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The Development of a Fluorescence-based Reverse Flow Injection Analysis (rFIA) Method for

Quantifying Ammonium at Nanomolar Concentrations in Oligotrophic Seawater

by

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A thesis submitted in partial fulfillment
of the requirements for degree of
Master of Science
College of Marine Science
University of South Florida

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Date of Approval:
November 4, 2015

Keywords: Ammonia, Formaldehyde, Marine Environment, Nutrients, o-Phthaldialdehyde, Sulfite

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Abstract

The goal of this thesis was to adopt a reverse flow injection analysis (rFIA) technique to the fluorometric analysis of the reaction o-phthaldialdehyde (OPA) with ammonium, allowing accurate measurements of ammonium concentrations lower than the detection limit of the widely used indophenol blue (IPB) colorimetric method while accounting for the background fluorescence of seawater. Ammonium is considered an essential nutrient for primary productivity, especially in the nutrient depleted surface ocean where as the most reduced form of dissolved inorganic nitrogen, it is readily assimilated via metabolic pathways. Challenges in the quantification of ammonium require more sensitive analytical techniques for a greater understanding of the biogeochemical cycling of ammonium in the oligotrophic ocean. On-line and automated flow analysis techniques are capable of mitigating some of the challenges. Fluorescent-based methods out-perform colorimetric methods in terms of detection limits and sensitivity. Presented here is the development of an rFIA technique paired with an OPA-sulfite chemistry. For this method, a sulfite-formaldehyde reagent is mixed with the sample stream and then injected with the OPA reagent before being heated. Fluorescence is measured before and at the peak of the OPA injection, differentiating the background fluorescence from the analyte signal. Experiments to optimize reaction parameters and characterize the effects of salinity and potentially interfering species were conducted. The newly developed method offers a reasonable throughput (18 samples per hour), low limit of detection (1.1 nM) ammonium analysis technique with automatic background fluorescence correction suitable for oligotrophic seawater as a preferable alternative to the low sensitivity and high limit of detection IPB colorimetric method.

Chapter 1: Introduction and Objectives

The open ocean accounts for 90% of the ocean surface area while coastal zones and upwelling areas make up the remaining 9.9 and 0.1%, respectively (Schlesinger 2007). A distinguishing feature of the open ocean is the limitation of primary productivity in the euphotic zone by nutrient availability, frequently by nitrogen (Redfield et al. 1963). Fixed nitrogen is an essential nutrient for the formation of DNA, amino acids, and proteins. Of the inorganic forms of fixed nitrogen, ammonium is the most reduced and is readily assimilated into phytoplankton (Dortch 1990, Pilson 1998), leading to a depletion of the nutrient in surface waters when not replenished by autochthonous processes, N-fixation, atmospheric deposition, remineralization and photo-oxidation of dissolved organic matter, or by other allochthonous input from terrestrial sources or subsurface upwelling (Menzel and Spaeth 1962, Bushaw et al. 1996, Johnson et al. 2007, Schlesinger 2007, Johnson et al. 2008). Nitrogen, and specifically ammonium (NH_4^+), availability in the oligotrophic surface ocean is not widely measured due to the inherent challenges of these measurements. Improved measurement techniques as offered by the reverse flow injection analysis method presented here can enhance the understanding of the biogeochemical cycling of nitrogen that affects such a large portion of the ocean and its ecosystems (Harrison et al. 1996, Schlitzer et al. 2003, Johnson et al. 2007, Johnson et al. 2008).

Ammonium concentrations in the oligotrophic ocean are difficult to characterize due to sampling infrequency, analytical insensitivity of traditional measuring techniques, and the complications of the seawater matrix on analytical techniques (Šraj et al. 2014). The wide variety

of ammonium analysis tools available has resulted in a recent publication of a guide to assist in

the selection of a method for determining ammonium in different types of waters and diverse

applications from industrial waste to drinking water and environmental monitoring (Molins-

Legua et al. 2006). In oceanic applications, a colorimetric method based on the Berthelot

reaction of ammonium with phenol-hypochlorite, forming indophenol blue (IPB) product, is

most frequently used and has been modified for automated segmented flow spectrophotometric

analysis (SFA) (Solorzano 1969, Grasshoff and Johannsen 1972, Mantoura and Woodward 1983,

Kerouel and Aminot 1997). Other methods (Nessler, indothymol, ion selective electrodes, solid

phase extraction) have sensitivities and ranges suitable for ammonium measurement in other

applications (Molins-Legua et al. 2006). The IPB method is suitable for coastal and estuarine

applications (with consideration given to matrix effects), where ammonium concentrations can

be in the micromolar range, but it suffers from poor interlaboratory comparisons at low

concentrations (e.g. 56% relative standard deviation for a 0.34 μM sample reported from a

survey of 106 marine labs conducted by Aminot et al. (1997)) and an insufficiently low limit of

detection (LOD between .004 and 1.4 μM NH_4^+ (Šraj et al. 2014)) for seawater applications.

Increasingly, the fluorogenic reaction of *o*-phthaldialdehyde (OPA) with ammonium has been

explored in manual and automated systems for measurement of nanomolar ammonium

concentrations, as fluorometry can offer a 10- to 100-fold increase in sensitivity relative to

spectrophotometric methods. (Table 1) (Roth 1971, Genfa and Dasgupta 1989, Jones 1991,

Kerouel and Aminot 1997, Holmes et al. 1999, Masserini and Fanning 2000, Aminot et al. 2001,

Watson et al. 2005, Frank and Schroeder 2007, Amornthammarong and Zhang 2008, Bey et al.

2011, Ma et al. 2014). However, natural fluorescence of dissolved organic matter and the

reaction of OPA with primary amines and amino acids creates analytical challenges for fluorescent OPA detection of ammonium (Roth 1971, Masserini and Fanning 2000).

In addition to these analytical challenges for measuring the nanomolar ammonium concentration in surface seawater, ammonium contamination of samples also complicates measurements. Handling and preparation of reagents and ammonium samples exposes the samples to sources of errors including atmospheric contamination, sample bottle surface adhesion of ammonium, and uptake or release of ammonium during sample storage. Ammonium is particularly difficult to measure due to its propensity to adsorb onto glass and plastic surfaces and its high solubility in water (0.515 g NH_4^+ g^{-1} of water at 20 °C compared to 0.00172 g CO_2 g^{-1} water for carbon dioxide solubility (Van Der Linden 1983, Kerouel and Aminot 1997)). Volatile nitrogenous compounds present in laboratory environments or as part of the reagents for the analysis of other nutrients are common concerns among ammonium analysts (Aminot et al. 1997). Additionally, ammonium samples are subject to continued microbial utilization after collection and should ideally be analyzed within 3 hours of collection, or within 1 week if frozen (Zhang et al. 1997). Analysis of samples in automated systems, on-line or *in-situ*, bypasses many of these sources of error in the handling process. Therefore, automated flow analysis techniques paired with fluorometry offer the advantages of higher precision, lower contamination risk, lower detection limits, and higher sample throughput relative to manual techniques.

Table 1. Composite summary of colorimetric and fluorometric methods of ammonium detection.

Reference	Detection Method	Chemistry	Technique	Analytical Performance	Comments
Solorzano (1969)	Colorimetry λ_{abs} 640 nm	Phenol-hypochlorite, nitroprusside, citrate pH = 9.3, 22-27 °C	Manual	LOD 0.008 µM NH_4^+, linear to 714 µM NH_4^+; 1 hr sample^{-1}	Indophenol Blue (IBP)
EPA standard method (Zhang et al. 1997)	Colorimetry λ_{abs} 640 nm	Nitroferricyanide, dichloroisocyanuric acid, citrate, EDTA, pH = 13, 60 °C	SFA	LOD 0.02 µM NH_4^+, linear to 290 mM NH_4^+;60 samples hr^{-1}	(Solorzano 1969, Grasshoff and Johannsen 1972, Mantoura and Woodward 1983)
Roth (1971)	Fluorometry $\lambda_{ex}/\lambda_{em}$ 340/445 nm	OPA-2-mercaptoethanol, Tetraborate buffer pH = 9.5	Manual	Analysis of amino acids; 25 min sample^{-1}	Ammonia response << amino acid response
Jones (1991)	Fluorometry $\lambda_{ex}/\lambda_{em}$ 335/470 nm	OPA-2-mercaptoethanol, borate buffer pH = 9.5, 35 °C	GD-FIA	LOD < 1.5 nM NH_4^+, linear to >2,000 nM NH_4^+, 30 samples hr^{-1}	Fresh and seawater
Masserini and Fanning (2000)	Fluorometry $\lambda_{ex}/\lambda_{em}$ 335/470 nm	OPA-2-mercaptoethanol, borate buffer pH = 9.5, 35 °C	GD-FIA	LOD ~1 nM NH_4^+, 18 samples hr^{-1}	Modification of Jones (1991) Shipboard, seawater
Genfa and Dasgupta (1989)	Fluorometry $\lambda_{ex}/\lambda_{em}$ 360/420 nm (365/425 nm ideal)	OPA-sulfite, phosphate buffer pH = 11.0, 85 °C	FIA	LOD 20 nM NH_4^+, linear: 0.25-20 µM NH_4^+, 25 samples hr^{-1}	Rain water
Kerouel and Aminot (1997)	Fluorometry $\lambda_{ex}/\lambda_{em}$ 365/425 nm	OPA-sulfite, tetraborate buffer pH = 9-9.5, 75 °C	SFA	LOD 1.5 nM NH_4^+, linear up to 12 µM NH_4^+; 20 samples hr^{-1}	Estuarine and seawater
Holmes et al. (1999)	Fluorometry $\lambda_{ex}/\lambda_{em}$ 350/420 nm	OPA-sulfite, tetraborate buffer, ambient temp.	Manual	LOD 31 nM NH_4^+; linear up to 6.2 µM NH_4^+; 3 hr sample^{-1}	Field and lab protocol
Aminot et al. (2001)	Fluorometry $\lambda_{ex}/\lambda_{em}$ 370/418 nm	OPA-sulfite Tetraborate buffer, 30 °C	FIA/stop-flow	LOD 30 nM NH_4^+; linear up to 50 µM NH_4^+; 9 samples hr^{-1}	*In-situ;* coastal, estuarine, fresh water
Watson et al. (2005)	Fluorometry $\lambda_{ex}/\lambda_{em}$ 310/390 nm	OPA-sulfite phosphate buffer pH = 9.3, 70 °C	GD-FIA	LOD 7 nM NH_4^+, linear to 4 µM NH_4^+; 30 samples hr^{-1}	Combination of GD-FIA with OPA-sulfite chemistry Seawater
Amornthammarong and Zhang (2008)	Fluorometry $\lambda_{ex}/\lambda_{em}$ 362.5/423 nm	OPA-sulfite-formaldehyde, no buffer, pH = 11, 65 °C	FIA/cFIA	LOD 1.1 nM NH_4^+, linear to 600 nM NH_4^+ 8 samples hr^{-1} (3600 hr^{-1} CFA)	Shipboard, seawater
Bey et al. (2011)	Fluorometry $\lambda_{ex}/\lambda_{em}$ 370/427 nm	OPA-sulfite, tetraborate buffer, pH = 9.4, 65 °C	FIA	LOD < 5 nM NH_4^+, linear to 1 µM NH_4^+; 12 samples hr^{-1}	Field application, seawater
This method	Fluorometry $\lambda_{ex}/\lambda_{em}$ 365/425 nm	OPA-sulfite-formaldehyde, borate buffer pH = 9.5, 60 °C	rFIA	LOD 1.1 nM NH_4^+, linear to 1000 nM NH_4^+, 18 samples hr^{-1}	Oligotrophic seawater

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This thesis research seeks to continue to improve the methodology for the quantification of ammonium in oligotrophic seawater at nanomolar concentrations. OPA chemistries were evaluated here for salinity and matrix effects, potential amine and amino acid interferences, and dissolved organic matter fluorescence. A reverse flow injection analysis (rFIA) technique was employed to account for the background fluorescence of ambient dissolved organic material in samples.

