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Improving the fluorometric ammonium method: matrix effects, background fluorescence, and standard additions

Brad W. Taylor[1], Christine F. Keep[2], Robert O. Hall, Jr.[3], Benjamin J. Koch[4], AND Lusha M. Tronstad[5]

Department of Zoology and Physiology, University of Wyoming, Laramie, Wyoming 82071 USA

Alexander S. Flecker[6] AND Amber J. Ulseth[7]

Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York 15853 USA

Abstract. Our understanding of the N cycle is affected by how accurately we can measure NH_4^+ in natural waters. Measuring NH_4^+ concentrations requires accounting for matrix effects (ME) that are caused by substances in the sample that attenuate or intensify the signal of the samples relative to the standards. We show that the ME calculation in the recently published fluorometric NH_4^+ method is mathematically incorrect, producing results that consistently underestimate NH_4^+ concentration as a nonlinear function of the ME. We provide the correct equation and offer an alternative approach that accounts for ME by using sample water rather than deionized water to make the standards, thereby producing a standard curve that contains the same background chemical properties as the samples. In addition, we show that the previous method for measuring a sample's background fluorescence does not include the background signal of the reagent or its interaction with the matrix constituents of the sample. We provide a new method for measuring a sample's background fluorescence that includes the background fluorescence of the sample, reagent, and their interaction. The simple changes we suggest produce more accurate and precise NH_4^+ measurements.

Key words: fluorometry, nitrogen cycle, quenching, standard additions.

NH_4^+ is often difficult to measure in natural waters (Aminot et al. 1997), but it is ecologically important. NH_4^+ is preferred over NO_3^- by autotrophs and heterotrophs (Dodds and Priscu 1989, Kirchman 1994), controls nitrification rates (Schlesinger 1997), is excreted by animals, and has increased globally in fresh and marine waters because of human activities (Howarth et al. 1996). ^{15}N-enriched NH_4^+ (e.g., ^{15}N-NH_4Cl) also is used widely as a tracer to understand the N cycle (e.g., Peterson et al. 2001). Despite widespread attention and modern analytical instruments with high sensitivity, quantifying NH_4^+ remains difficult. For example, NH_4^+ can be relatively unstable during sample preservation, and numerous sources of contamination during sample collection, preservation, and analysis hinder measuring low concentrations of NH_4^+ (Eaton and Grant 1979, Aminot et al. 1997).

Recently, Kérouel and Aminot (1997) and Holmes et al. (1999) introduced an elegant fluorometric method for measuring low NH_4^+ concentrations that solves many of these problems. The fluorometric method works well because samples can be combined with reagents and analyzed immediately in the field, alleviating problems with the instability of NH_4^+ during storage (Avanzino and Kennedy 1993, Zhang et al. 1997). The method also decreases contamination from laboratory materials and atmospheric NH_3 by using a single working reagent (WR) and a time-

[1] Present address: Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755 USA. E-mail: brad.taylor@dartmouth.edu

[2] E-mail addresses: chrisfish11@gmail.com

[3] bhall@uwyo.edu

[4] bkoch@uwyo.edu

[5] tronstad@uwyo.edu

[6] asf3@cornell.edu

[7] Present address: Department of Zoology and Physiology, University of Wyoming, Laramie, Wyoming 82071 USA. E-mail: aulseth@uwyo.edu

dependent reaction. Moreover, like the salicylate-hypochlorite NH_4^+ method (Bower and Holm-Hansen 1980), the fluorometric method does not produce toxic carcinogenic phenol-based waste, as does the phenol-hypochlorite (or indophenol-blue) method (e.g., Solórzano 1969).

Measuring NH_4^+ in natural waters requires accounting for matrix effects (ME) caused by substances other than the analyte in the sample that suppress or intensify the response signal (Strickland and Parsons 1972, Taylor 1989, ASTM 1996). ME are the result of differences between the background chemical composition of the sample water and the water used to make the standards. For example, standards made with deionized (DI) water may differ considerably from sample water with respect to pH, dissolved organic matter, salts, and other dissolved substances that can affect the response signal. ME are not unique to the fluorometric NH_4^+ method; they are an issue for most analytical chemistry methods, including the indophenol-blue and salicylate methods for measuring NH_4^+ (Loder and Glibert 1977, Bower and Holm-Hansen 1980, Stewart and Elliot 1996, Zhang et al. 1997). Accounting for ME is important because substances that cause ME vary through space and time in natural waters (Stewart and Elliot 1996). In addition to ME, substances in the sample and reagent can autofluoresce, producing background fluorescence (BF) that should be measured and subtracted to estimate accurately the fluorescence caused by NH_4^+. Thus, correcting for ME and BF properly is important because the accuracy of NH_4^+ measurements affects our progress toward understanding N cycling.

The approach suggested by Holmes et al. (1999) does not correctly adjust NH_4^+ concentrations for ME or BF. Here we show why their ME and BF methods are incorrect and provide an alternative approach that improves the accuracy and precision of measuring NH_4^+.

Concerns with Holmes et al.'s (1999) Method

Calculating matrix effects

Holmes et al. (1999) provide the following equation to calculate ME when standards are prepared in DI water:

$$\%ME = \left\{ \frac{[(F_{std_{spike}} - F_{std_{obs}}) - (F_{sample_{spike}} - F_{sample_{obs}})]}{(F_{std_{spike}} - F_{std_{obs}})} \right\} \times 100 \quad [1]$$

where $Fstd_{spike}$ and $Fstd_{obs}$ are the fluorescence of standards with a known amount of NH_4^+ added and no NH_4^+ added, respectively. $Fsample_{spike}$ is the fluorescence of a sample with the same amount of NH_4^+ added as the $Fstd_{spike}$. If the fluorescence of the spiked standard and the spiked sample increase by exactly the same amount then the ME is 0 because 100% of the added NH_4^+ is recovered. Equation 1 is similar to calculating the % recovery of an analyte (e.g., Zhang et al. 1997). Assumptions of equation 1 are that only 1 standard and sample spike are needed to precisely and accurately estimate the ME, and that measurement error (e.g., instrument or pipette error) is not propagated by the equation. We show that equation 1 may violate these assumptions, resulting in highly variable ME values and NH_4^+ concentrations even for samples with small ME; therefore, we recommend using the method of multiple standard additions (ASTM 1996) to account for ME, particularly for measuring low NH_4^+ concentrations.

