# Analyses of Dissolved Organic Nitrogen in Forested and Estuarine Ecosystems: Black Rock Forest and Hudson River

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Abstract: Nitrogen is considered a limiting nutrient for primary production in both forested and marine ecosystems, and estimates of primary production can be based on nitrogen budgets. Often in these past studies, dissolved organic nitrogen (DON) has been ignored. However, DON has proven to be a significant component of the fixed nitrogen cycle in both forested and marine environments. This thesis seeks to investigate the concentration and the chemical composition of DON in two non-marine environments in an attempt to more accurately describe the nitrogen budgets of these ecosystems. Two different environments were studied. The first study compared the concentration of dissolved organic nitrogen and the characteristic dissolved proteins released from two watersheds in Black Rock Forest. Cascade Brook watershed was characterized by a wetland and by primarily deciduous vegetation; Black Rock Brook watershed had a distinctive stand of coniferous hemlocks. The second study examined the concentration of dissolved organic nitrogen and characterized the proteinaceous component of DON from Hudson River water collected at Erie Pier. Data for both studies were collected from 2/12/99- 3/27/99.

Dissolved organic nitrogen was a significant component of the total dissolved nitrogen in both the forest and the estuary. In Black Rock Forest, DON accounted for between 45% to 55% of total dissolved nitrogen. Cascade Brook Watershed had a lower concentration of DON (1.2-1.7μM) than Black Rock Brook Watershed (3.7-4.1μM). Samples collected after the Cascade Brook wetland had an increased concentration of DON. The watersheds had similar characteristic proteins. Cascade Brook had proteins of 62kDa, 66kDa, and 150kDa. Black Rock Brook had proteins of 118kDa, 64kDa, and 62kDa. Water that passed through the Cascade Brook wetland had a higher molecular weight protein (150kDa) than water that had not passed through the wetland; this suggests DON production in the wetland. Water that passed through the hemlock stand had no distinct protein bands; this suggests protein degradation. The Hudson River samples had DON concentrations of 3-20μM; these values represented between 5% to 50% of total dissolved nitrogen. The Hudson River samples had some proteins in common with the Black Rock Forest samples (116kDa, 65kDa, 62kDa, 118kDa). These samples also had some unique proteins (85kDa, 29kDa, 17kDa, 10kDa). The quantitative information about the DON contribution to total dissolved nitrogen indicate that DON should be considered in nitrogen budget calculations. The qualitative information about DON provides insight into the biogeochemistry of ecosystems that can be useful in constructing nitrogen cycles and nitrogen budgets.

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# Introduction

## Nitrogen in the Environment

Nitrogen is an essential nutrient for all organisms. Although it is present in the atmosphere as $N_2$, only a few types of bacteria can use this form of nitrogen. Bioavailable nitrogen exists in both inorganic and organic forms. Plants can utilize the major inorganic forms of nitrogen: ammonium ion ($NH_4$), nitrate ($NO_3$), and nitrite ($NO_2$). Bacteria and microorganisms metabolize organic forms of nitrogen and can remineralize organic nitrogen to inorganic nitrogen. Organic nitrogen can be either particulate or dissolved. Particulate organic nitrogen can be in the form of amino acids, proteins, or other more complex molecules. Because the compounds which compose dissolved organic nitrogen are not easily isolated, the chemical composition of DON is not well known. Burdige 1998 describes dissolved organic nitrogen as "a heterogeneous class of organic compounds that ranges from well-defined biochemicals such as urea or amino acids to more complex (and poorly characterized) compounds such as humic and fulvic acids."

In forested, estuarine, and marine ecosystems nitrogen is involved in complicated cycling between inorganic and organic forms. There also are significant fluxes both into and out of each ecosystem. A nitrogen budget for a particular ecosystem can be constructed by calculating the fluxes of nitrogen into and out of the ecosystem. Nitrogen budgets can provide information about some important biological and geochemical processes of the ecosystem.