1. Ammonium Analysis

Recent summaries of ammonium analysis techniques include the previously discussed guide by Molins-Legua et al. (2006), a comparison of flow-based techniques (IPB, gas-diffusion, OPA) by Šraj et al. (2014), and a comprehensive investigation of nanomolar determination of nutrients by Ma et al. (2014). Fluorescent OPA-based methods have a relatively low limit of detection (30 nM NH_4^+) suitable for seawater analysis compared to IPB (0.1 μM NH_4^+). Though costs did not vary widely across examined methods, the modified Roth's OPA technique had the advantage of lowest reagent toxicity and low waste production over the other techniques, whereas IPB had high toxicity associated with the phenol reagent and high volume waste generation. Though overall the guide is of little use for the rFIA OPA research, it highlighted the attractiveness of the OPA chemistry as an alternative means of ammonium detection to IPB or other methods listed.

Šraj et al. (2014) evaluated existing ammonium/ammonia flow analysis techniques from a regulatory and environmental monitoring perspective. At pH > 9.75, ammonia existed predominantly in its more toxic form, NH_3. In natural waters at pH < 8.75, ammonium (predominantly NH_4^+) can stimulate production, and in eutrophic conditions may cause algal

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blooms with ramifications throughout ecosystems. The need for rapid, sensitive analytical methods to measure ammonium as an ecological stressor (e.g. acute toxicity for the oyster mussel is 23.42 total ammonium nitrogen per liter, ~2.2 μM NH_4^+ as published by the U.S. Environmental Protection Agency (US 2013))and water quality indicator has been met with the development of flow analysis techniques for the colorimetric IPB methods (2 segmented flow analysis, SFA, 6 FIA), gas diffusion techniques (4 chemistries, 9 flow techniques), and fluorometric methods (2 OPA chemistries with 13 flow techniques employed) examined by Šraj et al. (2014). Differences between the flow techniques were discussed: air segmentation in SFA suffered slow start-up times and generated more waste but was widely used for ocean chemistry analysis; FIA used controlled dispersion of reagents in an non-segmented stream for reproducibility, portability, and higher sample throughput (Šraj et al. 2014). Additional flow injection systems were reviewed as each system attempted to improve some aspect of the analysis. Relevant to the progress of the rFIA technique for ammonium, the classical IPB SFA and most of the OPA-based chemistries for fluorometric FIA were further investigated in this research.

Ma et al. (2014) examined 23 methods for ammonium analysis at nanomolar concentrations in seawater, of which 15 were fluorometric methods using OPA with either of two reducing agents (2-mercaptoethanol or sulfite), totaling 3 gas-diffusion FIA (GD-FIA), 1 SFA, 3 FIA/continuous flow analysis (CFA), 2 batch analyzers, and 1 of each sequential injection analysis, multi-pumping flow analysis, ion chromatography, well-microplate, solid phase extraction/batch analyzer and one manual technique (Ma et al. 2014). Eight of the OPA FIA/CFA techniques were investigated further for their characteristics and suitability for rFIA application.

The OPA chemistries have been divided into three groups by their reducing agent: 2-mercaptoethanol, sulfite, and sulfite-formaldehyde. The first use of OPA for measurement of ammonium was by Roth in the analysis of amino acids (1971). It was found that 2-mercaptoethanol more favorably produced fluorogens in the reaction with a suite of amino acids. Ammonium produced fluorescent intensities not much greater than the blank. Jones (1991) designed a sensitive (LOD 1.1 nM) flow injection analysis system using gas diffusion (GD-FIA) across a Teflon membrane to enhance the selection of the ammonium ion over amino compounds. An acidified carrier stream prevented ammonium ions present in the carrier from converting to ammonia and contributing to the background signal. Once the sample was injected, ammonium ions were converted to ammonia in sodium hydroxide/sodium citrate solution and subsequently driven across the membrane by a pH gradient into the OPA-2-mercaptoethanol receptor stream at a pH of 9.5. Ammonium reacted with the OPA in the presence of the reducing agent before detection by a fluorometer (Jones 1991). Of the fluorescent OPA methods, the GD-FIA technique with the OPA-2-mercaptoethanol chemistry (Jones 1991) is the most well-known, modified, and tested (Harrison et al. 1996, Aiken et al. 1997, Aiken and Woodward 1998, Masserini and Fanning 2000, Woodward and Kitidis 2000, Jickells et al. 2003, Robinson and Woodward 2003, Holligan et al. 2005, Woodward and Harris 2008). The Atlantic Meridional Transect cruises have recently relied upon a modification of this technique and only performed analysis with the colorimetric segmented flow auto-analyzers when ammonium concentrations reached greater than1 micromolar (Aiken et al. 1997, Aiken and Woodward 1998, Woodward and Kitidis 2000, Jickells et al. 2003, Robinson and Woodward 2003, Holligan et al. 2005, Woodward and Harris 2008). A particular modification of the GD-FIA OPA-2-mercaptoethanol

chemistry has been developed for field applications and served as the control technique for this study (Masserini and Fanning 2000).

The replacement of 2-mercaptoethanol with sulfite as a reducing agent enabled Genfa and Dasgupta to detail advantages of the chemistry for both absorptive and fluorescent detection methods of ammonium in fresh water and atmospheric studies. A FIA technique was used to achieve a 20 nM NH_4^+ limit of detection, but the analysis experienced curvature in its calibration below 250 nM, which the authors attributed to enhanced salt effects on the blanks and lower standard concentrations. Considerable discrimination for ammonium against amino acids (16 to 588 times the 10 µM relative signal of 11 amino acids) were also observed (Genfa and Dasgupta 1989). Kerouel and Aminot (1997) deviated from the previous technique in the use of a phosphate buffer to allow for seawater analysis without the precipitation of magnesium hydroxide and calcium phosphate from the matrix. Using a fluorometric SFA technique on the OPA-sulfite chemistry, they achieved a LOD of 1.5 nM NH_4^+. The authors enumerated advantages over the SFA IPB technique in terms of simplicity of operation, low interference ($<$ 0.5% for 16 N-organic standards at 5 µM), and low salt effects ($<$ 3%) (Kerouel and Aminot 1997). The group continued with a manual technique for field analysis using the same chemistry with a LOD of 31 nM paying particular attention to sample collection and handling techniques, though matrix effects reduced response for seawater by 17% compared to a similar 1 µM sample in freshwater (Holmes et al. 1999). Another technique further expanded the use of the OPA-sulfite chemistry from the same group with stop flow analysis (SFIA) for approximately 20-30 nM LOD. Interestingly, the salinity effects deviated in the opposite direction at low salinities (freshwater standards had a reduced response relative to standards prepared in seawater (Aminot

et al. 2001)). SFIA offered advantages in terms of reduced reagent consumption and lower power requirements for the development of a remote *in-situ* system.

As a revisit to the GD-FIA technique, Watson et al. (2005) employed the sulfite-OPA chemistry with a detection limit of 7 nM ammonium. In his discussion on the consistency of pore size for membrane diffusion, the troubles with membrane separation reemphasized Van Der Linden's (1983) observations on ammonia diffusion with Nessler's reagent including manufacturing defects, susceptibility to clogs and ruptures for polytetrafluoroethylene (PTFE) plumbers' tape, fragility of the higher quality PTFE membranes, and the ability for methylamines to diffuse across the membrane (Van Der Linden 1983, Watson et al. 2005).

The third OPA chemistry technique relied upon the addition of formaldehyde to the sulfite reagent, forming a stable α-hydroxymethanesulfonate complex (Olson and Hoffmann 1989) and resulted in a lowered reagent blank contribution, increased reaction response of OPA to ammonium, and improved selectivity for ammonium. Amornthammarong and Zhang (2008) developed a shipboard FIA system capable of 1.1 nM LOD. Additionally, through continuous flow analysis (CFA), they claim a very high sample throughput at 3600 samples hr^{-1}. Reagent blanks were analyzed and low nutrient seawater (LNSW) was found to be inappropriate for use as a blank for the technique due to the potential for increasing ammonium concentrations in the LNSW when stored for long periods of time. Potential interferences were reported at higher relative responses than other techniques, but the concentrations that were examined were more environmentally applicable (1 µM amino acid concentration compared to 5 and 10 µM previously discussed). Further adaption of the formaldehyde addition to the OPA-sulfite chemistry by this group has applied it to autonomous batch analyzers and portable batch analyzers (ABA) with 1 and 10 nM LOD, respectively (Amornthammarong et al. 2011,

Amornthammarong et al. 2013). The ABA techniques sacrifice sample throughput for reduced

reagent and sample consumption and smaller instrument size. Due to the improvements offered

in terms of sensitivity and selectivity, the OPA-sulfite-formaldehyde (Amornthammarong and

Zhang 2008) combination was ultimately chosen for adaption to the rFIA technique in this study.

