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Determination of Nanomolar Concentrations of Nitrite and Nitrate in Natural Waters Using Long Path Length Absorbance Spectroscopy

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The concentrations of nitrite and nitrate in many natural waters are below the detection limits of conventional colorimetric analysis. A liquid core waveguide (LCW) has been used to extend the sensitivity of conventional colorimetric nitrite and nitrate determinations by more than an order of magnitude. Long path length absorbance spectroscopy (LPAS) with a 4.5 m path length LCW made of Teflon AF-2400 provides detection limits for NO_2^- and NO_3^- equal to 0.5 and 1.5 nM, respectively. The absorbance response of the LPAS system varies linearly with concentration. Calculations of azo dye molar absorbance using LPAS observations at nanomolar concentrations are in excellent agreement with molar absorbance results obtained with conventional measurement systems at much higher concentrations. For 1 nM NO_2^- and NO_3^- concentration differences, the 4.5 m path length LCW used in this work produces absorbance differences on the order of 0.02. No significant changes in the behavior of the LPAS system have been observed for periods of 6 month and more. The system is simple, rugged, and amenable to field studies.

Introduction

Dissolved inorganic nitrogen (DIN), in the form of nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+), is an essential nutrient controlling biomass in natural waters. At high concentrations, DIN contributes to freshwater and estuarine eutrophication. At very low concentrations, DIN is the nutrient that most frequently limits phytoplankton primary productivity in the oceans (*1*). Although NO_3^- and NO_2^- are generally more abundant than NH_4^+ in the euphotic zone of the world's oceans, the concentrations of these ions in the ocean's zone of photosynthesis are generally below the detection limit of the conventional colorimetric technique (*2*) that is used in quantifying their concentrations (*3*). High surface productivity in stratified Sargasso Sea water has been observed in response to nanomolar changes in nitrate concentrations (*4*). In view of the 10−50 nM detection limits of the colorimetric procedures used in determinations of NO_2^- and NO_3^-, changes in the concentration of DIN will typically pass unobserved in the zone where DIN exerts a controlling influence on ocean productivity. Highly sensitive nutrient analyses are needed to better understand DIN−phosphate relationships in the euphotic zone and provide much needed insights into the mechanism of new production (*5*).

Many analytical methods have been proposed for the determination of nitrite/nitrate (*6*−*13*). The widely used colorimetric method for analysis of natural waters (including seawater, lake water, and rainwater) is based on the reduction of nitrate to nitrite and a subsequent chemical reaction resulting in the formation of an azo dye (*6*, *7*). The conventional colorimetric method is inaccurate below 50 nM. A sensitive method involving formation of a fluorescent azo dye has a reported detection limit of 1−5 nM (*8*, *9*). However, this claimed sensitivity is difficult to achieve for samples such as surface seawater that have high dissolved organic contents and thereby strong background fluorescence. DIN analyses via chemiluminescence have detection limits and precisions on the order of 2 nM (*10*, *11*). However, this technique requires sophisticated equipment and is quite complex as compared to colorimetric analyses. Zafiriou and co-workers (*12*) extended the DIN detection limit by using large sample sizes with concomitant lengthy analysis times. A very sensitive procedure involving HPLC for analysis of nitrite has a reported detection limit of 0.1 nM (*13*). This technique, involving preconcentration, is also complex and demands careful chemical preparation and purification of derivatizing reagent.

The molar extinction coefficient of the azo dye formed in NO_3^- and NO_2^- analysis is quite large (4.6×10^4). The inherent sensitivity of colorimetric NO_3^- and NO_2^- analysis is limited principally by optical path length. Liquid core waveguides (LCW) provide long optical path lengths by constraining light propagation within a liquid medium that has a higher refractive index (RI) than the surrounding solid tubing (*14*). There are very few materials that have an index of refraction less than that of water, and even fewer that are chemically stable and inert. However, an amorphous fluoropolymer form of Teflon designated AF-2400 (Dupont), has an RI of ∼1.29 and many of the desirable chemical properties of Teflon (*15*, *16*). An LCW constructed of Teflon AF-2400 with a water core provides total internal reflection for light rays intersecting the water/tubing interface at angles less than or equal to 19°. We have recently used a Teflon AF-2400 LCW to lower the detection limit of colorimetric Fe(II) analysis by 2 orders of magnitude (*17*). In the work described here, we apply long path length absorbance spectroscopy (LPAS) to the colorimetric determination of nitrite/nitrate at nanomolar concentrations. The methods outlined in this work are simple and sensitive. The simplicity and sensitivity of colorimetric LPAS analysis makes this technique particularly well-suited to miniaturization and in-situ analysis.

