Analytical tools for studying phosphorus and nitrogen lake nutrient dynamics and their application to the field

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Analytical Tools for Studying Phosphorus and Nitrogen Lake Nutrient Dynamics and Their Application to the Field

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A Thesis Presented to the Department of Chemistry, Colby College, Waterville, ME
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Analytical Tools for Studying Phosphorus and Nitrogen Lake
Nutrient Dynamics and Their Application to the Field

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Vitae

Ivan Mihajlov was born in 1982 in Pančevo, a bohemian city on the Tamiš river, not far from the capital of Serbia, Belgrade. He spent his childhood years in the safe and loving family cradle of his parents, Mirjana and Tomislav, and sister Nataša. While Ivan’s childhood was “spiced up” with the wars of the nineties that saw his former country of Yugoslavia fall apart, and despite the international sanctions and isolation, he continued to hope that one day he would travel and meet people from all over the world. He also developed strong interests in natural sciences, especially biology and chemistry. The success at school in Serbia earned him a scholarship in Li Po Chun United World College of Hong Kong where he finished secondary studies and met peers from many different continents. This was a life-changing experience that shaped Ivan’s ideals for the future and allowed him to attend Colby College in Waterville, Maine. At Colby, he furthered his interests in natural sciences by majoring in chemistry: biochemistry, and also confirmed the enthusiasm for international experience by taking up a second major in French Studies. The long-term passion for environment contributed to his dedication to the studies of the Belgrade Lakes and resulted in the present honors thesis. Ivan hopes to remain an active player in the sphere of environment upon graduation from Colby in May 2005.
Abstract

In order to evaluate the causes and conditions that lead to cyanobacteria blooms in northern temperate lakes, studies of nutrients such as total phosphorus, total nitrogen, phosphate, and nitrate are necessary. Sensitive, environmentally friendly and cost-effective techniques were developed to detect ppb-level nitrates and phosphates in the lake water. Phosphate was analyzed by luminol chemiluminescence, while the quantification of nitrate was performed by capillary electrophoresis (CE) using indirect UV detection, with a background electrolyte consisting of pyromellitic acid, hexamethonium hydroxide and triethanolamine. Sample injection methods for the CE were optimized for the analysis in Belgrade Lakes, Maine. Vertical profiles of four different lakes in this watershed were taken in winter period, including the data for nitrate, phosphate, dissolved oxygen (DO) and temperature. While temperature profiles were characteristic of ice-cover season, and phosphate was uniformly distributed between different depths, nitrate and DO showed a high degree of vertical stratification. Elevated levels of nitrate, in comparison to other lakes, were noted in East Pond and, to a lesser extent, in North Pond. A link may exist between this discovery and the fact that East Pond suffers from the most severe summer algal blooms.
I. Introduction

Algal blooms represent an increasingly important problem for limnologists as well as for every-day lake enthusiasts. With rising nutrient inputs (e.g. nitrogen and phosphorus), the species that cause lake blooms progress from chrysophytes and cryptophytes to chlorophytes and diatoms, until the relative abundance of cyanobacteria rises as a final stage of lake eutrophication (Watson et al. 1997). Cyanobacteria, or bluegreen algae, can grow as large cells, filaments, or colonies, and contain vacuoles for buoyancy regulation. They are subdivided into heterocystous cyanobacteria (*Aphanizomenon* sp., *Anabaena* sp., *Gloeotrichia* sp.) that contain N$_2$-fixing cysts, and non-heterocystous cyanobacteria that are incapable of fixing atmospheric nitrogen (for example, *Microcystis*, *Oscillatoria*, *Pseudoanabaena* and *Lyngbya*) (Ferber et al. 2004). Blooms of cyanobacteria are particularly problematic because they impact recreation, as well as human and animal health, and produce taste and odor problems that cannot be resolved by conventional drinking water treatments (Downing et al. 2001). Accumulations of these algae are unsightly and bad-smelling, while cyanobacterial poisoning of livestock, pets and humans has also been documented (Sivonen 1996).

1. Lake water nutrients and cyanobacteria blooms

A baseline for nutrient control of algal blooms was laid by the work of Redfield (1963) who demonstrated a close to constant ratio of major nutrients, C, N and P, in all plankton, that is C:N:P = 106:16:1 in molar units. Measuring this ratio has been the focus of researchers attempting to control blooms by manipulating the limiting nutrients. In particular, Smith (1983) contends that a low N:P ratio selectively favors cyanobacteria due to their ability to fix atmospheric nitrogen and thus outcompete other algal species.
that are nitrogen-limited. From data sets for several lakes, it appears that cyanobacteria dominance is low when total P (TP) : total N (TN) ratios were greater than 29:1 by weight, while the potential for this dominance is very high at ratios below the 29:1 cut-off. Many other cut-off ratios have since been proposed by other scientists.

This low N:P hypothesis has been modified, however, in particular with respect to its relevance to heterocystous versus non-heterocystous bluegreens' dominance. While all cyanophytes can store surplus N in cyanophytin and thus may be able to compensate for short-term N shortages, long-term N-limitation would only favor the heterocystous N$_2$ fixers, and not all cyanobacteria (Levine and Schindler 1999). However, other studies have also shown that vacuolated, filamentous or colonial, non-heterocystous cyanobacteria (Microcystis and Oscillatoria) can also dominate at low N:P ratios because of their ability to migrate vertically at low energy cost compared to eukaryotes, bringing benthic N to the surface (Reynolds 1984). An additional complication pointed out by Ferber et al. (2004) is that even N$_2$-fixing cyanobacteria can migrate vertically [notably, Gloeotrichia (Nøges et al. 2004)], so the migration mechanism, rather than their N$_2$-fixing ability, may be responsible for their dominance. Indeed, their study shows that while some major blooms may be supported by N$_2$ fixation, at most times the contribution due to fixation of N$_2$ is much lower than that of nitrate or urea, and in their study on a bloom-prone Vermont lake only 2% of the N required was supplied by fixation.

Moreover, the mere applicability of low N:P in the prediction of cyanobacteria blooms has been questioned and the concept has been shown to be incomplete at best. Rather than the ratio of these nutrients, the analysis of 269 observations from 99 lakes around the world, with data from multiple years or sites (Downing et al. 2001), shows
that the best variables that correlate with cyanobacteria blooms are total N and total P as separate entities. Indeed, the study by Levine and Schindler (1999) confirmed that N:P ratios do a very poor job predicting the dominance of the bluegreens and conclude that phosphorus inputs remain the main variable to control. Schindler (1977) demonstrated earlier that phosphorus deficiencies may be the ultimate limitation to a bloom, while C and N deficiencies can be compensated for by natural mechanisms in eutrophic lakes.

2. Other factors contributing to cyanobacteria blooms

In addition to the dubious governance of the system by N:P ratios, nutrient analysis alone does not suffice to predict cyanobacteria blooms. Several physical and chemical factors that impact algal blooms have been identified, such as the amount of wind and mixing in the lake, the light intensity, the concentration of carbon dioxide (CO₂) and pH, and the food-web structure. Decreased cyanobacterial blooms seem to coincide with mixing events caused by strong winds (Nõges et al. 1999; Ojala et al. 2003). The risk of cyanobacteria blooms is higher when the usually well-mixed column develops thermal stratification due to warm and calm weather and the eplimnion gets depleted of nutrients. In that situation, Gloeotrichia sp., for example, can effectively transport nutrients from the hypolimnion and spread, while the wind event would cause a competition for potentially scarce nutrients with all other species. This explanation only works for small, shallow lakes in the temperate zone because the vertical migration of algae and nutrient mixing by wind is possible, while the large lakes rarely develop bluegreen algae blooms (Elser 1999).