2. Flow Injection Analysis and Reverse Flow Injection Analysis

      Flow injection analysis detects an analyte through dispersion patterns of a precise

quantity of sample injected into a continuously flowing reagent stream, allowing for the

measurement of incomplete but reproducible reactions that can be reliably quantified. Růžička

and Hansen (1978) provided a set of rules for FIA theory. The rule for minimizing flow

dispersion and dilution of the sample by limiting manifold length, however, should be broken in

the case of reverse flow injection analysis. In rFIA, the sample is part of the carrier stream while

a reagent is injected; this approach maximizes dispersion of the reagent and increases the

reaction time with the analyte, as discussed by Masserini and Fanning (2000)in a rFIA technique

for nanomolar nitrate and nitrite measurements. In this study, it was found that leachate from

Tygon ® tubing and dissolved organic material naturally present in seawater fluoresces at similar

wavelengths for nitrate and nitrite analysis and can account for some contribution to the analyte

signal (Masserini and Fanning 2000). Reverse flow injection analysis technique removed the

contribution of the background fluorescence without having to run a separate check for each

sample. Advantages of rFIA include high selectivity for the analyte and low reagent

consumption. The rFIA technique has also been developed for phosphorous at nanomolar

concentrations with a long path-length liquid waveguide capillary cell (Ma et al. 2009). Though

technique has also been used to analyze ammonium spectrophotometrically using the IPB

chemistry, the limits of detection were high (16.6 μM) (Bucur et al. 2006). rFIA has been paired

in this research with the OPA-sulfite-formaldehyde combination as a new method for the

analysis of ammonium at nanomolar concentrations in seawater.

3. Objectives

The OPA-based approaches discussed above provided the basis for this study, which has

several objectives in order to measure ammonium in seawater at nanomolar concentrations. 1. By

employing a fluorescence method to measure ammonium, lower detection limits than the widely

used IPB colorimetric method can be achieved. 2. rFIA enables the correction for the background

fluorescence of dissolved organic material in samples. 3. The OPA-sulfite-formaldehyde

chemistry selects for ammonium against potentially interfering species, avoiding the need for a

membrane separation technique. With chemical optimization and through deliberate testing of

the manifold parameters to select the most reproducible setting, the new method presented here

provides a reliable means to measuring naturally low ammonium concentrations in oligotrophic

surface seawater.

Chapter 2: A Reverse Flow Injection Analysis Technique for the Fluorometric Determination of

Nanomolar Ammonium Concentrations in Seawater

1. Background Information

The inorganic nitrogen compounds nitrate, nitrite, and ammonium are essential nutrients

for primary productivity. In the oligotrophic surface ocean, these compounds are present in such

low concentrations that they can limit productivity (Sarmiento and Gruber, 2006).

Concentrations of ammonium in the oligotrophic surface ocean are not well characterized

because they frequently occur at or below the detection limit of the widely used indophenol blue

colorimetric analytical method (Solorzano 1969): 0.1 micromolar (μM) ammonium (Molins-

Legua et al. 2006). As the oligotrophic open ocean accounts for 90% of the total surface ocean,

the accurate measurement of ammonium in these waters is critical to our understanding of these

ecosystems (Schlesinger 2007).

Interest in improving the analysis of ammonium has resulted in the development of

sensitive fluorometric analytical techniques using the reaction of o-phthaldialdehyde (OPA) with

ammonium to lower the detection limit to the nanomolar (nM) level, summarized recently by Ma

et al. (2014). Among these developments, the OPA-2-mercaptoethanol chemistry initially

investigated for ammonium and amino acid analysis by Roth (1971) was improved upon with

modifications for rapid and more sensitive analysis of ammonium in oligotrophic systems by the

diffusion of ammonia gas through a PTFE membrane separation technique before flow injection

analysis (FIA) is applied (Aoki et al. 1983, Jones 1991). Sulfite was explored as an alternative to

the 2-mercaptoethanol reductant for measuring ammonium in rainwater (Genfa and Dasgupta 1989). The sulfite chemistry was subsequently applied to fluorometric ammonium measurement during segmented flow by Kerouel and Aminot (1997), achieving an improvement in the reagent stability from less than a week for 2-mercaptoethanol to several weeks for the OPA-sulfite based chemistry. The addition of formaldehyde to the sulfite reagent enabled greater protection against potentially interfering amino compounds while also lowering the blank signal, increasing the sample signal to noise ratio, and further stabilizing the reagent in an FIA system (Amornthammarong and Zhang 2008).

A problem common to all fluorescent ammonium methods is that natural dissolved organic material (DOM) in seawater samples fluoresces at wavelengths of the OPA-ammonium complex, contributing to a larger background fluorescent signal (Holmes et al. 1999, Bey et al. 2011). Without dedicated background signal correction estimates, the contribution of the background signal can raise the apparent ammonium sample signal by as much as 100 nanomolar equivalents (Aminot et al. 2001). Reverse flow injection analysis contrasts from conventional FIA in that instead of a continuous reagent stream with sample injection, the sample is first allowed to flow unreacted before injecting the OPA reagent. The detector thus produces a broad flat signal that defines the background fluorescence of the sample before the OPA reagent is added into the sample stream to produce the analyte signal. The ammonium concentration can be calculated from the difference between the analyte signal and background fluorescence. The contribution of background fluorescence in a sample prepared in LNSW compared to one prepared in DIW, as well as the OPA reagent contribution, is depicted in Figure 1, which illustrates that failure to correct for background fluorescence could potentially overstate the

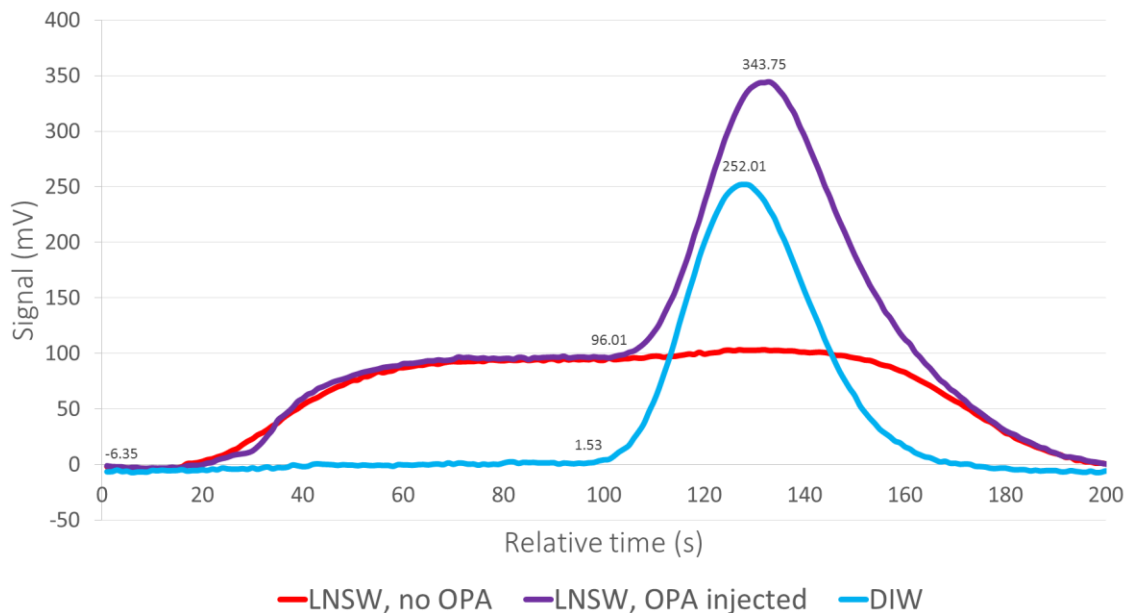ammonium concentration by ~100 nM calculated from a calibration curve of approximately 1

nM/mV.

Figure 1. rFIA Background Fluorescence The response of a sample prepared in de-ionized water (DIW, blue) and low nutrient seawater (LNSW, purple) shows the background fluorescence from dissolved organic matter (DOM) present in seawater after the mixed sample-sulfite stream enters the fluorometer between 20-100 seconds and before OPA is injected and mixed during the 100-160 s timeframe. DIW wash subsequently flushes the manifold. The same sample in LNSW without OPA injected (red) shows that the contribution of DOM persists through the OPA-sample reaction timeframe of the sample shown in purple.

Here we present a reverse flow injection analysis (rFIA) technique that capitalizes on the benefits

of the OPA and sulfite-formaldehyde reagents, namely sensitivity to and selectivity for

ammonium to achieve low detection limits as well as a technique for correcting for the

fluorescence of DOM in each seawater sample. Characterization of the reaction settings, matrix

effects, and potential interferences that can influence both the background fluorescence and the

response of the method were explored. A summary of results of the standard calibration curves

tracking the progress of the rFIA technique is included in the appendix Table A1.

2. Materials and Methods

2.1 Reagents

All reagents were prepared in either de-ionized water (DIW) polished to > 18.2 MΩ-cm in a Millipore Milli-Q system or in a 0.25 M borate buffer. To prepare the buffer, boric acid (Fisher ACS grade) was dissolved in the same DIW and adjusted to a pH of 9.50 with 10 N sodium hydroxide (Fisher ACS grade). Glassware used for reagent and calibration standard preparations were thoroughly washed with DIW, rinsed with 10% HCl (Fisher trace metal grade), and rinsed three times with DIW rinses and three rinses of the solution to be used in order to minimize atmospheric ammonium contamination (Zhang et al. 1997).

2.1.1 rFIA Reagents

Ortho-phthaldialdehyde (Sigma ≥ 97% HPLC) was dissolved at a ratio of a tenth of a gram of OPA per 1 mL methanol (Sigma-Aldrich CHROMASOLV® gradient grade for HPLC, ≥99.9%) to minimize the contribution of methanol to the OPA blank signal. Aliquots of dissolved OPA in methanol were mixed into 1 L volumes of the 0.25 M borate buffer to make five different solutions with rFIA OPA reagent concentrations at 5 mM intervals from 5-25 mM. A 5 mM formaldehyde (HCHO) solution was prepared by dissolving 750 µL of HCHO stock (Fisher ACS grade 37% wt.) in 2 L of the 0.25 M borate buffer. To the HCHO solution, 2.52 g sodium sulfite (Fisher ACS grade) was added and shaken until dissolved to make a 10 mM SO_3^{2-}—5 mM HCHO reagent in the 0.25 M borate buffer. The resulting sulfite-formaldehyde reagent was found to be stable for several months when stored in dark, high density polyethylene (HDPE) containers at room temperature.