## Nitrogen and Ecosystem Primary Production

Primary production can be described as the transformation of inorganic carbon ($CO_2$) into organic carbon. The process of photosynthesis uses the sun's energy to accomplish this transformation. Photosynthetic organisms require nutrients in addition to sources of carbon and energy. Nitrogen is an essential nutrient for plant growth, and the availability of nitrogen often limits the primary production of ecosystems.

In forested ecosystems, nitrogen budgets have been used to calculate the inorganic carbon uptake and the corresponding organic carbon storage in biomass. The difference

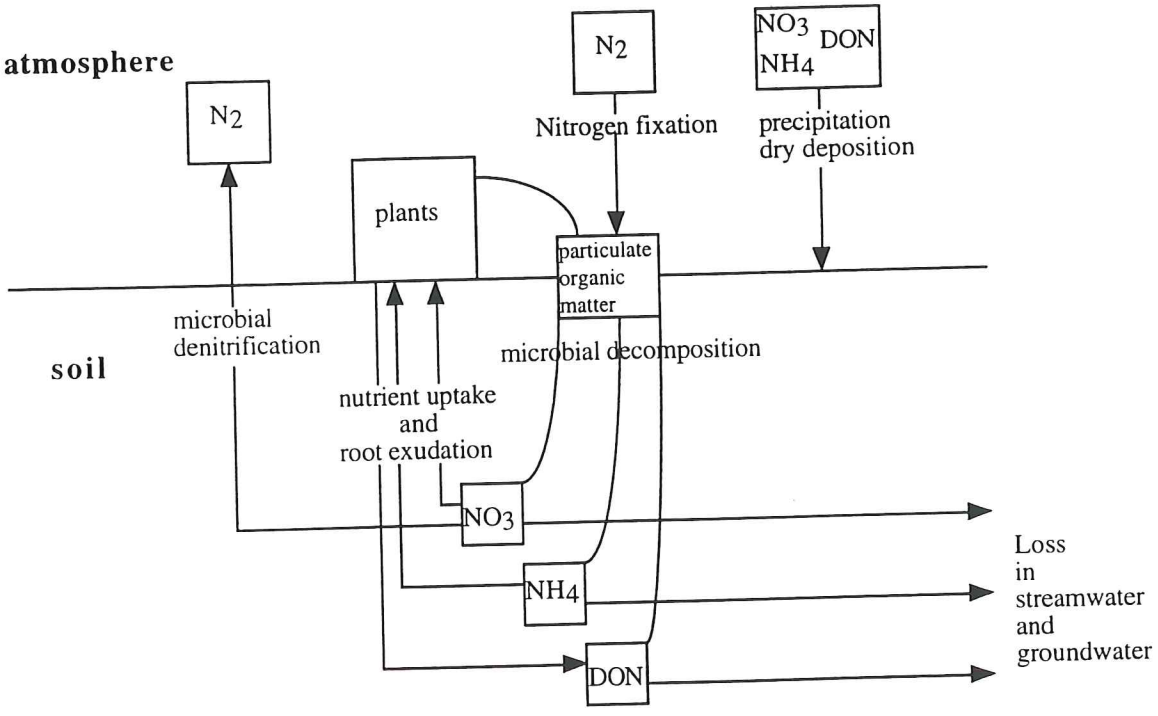
between the amount of fixed nitrogen entering and exiting the system is often assumed to represent primary production.

In the marine ecosystem, nitrogen budgets have been similarly used to estimate primary production.

**Nitrogen in Forested Ecosystems**

Nitrogen can enter a forested system through the fixation of atmospheric nitrogen, precipitation, and dry deposition. Nitrogen is stored on land in biomass, plant litter, and surface soils (Hedges 1997). Nitrogen is also recycled to inorganic nitrogen by microbial mineralization; this nitrogen can be reused or it can be lost. Nitrogen can leave forested systems by bacterial denitrification. Inorganic and organic forms of nitrogen can also be lost via surface water and groundwater which eventually feed into streams and rivers (Figure 1).