To correct a sample for ME, Holmes et al. (1999) provide the following equation:

$$F_{sample_{cor}} = F_{sample_{NH4}} + \left[F_{sample_{NH4}} \left(\frac{\mathrm{ME}}{100} \right) \right] \quad [2]$$

where $Fsample_{cor}$ is the fluorescence corrected for ME and is the value entered into the DI water standard-curve equation to compute the NH_4^+ concentration in a sample. $Fsample_{NH4}$ is the fluorescence of a sample corrected for BF (Holmes et al. 1999) caused by sample and reagent autofluorescence:

$$F_{sample_{NH4}} = F_{sample_{obs}} - F_{sample_{BF}} \quad [3]$$

where $Fsample_{obs}$ is the fluorescence of a sample when incubated for 3 to 5 h with WR, and $Fsample_{BF}$ is the fluorescence of a sample when incubated for 3 to 5 h with borate buffer only (i.e., no WR is added). We think equation 2 is incorrect because if, for example, the $Fsample_{NH4}$ is 50 fluorescence units and the ME is 50%, then only ½ the NH_4^+ was recovered; therefore, the matrix constituents are suppressing ½ of the sample's fluorescence caused by NH_4^+, and the $Fsample_{cor}$ should be 100. However, equation 2 computes an $Fsample_{cor}$ of 75, yielding an NH_4^+ concentration that is 25% too low. Equation 2 (or equation 3 in Holmes et al. 1999) systematically underestimates the true NH_4^+ concentration as a nonlinear function of the ME. The bias of NH_4^+ concentration estimated with equation 2 is: NH_4^+ concentration bias $= -([\mathrm{ME}/100]^2)$. We derived the valid equation for applying the ME adjustment by rearranging equation 1 and solving for the expected fluorescence of the recovered spike. The equation simplifies to

$$\mathrm{F}_{\mathrm{sample}_{\mathrm{cor}}} = \frac{\mathrm{F}_{\mathrm{sample}_{\mathrm{NH_4}}}}{\left(1 - \frac{ME}{100}\right)}. \quad [4]$$

Thus, equation 4 should be used to apply the ME adjustment, not equation 2.

Estimating BF

The approach provided by Holmes et al. (1999) to correct for BF using equation 3 assumes WR has no BF and that there is no interaction between WR and sample that would alter BF. We show these assumptions are false and result in overestimation of NH_4^+ concentration because WR contains DI water, orthophthaldialdehyde, sodium sulfite, borate buffer, and high-grade ethanol that can all produce BF (Kérouel and Aminot 1997, Holmes et al. 1999) and must be subtracted from the fluorescence of a sample to estimate the fluorescence caused by NH_4^+. Therefore, we suggest that fluorescence of a sample should be corrected for BF as

$$F_{sample_{NH4}} = F_{sample_{obs}} - F_{sample_{time-zero}} \quad [5]$$

where $Fsample_{NH4}$ and $Fsample_{obs}$ are as defined in equation 3. $Fsample_{time\text{-}zero}$ is the fluorescence of a sample with WR added and measured immediately (i.e., incubated <30 s). Because fluorescence of a sample increases rapidly after WR is added (e.g., fig. 2 in Holmes et al. 1999, Kang et al. 2003), the $Fsample_{time\text{-}zero}$ measurement must be made quickly to minimize any reaction of the WR with the ambient NH_4^+.

Methods

Chemical methods

We used similar analytical equipment, reagents, and procedures as Holmes et al. (1999), except we used 40 mL of sample water and 10 mL of WR for protocol A rather than 80 mL of sample water and 20 mL of WR. We prefer protocol A because our laboratory has measured a method detection limit ([MDL] concentration that can be measured and reported with 99% confidence as >0) of 0.2 μg NH_4-N/L ($t = 3.14$, df = 6) and a coefficient of variation (CV) of 3% with our samples, ME, procedures, instruments, and personnel. We did not use Holmes et al.'s (1999) protocol B or Kang et al.'s (2003) protocol I because we measured a MDL of 4.8 μg NH_4-N/L ($t = 3.14$, df = 6) and a CV of 30% using these protocols. Thus, these protocols may not be sensitive enough for low-level NH_4^+ concentrations (e.g., <20 μg NH_4-N/L) typical of many fresh waters.

Background fluorescence

We tested whether the time-zero BF was stable for several minutes (0–167 min) after adding WR by measuring the fluorescence of 30 DI water standards spiked with 100 μg NH_4-N/L and 30 replicate samples from Crow Creek, Wyoming, at second, minute, and hour intervals. Ideally, $Fsample_{time\text{-}zero}$ measures the autofluorescence of the sample, matrix constituents, WR, and their interaction but does not include fluorescence caused by the reaction of NH_4^+ and