2.1.2 Standards

A primary standard solution composed of 2.4995×10^{-2} M ammonium (ACS reagent grade ammonium chloride) was used to make a secondary standard (2.5072×10^{-4} M ammonium) for daily preparation of calibration standards at 0, 250, 500, and 1000 nM using gravimetrically calibrated volumetric flasks and pipets. Calibration standards were made in unfiltered and aged low-nutrient seawater (LNSW) collected from offshore Gulf of Mexico surface waters. Calibration standards of the same nutrient concentrations were also made in DIW and aged LNSW diluted with DIW for the comparison of the slopes of the calibration curves at a range of salinities (S) between 0 (DIW) and 38 (LNSW) for matrix effect investigation. The rFIA OPA reagent and calibration standards were contained in 240 mL polyethylene collapsible sleeves (Playtex) sealed within a polyvinyl chloride (PVC) housing by a silicone O-ring and PTFE Omnifit™ valve and adapter. Replicate standards and rFIA OPA reagent were drawn from the sleeves through flanged PTFE fittings with manual valves to minimize atmospheric contamination.

2.1.3 Interference Solutes

Primary amine and amino acid standards (summarized in Table 1) were made to ~2.5 x 10^{-4} M in DIW from stocks of ethanolamine (Arcos 99%), mono-methylamine hydrochloride (Aldrich), L-serine (Sigma SigmaUltra >99% TLC), L-tyrosine (Sigma), L-alanine (Sigma minimum 98%), L-leucine (Sigma minimum 98%), L-phenylalanine (Sigma minimum 98%), L-tryptophan (Sigma, sigma grade), B-alanine (Aldrich 99% assay), and glycine (Sigma Ultra >99% titration). Ammonium high standards prepared in DIW were spiked with each solutions of amino acids to make a solution that were 1 µM in ammonium and 1 µM in an amino acid for interference characterization. Additionally, DIW blanks were spiked with each primary amine or

amino acid to make 2 µM amino compound solutions that contained no added ammonium. Glass vials (30 mL screw cap) for these solutions were soaked in 10 % HCl overnight and rinsed with DIW prior to use.

2.1.4 GD-FIA Reagents

Reagents for the GD-FIA control technique were made with a modification of Jones (1991), doubling the OPA concentration in the OPA reagent to 1.5 mM. The 1.5 mM GD-FIA OPA reagent was made using 200 mg OPA (Sigma ≥ 99% HPLC) dissolved in 2 mL methanol and added into 1 L 0.25 M borate buffer and along with 500 µL 2-mercaptoethanol (Sigma). The reagent was aged for at least 48 hours to allow for a decay of the background signal and was stable for an additional 5 days before the reagent to become unusable. The 2% sulfuric acid (Fisher ACS plus) carrier was made fresh daily, while the 0.7 M NaOH-sodium citrate solution made from 200 g sodium citrate (Fisher certified) and 18.0 g sodium hydroxide (Fisher ACS) dissolved in 950 mL of DIW was stable.

2.2 Manifold Description

The fluorometric ammoniuim analyzer consisted of a manifold for the experimental rFIA technique and an additional manifold for the GD-FIA technique that served as a control. A schematic of the manifolds modified from the 3-channel nitrogen sensor developed by Masserini and Fanning (2000) is depicted in Figure 2. The upper panel depicts the rFIA technique for ammonium analysis. The lower panel shows the GD-FIA technique used as a control. For both manifolds, a 16 channel peristaltic pump (Ismatec IPC-16) drew the wash, sample/standards and reagent solutions from their reservoirs at 25% pump speed through PVC pump tubes. Pump tubes were fitted onto barbed connectors with ¼"-28 threaded zero-dead-volume flange fittings. The remainder of the analytical manifolds consisted of 0.8 mm inner diameter PTFE tubing. A

series of manual 2-way valves (Hamilton HV Plug Valve) at the beginning of the manifolds

allowed for selection between blanks, calibration standards, and samples for analysis drawn from

the collapsible sleaves as an alternative to the use of an autosampler probe drawing from glass
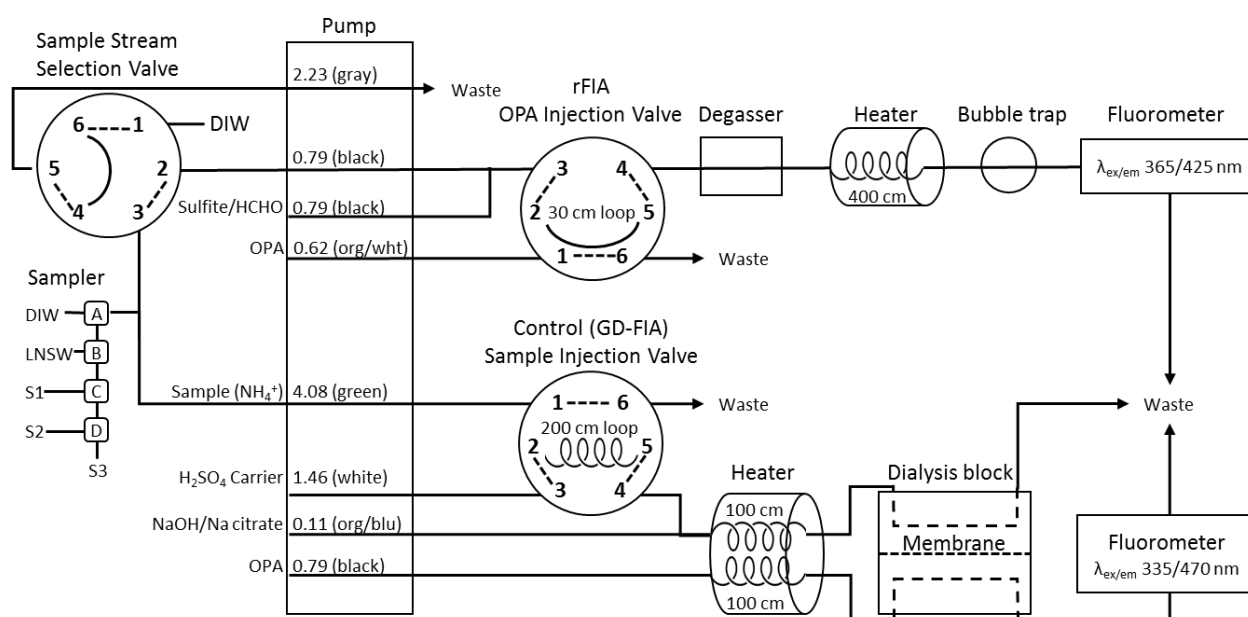
vials.

Figure 2. **rFIA and control (GD-FIA) manifold.** Manifold schematic for the reverse flow
injection analysis technique (rFIA, upper) and the control gas-diffusion flow injection analysis
technique (GD-FIA, lower). Standards are drawn from the PVC housed PETE collapsible
sleeves, manually selected with two way valves labeled A-D. An autosampler can also be fitted.
The sample stream selection valve alternates between drawing sample and DIW wash. Flow rates
within the pump diagram are in mL/min. The OPA injection loop is ~0.15 mL in volume. The
OPA injection valve loads the injection loop with OPA and adds the contents of the loop to the
carrier stream upon actuation. Dashed lines within the valves indicate the "sample" and "inject"
positions. The mixing coil in the heater is approximately 2 mL in volume. The overall length of
the rFIA system is ~5 m. The control manifold is described in Masserini and Fanning (2000).

2.2.1 rFIA Experimental Manifold

During the "wash" step, DIW was directed through the sample stream selection valve (2-

position 6-port valve, VICI Cheminert) to the manifold while the analyte stream was directed

from the sampler tee fitting, through the valve and pump to waste. The sample stream selection

valve in the "sample" position redirected the analyte stream to the manifold where it mixed with

the sulfite-formaldehyde reagent at a second tee fitting, timed to prevent the introduction of an intersample air bubble. The mixed analyte stream was directed through the OPA injection valve (2-position 6-port, VICI Cheminert) in the "load" position to the heater. Concurrently, the rFIA OPA reagent stream was directed through the 0.15 mL injection loop and to waste. In the "inject" position, the rFIA OPA reagent within the injection loop was incorporated within the mixed analyte stream and was directed to the heater.

Temperature control was achieved through a PID controller (Watflow Anafaze CLS204) which limits power to the heater to keep temperature within $\pm$ 0.1 °C. To minimize bubble formation at temperatures above 60 °C, the analyte stream was degassed (Alltech On-line Degassing System 2000) before passing through 400 cm of PTFE tubing coiled around a heating block assembly within an insulated enclosure (VICI® Valco Instruments Co. Inc. HVE2). A PTFE bubble trap (Omnifit) removed gas bubbles from the analyte stream formed in the heating process. The Hitachi L-7480 fluorometer equpped with adjustable excitation and emission monochrometers, a photomultiplier tube, and a 40 µL quartz flow cell served as the detector. Excitation and emission wavelengths were set to 365 and 425 nm, respectively. The photomultiplier tube was set at its highest sensitivity "1," and signal averaging was 2 seconds.

2.2.2 GD-FIA Control Manifold

The modified version of a GD-FIA technique (Jones 1991) was run simultaneously for experiments as a control method for comparative analysis of the rFIA performance. Upstream of the sample selection valve, a tee was fitted to split the analyte stream to both rFIA and the GD-FIA control manifolds. The manifold and operational procedure of the GD-FIA control technique were unchanged from their original configuration with the exception of a doubling of the OPA concentration (Masserini and Fanning 2000).

2.3 Procedure

Concord V2.8 software (A.I. Scientific) was programmed to run the AIM 1250 autosampler MkII relays (A.I Scientific), which then control the valve actuators (VICI®). At 0 seconds, the sample selection valve was set to "wash" and the rFIA OPA injection valve set to "load" to purge the manifold and rFIA OPA injection loop of any residual analyte. After 60 seconds of DIW wash, the sample selection valve was actuated to the "sample" position during which a background fluorescence signal of the mixed analyte (sample and sulfite) without OPA was established. At 185 seconds the rFIA OPA injection valve was actuated to "inject," allowing the reaction between the 0.15 mL rFIA OPA reagent and the mixed analyte to occur through the heating coil. After an additional 15 seconds, both valves were switched back to the respective "wash/load" position. The total elapsed time for each sample was 200 seconds. 10% HCl was used to flush the instrument manifold whenever baseline stability degradation was observed.

