concentration (see Fig. 1 in Cecchini and Caputo, 2012).

### *3.7. Detection limit, precision and sensitivity for MR and for low level samples using spectral photometry and large cuvettes*

For MR each calibration solution was prepared in triplicate, the blank thirty times and all samples were measured in triplicate. For CFA or conventional UV–VIS spectrometry using a large cuvette, thirty blank solutions were prepared and measured once. From these data mean values, standard deviations, detection limits and sensitivities were calculated (Table 2) according to Skoog and Leary (1992).

Since both reduction methods for nitrate ($VCl_3$, Cd/Cu column) are close to 100% in efficiency, detection limit and sensitivity for NOx, determination should be comparable when using the same instrument configuration. However the strong blue colour of the $VCl_3$ reduction agent results in a lower sensitivity. As expected, the $VCl_3$ method is most sensitive using a 5 cm cuvette, followed by a 2 cm cuvette. The MR technique has a slightly lower sensitivity comparable to conventional spectrophotometry systems equipped with 1 cm cuvettes. When required, even lower sensitivities can be achieved by filling the wells with smaller volumes of sample plus reagent. The most sensitive method, which we tested is CFA using a Cd column for NOx determination.

Due to the use of a higher sample to reagent ratio for nitrite, detection limit and sensitivity are generally higher for nitrite than for NOx for all instruments used in this work. Detection limits improve in the sequence 1 cm cuvette, MR, 2 cm cuvette, CFA, and 5 cm cuvette.

The proposed method is useful for the analysis of nitrate in rivers, lakes, marine and terrestrial porewater, estuarine water, coastal seas and open ocean waters with the MR technique, except for the most depleted surface water samples where a 5 cm cuvette should be used. Nitrite concentrations are much lower in these environments and therefore a larger cuvette or CFA technique should be used for the Griess reaction. Concentrations are sufficiently high for nitrite determination using the

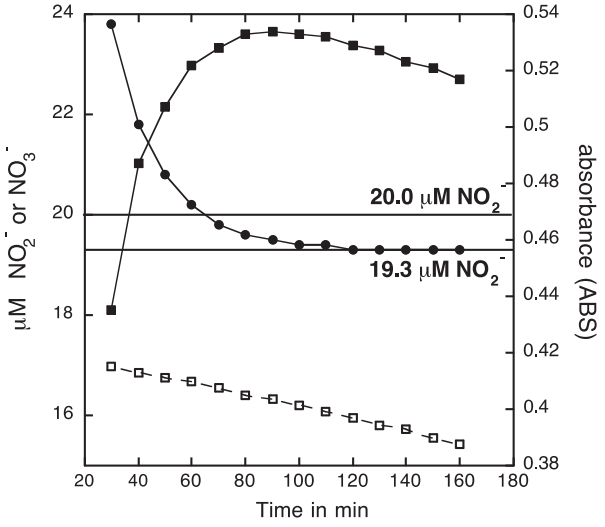


**Fig. 5.** Influence of different reaction times on the quantification of 20 μM $NO_2^-$ (filled dots) using a $NO_3^-$ calibration (right axis) and MR analyser. The absorbance (left axis) of a 25 μM $NO_3^-$ calibration solution (filled squares) and that of the 20 mM $NO_2^-$ solution is also shown (open squares) to demonstrate the temporal development. The different kinetics of the N-species leads to different final results for $NO_2^-$.

MR technique for marine porewater, suboxic to anoxic basins, streams and lakes contaminated with waste water.

The precision for nitrite and NOx measurements calculated from the combined relative standard deviation is <2% for all instruments within the concentration range given in Table 2.

### 3.8. NOx in seawater samples: CFA–MR comparison

To assess the comparability of the $VCl_3$– and the conventional Cd/Cu– reduction method, seawater samples were measured by using both methods. Seawater samples obtained from a cruise along a transect from the Jade Bay to the southern North Sea (Germany) in October 2009 were filtered onboard, poisoned with $HgCl_2$ (Kattner, 1999) and stored at 4 °C in the dark until measurement. A CFA analyser (Quattro, Seal Analytical) equipped with a copperised cadmium column as a reductant and a microplate reader using the $VCl_3$ reductant method was used (both described above). Measurements with both methods were done within three days and no dilution, except for the addition of the reagents, was applied.

