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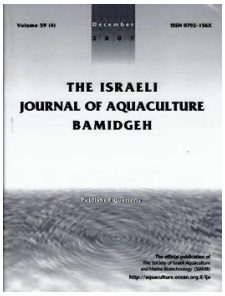

A Direct Spectrophotometric Assay for Evaluating Nitrate-Nitrogen in Intensive Aquaculture Systems

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Abstract

This paper describes a simple spectrophotometric procedure for determining NO_3^--N in water samples. In the proposed method, NO_2^--N of a water sample is first removed from the water with sulfamic acid. Then, NO_3^--N is reduced to NO_2^--N using vanadium (III) chloride and a detection reagent is added. When rapid collection of data is necessary, this method can be adapted to a microtechnique using a 96-well microplate, resulting in a detection limit of 5 NO_3^--N mg/l. Using a larger volume spectrophotometer cell (5 cm) lowers the detection limit to 0.5 NO_3^--N mg/l. The proposed method is applicable for routine analysis of water in aquaculture systems and wastewaters in which medium-high NO_3^--N concentrations are expected. Benefits of the method are reduction of the laborious preparation and avoidance of contact with the harmful substances typical of the cadmium reduction method.

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Introduction

Nitrate-nitrogen (NO_3^--N) is the oxidized form of ammonia-nitrogen and represents the major nitrogenous waste product excreted by fish (Wedemeyer, 1996). Its release into the environment by aquaculture plants is a side-effect of fish farming (Tovar et al., 2000; Islam, 2005). Although NO_3^--N is relatively non-toxic to reared fish, high concentrations can affect growth (Kamstra and van der Heul, 1998) and physiological parameters (Hrubec et al., 1996; Hamlin et al., 2008), and induce disease outbreaks (Burgess, 1995). In recirculating aquaculture systems (RAS), in which NO_3^--N can reach high levels, detrimental accumulations are normally prevented by water exchange or denitrification filters (van Rijn et al., 2006).

Among spectrophotometric assays to determine NO_3^--N, the reduction of NO_3^--N to nitrite-nitrogen (NO_2^--N) is a common procedure in brackish and salt waters. The most widely accepted method reduces NO_3^--N to NO_2^--N by passing a water sample through a cadmium (Cd) column (Morris and Riley, 1963). The reduction rate and precision of this method was improved by Wood et al. (1967), who introduced a column containing copper-coated Cd for reduction. In this method, NO_3^--N is analyzed as NO_2^--N, based on diazotization with sulphanilamide and subsequent coupling to N-naphthylethylenediamine to produce a rose-colored dye (Bendschneider and Robinson, 1952) that is quantifiable at 540 nm. Because methods based on NO_3^--N reduction provide the total nitrite/nitrate-nitrogen (NOx-N) concentration, the real NO_3^--N concentration of a sample is the difference between the NO_2^--N and NOx-N concentrations.

The Cd-reduction method, however, has disadvantages and limitations. Preparation of the column is laborious and toxic heavy metals remain in the treated water. Thus, other reducing agents have been proposed (Moorcroft et al., 2001; Campbell et al., 2006). Vanadium (III) chloride (VCl_3), introduced as a reducing agent for determining nitric oxide metabolites in biological samples (Miranda et al., 2001), was adapted for NO_3^--N determination by adding VCl_3 solution to a similar volume water sample to obtain complete NO_3^--N reduction within a few minutes at room temperature (Doane and Horwáth, 2003). However, the sensitivity of this method declines when samples contain an excess of NO_2^--N relative to NO_3^--N (Beda and Nedospasov, 2005). Thus, sulfamic acid is used to remove NO_2^--N from biological matrices before determining NO_3^--N. In this way, NO_3^--N is determined directly and more accurately.

In this paper, we adapt the assay proposed by Beda and Nedospasov (2005) for direct determination of NO_3^--N in water, compare results with the Cd-reduction method of Wood et al. (1967) as described by APHA (1989), and validate analyses of NO_3^--N concentrations in waters with different salt concentrations and in the presence of NO_2^--N. The proposed method can be used for routine monitoring of water quality in intensive systems such as RAS, where medium and high NO_3^--N concentrations are expected.