A Universal Data Collection (UDC) data acquisition package developed in-house on National Instruments LabView™ software (Masserini 2005) recorded the signal from the fluorometer, identifying the background fluorescence shoulder height and the uncorrected peak height of the rFIA OPA-analyte reaction. The peak heights were corrected for background fluorescence due to DOM in seawater samples and standards by subtracting the shoulder height from the uncorrected peak height value. Reagent blank corrections were applied to samples and standards by subtracting the average of the DIW replicates with no background fluorescence. The average of triplicate un-spiked LNSW blanks corrected for background and reagent (DIW blanks) fluorescence contributions was subtracted from standards to account for analyte present in the solvent matrix. Standard calibration curves were constructed and recorded by the UDC software when running standards. The software calculated the linear least squares regression of

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the corrected standards and displayed the results in a graphic user interface (Figure 3). The

corrected peak heights of samples were then multiplied by the slope of the curve (units in

nM/mV) to produce actual ammonium concentrations in the samples. The UDC software

allowed for discrete analysis of unknown samples, automatically correcting for background

fluorescence and reagent blank values. Raw data and processed data were stored in separate text

files (.raw, .pkv, .cnc). Similar calculations were performed for the GD-FIA technique with the

exception of the background fluorescence correction.
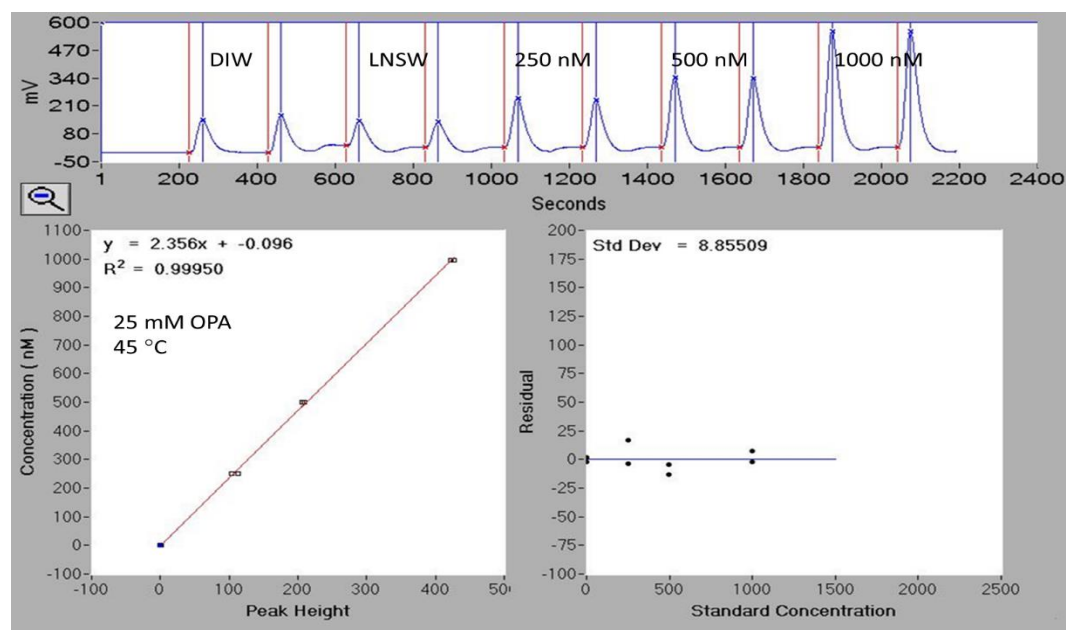
Figure 3. Universal Data Collection Ammonium Calibration Curve Display. Merged display of the Universal Data Collection (UDC) software graphic user interface displaying the raw data (top), linear regression (bottom left) and residuals (bottom right) of a standard calibration with duplicate standards made in low nutrient seawater using 25 nM OPA at 45 °C. The lower panels become available in separate windows post-processing. Peaks of the 250, 500, and 1000 nM ammonium standards are indicated by blue vertical lines in the upper panel, background fluorescence by red vertical lines. The equation for the calibration curve (in nM/mV) and the r^2 value for the linear regression are inset in the lower left panel (y = 2.356 nM/mV x – 0.096 nM; r^2 = 0.9995). The residuals (standard deviation of residuals = 8.9 nM) are inset in the lower right panel.

3. Results for the rFIA Ammonium Method

The results of even the initial rFIA reaction parameters (25 mM OPA, 45° C) displayed in Figure 3 suggested that these conditions are suitable for sub-micromolar ammonium measurements. Values for the average peak heights of LNSW were 162.5 ± 12.5 mV with a limit of detection (LOD) of 7 nM, calculated as three times the standard deviation of the blanks times the slope 2.356 nM/mV (IUPAC 1997). However, the sensitivity under these conditions was low and a 34% difference between the DIW and LNSW blank values was observed where DIW appeared to have greater ammonium present than LNSW, both of which were further examined. Saturation of the fluorometer occurs at signals beyond 1500 mV, and temperature, reagent concentration, and flow rates were investigated to produce a 1000 mV signal for the high standard while minimizing the signal of the blank. A calibration curve slope of 1 nM/mV was determined to be a reasonable compromise between the upper limit of the instrument response, resolution across the dynamic range, and sensitivity.

3.1 Reaction Conditions

Reaction temperature and rFIA OPA concentrations strongly influenced analytical response. Flow rates of the sample and sulfite streams also influenced system performance by affecting the mixing and dispersion of the sample and reagents and the duration of the analyte reaction within the heating manifold. These three parameters were systematically checked for optimal conditions. Discrete analysis of LNSW blanks and standard curves were run at 42.5, 45, 50, 55, 60, 65, and 70 °C and for OPA concentrations of 5, 10, 15, 20, and 25 mM. Results of the effects of temperature and rFIA OPA concentration are displayed in Figure 4. The response of DIW reagent blanks was explored at 60 °C for the 5-25 mM range of rFIA OPA concentrations

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(inset to Figure 4). The 55-65 °C temperature range and 10-15 mM OPA concentration range

meet the desired conditions for optimal method performance for sensitivity and dynamic range.
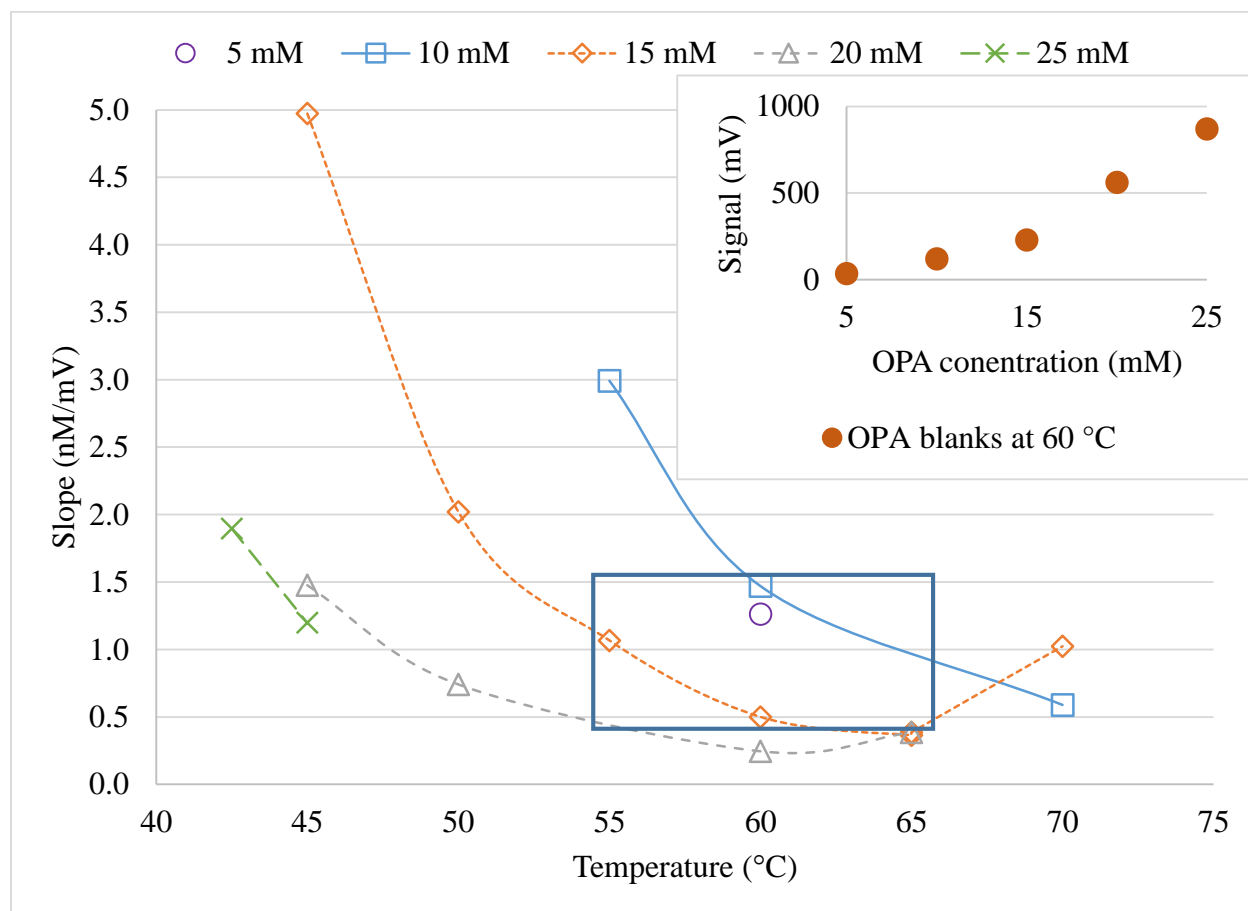

Figure 4. Slope variation with respect to temperature and OPA concentration. Standard calibration curve slopes (in nM/mV) were calculated from ammonium standards prepared in low nutrient seawater (LNSW) and run by the reverse flow injection analysis (rFIA) technique at temperatures between 42 and 70 °C and 5-25 mM OPA concentrations. The combination of higher temperature and OPA concentrations saturated the fluorometer, therefore slope interpolations were used from the blanks and lower concentration standards that were not off scale. Similarly, low temperatures and low OPA concentrations would not produce meaningful calibration curves, hence slopes at some concentrations were not directly comparable (i.e. 25 mM OPA and 10 mM OPA). Emphasis was placed on reactions that achieved a reasonable compromise between resolution and sensitivity identified as a slope of 1 mV/nM. The blue rectangle encloses conditions that were close to the desired sensitivity and were within the operating thresholds of the instrument: temperatures between 55 and 65 °C and 5-15 mM OPA. The inset indicates OPA reagent DIW blank values increased substantially with increasing OPA concentrations at 60 °C.