Experimental Section

Apparatus. The compact experimental setup for determination of nitrite/nitrate by long path length absorbance spectroscopy is shown in Figure 1. All of the components of Figure 1, exclusive of the pump and computer, can be purchased from Ocean Optics Inc. The heart of the setup is a Teflon AF-2400 LCW (Biogeneral) with an inner diameter equal to 560 μm and an outer diameter equal to 800 μm. A 4.5 m length of LCW was coiled and placed in a 10 cm diameter chamber to prevent introduction of ambient light into the LCW. A custom "T" was designed to interface the LCW to an optical fiber (Polymicro Technologies 150 μm core diameter) and standard 5 mm i.d. Tygon tubing (Figure 1). This "T" allows insertion of the optical fiber in the Teflon AF-2400 tubing. Sample solutions enter and exit the LCW

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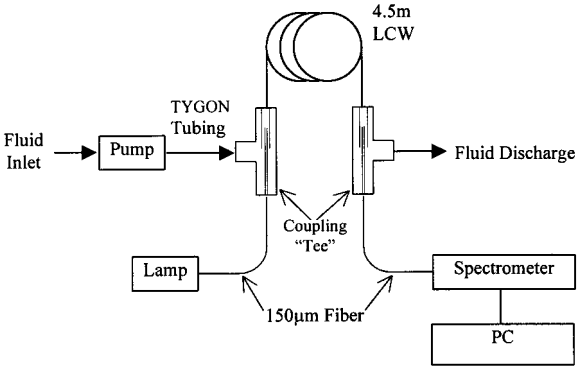

FIGURE 1. Overview of the LPAS experimental setup.

through an annular gap between the fiber and tubing. A palm-size CCD array spectrometer (Ocean Optics S2000) and fiber-coupled tungsten halogen lamp (Ocean Optics LS-1) provides spectral absorbance measurements. Continuous sampling is achieved with a peristaltic pump (Ismatec, model 78016-30) at a flow rate of ~2 cm^3/min. To avoid formation of bubbles in the system, the pump is active only during periods of sample introduction. Bubbles inadvertently introduced to the system are, however, easily flushed out by continuous pumping of sample.

Reagents. Sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, copper sulfate, and imidazole were purchased from Aldrich and used without further purification. Water was obtained as fresh ion-exchanged Millipore Super Q (18 MΩ). Sulfanilamide and aromatic amine reagents were prepared as described in Grasshoff et al. (*7*). Nitrite and nitrate stock standard solutions (10 mM) were prepared from analytical-reagent grade sodium nitrite and potassium nitrate and were dried at 100 °C before weighing. Serial dilutions of the stock solution with Milli-Q water and aged low-nutrient seawater were used to construct calibration curves. A 10 cm length of cadmium column was prepared in a 2 mm i.d. plastic tube using freshly prepared cadmium filings (*7*). This packed cadmium column as well as a commercially available Cd Coil (24 in. length, IRAMA) were used to reduce nitrate to nitrite. An anion exchange column (1 cm i.d., 25 cm length) was prepared by packing hydroxide form AG 1-X8 resin (mesh 20−50, Bio-Rad) in a glass dispensing buret. This column was used to remove trace amounts of nitrite and nitrate from Milli-Q water.

Analysis of Nitrite and Nitrate. Samples were prepared manually for nitrite/nitrate analysis following standard colorimetric procedures (*7*). Sulfanilamide reagent (reagent 1, 0.2 cm^3) and amine reagent (reagent 2, 0.2 cm^3) were combined in 25 cm^3 of each sample solution, and 15 min was allowed for development of the azo dye formed in the presence of NO_2^-. Azo dye absorbance at 540 nm was used for determination of nitrite concentrations. Absorbances at 540 nm (A_{540}) were compensated for baseline shifts by monitoring a nonabsorbing wavelength (700 nm). All sample absorbances were obtained from the ratio of transmitted light intensity (log I_0/I) using samples with (I) and without (I_0) combined reagents (reagent 1 + reagent 2). The difference in baseline absorbance between Milli-Q water and seawater at 540 nm was close to zero (~0.005) after referencing all A_{540} measurements to absorbance at 700 nm (A_{700}). Nitrate in water samples was analyzed after reduction to nitrite. The reduction step was effected by passing samples containing added imidazole buffer (1 mM) through a packed cadmium column or a Cd coil.