Figure 1. Simplified Forest Nitrogen Cycle (Modified from Berner and Berner 1996).




Hedges 1997 notes that much of the total dissolved organic matter in rivers appears to be soil derived. This suggests that much of the dissolved organic nitrogen in rivers is also soil derived. How does DON from the soil enter streams and rivers?

*DON Sources*

The major source of dissolved organic nitrogen in forested ecosystems is the decomposition of particulate organic matter. Microbial decomposition indirectly and directly produce DON. Microbes metabolize complex particulate organic matter to less complex organic matter called humus. Humic matter is defined as "soil organic matter which cannot macroscopically be recognized as plant or animal remains" (Tamm 1991). Based on this definition, humus always contains nitrogen (Tamm 1991). Humus has also been described as "amorphous resistant products of decomposition" (Schlesinger 1991). Although the chemical composition of humus is not well described, much of the nitrogen in humic matter is in aromatic rings which are resistant to microbial decomposition (Tamm 1991; Schlesinger 1991). Humic matter is a major component of forest soils, and organic nitrogen dissolved from woodland humic soils provides DON to groundwater and streams (Rudy 1994; Hedin 1995).

DON is also a direct product of microbial decomposition; DON and $NH_4$ are the two principal end-products of the decomposition of particulate organic nitrogen (Dickens et al. 1996). Actively growing plants excrete dissolved organic compounds directly from their roots. Cornell et al. 1995 also suggest that DON in rainwater could represent a significant input of DON to forested ecosystems. However, further study is needed to determine if plant root exudate and precipitation are major sources of dissolved organic nitrogen in forested ecosystems.

The rate of DON production by both microbes and plants depends on temperature. Microbial metabolism increases with temperature. Root exudate will only be produced during the growing season. These sources of DON contribute to a reservoir of DON which can be stored in humic soils.

The chemical composition of terrestrial DON may vary between watersheds. The chemical composition of the microbe-derived DON depends on the types of complex organic material available for decomposition (species of forested vegetation) and on the community of microbial species. The composition of forested vegetation has been shown to affect the composition of the DON exported from the ecosystem (Rudy 1994). The tissue and litter quality (defined as C:N or lignin: N ratios) may differ between coniferous and deciduous forests, and these different chemical structures will result in different

products of decomposition (Hedin et al. 1995). Ranges of microbial communities also inhabit different soil types. These different communities will metabolize organic matter differently; the chemical components of DON could differ according to the bacterial communities. In addition to providing organic matter which bacteria can decompose to DON, plants also directly produce DON through root exudate. The chemical composition of this root exudate will be species dependent.

*DON Losses*

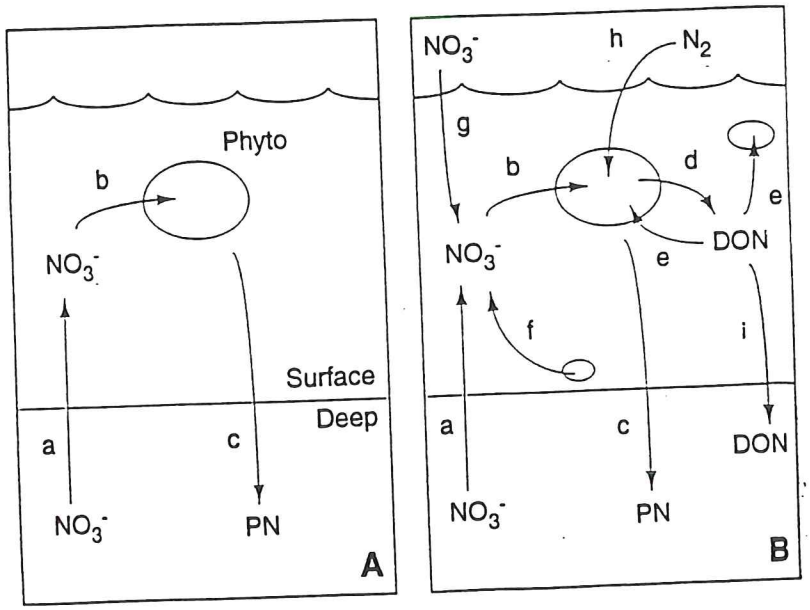
Unlike $NO_3$ and $NH_4$, DON is often biologically unavailable; it cannot be directly used by plants, and it is often resistant to microbial decomposition (Hedin et al. 1995). Because plants cannot utilize biologically unavailable compounds, DON can leach below the root zone. DON in soil below the root zone cannot be retained in the ecosystem biologically; its export depends on abiotic factors such as precipitation and groundwater flow (Clair 1996). DON can be immobilized in soil humus; it can also be released from humic soils (Hedin et al. 1995). In forested ecosystems, humic soils and other sources of DON contribute to soil organic pools (Hedin et al. 1995). Both surface water and groundwater can dissolve fulvic and humic acids from these pools and contribute to DON export (Hedin et al. 1995). "Losses of DON are more likely more directly linked to hydrological parameters, such as variations in water flow paths through soils and dissolution kinetics of humic soil components" (Hedin et al. 1995). The reservoirs of DON can contribute to DON export throughout the year.