3.2 Reagent Blanks and Matrix Effects

DIW blanks and LNSW blanks were analyzed at the beginning of every calibration to provide a value for the reagent blank and matrix correction for standards and samples. Discrepancies in the blank values of up to 34% difference between DIW and LNSW (as indicated in the summary of Figure 3 in the second row of Table A1) led to an investigation of the matrix effects on the rFIA technique: standards were made in LNSW and in LNSW diluted with DIW to cover a full salinity range (S = 2.5-38). The results of these matrix effects tests are displayed in Figure 5 and were obtained at 60 °C with 5 mM OPA and achieved calibration curve slopes across the salinity range at approximately 1 nM/mV with low reagent blank fluorescence values. The slope of the regression in Figure 5 was 0.002 nM/mV S^{-1} with a coefficient of determination (r^2) at 0.6624. The percent error of the calibration curve slopes of the dilutions compared to the slope of standards in LNSW were calculated with the equation 1.

$$\text{Equation 1: } \frac{Slope_{dilution} - Slope_{LNSW}}{Slope_{LNSW}} \times 100\%.$$

Percent error did not exceed the 5.9% of undiluted LNSW standard calibrations conducted two days apart (Table A1: rows 3-10, column 6). Salinity had minimal effect on the calibration curves at the 5 mM OPA concentration. The discrepancy in the DIW reagent blank and the solvent matrix blanks also decreased from approximately 20% to 1.2% as the salinity of the solvent matrix blank also decreased (Table A1: rows 3-10 column 15).
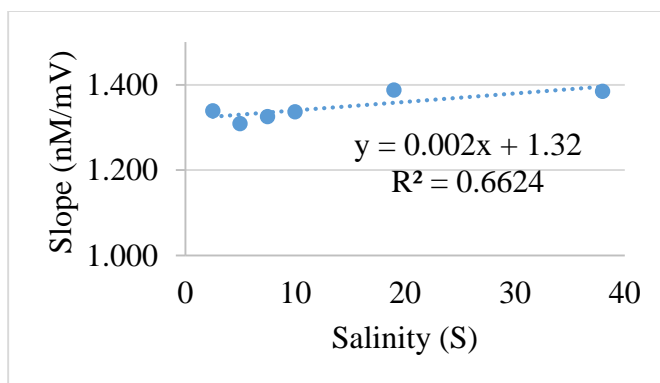
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Figure 5. Effect of salinity on calibration curve slope. Calibration curves were constructed from standards prepared in low nutrient seawater (LNSW) and LNSW dilutions made with deionized water. Effects of the salinity (S) on the slopes (in nM/mV) of standard calibrations were minimal as expressed by a nearly horizontal linear regression line plotted above.

3.3 Calibration Curve and High Resolution Analysis

Despite the limitations presented by a potentially large matrix effect between LNSW and DIW blanks, favorable results were obtained from calibration standards prepared at 5 mM OPA and analyzed at 60 ° C (Figure 6). The equation of the rFIA calibration curve was $y = 1.263$ nM/mV x + 7.485 nM with a linear regression coefficient of determination of 0.9995. The standard deviation of the residuals was 8.7 nM. For the control technique (Figure 6), the equation of the calibration curve was $y = 1.081$ nM/mV x + 4.918 nM with a linear regression r^2 value of 0.9998. The standard deviation of the residuals was 6.0 nM. Comparing the plots of the calibration standard concentrations calculated from each curve gave very good agreement between the two techniques and a linear regression equation of $y = 0.9998x - 0.1008$ and $r^2 =$ 0.9998 as shown in the lower panel of Figure 6. Replicate analysis of the 1000 nM ammonium standard at these reaction conditions indicate that the analyte signal is highly reproducible with a standard deviation of 10.6 nM (818.2 ± 8.4 mV, 2.4% rsd, n = 10).

A high resolution experiment examining ammonium concentrations between 0 and 550 nM added ammonium in LNSW at 25 nM intervals was conducted to verify the sensitivity and range of the selected reaction conditions (5 mM OPA and 60 ° C, Figure 7). Triplicates of each

addition had a very low inter-sample standard deviation (the standard deviations of each set of triplicate samples were averaged and multiplied by the slope for 2.7 nM rsd = 1.4%) and were clearly distinguishable between intervals. A single tailed s-pooled Student's t-test was conducted on the means of the reagent blank (DIW 42.4 ± 0.8 mV) and the matrix blank (LNSW 41.6 ± 0.3 mV); the t-experimental value (0.395) was less than the t-critical value (2.78), indicating that the DIW and LNSW blanks were similar at a 95% confidence level. The triplicate LNSW blanks had a standard deviation of 0.3 nM; three times the standard deviation for the replicate blanks gave a LOD of 1.1 nM.

3.4 Amine and Amino Acid Interference

The interferences from primary amines and amino acids were checked with duplicates of 1000 nM ammonium standards spiked with 1 µM of the target amino compound (listed in section 2.1.4 and Table 2) prepared in DIW. The equivalent ammonium concentration of the spiked ammonium standards was determined relative to replicates (n = 11) of 1000 nM ammonium standards prepared in DIW that had not been spiked with the given amino compound. The results displayed in column 3 of Table 2 ranged from 778 to 1044 nM. Additionally, DIW spiked with 2 µM of the target amino acid were also compared against replicate non-spiked DIW blanks (n = 11) in column 2, with no amino acid resulting in greater than 55 nM ammonium equivalents. The reaction temperature was reduced to 45 °C, and 10 mM rFIA OPA was used for the rFIA technique to allow sufficient dynamic range to accommodate for potential saturation of the fluorometer for combined interferences. Ranges (not listed in Table 2) for the control technique were very similar with the notable exception of mono-methylamine, which saturated the fluorometer for both the blank and the spiked standard in the GD-FIA measurement, but not for the rFIA technique.
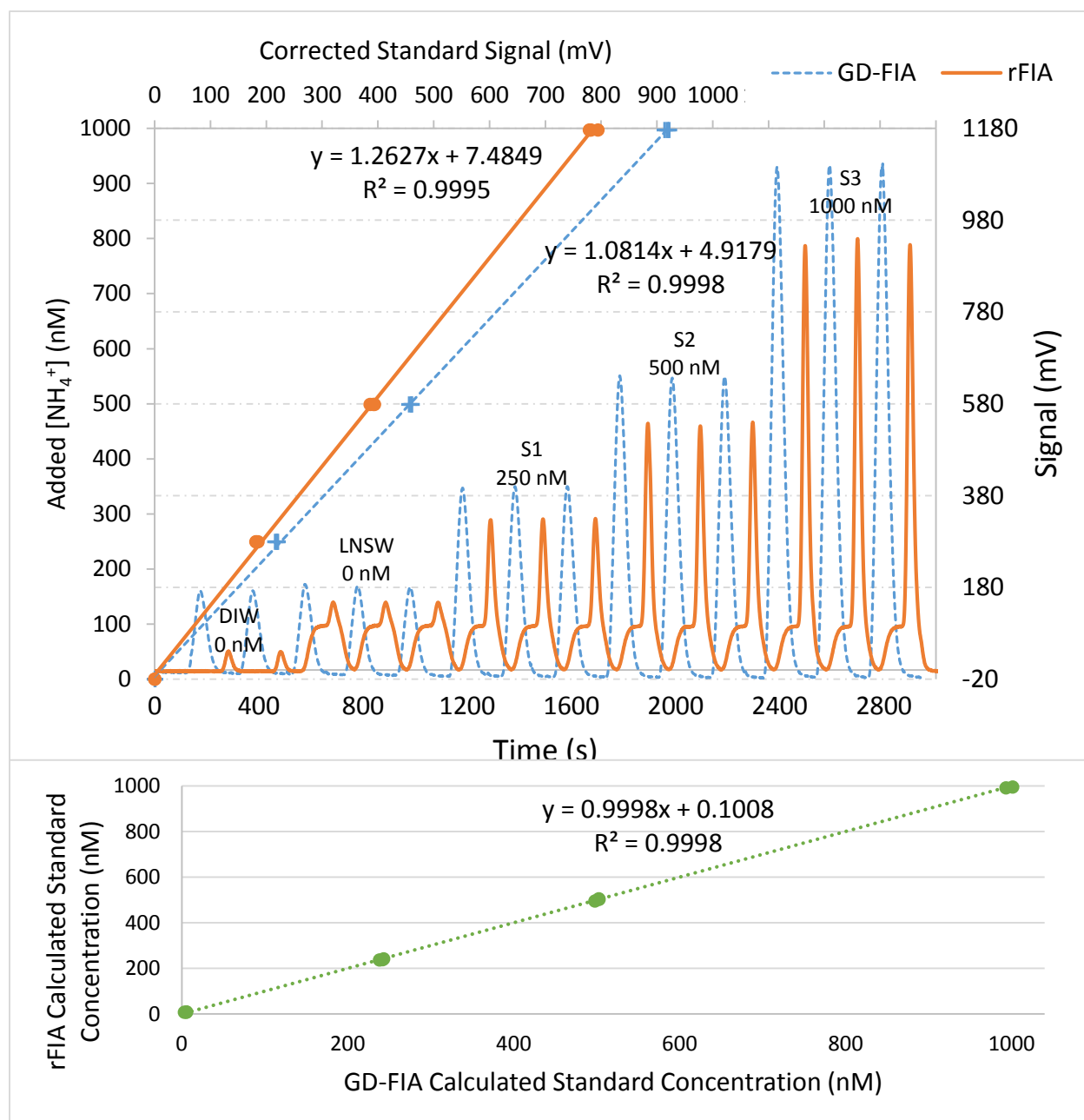
Figure 6. Comparison of OPA techniques. Calibration standards were compared on the reverse flow injection analysis (rFIA) and the gas diffusion flow injection analysis (GD-FIA) techniques simultaneously and are superimposed on the plot. Two deionized water blanks and 3 low nutrient seawater (LNSW) blanks were followed by triplicates of standards prepared in LNSW (250, 500, and 1000 nM ammonium respectively). The rFIA raw signals and calibration curves are indicated by the solid orange lines. GD-FIA is presented as the dashed blue lines. The axes for the calibration curves in the upper panel are along the top and left side of the chart, while axes for the raw signals are on the bottom and right side of the chart. The rFIA method was conducted at 60 °C using 5 mM OPA.

Figure 7. High resolution analysis. Triplicate of samples prepared in LNSW at 25 nM intervals from 0-500 nM ammonium concentrations were analyzed at 5 mM OPA and 60 °C to examine the resolution and sensitivity of the rFIA technique. The eleventh sample signal suffered from noise as the result of a bubble passing through the flow cell, and was removed from analysis.