NOx concentrations determined by the two methods show the same trend and agree within <5% for samples exceeding 5 μM NOx (Fig. 6a) with a coefficient of determination of $R^2 = 0.98$ (Fig. 6b). Samples with lower NOx concentrations (stations 13–15) exhibit a larger deviation of below 0.9 μM. Given the fact that the two data sets are the result of a routine procedure and based on different days, different calibration solutions, different reduction procedure and different sample transport (manual pipetting against automatic continuous flow analysis) the differences are considered as acceptable. Cecchini and Caputo (2012) found a similar fit ($R^2 = 0.99$) between the Cd-reduction method and the $VCl_3$ reduction method.

Stations represent a loop transect from the Jade Bay (stations 1 and 25) to the vicinity of the city of Wilhelmshaven (stations 7 and 19) towards the Outer Jade fading into the southern North Sea (stations 12 and 13). The highest NOx concentrations, most probably due to the municipal sewage plant outlet, are located close to Wilhelmshaven whereas the lowest concentrations were analysed at sampling locations close to the North Sea.

## 4. Conclusions and comments

The combination of $VCl_3$ reduction of nitrate and the nitrite Griess reaction allows simple, fast and cost-effective analyses of nitrate and nitrite in freshwater, brackish water and seawater. Thus, NOx determination using $VCl_3$ forms an alternative to the commonly used Cd column reduction technique. Results obtained by both methods are comparable but $VCl_3$ reduction is easier to handle, less prone to efficiency changes of the reduction agent and requires less reagents and toxic substances. There is no influence by varying salt contents and DOC has no effect on the efficiency of the reducing agent (contrary to the cadmium column reduction method). For the macro- as well as the micro-method (less than 0.5 mL sample consumption) robust standard laboratory equipment can be used and thus bears advantage not only in the laboratory but also in the field, in remote areas or on ships.

It is recommended that for NOx determination a single reagent solution consisting of the colour reagent and the reductant is added to the sample to minimize NO formation (except for direct nitrate determination).

For NOx analysis of samples containing nitrite a correction is necessary. While measuring NOx (using a nitrate calibration), a nitrite solution should always be analysed as a sample to control reduction efficiency of nitrate and/or nitrite loss. Low nitrite recoveries indicate nitrite loss by NO formation, e.g. by applying a too long reaction time or a too high reaction temperature. Nitrite recoveries exceeding 100% indicate that the reaction temperature is too low and/or the reaction time too short. In most open ocean environments nitrate is the major nitrogen species whereas in marine pore waters (e.g. nearshore beach environments, deep sea), waters within oxygen minimum zones of upwelling systems or the redoxcline of anoxic basins higher nitrite than nitrate concentrations may occur as well. Note, that the quantification error in NOx due to a low reduction efficiency is also valid for the conventional method using the cadmium column as a reductant.

So far, only few correctable limitations are evident for the NOx determination using the $VCl_3$ reduction technique in freshwater, seawater and pore water from terrestrial or marine environments. Self-absorbance by naturally coloured samples is present for the nitrite determination and can be corrected by the additional measurement of the sample without reagents. $H_2S$ in water samples can be precipitated before analysis using $HgCl_2$ to eliminate potential interferences. $HgCl_2$ as a commonly used poisoning agent does not lead to interferences. In strongly coloured samples the dissolved humic acid compounds may precipitate when adding the acidic reagent solutions. Such samples are easily identified visually and have to be diluted or require an additional filtering step.