Light intensity seems to negatively correlate with the bluegreen blooms. While these algae have gas vesicles and could potentially rise up and shade other phylums of algae, it is sometimes unclear whether light intensity is the cause that brings
cyanobacteria to dominance or the consequence of that very dominance (Levine and Schindler 1999). The concentration of $\text{CO}_2$ and pH are inversely related to each other and the conditions of high pH and low $\text{CO}_2$ may favor cyanobacteria over eukaryotic algae because the former are able to hydrolyze carbonate as a source of $\text{CO}_2$, can concentrate $\text{CO}_2$ before photosynthesizing, thus elevating $\text{C}$ fixation rates. If vacuolated, the bluegreens can also take advantage of the higher atmospheric $\text{CO}_2$ levels at air-water interface (Paerl and Ustach 1982). Low $\text{CO}_2$ and high pH levels correlated in certain years with cyanobacteria blooms in the study of Ontario Experimental Lakes (Levine and Schindler 1999), but by no means did these parameters guarantee the dominance of cyanobacteria.

Finally, Elser (1999) argues that changes in the food-web structure are the final parameter in the determination of bluegreen dominance, particularly those changes that induce $\textit{Daphnia}$ (a large Crustacean species of zooplankton) depletion. Although $\textit{Daphnia}$ does not significantly graze bluegreens, it could accomplish the control over cyanobacteria populations by grazing small colonies or filaments before the bluegreens reach dominance. Moreover, $\textit{Daphnia}$ has a low body N:P ratio, meaning that it tends to retain P in its biomass, but release N, enriching the water column with N and, thus, generating a higher N:P ratio that shifts the advantage away from cyanobacteria.

All factors considered, it is clear that cyanobacteria dominance is a result of a complicated combination of factors that could all contribute to, but hardly independently predict, the blooms. These factors are briefly summarized and presented by Elser (1999) as a hierarchical "decision tree" (Fig. 1). The relative importance of factors, according to this review, gives the greatest importance to absolute loading rates of N and P, followed by their ratios, and subsequently by the hydrodynamic, light and other physical
Is nutrient loading high?

Yes No

Is loading N:P low?

N Yes

Are hydrodynamic / light conditions 'correct'?

Yes No

Does food-web structure inhibit *Daphnia* dominance?

Yes No

**NOXIOUS CYANOBACTERIA BLOOM**

Figure 1: The hierarchical decision tree for the cyanobacteria blooms (Elser, J. J. 1999). In order for a lake to undergo a cyanobacteria bloom, several key factors have to be satisfied in a hierarchical order: P and N loading must be high, but with a low N:P ratio. Physicochemical conditions, such as pH, wind speed or light intensity must be right, and finally, due to food-web structure, zooplankton, such as *Daphnia*, must not be present in large quantities.
conditions, while the food-web structure intervenes last. From this hypothesis of hierarchical factors, it is obvious that N and P levels, as well as their ratios, are crucial to the lake management, and although they are not the only factors influencing the bloom, they do represent key players. But, keeping in mind that the determination of these nutrients is important, what could be the best variables to measure that would reflect their availability in lake water column?

3. Nutrient variables to analyze

Total Phosphorus (TP) and Total Nitrogen (TN) are clearly the best factors that indicate trophic state of a lake because they measure the total amount of each nutrient in the biomass or that is available to be incorporated into it (Dodds 2003). However, many researchers tend to measure Dissolved Inorganic Nitrogen (DIN) and Soluble Reactive Phosphorus (SRP) in order to evaluate the amounts of P and N. DIN, which includes nitrite (NO$_2^-$), nitrate (NO$_3^-$) and ammonia (NH$_4^+$), and SRP, which should reflect the levels of orthophosphate (PO$_4^{3-}$), are simpler to measure because the totals require an extra step of digestion in extreme basic or acidic conditions. However, Dodds (2003) strongly challenges this method of analyzing nutrient relationships. While inorganic N and P species are the major forms of nutrients that plants utilize, their concentrations in the water column may not reflect the actual nutrient supply in the lake because they can undergo a rapid turnover rate – nutrient regeneration can supply nutrients instantaneously taken up by biota, keeping the concentration low despite the high productivity of the system (Dodds 1993). As Hudson et al. (2000) demonstrated, the relative proportion of phosphate to TP decreases as TP increases, hence these two variables are not proportional. An additional problem with SRP is that it does not necessarily represent orthophosphate (PO$_4^{3-}$) either; instead, the usual colorimetric procedures, notably the
colorimetric molybdate blue method (Levine and Schindler 1999), tend to overestimate inorganic phosphate concentrations because they use highly acidic reagents that cleave phosphate from dissolved organic compounds such as ATP and other phosphate esters, or from acid labile P compounds like FePO$_4$ (Dodds 2003). Therefore, the best values to use as indicators of nutrient availability and limitation would be TN and TP.

4. Belgrade Lakes as area of study

The four lakes, subjects of this study, belong to Belgrade Lake watershed (Maine) in which lakes form a system of water bodies connected by ground water circulation and surface streams, with Messalonskee stream as the eventual outlet to the Kennebec River (Fig. 2). Arranged in a downstream manner, the four lakes included in this study were East Pond, North Pond, Great Pond and Snow Pond (also commonly referred to as Messalonskee Lake). These are low-nutrient, northern temperate lakes that tend to thermally stratify in summer and are ice-covered from December to April. The two lakes with large surface areas and deep bottom holes, Great Pond and Snow Pond, are generally dimictic, while the two shallower and smaller lakes, East and North Pond, can be polymictic, depending on the wind events. All four lakes exhibit signs of algal blooms, in particular East Pond, where the blooms have been very persistent and damaging to sports activities on the lake. Moreover, cyanobacteria, especially *Anabaena* sp. and *Gloeotrichia* sp. have been blamed for the most noxious blooms, but no data about the relative abundance of these species are currently available (personal communication with Maine Department of Environmental Protection and Dr. D. Whitney King, Colby College).

The physicochemical conditions of these lakes, such as pH, dissolved oxygen (DO), temperature, fluorescence and conductivity, have been closely monitored for
Figure 2. Area of Study: Belgrade Lakes watershed, a complex system of lakes interconnected by streams and groundwater fluxes. Marks indicate the position of “deep holes” from which the samples were taken: East Pond (N 44° 36.655', W 69° 46.992'), North Pond (N 44° 37.619', W 69° 50.275'), Great Pond (N 44° 32.675', W 69° 51.181'), and Snow Pond (N 44° 30.341', W 69° 45.993').
several summers by Dr. King and this work is likely to continue for several more years. For that reason, the four lakes are a good system for the studies of pathways that cause cyanobacteria and other algal blooms, and it would be useful to characterize the nutrient banks of P and N in order to understand what their absolute levels, as well as ratios, are, and what role they play in the bloom mechanism(s).