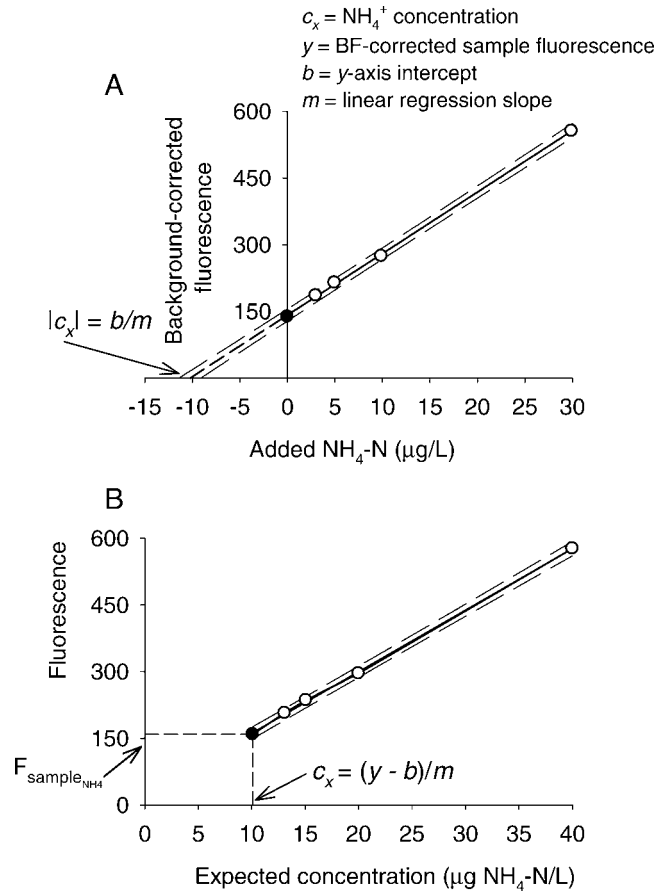

FIG. 1. Examples of the standard-additions method, protocol I and protocol II. A.—Protocol I: plot the background-corrected fluorescence of the standard additions against their nominal concentrations. Fit a linear regression, then extrapolate the line to the x-axis; the absolute value of the x-axis intercept ($|c_x| = b/m$) is the concentration of the sample (i.e., the standard addition with no NH_4^+ added), where b is the y-axis intercept and m is the slope. B.—Protocol II: add the NH_4^+ concentration of the 0 NH_4^+ added standard addition to the nominal concentration of all the standard additions including itself. Plot the fluorescence, uncorrected for background fluorescence, against the expected concentration of the standard additions. Fit a linear regression and apply the background-corrected fluorescence of the samples to the regression equation; use inverse prediction to estimate the concentration of the sample [i.e., $c_x = (y - b)/m$], where y is the background-corrected fluorescence of the sample. Dashed lines show the 95% confidence interval for the regression.

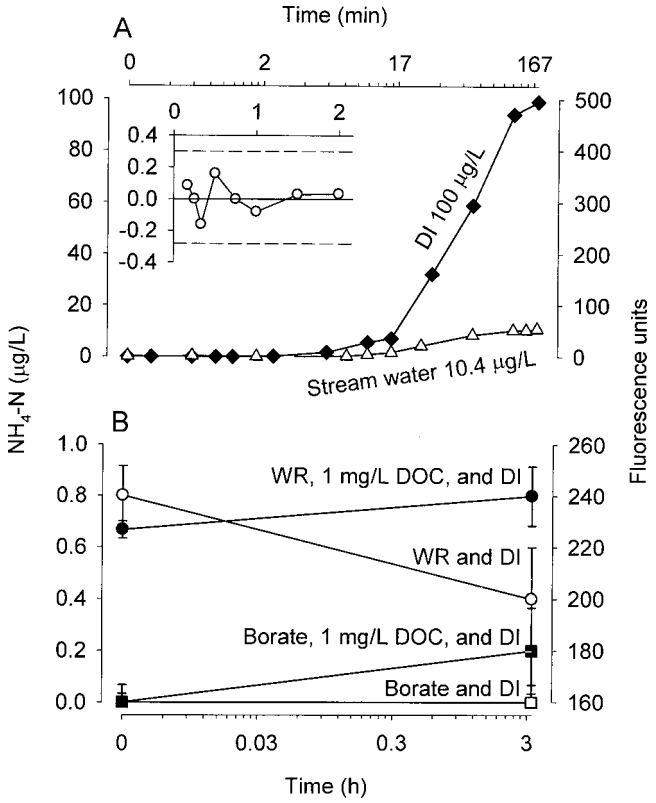

FIG. 2. Evaluation of the time-zero background fluorescence (BF) method. A.—Time series of mean (±1 SD, $n = 3$) fluorescence measurements for stream samples with 10.4 µg NH_4-N/L and a matrix effect of 18%, and deionized (DI) water with a concentration of 100 µg NH_4-N/L. The control chart (inset) shows that the mean NH_4^+ concentration of measurements made during a 120-s window after adding working reagent (WR) is within the upper and lower 95% confidence limits, which are at or below the method detection limit. B.—Comparison of time-zero BF of WR and borate buffer (added in the ratio of 1:4) to DI water only or DI water spiked with dissolved organic C (DOC), and measured at time zero (i.e., <30 s) and after incubating for 3 h. WR and borate buffer do not have the same time-zero BF, which may be altered by the presence of matrix constituents (e.g., DOC). The concentration of DOC was 1 mg/L, and DOC was obtained from the Eagle River, Colorado.

WR. Hence, our approach assumes no time-dependent interaction between the WR and sample that would affect BF. We tested this assumption by comparing the fluorescence of DI water combined with WR and a matrix constituent (e.g., 1 mg/L dissolved organic C from the Eagle River, Colorado) at time zero and after incubating for 3 to 5 h. In addition, Holmes et al. (1999) state that WR autofluorescence (or WR blank) decreases with age, but WR is stable for ~3 mo; however, they did not report whether the sensitivity of the WR changed with its age. To determine whether the age of the WR decreased the sensitivity of the time-zero BF method, we used WR that varied in age from 1 to 60 d and compared the fluorescence of DI water spiked with 10 and 140 µg NH_4-N/L at $Fsample_{time\text{-}zero}$ and after incubating 3 h with WR. We used repeated-measures analysis of variance (ANOVA) with bottles as subjects nested within NH_4^+ concentration and time of measurement (0 or 3 h) and measurement date as repeated effects in PROC MIXED (SAS, version 8.0; SAS Institute, Cary, North Carolina). If the sensitivity of WR to react with NH_4^+ declined with WR age, then we expected a significant interaction between WR age, time of measurement (i.e., $Fsample_{time\text{-}zero}$ or fluorescence after incubating 3 h with WR), and NH_4^+ concentration.

Standard-additions procedures

The standard-additions method accounts for ME by making the standards with sample water that contains the same background chemical properties as the samples (Bader 1980, Keith et al. 1983, Taylor 1989, ASTM 1996). We provide the procedures for 2 standard-additions protocols: Protocol I is the classic standard-additions method that is used to estimate the ME-corrected concentration of a single unknown, and protocol II is a modification of the standard-additions method in which the ME-corrected concentration of multiple unknowns with similar ME can be estimated.