Factors that reduce microbial metabolic activity could lead to preservation and accumulation of DON (Rudy 1994). Decomposition rates depend on temperature, moisture, and the chemical composition of soil litter (Schlesinger 1991). Anoxic conditions such as those found in wetland soils can also reduce microbial decomposition and increase the DON concentration in soil waters (Rudy 1994; Nelson et al. 1996). Davidsson et al. 1997 found that in peaty soils (soils with high organic content) dissolved organic nitrogen was released from anoxic soils; inorganic nitrate was consumed in these same soils. In general, the soil conditions in forested ecosystems could influence the quantity of DON exported from the ecosystem.

**Nitrogen in Estuarine Ecosystems**

Nitrogen fixation, precipitation, dry deposition, rivers, upwelling, and tides all can introduce nitrogen into an estuary. For a discussion of nitrogen cycling, I will define the estuarine ecosystem as the surface waters of the estuary, or any estuarine water that receives sunlight. Only nitrogen in the surface waters is available for primary production because photosynthesis depends on sunlight. I will consider nitrogen lost from the ecosystem if it enters water that sunlight cannot penetrate because this nitrogen is no longer available for primary production. Nitrogen that sinks below the photic zone can be reintroduced into the ecosystem by tidal mixing and upwelling (Fig. 2).

Figure 2. Simplified Estuary Nitrogen Cycle. (A) The traditional view of estuarine nitrogen cycling and primary production. (B) A revised view of estuarine nitrogen cycle where (g) can represent any flux of $NO_3$ into the estuary from rivers or the atmosphere. (Bronk et al. 1994).




Nitrogen is stored in biomass for a much shorter time in an estuary than in a forest because the types of primary producers differ. In an estuary phytoplankton are the major primary producers. These photosynthetic organisms are short-lived compared to forest vegetation. There is a much smaller reservoir of stored nitrogen in the surface waters of

an estuary than in a forest. The reservoir of nitrogen that exists in the surface waters of the estuary consists mostly of dissolved species of nitrogen; in forested ecosystems there are large DON reservoirs stored in the soil (Hedges 1997). Nitrogen can be recycled and reused in the surface waters of the estuary. It can also leave the surface waters in the form of sinking particles. Tidal mixing can also reduce the concentration of nitrogen.

There are at least three major sources of dissolved organic nitrogen in estuaries: microbial decomposition, phytoplankton, and river input. Hedges 1997 notes that most dissolved organic matter, and thus most dissolved organic nitrogen is derived from marine plankton. This derivation could be due to bacterial decomposition of dead plankton. Actively growing phytoplankton also can produce dissolved nitrogen, especially dissolved proteins (Paul 1990; Gilbert 1994).