Another curious piece of information is that winter sampling has not previously been done on these lakes. Assuming that little influence on the distribution of nutrients is present from biota in winter time, and that little thermal stratification occurs in the lake, with most water at about 4 °C, the lake water should be well mixed and contain a baseline nutrient level before the spring turnover when top layers of the lake warm up. As mentioned above, the best indicators of the lakes’ trophic status are TP and TN, therefore these measurements deserve attention.

5. Importance of phosphate and nitrate

Another specificity of the ice-cover, winter season is the redox chemistry of the lakes, especially as it compares to the summer time anoxia, thus resulting in different chemical equilibria between reduced and oxidized species. As an example of these redox processes, summer-time anoxia can result in the release of phosphate from sediments, as postulated by Mortimer (1941), by reduction of Fe(III) to Fe(II) in sediments containing Fe(OOH) coupled to $\text{PO}_4^{3-}$. Amirbahman et al. (2003) studied this process in eleven lakes in Maine, and proposed several new possible mechanisms for phosphate release in anoxic conditions. Thus, soluble inorganic P and N, in the form of phosphate and nitrate respectively, can provide a great deal of information in this study, not only because they are the nutrients readily available for algal growth, but also because their concentrations are correlated with chemical processes in the lakes. The other species of DIN, nitrite and
ammonia, are not considered relevant in this study because of the generally low levels of these nutrients in winter lake water. Ammonia can, as demonstrated for a cyanobacteria bloom-prone lake in Sweden by Szász and Pettersson (2000), constitute a major source of DIN in summer time when all other nitrogen species are depleted, and the highest uptake rates by plankton are achieved exactly with this nutrient. Studying this nutrient would be beneficial for summer time, but ammonia does not seem to be a major species in well-oxygenated winter water. Therefore, the species of major interest for the winter study are phosphate and nitrate, in addition to TP and TN.

6. Introduction to analytical methods

6.1. Phosphate methods

The most common method used to measure phosphate in lake water is the standard "molybdenum blue" method (Clesceri et al. 1998). This method utilizes the complexation of phosphate and molybdate in strongly acidic conditions to form 12-molybdophosphoric acid, which is subsequently reduced by ascorbic acid to produce a blue dye, readily quantified by spectrophotometry. However, the method suffers from some major disadvantages. Firstly, the concentration of sulfuric acid in the standard molybdate reagent is 2.5 M, dropping off to 0.34 M upon mixing with the sample. Since no basic buffers are used in the method, the technique generates a highly acidic waste. Secondly, highly concentrated acid in the reagent, as noted earlier, can cause the release of phosphate from organic and mineral material in the water column, introducing an uncertainty in the experimental values (Dodds 2003). Lastly, while optimizations of the method reported detection limits as low as 0.5 nM (Zhang and Chi 2002), the low sensitivity is obtained by utilizing a liquid waveguide capillary flow cell with extremely thin internal radius. From experience, this cell tends to get clogged, introducing problems
for flow in the cell when a flow-injection analysis (FIA) system is used. The alternative
of mixing reagents and introducing them manually into the cell introduced another
variable, the reaction time, resulting in a precise window of time when the absorbance
measurements have to be taken.

A new method for phosphate determination was recently described by Yaqoob et
al. (2004). This FIA method is based on the chemiluminescence of luminol when it reacts
with the 12-phosphomolybdate complex:

\[
\text{PO}_4^{3-} + 12 \text{MoO}_4^{2-} \rightarrow \text{H}_3\text{PO}_4(\text{MoO}_3)_{12}
\]

\[
\text{H}_3\text{PO}_4(\text{MoO}_3)_{12} + \text{luminol} \rightarrow \text{light}
\]

The reported detection limits for this method are also very low (1.0 nM) and the
complexation reaction is carried out in only 15 mM H_2SO_4. Moreover, 12-
phosphomolybdate acid reacts with luminol in a basic borate buffer, thus neutralizing
parts or all of the acid, depending on the buffer pH. Lastly, the method is straightforward
and easily applicable, thus it is the method of choice for this study.

6.2. Nitrate methods

The standard method for the determination of nitrate, somewhat similar to the one
for phosphate, is a spectrophotometric method. However, before applying this technique,
the sample needs to be reduced on a cadmium column because the method directly
measures nitrite, and not nitrate. Sample that contains nitrate, now reduced to nitrite, is mixed with sulfanilamide. This diazotized sulfanilamide reacts with N-(1-naphthyl) ethylenediamine (NED) dihydrochloride to produce a purple "azo" dye at pH 2.0-2.5 (Clesceri et al. 1998). While it is seemingly a simple, cost-effective, spectrophotometric method, the procedure involves dealing with a highly toxic cadmium metal. In addition to that, uncertainties are always tied to whether the reduction by Cd was complete, and the mere handling of the column involves an extra step in the analysis.

To quantify nitrate, therefore, a fairly different approach was taken by employing capillary electrophoresis (CE). The CE has become a more and more widely accepted method for the analysis of anions and cations in environmental samples, especially since 1990 (Pantsar-Kallio 1995), as attested by the large number of publications in this area to date. The technique involves very little sample preparation (only filtration is needed), and it is fully automated. In addition to that, very low sample volumes are needed since the only requirement is that the injector needle is submerged in the sample (this volume can go as low as several hundreds of microliters).

Capillary electrophoresis is used to separate anions based on their differential electrophoretic mobility, which in turn depends on the charge-to-size ratio of the anion migrating towards the positive terminal as well as on the degree of interaction of the anion with contents of the background buffer. Net flow through the separation capillary is ensured by the electroosmotic flow (EOF), normally operating due to the net movement of cations (in the direction of negative terminal) concentrated at the negative silanol groups of the silica wall (Fig. 3a). However, the electrophoretic migration of anions often exceeds the EOF, thus the compounds that can reverse the EOF, such as hexamethonium hydroxide (Fig. 4a), are added to the electrolyte buffer to coat the capillary in anion
analysis (Guan 1996). Reverse voltage (from that used in cation separation) can then safely be applied across the capillary ends and allow for a fast migration of anions in the direction of the flow.

Capillary electrophoresis can be used to separate and quantify several anions at a time using UV detection. Since many of these anions are non-absorbing, methods for indirect UV detection (Fig. 3b) are generally developed. These methods require a strongly absorbing background electrolyte (high molar absorptivity) that has a similar mobility as the analytes and that is displaced by the latter at the detector, causing a dip in absorbance (Kubán et al. 1999). One of these compounds is pyromellitic acid (Fig. 4b), and Hiissa et al. (1999) report detection limits of 20 ppb N for nitrate and 40 ppb P for phosphate using this method. It is possible to change the detection limits and linear range of quantification by varying the concentration of the absorbing species (Hiissa et al. 1999), however this path was not explored in the current work. Instead, different modes of sample injection were explored and more discussion on that follows in the Methods section.