Table 2. Potential interference from primary amines and amino acids. The equivalent ammonium concentration of the average values of listed potentially interfering species at 2 μM (n = 2) p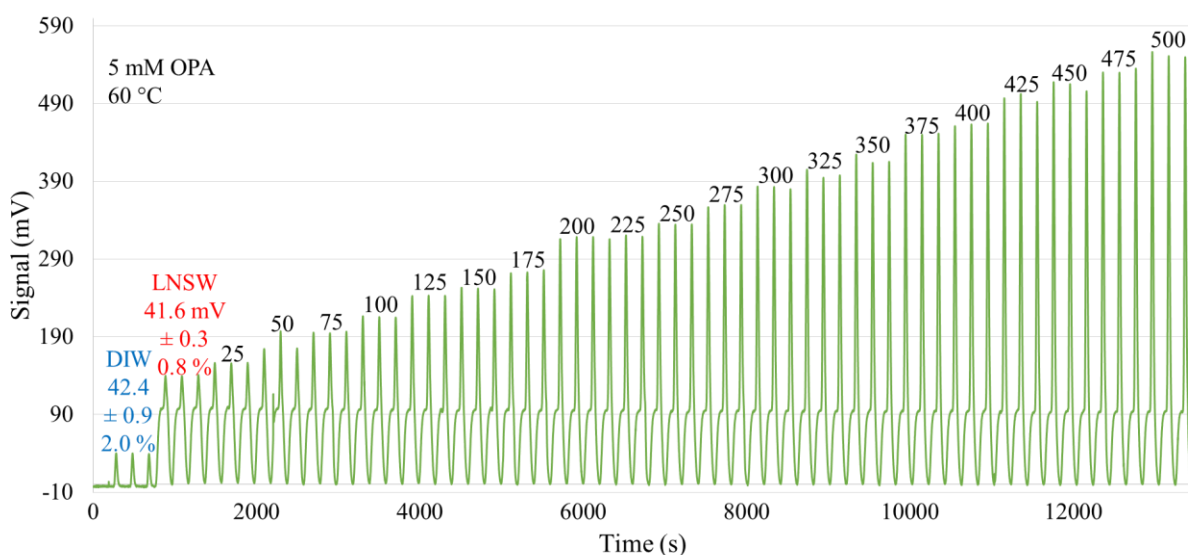repared in DIW are displayed in column 2. The third column displays the equivalent ammonium concentration of average signals of 1000 nM ammonium standards, spiked with 1 μM AC concentration, prepared in DIW. Replicates (n = 11) of DIW blanks and 1000 nM ammonium standards served as the calibration curve endpoints (rFIA slope = 2.359 nM/mV. The experiments were conducted at 45 °C with 10 mM OPA.

Primary Amine, Amino Acid	Ammonium equivalents (nM) of 2 μM interfering species in DIW	Ammonium equivalents (nM) of 1 μM interfering species spiked in 1 μM NH_4^+ standard
Ethanolamine	54.1 $\pm$ 36.2	945.1 $\pm$ 34.4
Methylamine	9.0 $\pm$ 14.5	815.7 $\pm$ 120.4
L-serine	23.5 $\pm$ 15.3	778.1 $\pm$ 173.6
L-tyrosine	12.2 $\pm$ 2.7	840.2 $\pm$ 51.0
L-alanine	14.6 $\pm$ 35.0	1044.9
L-leucine	19.3 $\pm$ 22.0	868.8 $\pm$ 17.8
L-phenylalanine	10.5 $\pm$ 3.5	881.9
L-tryptophan	19.1 $\pm$ 3.7	893.5 $\pm$ 66.4
B-alanine	0.4 $\pm$ 1.0	961.4 $\pm$ 23.3
Glycine	30.4 $\pm$ 60.4	912.0 $\pm$ 91.5

4. Discussion

Flow injection analysis and variations of FIA rely on carefully controlled timing. It is essential that the reaction starts and proceeds to the same extent, for the same period of time,

with the same delivery of reagents in order for every sample to be comparable (Ruzicka and Hansen, 1978). So long as these requirements are met, and there are few flow issues, no leakages (as commonly happens with the GD-FIA PTFE membranes (Van Der Linden, 1983), and reagent stability outlasts the duration of the analysis, it is not necessary to know the precise reaction conditions. Nonetheless, great effort has gone into understanding the reaction conditions for optimization of the rFIA technique for ammonium analysis. rFIA allows for the reproducibility of the reaction conditions common to FIA with the added benefit of producing a background signal for every sample, enabling corrections for the background fluorescence of seawater samples.

4.1 Temperature and OPA Concentration

The initial 34% difference between DIW reagent blanks and LNSW solvent blanks observed at OPA concentrations of 25 mM and a temperature at 45 °C led to experimentation to discover the cause of the difference. DIW reagent blanks produced too high of a signal, and subtraction of the reagent blank would result in negative values for the LNSW solvent blank and any low ammonium samples. Decreasing the OPA concentration lowers the reagent blank values, but also decreases sensitivity as the available OPA reacts less during the ~80 seconds of mixing and diffusion allowed to occur after the OPA is injected. Increased temperature at lowered OPA concentrations offered an appropriate compromise between lower blank values and adequate sensitivity. At OPA concentrations of 5-15 mM and temperatures between 55 and 65 °C, slopes were close to the ideal selected slope of 1 nM/mV. Temperatures above 60 °C forced dissolved gases out of solution and required the use of an on-line degasser and bubble trap to reduce noise in the baseline and occasional spikes in the signals due to air bubbles passing through the fluorometer. At the 60 °C and 5 mM OPA parameters, linear results were obtained and a low

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limit of detection of 1.1 nM were produced, a six fold improvement compared to the previous 45 °C, 25 mM OPA result (LOD = 7 nM). The differences in the blank values for both DIW and LNSW showed much less disparity and were insignificant at the 95% confidence level. . Validation of the new setting was conducted by running the GD-FIA control method concurrently and the techniques had very good agreement (r^2 = 0.9998 as shown in the lower panel of Figure 6).

4.2 Background Fluorescence

The importance of correcting for background fluorescence is well established. Background fluorescence of dissolved organic material was corrected for manually in Holmes et al. (1999) by subtracting the measured signal of the analyte mixed the sulfite reagent without OPA from the sulfite reagent with OPA. Similar corrections were conducted by Aminot et al. (2001) on the FIA system by allowing the analyte to continue without reacting with OPA. Bey et al. (2011) devised a multichannel instrument that could make the same measurements with and without OPA through a FIA technique using sulfite and perform a background fluorescence correction for all samples automatically. However, like the manual and FIA techniques, it requires the preparation of an additional reagent, potentially introducing errors from differences in the reagents with and without OPA. Reverse flow injection analysis simplifies the correction procedure in a two-step process: 1. it establishes a background fluorescence signal by streaming the sample with the sulfite-HCHO reagent through the fluorometer without OPA, and 2. it subtracts the background fluorescence signal from the height of the signal of the analyte stream that had reacted with injected OPA.

4.3 Blanks and Matrix Effects

Further investigation into the initial 34% difference in fluorescence between DIW and LNSW indicated minimal salinity effects ($< 6\%$) on the slopes of standard curves prepared in the various LNSW dilutions. Discrepancies in matrix effects have been previously reported, ranging from minimal effects (Amornthammarong and Zhang 2008), to an elevated response of DIW relative to seawater (Kerouel and Aminot 1997, Holmes et al. 1999, Watson et al. 2005), to a suppressed DIW response relative to LNSW (Jones 1991, Aminot et al. 2001, Bey et al. 2011). Although there were some inconsistencies in magnitude and direction of reported matrix effects, this general trend applies: matrix effects have greater impact on measurements of samples with lower concentrations of ammonium. The minimal effects reported here agree with the Amornthammarong and Zhang (2008) technique from which the OPA-sulfite-formaldehyde chemistry was adapted for rFIA. This technique is tailored for use in the oligotrophic ocean where salinity is relatively consistent across a given gyre or basin. Therefore, the use of LNSW as the solvent for standard preparation is recommended to minimize matrix effects for rFIA ammonium measurements in seawater. This recommendation runs contrary to the use of DIW by Amornthammarong and Zhang (2008), but agrees with Zhang et al. (1997) for colorimetric determination of ammonium below 1.2 µM. The selection of the LNSW solvent matrix as the reagent blank has a precedent in the Environmental Protection Agency Method 349.0 for colorimetric analysis of ammonium below 1.2 µM (Zhang et al. 1997).

4.4 Limit of Detection

Limits of detection in this work are three times the standard deviation of replicate blanks times the slope of the calibration curve, similar to the approach of Aminot et al. (1997) and Amornthammarong and Zhang (2008). This method had a LOD as low as 1.1 nM ammonium

from the 25 nM interval high resolution experiment. The background fluorescence correction permitted by rFIA makes these very low limits of detection possible. Without it, the calculated ammonium in the low nutrient seawater would be artificially high (118.1 nM) due to the background fluorescence, and these low limits of detection would be unattainable. Standard deviations of replicate blanks (DIW for other works, LNSW for this one) to determine detection limit do not reflect how temperature effects on mid- and high-range analyte values and their impacts on the calibration slope. The relative standard deviation of higher ammonium standards indicates consistency of performance at those concentrations. The average of the standard deviations for all the triplicates of the high resolution experiment was 3.2 nM, 1.4% of the signals, while the standard deviation for replicates of the 1000 nM ammonium standard was 10.6 nM, 2.4% relative standard deviation, indicating high reproducibility across the analytical range.

4.5 Potential Interferences

Amino acids and primary amines found in seawater may potentially react with OPA and modify the ammonium signal if the reaction product fluoresces at similar excitation/emission wavelengths (325/465 nm, respectively). The sulfite-formaldehyde reagent was adopted for the rFIA technique because it was reported to increase the selectivity of OPA for ammonium over potential interferences by amines and amino acids (Amornthammarong and Zhang 2008). Solutes of 2 μM of the potentially interfering species only produced a signal ≤ 34% of the DIW blank, and only 3.5% of the 1000 nM ammonium standard. The equivalent ammonium concentration of spiked standards presented in Table 2 ranged from 778 to 1044 nM compared to the 1000 nM ammonium standard. The general suppression of the ammonium signal by these amines has also been reported by Bey et al. (2011), who attributed the suppression to the formation of an adduct between the sulfite, OPA, ammonium, and the amine which may exhibit maximum fluorescence

at wavelengths shifted away from those selected for this method. The GD-FIA control method produced similar range and magnitude of results as the rFIA method with the exception of mono-methylamine, which saturated the fluorometer for all tests on the gas diffusion technique. The small molecule mono-methylamine has also been known to permeate PTFE membranes in the gas diffusion techniques (Jones 1991) and was also problematic in the results of Bey et al. (2011) producing an approximate 53% yield. Furthermore, they reported a 4 percent yield for ethanolamine (suppression of signal by 96%). A 72% response relative to an ammonium standard shown in Amornthammarong and Zhang (2008) differs in how the measurement was obtained (i.e. a comparison of the reaction of 1 µM ethanolamine independent of a 1 µM ammonium versus the direct analysis of the spiked standard analyzed here). The 2 µM ethanolamine blank analyzed via rFIA only had a 5.8 % relative response, a 12 fold reduction in potential interference. The sulfite-HCHO chemistry for rFIA OPA injection discriminates against the interaction of amines and amino acids with OPA, allowing for a nearly full signal across the selection of analyzed compounds. The most common amino acids comprising of dissolved free amino acid pool (glycine, serine, and alanine) in seawater are not usually present in excess of 50 nM in the Sargasso Sea (Suttle et al. 1991) and at these lower concentrations the effects of interference on ammonium analysis will be dramatically diminished relative to the 20 and 40 fold concentrations examined here.