## Previous Nitrogen Studies

In nitrogen budgets for both forested and estuarine ecosystems, dissolved organic nitrogen has often been ignored. Perhaps because photosynthetic organisms directly utilize inorganic nitrogen for growth, inorganic species of nitrogen have dominated the focus of nitrogen studies. Scientists have often focused on the import and export of inorganic nitrogen ($NO_3$ and $NH_4$).

### *Forest Nitrogen Studies*

A common assumption about forested ecosystems is that they are nitrogen limited and "characterized by efficient internal nitrogen cycling leading to a minimal loss of inorganic nitrogen in surface waters, groundwater, and gaseous loss through denitrification" (Williams et al. 1996). This assumption seems to imply that inorganic nitrogen is the major form of nitrogen cycling through forested ecosystems and the major form of nitrogen lost from these ecosystems. If this were true, the export of nitrogen could accurately be described based on measurements of inorganic nitrogen. However, dissolved organic nitrogen can represent a significant component of the nitrogen lost from forested ecosystems. J. Aber's 1998 models of nitrogen cycling in forest ecosystems also focus on inorganic nitrogen loss. He seems to equate total nitrogen loss with inorganic nitrogen loss. In the abstract of a paper entitled "What's missing from models of N cycling in forest ecosystems?" there is no mention of DON (Aber 1998).

Church and Driscoll 1997 suggest that "failure to consider DON may result in considerable error in watershed N mass balances." The flux of DON out of forests and into surface water and groundwater decreases the amount of nitrogen available in the ecosystem for primary production. This flux can also increase the amount of nitrogen available in the surface waters of estuaries and coastal marine environments.

*Estuary Nitrogen Studies*

Although many studies of primary production have occurred in the ocean, the concepts and models generated are also often used to describe coastal and estuarine environments (Bronk et al. 1994). Like forested ecosystems, estuaries are sometimes considered to be nitrogen limited, and for the past two decades studies of estuarine and marine primary production have usually considered only the fluxes of inorganic nitrogen. (Bronk et al. 1994). Bronk et al. 1994 assert that "one nitrogen pool conspicuously absent from this discussion, however, is DON." Including DON as a component of the nitrogen in surface waters could provide a more complete description of the nitrogen import, cycling, and export in the estuarine ecosystem.

## Role of DON in Ecosystems

*DON in Forested Ecosystems*

More recent studies of nitrogen budgets in forests indicate that DON plays a significant role in the nitrogen cycle. Hedin et al. 1995 measured the concentrations of the major forms of dissolved nitrogen in streams draining old-growth temperate forested catchments on a Chilean island (42S, 74W) (Fig. 3). They found that the hydrologic nitrogen losses occurred nearly exclusively (95% of total N) as dissolved organic forms of nitrogen (Hedin et al. 1995). The geometric mean concentration of DON was 153μg/L. The $NO_3$ geometric mean concentration was 0.37μg/L; $NH_4$ geometric mean concentration was 7.4μg/L. Measuring only the concentration of $NO_3$ in the streams would only have described about 1% of the nitrogen lost from the ecosystem (Table 1). Hedin et al. 1995 suggest that DON may represent a significant fraction (>10%) of hydrologic nitrogen losses in temperate forests.

Figure 3. Concentrations of Major Nitrogen Species in a Chilean Temperate Forest. Geometric mean concentrations of major dissolved forms of nitrogen, $NO_3$, $NH_4$, and dissolved organic nitrogen in streams draining forested watersheds at Cordillera de Piuchue, Chile (Hedin et al. 1995).