The goal of the current paper is to develop and adapt the phosphate and nitrate methods mentioned above to our purposes in the Belgrade Lakes. In addition to that, the aim is to apply these techniques to the measurement of phosphate and nitrate concentrations in the water of four Belgrade lakes sampled in winter period, and analyze the results. Total N and total P methods and results are not discussed in this paper because the efforts are still ongoing, and the method development and results are incomplete.
Figure 3.
A. Electroosmotic and electrophoretic flow between the negative and positive electrode:
Negative silanol groups (marked with minus signs) are saturated with positive surfactant such as HMOH (marked with plus signs). The surfactant prevents cations from sample to interact with silanol groups, thus the electroosmotic flow is in the direction of electrophoretic flow of anions (marked by small blue arrows). Anions move fast toward the positive electrode at the detector side of the capillary.
B. Indirect UV detection:
Absorbance is high when the background buffer containing pyromellitic acid (PMA) occupies the capillary. When analyte plug arrives (anions), absorbance drops and describes a peak.
Figure 4.
A. Hexamethionium hydroxide (HMOH). A flow modifier that binds to the negative silanol groups of the silica walls, and creates a bilayer with the help of its long non-polar chain.
B. Pyromellitic acid, the absorbing species in the background electrolyte buffer that permits indirect UV-detection of analytes.
II. Phosphate Methods

1. Experimental

1.1. Stock solutions

All reagents were of analytical grade and solutions made with ultrapure (18 MΩ) Milli-Q water (E-pure, Barnstead). All reagent bottles or volumetric flasks were thoroughly rinsed with the ultrapure water before use. Molybdate stock solution (10 mM) was prepared by dissolving 4.96 g of ammonium molybdate(VI) tetrahydrate (Acros) in 400 mL of Milli-Q water. Carbonate buffer (0.1 M, pH 10.5) was prepared by adding 5.30 g of anhydrous sodium carbonate (Sigma) to 500 mL of Milli-Q water. Borate buffer (0.1 M, pH 9.7) was prepared using 38.1 g of sodium borate decahydrate (Mallinckrodt) and dissolving it up to 1 L in water. Luminol stock solution (8.94 mM) was prepared by dissolving 0.356 g of 5-amino-2,3-dihydro-1,4-phthalazinedione (Sigma) in 40 mL of 0.1 M carbonate buffer, sonicating for 30 min, and filling up to the 200-mL mark with ultrapure water. It was stored refrigerated. Chelex column was made using chelating resin, “Chelex 100” (Sigma). Ferrozine stock solution (0.1 M) was obtained by dissolving 4.925 of the ferrozine salt (Sigma) in Milli-Q water. Finally, phosphate stock solution (100 ppm as P) was prepared by adding 0.4394 g of KH₂PO₄ (Acros) to a 1-L vol. flask and filling up to the mark.

1.2. Working solutions and sample preparation

Luminol working solution was prepared by mixing different volumes of luminol stock solution with about 25 mL of the stock carbonate buffer and filling up to 500 mL with borate buffer to produce luminol concentrations ranging from 4.6x10⁻³ mM to 1.8x10⁻¹ mM. When the pH was adjusted to 12.5, 51.5% w/w NaOH stock (Fisher) was
added in 200 μL increments. Molybdate working solution was made by diluting 100 mL of the molybdate stock solution up to 1 L using ultrapure water, and subsequently adding 1.6 mL of concentrated sulfuric acid (trace metal grade, Fisher) to make an overall 28.8 mM solution of H₂SO₄. Phosphate standards were made by dilutions in Milli-Q of appropriate volumes of the 100-ppm P stock solution. To 500 mL of each sample and standard, 500 μL of 0.1 M ferrozine stock was added to make a 100 μM ferrozine solution and complex any Fe²⁺ present in the samples.

1.3. Instrumentation

Waterville Analytical (Waterville, ME, USA) flow-injection analysis (FIA) instrument with chemiluminescence detection (Fig. 5) was used to mix sample with reagents and quantify the light given off by the reaction between luminol and the phosphomolybdate complex. For all pump lines in the FIA set-up, Tygon pump tubing of the same size (gray-gray) was used, thus the sample and working molybdate solution were mixed at equal volumes, spent some time in the reaction coil (if present) and then entered the flow cell. The other loop (labeled L2) was used to load the sample during the loading time and flushed by the pure water career into the system in the sample mode. Phosphomolybdate complex reacted with luminol (equal volumes entering again) in the flow cell. The detector was HC 135 Photon-Counting Photon Multiplier Tube (PMT), connected to a PC computer running Waterville Analytical software. PMT voltage was default (950 V) or 800 V, and the integration time for data points was 10 or 20 msec.
Figure 5. Waterville Analytical instrument for flow-injection analysis using chemiluminescence detection. Sample fills up the loop L2 while in the “load” mode. When switched to “sample” mode, sample from this loop is flushed by the pure water carrier to meet molybdate reagent at the T-section. Phosphate from the sample complexes with the molybdate forming 12-molybdophosphoric acid, subsequently reduced by luminol in the flow cell (box in the diagram). The light given off is quantified by HC 135 Photon Counting PMT.
2. Results and analysis for method standardization

Two initial method modifications were made to improve the system. First, the PMT signal was recorded using the system with the mixing coil in place between the sample/molybdate reagent T-cell and the flow cell, then the signal was recorded without the coil. No apparent difference was noted, meaning that the 12-phosphomolybdate complex formation is fast. Therefore, the loop was taken out of the system to shorten the time of analysis.

Another modification was needed to eliminate possibilities of interference with the phosphate signal. From the work of Yaqoob et al. (2004), the only important and a fairly potent interferent with this method is Fe(II), the reduced form of iron. Although the quantities of this cation tend to be fairly low in oxygenated surface water, significant amounts can be found in anoxic conditions; hence the standard method had to include a system to eliminate Fe(II). The initial attempt with an in-line column of chelating resin (Chelex 100) proved inconvenient because of the constant introduction of bubbles from the column and interference with the flow. Instead of Chelex, ferrozine (specific Fe(II) ligand) was added to all samples directly and in a sufficient amount (100 μM) to remove the possible interference from Fe(II) ions (Lin and Kester 1992).

The most important adjustment to be made was in the sensitivity of the system. The initial method used 20 ml of luminol stock per 100 ml working luminol solution (five-fold dilution), resulting in a great quantity of light that overwhelmed the PMT. The working solution was, thus, diluted to several different concentrations of luminol, ranging from 4.5x10^-6 M (2000-fold dilution) to 1.8x10^-4 M (50-fold dilution) (Fig. 6). Two PMT parameters were also varied. For 4.5x10^-6 M working solution, signal integration time of 10 milliseconds was used, giving the lowest signal. For all other runs, the integration
Figure 6. Reaction sensitivity as a function of changing concentration of luminol in the working solution buffered by 0.1 M borate at pH 12.5. Default PMT voltage (950 V) for all curves except blue square (800 V). PMT signal integration time $T = 20$ msec for all curves except blue diamond (10 ms).
time was set at 20 milliseconds. The run at the highest luminol concentration, \(1.8 \times 10^{-4}\) M, was performed with a PMT voltage of 800 V because the signal was out of range for the default voltage of 950.

Generally, the higher the luminol concentration, the higher the sensitivity of the method and the intensity of the light produced in the reaction. What seems to be an exception from this trend is the \(1.8 \times 10^{-4}\) M curve that almost overlaps with the data for \(8.9 \times 10^{-5}\) M luminol; however, the PMT sensitivity was lower due to the decreased voltage, so the effect of higher concentration was cancelled out. All standard curves were non-linear, following a second-order polynomial shape. All trendlines were curved and steep at low phosphate concentrations, slowly leveling off towards higher phosphate concentrations. This means the method was more sensitive at lower concentrations of analyte. The best quadratic fit was obtained with the 50-fold dilution \((1.8 \times 10^{-4}\) M), thus this proportion was used to prepare the solutions for analysis.