5. Conclusions

The reverse flow injection analysis technique presented here combines the stability and selectivity of a sulfite reagent amended by formaldehyde with the sensitivity of fluorometric ammonium detection with OPA. This method has demonstrated a detection limit of 1.1 nM

ammonium in seawater with simultaneous background fluorescence correction, suitable for the analysis of the oligotrophic surface ocean for sub-micromolar ammonium concentrations. This technique represents an improvement in sensitivity over the widely used indophenol blue colorimetric technique and simplifies corrections for background fluorescence relative to other fluorometric OPA FIA techniques. Although gas diffusion techniques also avoid background fluorescence, they suffer from a propensity to develop clogs and leaks within the gas diffusion block. rFIA needs no membrane separation step. Matrix effects were ameliorated by the use of aged LNSW for standard preparation. The method is tailored for measurements of sub-micromolar ammonium concentration in surface waters of the open ocean where salinity deviations are minimal and the concentrations of amino acids and primary amines are below 50 nM and not likely to interfere with the measurement of ammonium.

Future direction for the rFIA technique for ammonium analysis include a GD-FIA and rFIA comparison in a field deployment and the potential integration into the 3-channel nitrogen sensor to replace the GD-FIA technique (Masserini and Fanning 2000) from which the manifold was derived, making it an all rFIA nanomolar inorganic nitrogen analyzer. Such an analyzer could provide valuable information about the extent, concentration, and rates of uptake and release of dissolved inorganic nitrogen compounds in the oligotrophic surface ocean.

6. Acknowledgements

Chapter 3: Conclusion

The work presented in Chapter 2 is the culmination of the application of rFIA to the measurement of nanomolar ammonium concentrations in oligotrophic seawater. The reaction conditions settled upon in this technique were mixing the sample at a 1:1 flow rate with 10 mM sulfite-5 mM formaldehyde reagent, injecting the analyte stream with a 5 mM OPA reagent, and heating the mixed reagent-analyte stream to 60 °C. The results of standard calibration curves at these conditions indicate a 1.1 nM limit of detection, a slope (sensitivity) of 1.2 nM/mV with a least square regression fit coefficient of determination better than 0.999 up to 1000 nM ammonium, and excellent reproducibility (2.4% relative standard deviation for 10 replicate 1000 nM ammonium standards). The rFIA technique enables automatic background fluorescence corrections to be applied to each sample by streaming analyte through the fluorometer without OPA to establish the background fluorescence first and subsequently injecting OPA to develop the analyte signal. The potential contribution of background fluorescence can be as high as 118 nM ammonium equivalents, which if uncorrected would show a systematically and disproportionately high ammonium concentration at the low concentrations expected in oligotrophic seawater. The effects of salinity on the solvent matrix was found to be minimal (less than 6% error between standards prepared in dilutions of LNSW with DIW and standards prepared in LNSW) Additionally, the selection of the sulfite-formaldehyde chemistry minimizes the potential interference of 10 tested primary amines and amino acids.

The rFIA technique performs as accurately as the well vetted Jones (1991) gas-diffusion flow injection analysis method (a comparison of slopes of standard calibrations in LNSW between the two methods resulted in a linear least square regression fit coefficient of determination of 0.9998). It offers advantages over the GD-FIA method in terms of reagent stability, ease of use, and elimination of the need for isolating the ammonium using a leak-prone PTFE membrane separation diffusion block. Fluorescent OPA-based chemistries in general perform better at nanomolar ammonium concentrations than the widely used colorimetric indophenol blue (Šraj et al. 2014). As a part of the growing prevalence of FIA for fluorescent ammonium analysis, automatic batch analyzers, multi-pump sequential injectors, and solid phase extraction techniques have been paired with the fluorescent detection of ammonium with OPA. These procedures offer advantages such as power requirements, reagent/sample consumption, and cost. The 1.1 nM limit of detection offered by rFIA and its relatively high sample throughput (18 samples per hour) put it on par with these techniques. No other technique, however, offers the background fluorescence correction in quite the same simple and effective manner as rFIA does.

The technique still needs to be vetted in a natural setting. A multi-day cruise in the Gulf of Mexico would be sufficient to prove the validity of the method at sea. The comparison of samples analyzed on-line and continuously via rFIA and GD-FIA aboard the ship against discrete samples returned for laboratory analysis with the same techniques and IPB is a short-term goal. Long-term goals may include the integration of the rFIA technique for ammonium with the rFIA techniques for nitrate and nitrite for a high resolution multi-channel nitrogen analyzer from which the ammonium technique was derived (Masserini and Fanning 2000).

The future of OPA-based fluorescent techniques is in accurately characterizing the low concentrations of ammonium in the oligotrophic ocean, where boluses of relatively enriched ammonium may have a spatial and temporal distributions with ecological significance (Fanning et al. 2015) that are not measurable by less sensitive techniques such as the IPB method. This can be achieved through adapting the new technologies to smaller platforms that can operate independently of an analyst. Moorings and autonomous underwater vehicles are attractive and relatively less expensive platforms compared to ship-board sample collection. The *in-situ* collection of data avoids several potential sources of ammonium contamination and changes to the sample concentration due to metabolic processes. Mobile and near real-time trace ammonium data can also provide researchers with information useful for tracking small scale water masses otherwise indistinguishable by salinity or temperature changes (Masserini 2005, Fanning et al. 2015).

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Table A1: Summary of calibration curve results. The results of standard calibration curve analyses are displayed by their file name (first column) with information about the experiment including solvent matrix, OPA concentration and age, and temperature (columns 2-4, respectively), The slope, intercept, and r square value from the linear least square regression analysis (column 5, 7-8, respectively) indicate the sensitivity, solvent matrix concentration of ammonium, and fit of the curve. The standard deviation of the residuals (column 9) indicates the scatter of the residual difference between actual concentrations versus concentrations calculated from the curve for the standard samples around the curve. The average and standard deviation of signals for the reagent blank, background fluorescence, background fluorescence corrected solvent blank, and background fluorescence corrected 1000 nM and 500 nM ammonium high standard (columns 10-13; respectively).

rFIA Standard Curve	Solvent Matrix (S)	[OPA], age (days)	T (°C)	Slope (nM/mV)	Slope % error	Intercept (nM)	r square	Standard deviation of residuals (nM)	Reagent blank (n = 2)	Background fluorescence (n = 6)	Solvent blank corrected for background fluorescence (n = 2)	1000 nM standard corrected for background fluorescence (n = 2)	1000 nM % error	LOD (nM) = 3 times solvent blank standard deviation times slope	% difference of reagent and solvent blank	Background fluorescence ammonium equivalents (nM)	Figure number
STD022112171723	DIW S = 0	25 mM 26 days	45	2.360	0.2	5.947	0.9977	18.8	153.38 ± 17.78	-7.38 ± 1.07	140.14 ± 1.90	563.42 ± 8.98	-0.26	13.4	9.0	4.1	
STD022112175840	LNSW S = 38	25 mM 26 days	45	2.356		-0.096	0.9995	8.86	162.51 ± 12.56	18.01 ± 0.56	115.57 ± 0.99	539.95 ± 2.81		7.0	33.8	61.4	3
									(n = 2)	(n = 9)	(n = 3)	(n = 3)					
STD021013183611	LNSW S = 38	25 mM 30 days	45	1.385	0.0	7.131	0.9996	7.26	76.78 ± 1.21	67.62 ± 0.73	68.77 ± 0.39	787.56 ± 2.35	0.00	1.6	11.0	98.1	
STD021113124650	S = 19	25 mM 31 days	45	1.387	0.2	2.363	0.9999	4.50	79.74 ± 6.43	12.70 ± 0.52	67.53 ± 0.43	785.14 ± 6.09	-0.16	1.8	16.6	22.4	
STD021113134223	S = 10	25 mM 31 days	45	1.337	-3.5	10.725	0.9986	14.5	77.97 ± 1.34	3.97 ± 0.45	71.41 ± 0.90	816.91 ± 4.87	3.72	3.6	8.8	11.3	5
STD021113150227	S = 7.5	25 mM 31 days	45	1.325	-4.3	3.658	0.9999	3.41	86.76 ± 10.75	1.00 ± 0.42	71.86 ± 0.61	823.06 ± 2.29	4.51	2.4	18.8	8.0	
STD021113160134	S = 5	25 mM 31 days	45	1.309	-5.5	15.075	0.9971	20.80	85.60 ± 7.64	-0.96 ± 0.37	75.28 ± 0.44	827.82 ± 2.54	4.70	1.7	12.8	5.5	
STD021113170310	S = 2.5	25 mM 31 days	45	1.339	-3.3	1.554	0.9998	4.93	81.76 ± 0.47	-3.11 ± 0.40	83.25 ± 5.04	828.02 ± 5.15	3.61	20.2	1.8	3.2	

Column header spanning columns 10–13: *Average signals ± standard deviation (mV)*

					Slope % difference								500 nM standard	500 nM % difference				
STD021513144626	LNSW S = 38	25 mM 32 days	45	1.303	-5.9	1.343	0.9999	4.02	81.12 ± 0.26	67.66 ± 0.64	65.10 ± 1.17	829.92 ± 6.05	6.40	4.6	21.9	92.2	
STD080114162140	LNSW S = 38	5 mM 25 days	60	1.266	5.6	7.915	0.9994	9.12	43.34 ± 0.52	95.25 ± 0.95	50.42 ± 0.61	441.04 ± 4.06	-6.59	2.3	15.1	124.1	6
DIS080514130228	LNSW S = 38	5 mM 29 days	60	1.197		4.720	0.9990	6.97	42.36 ± 0.85	95.82 ± 2.34	41.65 ± 0.31	458.90 ± 3.04		1.1	1.7	118.1	7