**Table 3**
Direct $NO_3^-$ determination using sulfamic acid and $VCl_3$ reduction method at different reaction times of sample + sulfamic acid.

| Reaction time in min. | 10 | 15 | 40 |
|---|---|---|---|
| 0 μM $NO_3^-$ + 25 μM $NO_2^-$ | n.d. | n.d. | n.d. |
| 10 μM $NO_3^-$ + 0 μM $NO_2^-$ | 9.5 ± 0.2 | 9.9 ± 0.1 | 10.0 ± 0.1 |
| 10 μM $NO_3^-$ + 10 μM $NO_2^-$ | 10.0 ± 0.2 | 10.0 ± 0.1 | 10.0 ± 0.01 |
| 10 μM $NO_3^-$ + 25 μM $NO_2^-$ | 10.0 ± 0.1 | 10.1 ± 0.1 | 10.1 ± 0.1 |
| 10 μM $NO_3^-$ + 50 μM $NO_2^-$ | 10.1 ± 0.1 | 10.2 ± 0.1 | 10.0 ± 0.1 |

180 μL sample + 30 μL sulfamic acid (60 mM), shaking time of 5 min, then 120 μL reagent D, reaction time of 60 min at 45 °C.
n.d.: not detected.

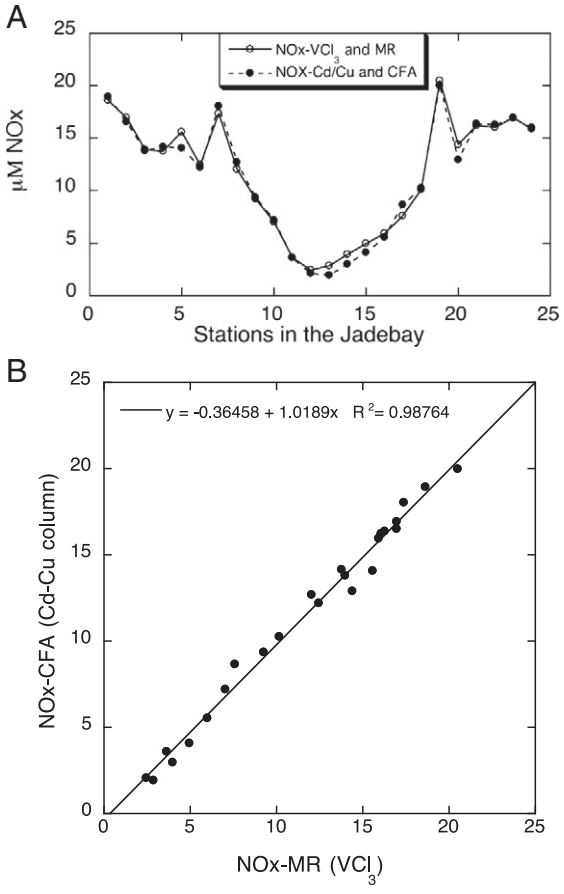


**Fig. 6.** A) Comparison of NOx concentration in seawater samples from a transect through Jade Bay (northern Germany) using the $VCl_3$ reduction method with MR detection and Cd/Cu column reduction with a continuous flow analyser (CFA), B) statistical relationship of the data set.

Other supplementary agents used by microbiologists during experiments may interfere and should be checked (e.g. sodium azide, ascorbic acid, antibiotics, several inhibitors: Doane and Horwath, 2003; Miranda et al., 2001, and references therein).

Sensitivity and detection limit of the $VCl_3$ reduction method cannot outcompete the Cd/Cu reduction coil technique but even the most insensitive detection method (MR or 1 cm cuvette) provides a detection limit of 0.4 μM (or 5.6 μg $L^{-1}$ NOx–N). For most environmental applications, pore water, estuarine and coastal seawater investigations this detection limit is sufficient.

To date, we adopted the NOx method also to a fully automated discrete analyser (Easychem 3000, Systea S.p.A.) as part of a sequential determination analysis of alkalinity, nitrite, phosphate and silicic acid) and to an on-line monitoring analyzer using the loop flow analyser technique (MICROMAC C, μMAC-1000, Systea S.p.A.). Further experiments in the future will be carried out to adopt the $VCl_3$ reduction technique to the NOx determination by automated CFA. First tests have shown that a more concentrated $VCl_3$ solution as well as a longer reduction coil is necessary to enhance efficiency of the nitrate reduction.

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