The reaction between luminol and 12-molybdophosphoric acid is highly pH-dependent. The standard curve with borate-buffered \(1.8 \times 10^{-4}\) M luminol working solution in fig. 6 was obtained at pH 12.5, the high pH being a result of adjustment using concentrated NaOH. The PMT peak integral at 10 ppb P was approximately \(6 \times 10^5\) with this set-up. However, the pH of working luminol used for lake data analysis was 9.7, solely due to the borate buffer, with no NaOH added. At this pH, the production of light was at a much lower scale, as attested by the lower PMT counts upon standard additions (Fig. 7) and much lower PMT peak integrals (only about \(4 \times 10^5\) at 10 ppb P). This lower sensitivity permitted the usage of default PMT voltage of 950 V with \(1.8 \times 10^{-4}\) M working luminol, and a typical standard curve for lake data analysis was obtained using this method (Fig. 8), with a limit of detection estimated at 0.25 ppb P.
Figure 7. Typical peaks for standard additions of phosphate (as ppb P). Working luminol solution contains 1.8x10^{-7} M luminol in 0.1 M borate buffer, pH = 9.7 (no NaOH added). PMT voltage was at default setting, 950 V, and the signal integration time 20 msec.
Figure 8. Standard curve for lake data analysis: integrals of the PMT signal peaks, such as the ones in fig. 7, are plotted against the known phosphate concentrations. LOD is estimated at 0.25 ppb P. Multiple points represent different runs of the same standard. For reaction conditions, refer to fig. 7.
3. Phosphate method summary

The luminol chemiluminescence method for phosphate determination was optimized for the needs of the lake analysis on Belgrade lakes by changing luminol concentration to $1.8 \times 10^{-4}$ M and the pH of this reagent to 9.7, taking out the reaction loop between the sample/molybdate T-section and the flow cell, and adding ferrozine (100 μM) to each sample before analysis. The improvements in low-range sensitivity can be made by bringing the pH up towards 12.5, by increasing luminol concentration, or by raising the integration time of the PMT. For our purposes, the LOD of 0.25 ppb P is satisfactory, but lower limits can be reached by applying some of the above suggestions. The method has several advantages over the standard colorimetric molybdenum blue method, such as the lower acidity of the reaction mixture and no need exists to use the long pathlength flow cell that gets clogged easily. Comparisons between the two techniques are shown in Table 1.
Table 1. Comparison of select properties of colorimetric and chemiluminescence methods for phosphate determination. The advantages of the chemiluminescence technique include the less acidic analysis mixture and waste, as well as the use of a small-sized flow cell. Both methods can be used in a flow-injection analysis set-up.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Mo-blue colorimetric</th>
<th>Luminol chemiluminescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>[sulfuric acid] upon mixing with sample</td>
<td>0.34 M</td>
<td>0.015 M</td>
</tr>
<tr>
<td>Buffer</td>
<td>none</td>
<td>0.1 M borate (pH 9.7-12.5)</td>
</tr>
<tr>
<td>Waste</td>
<td>highly acidic</td>
<td>less acidic</td>
</tr>
<tr>
<td>LOD</td>
<td>0.5 nm (0.016 ppb P)</td>
<td>1.0 nm (0.032 ppb P)</td>
</tr>
<tr>
<td>Long pathlength</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>flow cell needed</td>
<td>Slow, introduces drift if analysis is manual</td>
<td>Not investigated</td>
</tr>
</tbody>
</table>
III. Nitrate Methods

1. Experimental

1.1. Materials

Triethanolamine, hexamethonium hydroxide, and pyromellitic acid were obtained from Sigma/Aldrich (USA). Pre-made background electrolyte (BGE) was from Agilent Technologies, Inc. (Germany). KH₂PO₄, NaCl, and K₂SO₄ were obtained from Acros (USA), while NaNO₃, NaN₂O₂, and NaBr were from Fisher Scientific (USA). 1 N NaOH was produced by Acros.

1.2. Background electrolyte solution

The buffer solution for anion separation (pH 7.7 ± 0.2) contained 2.25 mM pyromellitic acid (absorbing species and flow carrier), 0.75 mM hexamethonium hydroxide (flow modifier), 1.60 mM triethanolamine (pKa 7.76), and 6.50 mM NaOH. Two background buffers were used: pre-made from Agilent and self-made. The buffers were made using Milli-Q water (18 MΩ, E-pure, Barnstead) and filtered through HV 0.45 μm Durapore PVDF Membrane (Millipore, Bedford, MA) prior to use.

1.3. Standard solutions

Nitrate and phosphate stock solutions of 1 g NO₃⁻/L and 1 g PO₄³⁻/L were made using Milli-Q water with NaNO₃ and KH₂PO₄, respectively, and stored at 4 °C. Stock solutions were appropriately diluted to the concentrations needed for standards using Milli-Q water and filtered before use through 0.45 μm membranes (Millipore).
1.4. Instrumentation

Agilent Capillary Electrophoresis system with indirect UV detection at 350 nm and referenced at 245 nm (changing absorbance is at 245 nm; the referencing makes peaks appear positive) was used for analysis. The bare fused-silica extended light path capillary (Agilent) had a total length of 64.5 cm, but a length of only 56 cm to the detector. The internal diameter was 75 μm, with an optical path length of 200 μm at the widening ("bubble"). The separation voltage was -20 kV. The sample injection was executed using two modes: hydrostatic (pressure) injection at 50 mbar for 4, 40, and 80 seconds, and electrokinetic injection at -5 kV for 10 seconds and -10 kV for 20 seconds. The temperature was maintained at 20 °C by an air fan cooling system.

1.5. Conditioning of the capillary

Before the first use, after storage, or if contaminated, the capillary was conditioned by purging with 1 M NaOH for 10 min, followed by Milli-Q water for 10 min, and finally with the background electrolyte mixture for 30 min. Before each analysis, the capillary was rinsed with 0.1 M NaOH for 3 min and with the background buffer for 5 min in order to keep the migration time reproducible.

2. Results and analysis for method standardization

Initial trials to separate a standard mixture of anions (Br⁻, Cl⁻, SO₄²⁻, NO₂⁻, NO₃⁻, and H₂PO₄⁻, 10 ppm) with the homemade background buffer using pressure injection for
Figure 9. Electropherograms of standard mixtures of anions (10 ppm), injected by pressure injection for 4 sec: A. and B. are mixes with different contents. Peak identities. 1. Bromide; 2. Chloride; 3. Sulfate; 4. Nitrite; 5. Nitrate; 6. Phosphate; U. Unknown. Electropherogram B was taken after A, and shows evidence of trace amounts of chloride (contamination). Also, note that sulphate and nitrite peaks are not resolved.
4 seconds were successful, but showed a poor resolution of sulfate and nitrite peaks (Fig. 9). However, this was deemed satisfactory because of the primary interest in quantifying nitrate and, possibly, phosphate.