Results and Discussion

Figure 2 shows LPAS absorbance response (A_{540}) as a function of nitrite concentration. The simple analytical apparatus

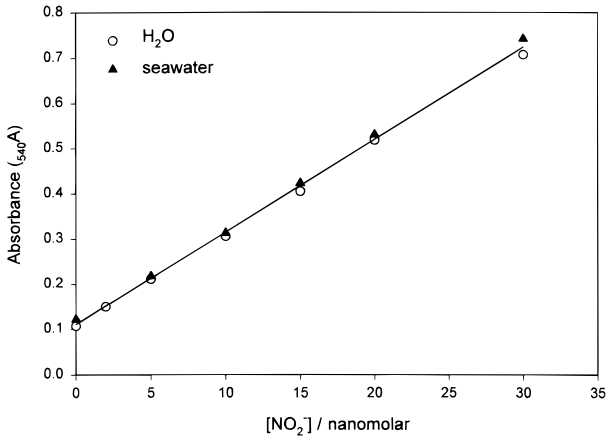

FIGURE 2. LPAS (4.5 m) nitrite standard curve (0−30 nM) for Milli-Q water and low-nutrient seawater (taken from Gulf of Mexico and aged for 1 month): A_{540} = 0.110 (± 0.004) + 0.0204 (± 0.0003) × [NO_2^-], where [NO_2^-] denotes the concentration of nitrite in nanomoles per liter.

shown in Figure 1 produced a linear absorbance response for nitrite concentrations between 0 and 30 nM. Samples with higher concentrations of nitrite (and nitrate) can be analyzed using shorter path lengths than the 4.5 m LCW used in this work. Standard curves in water and seawater are identical (Figure 2), indicating that there are no significant ionic strength effects and no specific ion effects from the major components of seawater. The azo dye molar adsorptivity obtained using the 4.5 m path length (4.5×10^4) is in good agreement with the value (4.6×10^4) determined using conventional 10 cm spectrophotometric cells (*6*). This close conformity to expectations based on Beer's law demonstrates the inherent simplicity and accuracy of LPAS analysis. The behavior of the LPAS system is very consistent. No significant loss or change in AF-2400 waveguiding properties has been observed over a period of 6 months or more.

Reagent absorbance is often negligible for colorimetric analysis at micromolar concentration levels. LPAS analysis is, however, capable of detecting absorbance contributions from species that have very low concentrations or molar absorptivities. The nonzero intercept in Figure 2 is attributable to either reagent absorbances, trace levels of NO_2^- in seawater and Milli-Q samples, or both. Further analyses were conducted to identify the source of the intercept in Figure 2. These analyses indicated that, under the conditions of our analyses (reagent 1 concentration = 460 μM, reagent 2 concentration = 30 μM), the Figure 2 intercept is entirely attributable to reagent absorbance. Figure 3 shows absorbance spectra obtained using fresh Milli-Q water. The four spectra shown in this figure depict absorbances of (a) water plus combined reagents, (b) water (which had been passed through an AG 1-X8 column) plus combined reagents, (c) water plus 5 nM NO_2^- plus combined reagents, (d) water plus 5 nM NO_2^- (which had passed through an AG 1-X8 column) plus combined reagents. Comparison of c and d indicates that the resin column effectively removes NO_2^- from solution. The close similarity of spectra a, b, and d indicates that the Milli-Q water used in our analysis contains negligible NO_2^- concentrations. Consequently, the absorbance observed at 540 nm in Figures 2 and 3 (A_{540} = 0.110) is solely attributable to reagent absorbance and/or NO_2^- added with the reagents.