Standard-additions protocol I.—The background-corrected fluorescence of 3 to 5 samples with known amounts of NH_4^+ added and 1 sample with no NH_4^+ added are regressed against their nominal spike concentrations, and the ME-corrected concentration of the sample is estimated by extrapolating the curve to the x-axis (Fig. 1A). We recommend spiking 3 to 5 samples because accounting for the ME depends on accurate and precise estimates of the slope and intercept, which can be unstable in regressions with only 2 to 3 points (Keith et al. 1983, Zar 1984, Taylor 1989). The number of bottles needed for each sample corresponds to the number of standard additions (including the 0 NH_4^+ added standard, or ambient concentration) plus at least 1 bottle for BF. Using a 60-mL graduated plastic syringe, we added 40 mL of sample to 60-mL amber-colored plastic bottles and stored them in the dark in a cooler until analysis. To keep sample bottles clean, we left the used WR in the bottle. We prepared standards in the field so that the added NH_4^+ equilibrated with the matrix components (Keith et al. 1983) and samples could be analyzed within several hours after collection, steps that are important for concentrations <20 µg/L (Avanzino and Kennedy 1993, Zhang et al. 1997). We pipetted NH_4^+

stock solution into the sample bottles to increase their concentration 0, 0.25, 1, 2, or 3× above the expected ambient concentration (ASTM 1996). We did not use higher concentrations because the farther the mean NH_4^+ concentration of the standard additions is from 0, the greater are the confidence intervals of the extrapolated concentration (Zar 1984). NH_4^+ stock was not added to the BF sample or to the sample that serves as the 0 NH_4^+ added standard. We added 10 mL of WR to all of the samples except the BF sample and incubated them in the dark at ambient temperature. After incubating 3 to 5 h, we measured the standard-additions fluorescence. We also measured the time-zero BF by adding WR to the BF sample and measuring its fluorescence immediately. We used the same test tube to measure each sample by rinsing the test tube with the next sample before taking a reading. At this stage in the analysis, contamination from test tubes was minimal because the reaction is time-dependent.

We calculated the sample NH_4^+ concentration by first correcting the standard additions for BF using equation 5 and then regressing the background-corrected fluorescence of the standard additions against their nominal concentration using linear regression. The sample concentration, $|c_x|$, was computed by extrapolating the estimated regression line to the x-axis intercept and solving $|c_x| = b/m$, where b is the y-axis intercept or the background-corrected fluorescence of the standard addition with ambient NH_4^+ concentration, and m is the slope of the regression line. The standard error and confidence intervals for the extrapolated NH_4^+ concentration of a single sample can be calculated using the procedure for a negative x-axis intercept prediction (see equation 13 in Bruce and Gill 1999).

Standard-additions protocol II.—This protocol can be used to estimate the NH_4^+ concentration of multiple samples if their ME are similar, for example, multiple samples collected during a stream NH_4^+ uptake length measurement. We recommend first testing the assumption of similar ME among samples or sites by comparing the slopes of standard-additions curves from each site using a homogeneity of slopes test (Zar 1984). A nonsignificant interaction between the covariate, which is the added NH_4^+ concentration, and the site term in the model indicates that the slopes and, therefore, the ME are similar. Protocol II standards are prepared similarly to protocol I, except the NH_4^+ concentration of the 0 NH_4^+ added standard is first estimated by protocol I and then arithmetically added to the nominal concentration of all the standard additions. A calibration curve that adjusts for the matrix properties of the samples is created by regressing the fluorescence of the standard additions against their expected concentration, which is the sum of their spike and ambient concentration (Fig. 1B). We recommend using the sample with the lowest concentration to make the standard-additions calibration curve to ensure that the sample concentrations are bracketed by the standard additions. We corrected each sample for BF using equation 5. To estimate NH_4^+ concentration, we regressed the fluorescence of the standard additions, uncorrected for BF, against their concentrations and solved for the concentration of the sample, c_x, by inverse prediction (i.e., $c_x = [y - b]/m$, where y is the BF-corrected sample fluorescence obtained from equation 5, b is the y-axis intercept, and m is the slope; Neter et al. 1996). The standard error and confidence intervals for the predicted NH_4^+ concentration of a single sample can be computed using the procedure for inverse predictions (see equation 4.32a in Neter et al. 1996).

To test whether protocol II can be used to estimate the NH_4^+ concentration of multiple samples, we used a homogeneity of slopes test (Zar 1984) to determine whether the slopes of standard additions from different locations were similar. We used the AT, LSMEANS, and TUKEY options in SAS to test for differences in >2 slopes. We collected enough samples to create individual standard-additions curves from 2 sites located 4 km apart on the Middle Fork of Crow Creek and 1 site located on the South Fork of Crow Creek, as well as from upstream and downstream sampling sites located 0.25 km and 2 km apart in Rio Las Marias, Venezuela.

To estimate the MDL of protocol I and II, we collected enough replicate samples from one location in Crow Creek to generate 8 sets of standard additions. We added enough NH_4^+ to increase sample concentrations 2× above the limit of quantitation ([LOQ] concentration above which values may be obtained with 99% confidence). For protocol I, we used 7 of these standard additions to predict the NH_4^+ concentration by extrapolating to the x-intercept. For protocol II, we used 1 set of the standard additions to predict the NH_4^+ concentration of 7 replicate samples from Crow Creek. We computed a MDL with 99% confidence for each protocol by multiplying the standard deviation of the 7 measurements by the critical t-value of 3.14 (Zar 1984).

Comparison of accuracy and precision among methods

Last, we also tested whether equation 1 produced highly variable ME values and NH_4^+ concentrations because equation 1 relies on the accuracy and precision of a single-spike addition and has several terms that

can propagate error. We collected triplicate samples from 2 streams (Spring Creek and Crow Creek) and 1 pond (La Bonte Pond) near Laramie, Wyoming, and made 5.0-μg NH_4-N/L DI water standards. We compared the means and variances of ME and NH_4^+ concentrations calculated by equation 1 to those calculated by the standard-additions method using a 2-sided *t*-test and a variance ratio test (Zar 1984).

Results and Discussion

Background fluorescence

Fluorescence of samples and DI water with NH_4^+ added was stable for ~2 min after WR was added (Fig. 2A). The variation in fluorescence measurements made within 2 min after adding WR was low and equivalent to ±0.2 μg NH_4-N/L (Fig. 2A, inset graph), which is typically at or below our measured MDL, and hence, not statistically different from 0. There was no difference in the time-zero BF of samples with 10.4 μg NH_4-N/L and DI water standards with 100 μg NH_4-N/L during the 2-min interval ($t_{10} = 0.206$, $p = 0.84$). Thus, the time-zero BF can be measured reliably within 2 min after adding WR to samples, and BF can be measured easily within 20 to 30 s.