When the lake water samples from Snow Pond were analyzed for the first time, it was apparent that detection limits with pressure injection were not be low enough, therefore the approach of electrokinetic injections was taken. Electrokinetic injection operates by applying a voltage to the sample in order to attract the ions of opposite charge to the detector electrode into the capillary. This method selectively introduces anions into the capillary, and its selectivity is proportional to the total mobility of each anionic species. Moreover, when this injection mode is utilized for low ionic strength samples (e.g. Belgrade Lakes water), the voltage drop across the length of the capillary is small due to the high conductance of the background electrolyte. Almost the entire voltage drop occurs at the low conductance plug from the sample, resulting in stacking of analyte at the sample/buffer interface (Jackson and Haddad 1993).

Electrokinetic injections at -10 kV for 20 seconds permitted the detection of nitrate peaks in lake water, albeit with a poor resolution from sulfate (fig. 10a) because of the high concentration of this anion in lake water. The problem was circumvented by using the pre-made Agilent anion buffer that had enough resolving power to produce distinct peaks for nitrate and sulfate (fig. 10b). The buffer supplied by Agilent may have worked better because triethanolamine (TEA) was highly viscous during the homemade buffer preparation and hard to quantitatively transfer into the solution. Also, TEA may be responsible for peak migration patterns that clearly did not follow the charge-to-size ratios, but seemed to be interacting with one of the buffer components. The amine group
Figure 10. Electropherograms from two runs of East Pond lake water (collected September 2004) using electrokinetic injection at -10 kV for 20 sec:
A. Background buffer was self-made.
B. Background buffer from Agilent.
Peak identity: 1. Chloride; 2. Sulfate; 3. Nitrate; U. Unknown. Lake water contains much more chloride and sulphate than nitrate. Note that the nitrate peak is resolved in electropherogram B.
of TEA is a candidate for this interaction. Another way to improve the resolution would also be to use a longer column, although at a cost of analysis time.

The exploitation of lower detection limits with electromigrative injection mode introduced another variable in the procedure because the injection of anions is biased towards ions of higher mobility. Due to this interdependence between the sample matrix conductivity and the amount of analyte injected, both standards and sample analysis would have to be performed at the same background conductivity (Pantsar-Kallio 1995). Since this is impossible in practice for samples from varying lakes and depths, internal standard or standard addition methods had to be performed. Standard additions were the method of choice.

The choice of parameter to measure was initially the nitrate peak area. However, despite the fact that standard additions were performed to each lake water sample from the same bottle and that the volume of nitrate/phosphate solution added to the lake water was at most 10% of the total volume of each standard, sulfate and chloride peak areas varied considerably and without a clear pattern between runs of standard additions to the same sample. Since the concentration of sulfate was the same in each standard addition sample made of the identical lake water sample, electrokinetic injection clearly introduced varying quantities of anions into the capillary between different runs. This problem was resolved by using the sulfate peak area as a reference to that of the nitrate peak. Area correction was applied to each sample by the following formula:

\[
\text{Corrected area} = \left( \frac{\text{nitrate peak area}}{\text{sulfate peak area}} \right) \times 1000
\]

Data processed in this way was clearly much more linear. For example, standard addition curve for Snow Pond sample at depth of 4 m using only the nitrate peak areas resulted in a linearity coefficient of 0.9908, while the plot of corrected area gave
Figure 11. Corrected versus uncorrected nitrate peak area in standard addition samples using electrokinetic injection. Nitrate peak area was divided by sulfate peak area and multiplied by 1000 to get the corrected peak area. The example shown is of Snow Pond, sample from 4 m deep. Clearly, correcting the peak area for fluctuations in injection amounts improves the linearity of the curve.
$R^2=0.9992$ (Fig. 11). Phosphate analysis was not performed because no peaks corresponding to the migration time of phosphate exhibited a proportional increase in area, therefore making it impossible to conclusively assign phosphate peaks. This was also true when injecting samples hydrostatically, so the analysis of phosphate was not performed using CE. The reasons why this method did not work for phosphate are not clear.

For each lake water sample analyzed using electrokinetic injection, a series of four standard addition samples was made. It was generally noted that the precision of these analyses decreased as the initial concentration of nitrate in the lake water decreased. One of the ways to avoid this problem was to use larger standard additions – additions of 10, 20 and 40 ppb P worked better for the Great Pond sample from 19 meter deep than the additions of 5, 10 and 20 ppb (Fig. 12). However, the limits can sometimes be reached where the capillary is overloaded with analyte, and no improvement in standard addition analyses can be made without changing the concentration of the absorbing species (PME). In that case, reverting back to pressure injections was useful.

Pressure injection method was by far simpler than the electrokinetic method because independent set of nitrate standards can be run and the lake samples’ concentration calculated from the same standard curve. The injection of 40 seconds produced a very linear standard curve ($R^2=0.9946$), with a dynamic range of up to 400 ppb N, limit of detection (LOD) of 12 ppb N, and limit of quantification (LOQ) of 40 ppb N (Fig. 13a). LOD and LOQ were calculated from the intercept plus 3 or 10 standard deviations of the intercept (LINEST values), respectively. Concentrations of nitrate in the range of 0-100 ppb lay in the part of the curve that deviates most from linear fit, therefore a series of standards injected by pressure for 80 seconds, and ranging up to 80 ppb N,
was used for the determination of the samples that contained nitrate at the levels between 30 and 80 ppb. This curve (Fig. 13b) was less linear ($R^2=0.9808$), but the results for lower nitrate levels were clearly more confident with LOD of 7 ppb N and LOQ of 22 ppb N. For comparison with the electrokinetic injection mode, a series of standard addition curves for Snow Pond samples from different depths is included here and shows a detection of nitrate as low as 2.4 ppb for the 2 meter sample (Fig. 14).

3. Nitrate method summary

A method was developed to quantify nitrate in Belgrade lakes water using the CE with 56-cm extended pathlength capillary and the buffer consisting of PMA, HMOH and TEA (see Experimental). For higher nitrate concentrations in lake samples (>20 ppb N), pressure injection was used for sample introduction, and the samples were quantified using a calibration standard curve. For a lower range of nitrate concentrations (2-40 ppb N), the sample was injected in electrokinetic mode, and series of standard addition samples were used for quantification. Capillary electrophoresis has several advantages over the standard colorimetric, azo dye method (Table 2). It is fully automated, does not use the toxic cadmium column and requires very small sample volumes. The sample preparation is minimal, limited to the filtration of the collected lake water only.
Figure 12. Changes in linearity with nitrate standard addition size for 19 m Great Pond sample. The concentration of nitrate in this sample is relatively high (41 ppb N), so adding larger amounts of analyte in standard additions works better.
Figure 13. NO$_3^-$ Standard curve using pressure injection:
A. For 40 sec. LOD = 12 ppb N, LOQ = 40.
B. For 80 sec. LOD = 7 ppb N, LOQ = 22 ppb N.
Figure 14. Standard additions curves for Snow Pond water samples. The concentrations of nitrate range from 2.4 ppb N (2 m sample) to 31 ppb N (18 m sample).
Table 2. Comparison between the standard analytical method for nitrate and the CE method. The advantages of the CE stem clearly from the little amount of sample preparation needed, full automation, low sample volume and toxicity.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Method</th>
<th>of analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colorimetric (azo dye)</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>Sample preparation</td>
<td>Reduction on</td>
<td>filtration (0.45 um)</td>
</tr>
<tr>
<td></td>
<td>cadmium column</td>
<td></td>
</tr>
<tr>
<td>Toxicty</td>
<td>high</td>
<td>virtually none</td>
</tr>
<tr>
<td>Sample volume</td>
<td>tens of mL</td>
<td>&lt; 1 mL</td>
</tr>
<tr>
<td>Operation</td>
<td>Flow-Injection Analysis</td>
<td>Fully automated</td>
</tr>
<tr>
<td></td>
<td>system possible</td>
<td></td>
</tr>
<tr>
<td>Compatibility with TN/TP</td>
<td>Compatible</td>
<td>Incompatible (digestion</td>
</tr>
<tr>
<td>digestions</td>
<td></td>
<td>introduces too many ions)</td>
</tr>
</tbody>
</table>
IV. Lake Water Data and Analysis