Nitrate analysis is identical to that described for nitrite with the exception of an additional step involving conversion (reduction) of NO_3^- to NO_2^-. Figure 4 shows the calibration curve for nitrate in Milli-Q water for NO_3^- concentrations between 0 and 30 nM. No calibration curve for nitrate in

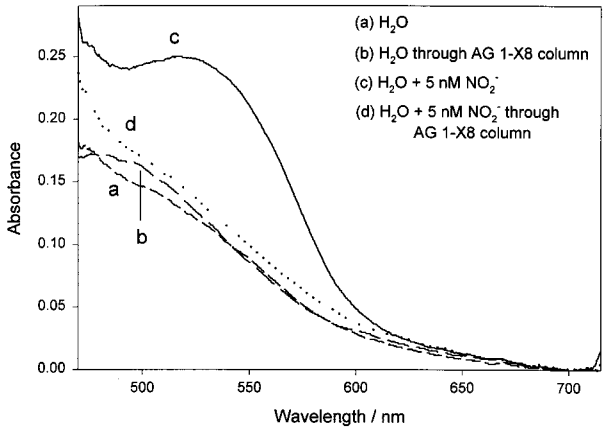

FIGURE 3. Absorbance spectra for samples of (a) Milli-Q water and (c) Milli-Q water plus added 5 nM nitrite. Curves b and d show spectra obtained after passing these solutions through an AG 1-X8 column (prior to addition of combined reagents).

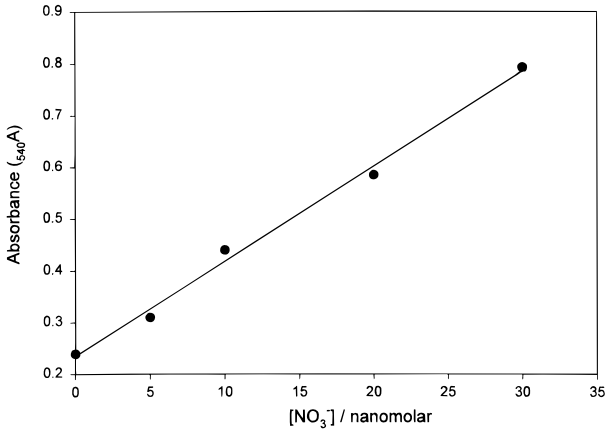

FIGURE 4. LPAS (4.5 m) nitrate standard curve (0−30 nM) for Milli-Q water. A packed cadmium column was used to reduce nitrate to nitrite: $A_{540} = 0.234 (\pm 0.013) + 0.0184 (\pm 0.0008) \times [NO_3^-]$, where $[NO_3^-]$ denotes the concentration of nitrate in nanomoles per liter.

seawater was obtained because of the relatively high NO_3^- concentrations (~30 nM) in our seawater samples. The slope of the Figure 4 calibration line is similar (Figure 4 slope/Figure 2 slope = 0.90) to that obtained for NO_2^-. Thus, these data indicate that efficiency of the Cd reduction column is on the order of 90% over this range of concentrations. Our analyses indicate that Cd column reduction efficiencies are dependent on column construction, sample flow rate, and sample concentration. Sample pumping rate must be adjusted empirically to achieve maximum efficiency. We observed that, while the reduction efficiency of rapidly pumped samples diminishes due to incomplete reduction, samples pumped too slowly also exhibit a reduction in the efficiency of NO_3^- conversion to NO_2^-. The apparent implication here is that, under the conditions used in our work, NO_2^- can to some degree be reduced in Cd columns. The optimal pumping rate through the commercial Cd coil (IRAMA) was slower than the optimal pumping rate for our freshly prepared Cd column. This demonstrates the necessity of empirically assessing nitrate reduction efficiency as a function of column type, pumping rate, and sample concentration.

The intercept in Figure 4 is larger than the intercept obtained in nitrite analysis (Figure 2). By using the AG 1-X8 resin in a manner closely comparable to the analyses performed for NO_2^- we determined that our fresh Milli-Q water contained no detectable NO_3^-. The absorbance spectrum obtained for the intercept (no added NO_3^-) in Figure 4 closely mirrored the characteristic absorbance specrum of the azo dye produced in nitrite analyses. The Figure 4 intercept is due to approximately equal contributions from (a) the absorbance contributions of reagent 1 and 2 and (b) the addition of NO_3^- with imidazole buffer (creating a sample NO_3^- concentration approximately equal to 6 nM). This observation indicates that the intercept obtained in Figure 4 can, in principle, be further reduced and made comparable to that shown in Figure 2.