The time-zero BF of DI water with WR was greater than the time-zero BF of DI water with borate buffer (reagent type: $F_{1,16} = 65.15$, $p < 0.0001$; Fig. 2B), indicating WR has some autofluorescence or that there was an interaction between the BF properties of the sample and the WR that affected fluorescence. The time-zero BF of WR and dissolved organic C (DOC) was 8× higher than the time-zero BF of borate buffer and DOC (reagent type × DOC: $F_{1,16} = 11.46$, $p = 0.0038$). Fluorescence of samples with DOC added increased with time (DOC × time: $F_{1,16} = 2.86$, $p = 0.01$), regardless of the reagent type (reagent type × time: $F_{1,16} = 1.69$, $p = 0.21$; reagent type × time × DOC: $F_{1,16} = 0.26$, $p = 0.79$). Measuring the time-zero BF did not account for this increase with time, but the effect of time on BF was small (e.g., 17% of the total fluorescence) compared to the effect of using borate buffer rather than WR (e.g., 75% of the total fluorescence) to estimate BF (Fig. 2B). Thus, equation 3 provided by Holmes et al. (1999) probably will underestimate the BF of a sample and, thus, overestimate NH_4^+ concentrations.

BF can account for a large % of the total fluorescence of a sample. BF estimated using equation 5 was 20% of the total fluorescence in La Bonte Pond, 50% in Spring Creek, and 90% in Crow Creek. BF estimated using equation 3 (or equation 1 from Holmes et al. 1999) was 15% lower than the time-zero BF estimated using equation 5 for all 3 Wyoming sites ($F_{1,12} = 15.84$, $p < 0.0018$). Thus, depending on the BF properties and NH_4^+ concentration of a sample, the BF can be a substantial adjustment to NH_4^+ concentration that is best estimated by the time-zero BF technique presented in equation 5.

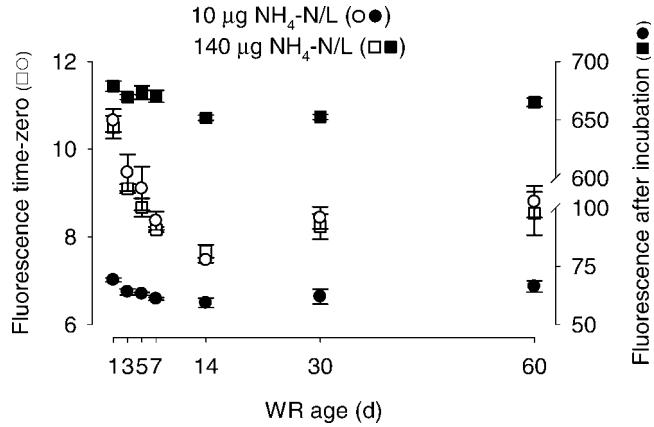

FIG. 3. Effect of the age of the working reagent (WR) on mean (±1 SD) fluorescence of WR added to deionized water with low and high NH_4^+ concentrations, measured at time zero and after incubating with WR for 3 h, as a function of the age of the WR. One batch of WR was prepared on day 0, stored at room temperature, and used for all of the measurements. New standards and samples were prepared each day from the same 10,000-μg NH_4-N/L solution. Sensitivity of the fluorometer was set at 32% using the 100-μg NH_4-N/L standard and was not altered between measurements. The signal from the instrument with nothing in it varied ±0.2 fluorescence units. $n = 3$ replicates.

The fluorescence of the WR decreased as a function of its age (age: $F_{6,48} = 28.29$, $p < 0.0001$; Fig. 3), a pattern that was similar for both low and high NH_4^+ concentrations (age × NH_4-N: $F_{6,48} = 1.02$, $p = 0.43$). The sensitivity of the WR to react with NH_4^+ was not affected by interactions among WR age, the concentration of NH_4^+, and fluorescence measured at time-zero and after incubating for 3 h (age × NH_4-N × $time_{0-3}$: $F_{6,48} = 1.48$, $p = 0.21$). Taken together, these results show WR fluorescence decreases with age, but the sensitivity of WR to react with NH_4^+ does not decline with its age. Thus, the age of the WR does not alter the precision and accuracy of the time-zero BF measurement.

Standard-additions procedures

The slopes of the standard additions for the 3 replicate samples collected at a single location were similar to one another for Crow Creek (replicate × NH_4-N: $F_{1,11} = 0.128$, $p = 0.72$), La Bonte Pond (replicate × NH_4-N: $F_{1,11} = 0.851$, $p = 0.38$), and Spring Creek (replicate × NH_4-N: $F_{1,11} = 2.75$, $p = 0.13$). Hence,

TABLE 1. Comparison of standard-additions slopes, and protocol I and II NH_4^+ concentrations for 3 sites in Crow Creek, Wyoming; 4 sites in Rio Las Marias, Venezuela; and 3 streams near Yellowstone Lake, Wyoming, 1 of which was sampled on 3 dates. Within each site and sampling date, slopes with the same uppercase letter are not significantly different ($p < 0.05$), based on either Tukey's multiple comparisons test among standard-additions slopes or Dunnett's pairwise comparisons between the standard-additions slope and the deionized (DI)-water standards slope. – indicates concentrations were not computed by protocol II on these dates. See text for equations 1 and 4.

			μg NH_4-N/L		
Site	Slope	ME	Protocol I	Protocol II	Equations 1 and 4
Crow Creek, Wyoming					
Standards in DI water	24.1^{A}				
Middle Fork 0 km	19.6^{B}	20	10.3	9.9	14.9
Middle Fork 4 km[a]	20.8^{B}	14	3.0	3.2	5.9
South Fork	23.0^{C}	5	9.4	9.3	8.1
Rio Las Marias, Venezuela					
Standards in DI water	1.5^{A}				
0.25-km-long reach, upstream	1.0^{B}	33	11.9	11.0	8.5
0.25-km-long reach, downstream	1.1^{B}	27	7.0	7.2	3.3
2-km-long reach, upstream[a]	0.91^{C}	39	5.7	5.4	3.8
2-km-long reach, downstream	0.73^{D}	51	15.7	8.1	20.6
Yellowstone National Park, Wyoming					
8 July 2004					
Standards in DI water	44.2^{A}				
Clear Creek[a]	$40.4^{A,B}$	9	2.1	2.0	1.8
Cub Creek	39.8^{C}	10	3.8	3.7	4.4
Little Cub Creek	37.8^{C}	15	4.2	3.9	5.2
19 July 2004					
Standards in DI water[b]	12.1				
Little Cub Creek	6.7^{A}	45	11.0	–	13.9
20 July 2004					
Standards in DI water[b]	40.6				
Little Cub Creek	26.2^{B}	36	10.9	–	14.7
27 July 2004					
Standards in DI water[b]	44.5				
Little Cub Creek	35.8^{C}	20	4.8	–	6.0