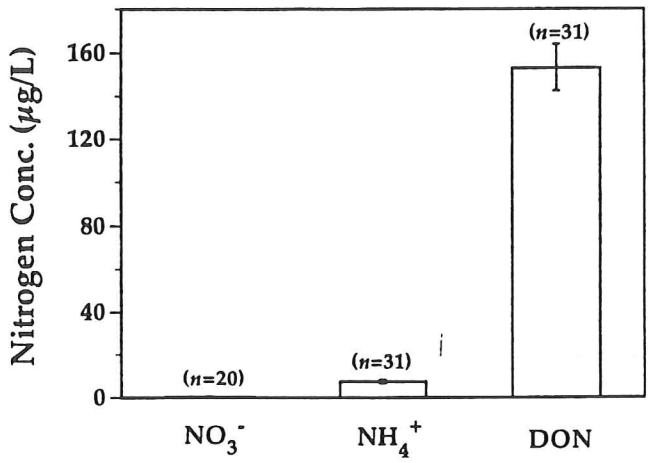



Table 1. Concentrations of Major Dissolved Nitrogen Species in a Chilean Temperate Forest. $NO_3$, $NH_4$, and dissolved organic nitrogen in streams draining forested watersheds at Cordillera de Piuchue, Chile (Hedin et al. 1995).

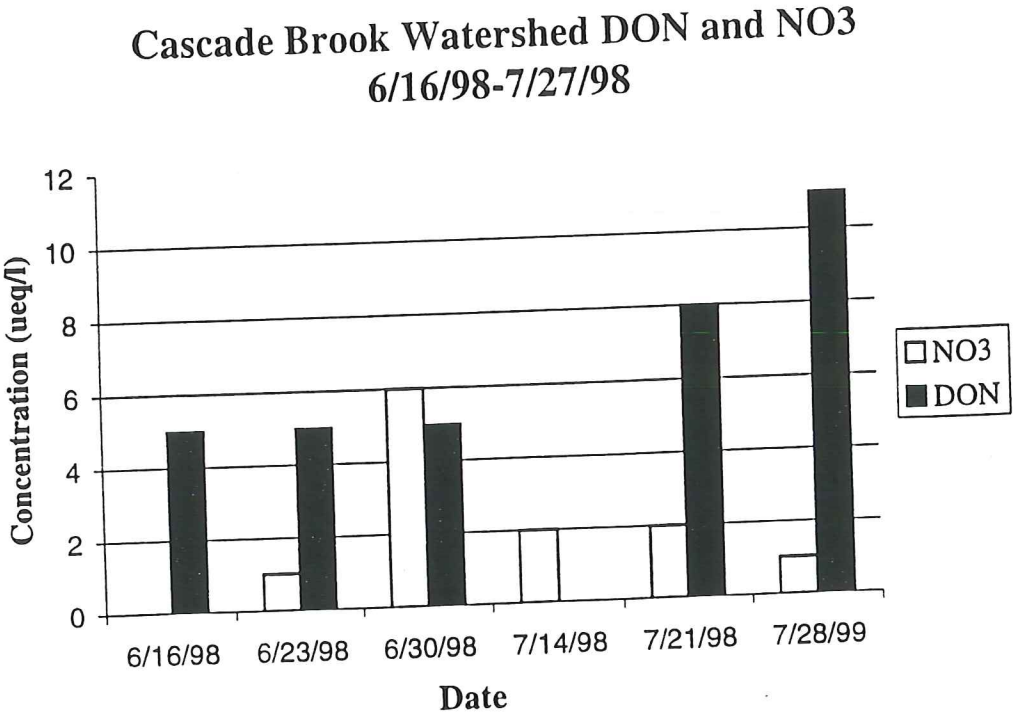
| | High-elevation forests ($n$ = 15) | Mid-elevation forests ($n$ = 11) | Coastal forests ($n$ = 5) |
|---|---|---|---|
| $NO_3^-$-N (μg/L) | 0.10 (0.05–0.24) | 0.30 (0.08–1.38) | 4.20 (1.16–12.32 |
| $NH_4^+$-N (μg/L) | 8 (4–23) | 5 (3–20) | 10 (5–25) |
| DON (μg/L) | 151 (121–170) | 133 (83–421) | 217 (121–350) |
| % DON | 95 | 96 | 94 |
| % $NO_3^-$ | 0.06 | 0.2 | 1.8 |
| DOC (mg/L) | 6.6 (2.5–10) | 6.5 (3.9–26) | 8.1 (2.2–16) |
| Organic anion charge density (μmol/mg C) | 6.3 (3.6–17) | 8.1 (4.8–15.7) | 12.5 (9.2–16.6) |
| pH* | 5.3 | 5.6 | 5.3 |
| DOC/DON | 49 | 48 | 39 |

* Values for streamwater pH are arithmetic averages based on $H^+$ concentrations. DON = dissolved organic nitrogen.