1. Sample collection

Before collecting samples in the field, every plastic bottle was filled overnight with 1% sulfuric acid, then washed thoroughly with Milli-Q water and filled with this pure water until the sample was taken in the field. Samples from Snow Pond were collected on February 17th, 2005 through a hole drilled by auger. East Pond and North Pond samples were collected on March 15th and 16th, 2005, in a joint effort with Maine Department of Environmental Protection (DEP). Great Pond samples from March 21st were kindly supplied by the DEP. Temperature and dissolved oxygen (DO) profiles from East Pond and Great Pond were collected by the DEP, while the profile from North Pond was recorded using a YSI sonde with temperature and DO probes. A temperature and DO profile for Snow Pond was not taken. Each lake was sampled at a deep “hole”, the deepest part of the lake, from surface to the bottom, apart from Snow Pond where the bottom was not reached (>30 m) (exact sampling information in Table 3). Two bottles of sample from each depth at each lake (apart from Great Pond) were collected. The contents of one bottle were refrigerated, while the contents of the other were frozen, both unfiltered and filtered through a 0.45 μm membrane in order to prevent the rupturing of cell membranes and, thus, preserve the phosphate and nitrate speciation.

2. Results

Temperature and DO profiles showed a similar pattern across the three lakes examined (Fig. 15). The very top layer of water lying beneath the ice was at about 0 °C, increasing with depth to about 4 °C, forming a mini winter thermocline at about 1.0 m
Table 3. Table listing the sampling dates, exact coordinates of each station and water sample depths. Shaded in gray on the side is the vital lake information such as the surface area, and average and maximum depth.

<table>
<thead>
<tr>
<th>Lake / Lake depth (m)</th>
<th>Sampling date</th>
<th>Station latitude and longitude</th>
<th>Sample depth (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snow Pond</td>
<td></td>
<td>17-Feb-05</td>
<td>2</td>
</tr>
<tr>
<td>10.05</td>
<td></td>
<td>N 44° 30.341'</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W 69° 45.993'</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Great Pond</td>
<td></td>
<td>21-Mar-05</td>
<td>1</td>
</tr>
<tr>
<td>6.4</td>
<td></td>
<td>N 44° 32.675'</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W 69° 51.181'</td>
<td>7</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19</td>
</tr>
<tr>
<td>North Pond</td>
<td></td>
<td>16-Mar-05</td>
<td>1</td>
</tr>
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<td>21.03</td>
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</tr>
<tr>
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<td>W 69° 50.275'</td>
<td>3</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.5</td>
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<td>East Pond</td>
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<td></td>
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</tbody>
</table>
Figure 15. Temperature and DO profiles of: A. East Pond, March 15, 2005; B. North Pond, March 16, 2005; C. Great Pond, March 21, 2005. For the exact coordinates of each station, refer to Table 3.
depth in East and North Ponds and about 5.0 m depth in Great Pond. The temperature then stayed more or less uniform with depth, albeit with one surprising feature: at the bottom of each lake, water temperature was higher than 4 °C, approaching nearly 5 °C. This feature was surprising since the maximum density of water is at 4 °C, so it would be expected that the lower density, 5 °C water would not stay at the bottom. Dissolved oxygen is inversely related to temperature and the temperature-depth and DO-depth curves mirror each other. The highest concentration of DO, 15-20 ppm for each lake, was recorded in the surface layer. Deeper in the lake, DO was 6-10 ppm in all lakes, and was below 5 ppm at the bottom of both Great Pond and North Pond (Table 4).

Nitrate and phosphate lake profiles (Fig. 16) showed more variation between the lakes. Phosphate was uniformly distributed throughout different depths in each lake, with the values close of about 8-10 ppb P (Table 4). Slightly higher phosphate concentrations (10-20 ppb P) were present in North Pond. Nitrate concentrations varied more with the depth, generally exhibiting a vertical stratification. The top layers of each lake (exception of North Pond, 1 m sample) generally contained less nitrate, but this nitrate concentration increased with depth. The levels of nitrate were elevated in the two smaller lakes (East Pond and North Pond). East Pond nitrate concentrations were about 70 ppb N through several meters of water column, then increased sharply to 400 ppb N at 6 m depth (Table 4). Also, the bottom sample from East Pond (6 m) contained nitrite (data not presented), but this nutrient was not quantifiable due to the co-elution of sulphate and nitrite. North Pond nitrate concentrations increased linearly with depth from 20 ppb N at 2 meters to >80 ppb N at the bottom of the pond. Both Great Pond and Snow Pond had much lower nitrate concentrations – ranging from 2-5 ppb N at the surface to a maximum of about 40
ppb N at depth (this was at the bottom of Great Pond; the bottom of Snow Pond was not reached).

The ratios of nitrate to phosphate were below the Redfield mass ratio of 7:1 for all lakes, with the exception of East Pond where the Redfield ratio was matched in lower depth samples, and exceeded in the deepest two samples (Fig. 17; see also, Table 4).
Figure 16. Phosphate and nitrate vertical profiles of: A. East Pond; B. North Pond; C. Great Pond; D. Snow Pond. For the exact coordinates of each station and sample collection dates, please refer to Table 1. East and North Pond nitrate data were obtained using pressure injection, Snow Pond nitrate data using electrokinetic injection, and Great Pond nitrate data with the combination of both. Phosphate data was obtained using the luminol chemiluminescence method. Multiple points for the same depth and nutrient (unless otherwise indicated) correspond to different runs of the same water sample, therefore indicating the analytical imprecision.
Table 4. The summary table of nitrate, phosphate, nitrate:phosphate ratio, DO and temperature data for all lakes studied.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Depth (m)</th>
<th>Nitrate (ppb N)</th>
<th>Phosphate (ppb P)</th>
<th>Nitrate:Phosphate ratio (mass)</th>
<th>Dissolved O2 (ppm)</th>
<th>Temperature (deg. Celsius)</th>
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</thead>
<tbody>
<tr>
<td>Snow Pond</td>
<td>2</td>
<td>2.4</td>
<td>9.1</td>
<td>0.3</td>
<td>N/D</td>
<td>N/D</td>
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<td>4</td>
<td>8.8</td>
<td>9.8</td>
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<tr>
<td></td>
<td>7</td>
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<tr>
<td></td>
<td>12</td>
<td>23</td>
<td>9.1</td>
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<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>31</td>
<td>10</td>
<td>3.1</td>
<td>N/D</td>
<td>N/D</td>
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<td>Great Pond</td>
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<td>9.3</td>
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Figure 17. Nitrate to phosphate mass comparison:
A. Data for all lakes. The Redfield ratio of N:P = 7:1 (in mass units) is indicated by the dashed line.
B. The two extreme cases: East Pond and Snow Pond.
3. Discussion

East Pond, followed by North Pond, is the lake that has the most recurring and strongest blooms of the four lakes, as indicated by the Secchi depth data collected since the 1970s (Figs. 18 and 19). The average yearly visibility through the water columns of East Pond and North Pond is lower compared to that of Great and Snow Pond. In addition to that, the values of minimum Secchi depth reading for each year, indicating the most serious blooms, are much lower for East Pond and undoubtedly confirm that East Pond has the most serious problem with algal blooms.