[a] Sampling location or stream with the lowest ambient NH_4^+ concentration, which was used as the protocol II standard-additions calibration curve to predict the NH_4^+concentrations within a site (e.g., Crow Creek)

[b] DI water standards slope and the standard-additions slope were significantly different at $p < 0.05$ for each pairwise comparison in Little Cub Creek in late July

we find it valid to assume the ME is similar for replicate samples taken from the same location.

Can a single standard-additions calibration curve be used to predict the concentration of samples from different locations (i.e., protocol II)? The standard-additions slopes of the 2 sites separated by 4 km on the Middle Fork of Crow Creek were similar but different from the standard-additions slope of the South Fork (site × NH_4-N: $F_{3,12} = 1565.22$, $p < 0.0001$, Tukey's $p < 0.05$; Table 1); however, violating the constant slopes assumption had a small effect (<1.5 μg NH_4-N/L) on NH_4^+ concentrations within the range of the samples collected. In contrast to the Wyoming streams, the standard-additions slopes for locations 2 km apart on the same branch of Rio Las Marias were not equal (site × NH_4-N: $F_{4,16} = 168.28$, $p < 0.0001$, Tukey's $p < 0.05$; Table 1), and using protocol II resulted in an ~2-fold underestimate of NH_4^+ concentration at the downstream site. For sites located 0.25 km apart in Rio Las Marias, the standard-additions slopes were equal (Tukey's $p > 0.05$; Table 1); nevertheless, protocol II underestimated the NH_4^+ concentration of the site located 2 km downstream because the slope of the standard-additions used as the calibration curve (i.e., the site located 2 km upstream) was steeper. In streams flowing into Yellowstone Lake, the standard-additions slopes and, therefore, the ME were different between 2 of the 3 streams sampled on 8 July 2004 (site × NH_4-N: $F_{3,9} = 791.00$, $p < 0.0001$, Tukey's $p < 0.05$; Table 1); however, the effect of unequal slopes on NH_4^+ concentrations was small. In 1 of these streams (Little Cub Creek), the slope of standard additions made on 3

TABLE 2. Comparison of matrix effects (ME, %) and NH_4^+ concentrations (μg/L) computed using equations 1 and 4 (see text for equations) or standard-additions protocol I with triplicate spike series or 1 spike series for samples collected from 2 streams and 1 pond in Wyoming and for $n = 21$ deionized (DI) water standards spiked with 5.0 μg NH_4-N/L. Individual standard-additions series consist of 1 ambient, 1 background fluorescence (BF), and 4 spiked samples ($n = 6$). The standard-additions ME was computed as %ME = ([DI water standards slope − standard-additions slope]/DI water standards slope) × 100. Concentrations based on equations 1 and 4 are derived from 3 samples, 3 spikes, 3 BF, and 5 DI water standards ($n = 14$). DOC = dissolved organic C, CV = coefficient of variation of 3 replicates, CI = 95% confidence interval.

Site	DOC (mg/L)	Conductivity (μS/cm)	Response	Equations 1 and 4 (n = 3 samples) Mean (CI)	CV	Protocol I (n = 3 spike series) Mean (CI)	CV	Protocol I[a] (n = 1 spike series) Mean	t-test[b] p-value	Variance ratio test[c] p-value
Crow Creek	12.3	145	ME	−2.0	127	45.0	11	41.0	0.001	0.006
			NH_4-N	2.0 (1.5–2.6)	11	3.8 (3.3–4.4)	6	3.6 (3.4–3.7)	0.002	0.88
Spring Creek	0.7	549	ME	3.0	402	18.0	9	21.0	0.2	0.009
			NH_4-N	9.9 (5.9–13.8)	16	11.5 (10.8–12.3)	3	11.7 (11.4–12.1)	0.11	0.16
La Bonte Pond	14.2	1200	ME	−56.0	66	9.0	13	12.1	0.03	0.004
			NH_4-N	16.4 (2.7–30.1)	34	27.3 (23.3–31.4)	6	29.7 (25.9–33.4)	0.05	0.06
21 standards			ME	−3.61	393	2.0	10	1.3	0.26	<0.0001
			NH_4-N	5.3 (4.8–5.8)	20	5.1 (4.9–5.4)	8	5.4 (5.1–5.7)	0.33	<0.0001

[a] CV not calculated
[b] 2-sided unequal variance t-test of the means between methods at each site
[c] Test for differences in the variances between methods at each site

dates in July changed significantly following severe flooding (date × NH_4-N: $F_{3,9} = 154.13$, $p < 0.0001$, Tukey's $p < 0.05$), demonstrating that the ME can vary temporally, even in streams that normally have low ME (Table 1). Typically, a new standard-additions curve is made on each sampling date. Therefore, we did not estimate NH_4^+ concentrations using protocol II for the 3 dates in Little Cub Creek. Taken together, these results suggest that, as the spatial and temporal domain of sampling increases, the assumption of similar ME may not be valid, and using standard-additions protocol II for analyzing samples from different locations should be assessed on a site-by-site basis by first using protocol I and then testing the equality of the standard-additions slopes among sites. It is important to note that, even if protocol I slopes are statistically different, protocol II may provide acceptable NH_4^+ concentrations depending on the magnitude of the difference in slopes and sample concentrations.

We computed MDLs of 0.5 and 0.8 μg NH_4-N/L ($t = 3.14$, df = 6) for standard-additions protocol I and II, respectively. These values were slightly less than the MDL of 1.1 μg NH_4-N/L estimated using DI water standards and equations 1 and 4, but they were greater than the MDL of DI water standards spiked with NH_4^+ and estimated without using equations 1 and 4 (i.e., 0.2 μg NH_4-N/L; see Chemical methods). Hence, equation 1 alone increased the MDL. Similarly, the LOQs of protocol I and II (1.5 and 2.5 μg NH_4-N/L, respectively) were lower than the LOQs of the DI water standard curve and equations 1 and 4 method (3.4 μg NH_4-N/L).