TABLE 1. LPAS Determination of Nitrite and Nitrate in Natural Waters

sample	nitrite	nitrate
surface seawater (Gulf of Mexico)	5.9 nM	33.0 nM
rainwater 1 (~10 min after onset of rain event)	110 nM[a]	2.5 μM[a]
rainwater 2 (after heavy overnight rain)	9.0 nM	0.75 μM[a]
spring waters (bottled)		
brand 1	5.2 nM	14.7 μM[a]
brand 2	7.6 nM	14.2 μM[a]
brand 3	60 nM[a]	13.1 μM[a]

[a] Measured using conventional 10 cm spectrophotometric cell.

Detection limits for nitrite and nitrate, defined as three times the standard deviation of measurement blanks (Milli-Q water + reagents), are 0.5 and 1.5 nM, respectively. The larger detection limit of nitrate is due to the higher blank value of imidazole buffer (~6.0 nM NO_3^-). The relative standard deviation for six measurements of 5.0 nM nitrite is 6%. The relative standard deviation for six measurement of 10 nM nitrate is 8%.

The LPAS analysis described above was used to determine concentrations of nitrite and nitrate in a variety of natural samples (Table 1). Surface seawater collected in the Gulf of Mexico had low NO_2^- and NO_3^- concentrations that were, nonetheless, easily detectable (Table 1) through LPAS analysis. The nitrite concentration in rain samples was relatively high (110 nM) during early stages of precipitation but, through time, decreased substantially (i.e., by more than a factor of 10). The observed decrease in nitrite and nitrate concentrations as well as the change in the ratio of concentrations are consistent with rain samples that were derived from different air masses over the greater than 24 h period of this rain event. Nitrate levels in various bottled spring waters were relatively large (approximately 10 μM) and did not require LPAS analysis. Nitrite levels were much lower (5.2−60 nM) in bottled water samples and, in some cases, required LPAS analysis. The capability of examining not only low NO_2^- and NO_3^- concentrations but also variations in their ratios, despite 3 orders of magnitude concentration differences (i.e., $[NO_3^-]/[NO_2^-] > 10^3$), highlights the potential use of LPAS analysis in monitoring the quality of public drinking water. Changes in NO_2^-/NO_3^- concentrations and concentration ratios can be indicative of source water contamination from animal waste, septic tanks, and sewage. An ability to monitor chemicals at concentrations well below maximum allowable contaminant levels allows for identification of undesirable trends long before public health is put at risk and rapid remediation is mandated.

The sensitivity of LPAS heightens the significance of potential sample contamination. We observed that sample blanks (20 mL, Milli-Q water + reagents) left directly exposed to air in two laboratories overnight acquired nitrite concentrations between 73 and 170 nM. The same blank samples in closed bottles acquired nitrite concentrations of only 4 nM. This suggests that nitrite can be acquired from airborne sources (i.e., nitrous gases and/or deposition of particles). This airborne contamination demonstrates that, for trace nitrite/nitrate analysis, Milli-Q water and reagents should

be freshly prepared and that exposure to air should be minimized. Air contamination during sampling and sample transfer is also a major concern in field studies (*12*). Contamination problems can be eliminated by performing nitrite and nitrate analyses in situ.

One of the most significant advantages of LPAS nitrite/nitrate analysis is the substantial sensitivity that can be achieved without sacrificing the inherent simplicity of spectrophotometric analysis. Shorter path length LCWs can be used to measure NO_2^-/NO_3^- concentrations larger than 50 nM. Since light throughput is not a limiting factor for path lengths even as large as 20 m, the practical upper limit path length for LCW analysis appears to be substantially larger than 4.5 m. The sample size requirement for LPAS analysis obtained using a liquid core waveguide is very low. The internal volume of a 4.5 m LCW (560 μm i.d.) is approximately 1.1 cm^3. For on-line flow injection analysis, a 2 cm^3 sample volume is adequate. The transition time between samples is approximately 1 min. LPAS analysis is amenable to miniaturization and autonomous in situ analysis. The spectrometer used in this analysis has a volume of $14 \times 10 \times 4$ cm^3. The 4.5 m LCW can be accommodated in a volume on the order of 25 cm^3. We are currently constructing an LPAS module for autonomous operation in the upper ocean.

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