Materials and Methods

Chemicals were purchased from Sigma-Aldrich (Milan, Italy) and solutions prepared in distilled water. Optical densities were recorded on a microplate reader (Model 550, BioRad) or on a spectrophotometer with 5-cm pathway cell (6300, Jenway). Volumes indicated in the spectrophotometric methods are for 96-well microplate techniques. Where not indicated, volumes of samples, standards, and reagents for analysis using the 5-cm pathway cell spectrophotometer were adapted, keeping ratios constant.

NO_2^--N removal by sulfamic acid and determination. In a preliminary study, we tested the capacity of different concentrations of sulfamic acid to completely inhibit the formation of the colored complex of increasing NO_2^--N solutions. Volumes of 120 µl NO_2^--N solutions, ranging 0.75-75 mg/l, were placed in microtiter plates and mixed with 120 µl sulfamic acid solutions, ranging 0.625-50 mM in 2.5% HCl. NO_2^--N was analyzed following the method of Strickland and Parsons (1972) with a few modifications, using sodium nitrite as the standard. Briefly, 240 µl samples, standards, and blanks were placed in a 96-well microtitre plate, mixed with 30 µl sulphanilamide (1% in 5% phosphoric acid) and 30 µl N-naphthylethylenediamine (0.1% in water), and incubated for 15 min at room temperature. Absorbance was read at 540 nm on a microplate reader

(Model 550, BioRad), with a measurable range of 0.05-1 NO_2^--N mg/l (r^2 = 0.9997). Analytical data are expressed as the lowest sulfamic acid concentration necessary for complete NO_2^--N removal in solutions at known NO_2^--N concentrations.

NO_3^--N determination by the proposed method. NO_3^--N was determined as suggested by Beda and Nedospasov (2005) for the evaluation of nitric oxide metabolites in oxidative stress, using sodium nitrate as the standard. Briefly, 60 µl samples, standards, and blanks were placed in a 96-well microtitre plate, mixed with the same volume of sulfamic acid (10 mM in 2.5% HCl) solution, and stirred. After 5 min, 60 µl sulphanilamide, 60 µl N-naphthylethylenediamine, and 60 µl reducing agent (50 mM VCl_3 in 1N HCl) were added and incubated for 45 min at room temperature before absorbance was read at 540 nm on the microplate reader.

NO_3^--N determination by Cd-reduction method. NO_3^--N was determined by the Cd-reduction method based on the assay described by Wood et al. (1967) as indicated by APHA (1989), in which the applicable analytical range is 0.01-1 NO_3^--N mg/l. After reduction of NO_3^--N, the NOx-N concentrations of samples were analyzed as described above. Finally, the NO_3^--N concentration of the sample was obtained by calculating the difference between the NOx-N and NO_2^--N concentrations.

Comparison of methods. Aliquots of artificial seawater, prepared from distilled water according to Nakamura et al. (2008), were added to different amounts of sodium nitrate to obtain NO_3^--N concentrations ranging 2-120 mg/l. The NO_3^--N concentrations of the solutions were analyzed by the proposed direct microtechnique and the Cd-reduction method. Samples analyzed by Cd-reduction were diluted if necessary.

Recovery of NO_3^--N in water samples. Solutions containing different salt concentrations (0, 5, 10, 20, 35 g/l) were prepared from artificial seawater by dilution with distilled water. Different NO_3^--N concentrations were obtained by adding sodium nitrate, resulting in nine NO_3^--N levels (0.2, 0.5, 1, 2, 5, 10, 25, 50, 100 mg/l) for each water solution. To determine the accuracy of the proposed method in the presence of NO_2^--N, 5 NO_2^--N mg/l was added to each solution; this concentration is highly toxic to fish in salt (Blancheton, 2000) and fresh (Wedemeyer, 1996) water. The assay was performed as above, using both the 96-well microplate technique and 5-cm pathway cell spectrophotometry in the ranges of 100-2 mg/l and 10-0.2 mg/l, respectively.

Statistical analysis. Analytical data (means±standard deviations) are the averages of five analyses, each performed in triplicate. Correlation and linear regression analyses were performed using SigmaPlot 2000 ver 6.0, statistical software. Data regarding recovery of NO_3^--N in solutions with different salinities containing 5 NO_2^--N mg/l are presented as concentrations and were processed by analysis of variance (ANOVA) using Systat 5.05 for Windows to evaluate how the different NO_3^--N concentrations and salt concentrations affected NO_3^--N recovery. When this analysis indicated significant differences ($p \leq 0.01$), Student's *t* test was performed.