East Pond also stands out in the current study as the lake where the highest nitrate to phosphate ratios were found. These ratios, apart from the 6 m sample from East Pond, all fall below the magic cut-off number of 29 (for TN:TP), proposed by Smith (1983), and according to which all the lakes studied should be supporting noxious cyanobacteria blooms. However, observations from the field indicate that the lake with the highest nitrate to phosphate ratio blooms the most, meaning that low nitrate:phosphate ratio does a poor job predicting the blooms of bluegreen algae on Belgrade lakes. Another uncertainty associated with the nitrate to phosphate ratio (and, realistically, with TN:TP ratios) lies in the way the numbers are reported. From the data for Belgrade Lakes, these ratios differ between samples from higher and lower depths. Yet another value could be reported if an integrated column sample were taken. Hence, it is clear that a lot of ambiguity is tied to reporting nutrient ratios.

Nevertheless, some general trends could be observed by performing the analysis of nitrate to phosphate ratios. Although these ratios do not represent TN to TP relationship,
Figure 18. Mean yearly Secchi depth readings with each year’s maxima and minima for Great Pond (pink square), and Snow Pond (green triangle). These lakes’ water column transparency is generally greater than that of North Pond and East Pond (Figure 19). Data from PEARL 2005.
Figure 19. Mean yearly Secchi depth readings with each year’s maxima and minima for North Pond (red triangle), and East Pond (blue diamond). These lakes’ water column transparency is generally lower than that of Great Pond and Snow Pond (Figure 18). In addition to that, East Pond’s minimum Secchi readings are consistently and significantly lower, indicating that the blooms are particularly severe on this lake. Data from PEARL 2005.
they indicate that East Pond has a higher amount of nitrate available for plankton growth early in the season. The high nitrate concentration, while not selectively favoring cyanobacterial growth, could allow a significant amount of all phylums of algae to grow until the nitrate limitation starts appearing. At that point, later in the season, nitrogen limitation may cause the appearance of cyanobacteria blooms. Data to substantiate this hypothesis is, unfortunately, lacking at the time.

Temperature in all lakes was fairly uniform throughout the water column, indicating that barriers to mixing of the water were low. In line with this observation were the fairly constant concentrations of phosphate in the water column of each lake. However, the apparent stratification of DO and nitrate data points out that mixing, if it occurred, was slow and insufficient to prevent DO depletion at greater depths, to prevent the occurrence of nitrite (reduced species) at the bottom of East Pond, or to redistribute nitrate from the bottom layers towards the surface.

The appearance of water warmer than 4 °C at the bottom of each lake, and below the column of denser, 4 °C water, suggests that there was a source of warmer water at the bottom of the lakes, possibly from ground water fluxes. This ground water may also be anoxic and contain higher levels of nitrate, which would contribute to the low DO and high nitrate levels near the bottom.

Comparing phosphate and nitrate data, it is clear that the processes resulting in addition or removal of phosphate from the water column were inactive at the time of sampling. In contrast to that, nitrate levels increased significantly with depth, especially in the small lakes, East and North Pond. For that reason, it appears that nitrate was effectively being released from the sediments. It would be interesting to compare these
trends in phosphate and nitrate levels with summer-time data when temperature and redox conditions are different, however these data are not currently available.

As discussed earlier, phosphate and nitrate are not necessarily representative of total P and total N, the parameters that are needed in order to evaluate the total nutrient banks of phosphorus and nitrogen in a lake (Dodds 2003). Nevertheless, if we assumed that the current levels of the two monitored species, phosphate and nitrate, stayed more or less unaltered after the spring turnover of the lake, these are the nutrients that will immediately be available to the newly-growing biota. When comparing the results from current study, it is clear that East Pond has the highest supply of nitrate, followed by North Pond that also has a higher concentration of phosphate compared to the other three lakes. These lakes, therefore, may be able to support larger algal populations early in the season.

Besides the analyses of phosphate and nitrate, proposed and demonstrated in this work, it is essential that total N and total P levels be regularly monitored as well in order to have the data that represents total N and P loading, and allows for N:P ratio calculations. These parameters are crucial in predicting and understanding the occurrence of cyanobacteria blooms (Dodds 2003; Elser 1999). Moreover, the search for causes and conditions of blooms on Belgrade lakes does not end at nutrient analysis by itself; other conditions such as wind speed, occurrence of storms, and the density of zooplankton must also be monitored (Elser 1999, Nøges et al. 1999; Ojala et al. 2003). These factors are pertinent, especially if we know that the two lakes with the highest potential for blooms, East and North Pond, are both small and shallow lakes that can easily be mixed in a wind event. Moreover, plans have been made to trap smaller-sized fish from East Pond in order to, in a sequence of food chain events, increase the population of
zooplankton and diminish the population of phytoplankton (cyanobacteria included).

Before any such decisions are made and executed, it is important to understand all the key players in the ponds.
V. Conclusions

Efficient, cost-effective and environmentally-friendly techniques for nitrate and phosphate detection were developed using capillary electrophoresis and luminol chemiluminescence, respectively. These methods have a number of advantages over the standard colorimetric methods, while maintaining low detection and quantification limits necessary for water analysis in clean, northern temperate lakes. The analysis of phosphate and nitrate in Belgrade Lakes water was successfully performed and contributed information that can be used in further nutrient studies on these lakes. Information on total P and N, as well as other lake parameters, is needed to properly assess the causes, and predict the occurrence of, cyanobacteria blooms in these lake basins.

1. Future work

Future work is necessary in three domains in order to successfully explore all the possible links in the enigma of cyanobacteria blooms on Belgrade Lakes. Firstly, analysis of the algal and zooplankton species distribution and density over summer season is crucial in order to understand which species dominate. Secondly, continued monitoring of physicochemical conditions on the lakes, and their relationship to bloom periods is advised. Lastly, but not less importantly, periodic TN and TP measurements are necessary in all seasons, as well as continuous monitoring of nitrates and phosphates throughout the year, and of ammonia in summer season.

As far as method development is concerned, more work is necessary to develop convenient procedures for TN and TP analysis. Furthermore, the use of CE can be further explored for the analysis of nitrates, phosphates, and cations, such as ammonia.
VI. References


Dodds, W. K., What controls levels of dissolved phosphate and ammonium in surface waters? *Aquatic Sciences* 1993, 55, 132-142.


Jackson, P. E.; Haddad, P. R., Optimization of injection technique in capillary ion electrophoresis for the determination of trace level anions in environmental samples. *Journal of Chromatography* 1993, 640, (1-2), 481-7.


PEARL: Public Educational Access to Environmental Information in Maine.