Comparison of accuracy and precision among methods

Although equation 4 is mathematically correct for adjusting the NH_4^+ concentration for ME, the ME is still based on a single spike estimate (i.e., equation 1) that produces ME values and NH_4^+ concentrations that are more variable than those calculated using standard-additions protocol I and II (Tables 1 and 2). Equation 1 either over- or undercorrected NH_4^+ concentrations because the slope of a single spike can differ slightly from the slope of an entire set of standards (Table 1). The only case in which concentrations were similar among methods was for the 21 DI water standards with NH_4^+ added (Table 2), a result probably caused by the small ME or the large sample size. However, both of these conditions are unrealistic practical expectations for improving accuracy and precision. Nonetheless, even for 21 DI water standards, the variability in ME and NH_4^+ concentration was significantly greater for estimates derived from equation 1 (Table 2). Therefore, we do not recommend using a method involving equation 1 to estimate and account for ME. The importance of correctly accounting for the ME cannot be overemphasized because differences of the magnitude and variability caused by the ME have been attributed to ecological and environmental factors. For example, the variability associated with concentrations estimated by equations 1 and 4 was ±4.0 NH_4-N/L in Spring Creek and

±13.7 μg NH_4-N/L in La Bonte Pond (Table 2), variation that is frequently viewed as biologically interesting. Thus, the variability of NH_4^+ concentrations generated by using equation 1 may equal or surpass the spatial or temporal variability being studied, thereby masking interesting natural patterns and processes.

When can a DI water standard curve be used rather than standard additions? It is generally recommended that samples should be corrected for ME if the ME is >10% (Keith et al. 1983, Zhang et al. 1997); otherwise, NH_4^+ concentrations usually can be estimated directly from a DI water standard curve without any ME correction. However, we think that determining when to correct samples for ME should be based on the performance of the analytical method or the resolution that is needed to address the particular question. Based on this criterion, we suggest that ME corrections should be applied to samples only when the change in NH_4^+ concentration caused by the ME is greater than the resolution of the method (e.g., LOQ) because applying an ME that is below the resolution of the method may add spurious variability to NH_4^+ concentrations.

Possible tradeoffs among methods

Like other techniques, the method of standard additions has some limitations. Foremost is the assumption that the recovery of the added NH_4^+ is the same as the recovery of the endogenous NH_4^+ (ASTM 1996). This assumption is especially important for NH_4^+, which is easily adsorbed by particles that may bind differently with the added NH_4^+ than with the endogenous NH_4^+. However, our data show that NH_4^+ concentrations estimated by the standard-additions method were greater than or equal to concentrations estimated using DI water standards. Nevertheless, to minimize any differential NH_4^+ sorption to particles, we recommend filtering samples. The filter should be prerinsed to minimize adsorption of NH_4^+ onto it (Eaton and Grant 1979).

Other concerns include the increased number of samples required if a standard-additions curve is needed for every sample, as in protocol I. Protocol II reduces the number of samples required for standard additions, but requires that the assumption of similar ME among samples is addressed a priori. In addition, a possible tradeoff with the standard-additions method is that fewer samples can be collected in space or time because of the additional effort given to an individual sample. However, there may not be a large difference among methods in the number of samples required to achieve the same accuracy and precision. For example, using equation 1 to account for ME may require a greater number of samples and spikes than the standard-additions method for similar accuracy and precision (Table 2). Moreover, the number of samples required for the standard-additions method can be reduced in several ways. One technique is protocol II. The other technique is to estimate the concentration of a sample using 1 spike series rather than multiple spike series. The concentration of a single sample is already based on replicate samples when using the standard-additions method (i.e., spikes with different amounts of NH_4^+ added), so multiple spike series may not be needed to achieve similar accuracy and precision. In Table 2, we randomly selected 1 spike series (1 ambient, 1 BF, and 4 spiked samples; $n = 6$) from the 3 standard-additions series within each site and compared this estimate and confidence intervals to those computed with 3 replicate samples using DI water standards and equations 1 and 4 (3 samples, 3 spikes, 3 BF, and 5 DI water standards; $n = 14$). The results show that, even with fewer samples, the standard-additions method produces more accurate and precise estimates of NH_4^+ concentrations. Hence, it is unlikely that a negative tradeoff exists in sampling associated with the standard-additions method, particularly if substantial matrix effects are present. Indeed, using DI water standards and equations 1 and 4 produces concentrations with such high variability that the opportunity to sample more sites may not afford any advantage over the standard-additions method. For example, La Bonte Pond concentrations for a single sampling location had an uncertainty of ±13.7 μg NH_4-N/L using equation 1 (Table 2), which could be equal to or greater than the variability among sites in this pond. Thus, a method that enables the collection of more samples but has higher analytical variability than the environmental variability may not improve the mean estimate of a site.

The standard-additions method and the fluorometric method have several other advantages: concentrations are both accurate and precise, only 1 working reagent is needed, processing and analyzing samples is not labor intensive relative to other methods (Brzezinski 1987), samples can be analyzed within hours after collection, portable field fluorometers (e.g., Turner Designs 10-AU or Aquafluor) can be used at remote field sites, and high-grade DI water is not needed.

Final comments

The fluorometric NH_4^+ method is in widespread use and represents an important advance in measuring low-level NH_4^+ in natural waters. There are some problems with the previously published equations and approaches for adjusting for BF and ME, but we have suggested and tested the necessary changes that

should enhance the method. Equation 2 (correcting samples for ME) is invalid and underestimates the concentration of NH_4^+, whereas equation 3 (correcting samples for BF) underestimates BF, resulting in overestimates of NH_4^+ concentration. We provide equation 4 as the valid equation for correcting samples for ME and offer equation 5 as a better way to correct for BF. Equation 1 produces highly variable ME values that increase the variability of NH_4^+ concentrations because the ME is based on the slopes of standards and samples from single-spike additions and it has several terms that can propagate error. ME estimated by a single-spike addition are commonly used because this method requires relatively little additional sampling and analytical effort (ASTM 1996, Holmes et al. 1999). However, our results show the single-spike method should not be used to correct NH_4^+ concentrations that have been estimated with high precision using multipoint calibration curves. Instead, we concur with others (Bower and Holm-Hansen 1980, Keith et al. 1983, Zhang et al. 1997) who have suggested that the method of standard additions should be used to estimate NH_4^+ concentrations when ME are large enough to affect our interpretation of NH_4^+ cycling. For example, in the case of seawater samples, standard additions can be prepared using artificial reagent-grade seawater (Bower and Holm-Hansen 1980) or low-nutrient water from the Sargasso Sea (Holmes et al. 1999) in place of using DI water to prepare standards. Overall, our study results suggest that changing how the ME and BF are measured will improve the accuracy and precision of the fluorometric NH_4^+ method.