Results

The sulfamic acid concentration (mM) necessary to completely remove NO_2^--N from solutions at known NO_2^--N concentrations depends on the NO_2^--N concentration ($p \leq 0.01$) and is described as sulfamic acid = -0.6746 + 0.6742 x NO_2^--N, r^2 = 0.9968 (Fig. 1). Following the method described by Beda and Nedospasov (2005), the measurable range of NO_3^--N in the absence of NO_2^--N was 2-128 mg/l (r^2 = 0.9997) using the microplate technique and 0.2-10 mg/l (r^2 = 0.9998) using 5-cm pathway cell spectrophotometry (Fig. 2). Regression analysis shows that the relationship between NO_3^--N values obtained by the proposed method and by the Cd-reduction method are highly correlated ($p \leq 0.001$; Fig. 3).

The NO_3^--N concentration significantly influenced NO_3^--N recovery ($p \leq 0.01$) while the salt concentration did not (Table 1). Because the lowest detected NO_3^--N concentrations (2 and 0.2 mg/l, respectively) significantly differ ($p \leq 0.01$) from the known NO_3^--N concentrations, the microplate technique reliably detected 5 NO_3^--N mg/l and 5-cm pathway cell spectrophotometry reliably detected 0.5 NO_3^--N mg/l in solutions of different salinities containing 5 NO_2^--N mg/l.

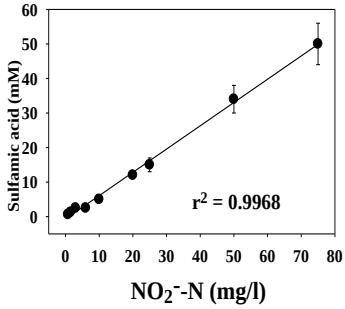

Fig. 1. Sulfamic acid concentration (mM) necessary to completely remove NO_2^--N from water with different NO_2^--N concentrations.

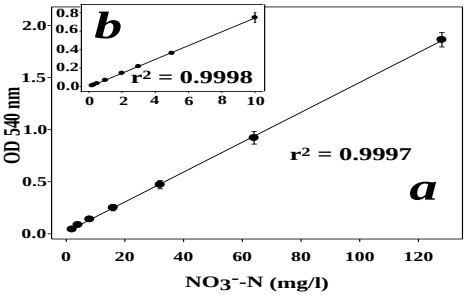

Fig. 2. Proposed methods for determining NO_3^--N: standard curves using (a) 96-well microplate technique (analytical range 2-128 mg/l) and (b) 5-cm pathway cell spectrophotometer (analytical range 0.2-10 mg/l).

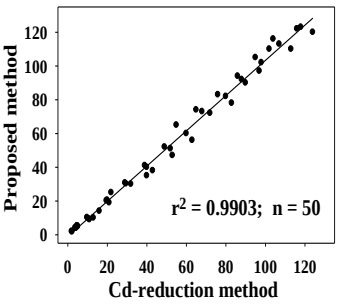

Fig. 3. Relationship between NO_3^--N values measured by the proposed method and values obtained with the Cd-reduction method.

Table 1. Recovery of NO_3^--N in solutions containing 5 NO_2^--N mg/l but with different salinities, using two methods.

NO_3^--N	*Salinity (g/l)*				
(mg/l)	*0*	*5*	*10*	*20*	*35*
96-well microplate technique					
100	98.45±2.56	99.65±1.21	99.13±0.96	98.89±1.34	99.75±0.77
50	48.88±1.13	49.43±1.25	48.92±1.37	50.51±0.58	49.76±0.66
25	25.57±0.88	24.88±0.35	25.49±0.51	25.20±0.32	24.89±0.41
10	10.40±0.64	10.23±0.37	9.96±0.23	10.33±0.37	9.92±0.19
5	5.05±0.12	5.10±0.20	4.98±0.14	5.11±0.12	5.05±0.08
2	3.15±1.23*	3.08±1.43*	2.88±0.68*	2.95±0.93*	3.23±1.55*
5-cm pathway cell spectrophotometry					
10	10.15±0.33	9.78±0.24	10.46±0.55	10.31±0.42	9.95±0.22
5	5.06±0.16	4.93±0.18	4.89±0.21	5.09±0.22	4.99±0.14
2	2.02±0.08	2.08±0.12	1.92±0.19	2.00±0.10	2.04±0.08
1	0.96± 0.08	1.05±0.06	0.99±0.05	1.04±0.07	1.06±0.07
0.5	0.48±0.04	0.48±0.05	0.52±0.05	0.50±0.06	0.52±0.04
0.2	0.38±0.09*	0.35±0.13*	0.40±0.18*	0.31±0.15*	0.33±0.08*