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Literature Cited

AMINOT, A., D. S. KIRKWOOD, AND R. KÉROUEL. 1997. Determination of ammonia in seawater by indophenol-blue method: evaluation of the ICES NUTS I/C5 questionnaire. Marine Chemistry 56:59–75.

ASTM (AMERICAN SOCIETY FOR TESTING AND MATERIALS). 1996. D5810: standard guide for spiking into aqueous samples. American Society for Testing and Materials, West Conshohocken, Pennsylvania.

AVANZINO, R. J., AND V. C. KENNEDY. 1993. Long-term frozen storage of stream water samples for dissolved orthophosphate, nitrate plus nitrite, and ammonia analysis. Water Resources Research 29:3357–3362.

BADER, M. 1980. A systematic approach to standard addition methods in instrumental analysis. Journal of Chemical Education 57:703–706.

BOWER, C. E., AND T. HOLM-HANSEN. 1980. A salicylate-hypochlorite method for determining ammonia in seawater. Canadian Journal of Fisheries and Aquatic Sciences 37:794–798.

BRUCE, G. R., AND P. S. GILL. 1999. Estimates of precision in a standard additions analysis. Journal of Chemical Education 76:805–807.

BRZEZINSKI, M. A. 1987. Colorimetric determination of nanomolar concentrations of ammonium in seawater using solvent extraction. Marine Chemistry 20:277–288.

DODDS, W. K., AND J. C. PRISCU. 1989. Ammonium, nitrate, phosphate, and inorganic carbon uptake in an oligotrophic lake: seasonal variation among light response variables. Journal of Phycology 25:699–705.

EATON, A. D., AND V. GRANT. 1979. Sorption of ammonium by glass frits and filters: implications for analyses of brackish and freshwater. Limnology and Oceanography 24:397–399.

HOLMES, R. M., A. AMINOT, R. KÉROUEL, B. A. HOOKER, AND B. J. PETERSON. 1999. A simple and precise method for measuring ammonium in marine and freshwater ecosystems. Canadian Journal of Fisheries and Aquatic Sciences 56:1801–1808.

HOWARTH, R. W., G. BILLEN, D. SWANEY, A. TOWNSEND, N. JAWORSKI, K. LAJHTA, J. A. DOWNING, R. ELMGREN, N. CARACO, T. JORDAN, F. BERENDSE, J. FRENY, V. KUDEYAROV, P. MURDOCH, AND Z. ZHAO-LIANG. 1996. Regional nitrogen budgets and riverine N and P fluxes for the drainages to the North Atlantic Ocean: natural and human influences. Biogeochemistry 35:75–139.

KANG, H., E. H. STANLEY, AND S. S. PARK. 2003. A sensitive method for the measurement of ammonium in soil extract and water. Communications in Soil Science and Plant Analysis 34:2193–2201.

KEITH, L. H., W. CRUMMETT, J. J. DEEGAN, R. A. LIBBY, J. K. TAYLOR, AND G. WENTLER. 1983. Principles of environmental analysis. Analytical Chemistry 55:2210–2218.

KÉROUEL, R., AND A. AMINOT. 1997. Fluorometric determination of ammonia in sea and estuarine waters by direct segmented flow analysis. Marine Chemistry 57:265–275.

KIRCHMAN, D. L. 1994. The uptake of inorganic nutrients by heterotrophic bacteria. Microbial Ecology 28:255–271.

LODER, T. C., AND P. M. GLIBERT. 1977. Blank and salinity corrections for automated nutrient analysis of estuarine and sea waters. University of New Hampshire Sea Grant No. UNH-5G-JR-101 and Woods Hole Oceanographic Institution No. 3897. University of New Hampshire, Durham, New Hampshire. (Available from; http://nnsgl.gso.uri.edu/)

NETER, J., M. H. KUTNER, C. J. NACHTSHHEIM, AND W. WASSER-

MAN. 1996. Applied linear regression models. Irwin, Chicago, Illinois.

PETERSON, B. J., W. M. WOLLHEIM, P. J. MULHOLLAND, J. R. WEBSTER, J. L. MEYER, J. L. TANK, E. MARTÍ, W. B. BOWDEN, H. M. VALETT, A. E. HERSHEY, W. H. MCDOWELL, W. K. DODDS, S. K. HAMILTON, S. GREGORY, AND D. D. MORRALL. 2001. Control of nitrogen export from watersheds by headwater streams. Science 292:86–90.

SCHLESINGER, W. H. 1997. Biogeochemistry. 2nd edition. Academic Press, New York.

SOLÓRZANO, L. 1969. Determination of ammonium in natural waters by the phenolhypochlorite method. Limnology and Oceanography 14:799–801.

STEWART, B. M., AND P. A. W. ELLIOT. 1996. Systematic salt effects in the automated determination of nutrients in seawater. Water Research 30:869–874.

STRICKLAND, J. D. H., AND T. R. PARSONS. 1972. A practical handbook of seawater analysis. Bulletin of the Fisheries Research Board of Canada 167:311.

TAYLOR, J. K. 1989. Quality assurance of chemical measurements. 6th edition. Lewis, Chelsea, Michigan.

ZAR, J. H. 1984. Biostatistical analysis. 2nd edition. Prentice–Hall, London, UK.

ZHANG, J. Z., P. B. ORTNER, C. J. FISHER, AND L. D. MOORE. 1997. Determination of ammonia in estuarine and coastal waters by gas segmented continuous flow colorimetric analysis. Method 349.0, version 1.0. EPA/600/R-97/072. Office of Research and Development, US Environmental Protection Agency, Cincinnati, Ohio.

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