* significantly differ ($p \leq 0.01$) from respective known NO_3^--N concentrations

Discussion

This paper describes a direct spectrophotometric method for determining NO_3^--N in water and wastewater adopting an assay developed to evaluate nitric oxide metabolites in biological samples (Beda and Nedospasov, 2005). In our proposed method, water samples are incubated for a few minutes with sulfamic acid to remove NO_2^--N, then sulphanilamide and N-naphthylethylenediamine reagents are added, leaving the solution colorless. A rose-colored dye is obtained when NO_2^--N derived from NO_3^--N reacts with the strong reducing reagent - 50 mM VCl_3 in 1N HCl.

Results of the proposed method highly correlated with results of the Cd-reduction method for determining NO_3^--N in artificial seawater in the absence of NO_2^--N. The Cd-reduction method has a lower analytical range (0.01-1 NO_3^--N mg/l), making this method recommended when the NO_3^--N concentration is low and other methods are not sufficiently sensitive (APHA, 1989). The proposed method (both the microplate technique and 5-cm pathway cell spectrophotometry) has a higher analytical NO_3^--N range (detection limits are 5 and 0.5 NO_3^--N mg/l, respectively). As confirmed by statistical analysis, the proposed method is valid for solutions with salt concentrations of 0-35 g/l.

Although the sulfamic acid concentration is dependent on the NO_2^--N value of the tested sample, we used sulfamic acid with low molarity (10 mM) because it proved sufficient to remove NO_2^--N concentrations greater than 10 mg/l, a level that is 5-10 times higher than the safe level for most fishes (Wedemeyer, 1996; Blancheton, 2000).

The described microplate technique has a detection limit of 5 NO_3^--N mg/l and seems reliable for measuring NO_3^--N levels in waters and wastewaters from intensive aquaculture systems in which NO_3^--N concentrations are usually high. Indeed, in RAS, NO_3^--N concentrations can exceed 100 mg/l (Tal et al., 2009), although concentrations below 100 mg/l are recommended (Blancheton, 2000). When lower NO_3^--N concentrations are expected, the use of 5-cm pathway cell spectrophotometer becomes necessary, as it has a detection limit of 0.5 NO_3^--N mg/l. In open culture systems where water retention time is usually limited, NO_3^--N concentrations in wastewater are generally so low that the proposed method would not be sufficiently sensitive and the Cd-reduction method remains recommended.

VCl_3 was proposed by Doane and Horwáth (2003) and adopted by Gou (2006) as a reducing agent in water sample analysis for determining total nitrogen after treatment of oxidative samples with potassium peroxodisulfate. The use of VCl_3 as a reducer prevents harmful contact with toxic metals, although the risk can also be reduced by applying the method of Hernández-Lopez and Vargas-Albores (2003) in which samples are reduced by Cd previously activated in a microtube. Compared to the routinely used screening ultraviolet (UV) method (Armstrong, 1963), the proposed method could be adopted even in the presence of dissolved organic matter that can interfere with the UV assay (APHA, 1989), given its original use in blood serum samples (Beda and Nedospasov, 2005).

In conclusion, the proposed method has several advantages compared to the accepted Cd-reduction method, although the latter remains the method of choice for analysis of low NO_3^--N concentrations. However, the Cd-reduction method requires laborious preparation of the column and results in residual toxic heavy metals in treated wastewater. Instead, the proposed method simultaneously removes NO_2^--N from water samples by the use of sulfamic acid and reduces NO_3^--N by the use of VCl_3, permitting direct and rapid NO_3^--N determination in waters and wastewaters from aquaculture plants in which NO_3^--N concentrations are in a medium or high range without the use of expensive and specialized equipment. The proposed method allows rapid data collection and is an excellent tool for routine analysis of waters in intensive aquaculture systems where NO_3^--N concentrations are medium or high.

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