Berner and Berner 1996 report total river output of the major forms of dissolved nitrogen. They estimate natural dissolved organic nitrogen flux of 10Tg/yr; they estimate the flux of natural dissolved inorganic nitrogen ($NO_3$ + $NH_4$) as 4.5 Tg/yr.

A recent study which measured the forms of dissolved nitrogen exported from Cascade Brook Watershed in Black Rock Forest suggested the potential significance of DON in that watershed (Fig. 4) (Nichols 1998).

Figure 4. (Nichols 1998)




*DON in Estuarine Ecosystems*

DON exported from forested ecosystems by streams and rivers affects estuaries and coastal marine ecosystems. This riverine DON can represent a source of new nitrogen for the ecosystem. Treguer and Queguiner 1989 found that estuarine DON concentrations fluctuated seasonally with lower levels in winter and higher levels in summer. Even with this fluctuation, DON accounted for between 1-40% of the total dissolved nitrogen (Treguer and Queguiner 1989). Much of this dissolved nitrogen was due to *in situ* regeneration rather than terrestrial inputs (Treguer and Queguiner 1989). A study of the Waquoit Bay estuary found that DON became a more dominant form of

nitrogen as the estuary neared the ocean (Dickens et al. 1996). Berg et al. 1997 claim that dissolved organic nitrogen comprises a substantial portion of total nitrogen in estuarine and marine environments. In some estuaries, the contribution of DON to the total riverine nitrogen input introduced has recently increased (Berg et al. 1997).

## Description of Present Study

This study is a preliminary exploration of dissolved organic nitrogen in a forested and in an estuarine ecosystem. The study seeks to answer both general and specific questions about the dissolved organic nitrogen of these ecosystems.

### *Forested Ecosystem Study- Black Rock Forest*

The questions pursued in this study are: (1) Is dissolved organic nitrogen a significant component of the total dissolved nitrogen in temperate forested ecosystems? (2) Does the concentration of DON and total dissolved nitrogen differ within or between watersheds? (3) Can proteins be identified as a component of the dissolved organic nitrogen? (4) Does vegetation or soil type significantly affect the protein signature of DON exported from forested watersheds?

### *Estuarine Ecosystem Study-Hudson River (Erie Pier)*

The questions pursued in this study are: (1) Is dissolved organic nitrogen a significant component of the total dissolved nitrogen in the Hudson River estuary? (2) How does the concentration of estuarine DON compare to the concentration of DON exported from a forested ecosystem? (3) Can proteins be identified as a component of the dissolved organic nitrogen? (4) Is the protein signature of the estuary similar to the protein signature of water exported from forested watersheds?

### *Types of Analyses*

This study makes both quantitative and qualitative analyses of dissolved organic nitrogen. Total dissolved nitrogen and dissolved organic nitrogen concentrations are quantified, and the proteinaceous component of dissolved organic nitrogen is qualitatively analyzed.

## Purpose of Present Study

The possibility that dissolved organic nitrogen plays a significant role in the nitrogen cycle of forested and estuarine ecosystems could significantly change our models of ecosystem processes. This thesis seeks to test this proposition. Data about the contribution of DON to total fixed nitrogen can provide information that could lead to a more complete description of the nitrogen cycle and the nitrogen budget for both ecosystems.

### *Importance of Forest Nitrogen Budgets*

Nitrogen budgets for forests can be used to calculate the primary production of the forest. There is a relationship between the amount of nitrogen stored in an ecosystem and the net primary production of the ecosystem (Tamm 1991). This calculation for forests can give an estimation of the amount of carbon stored in forest biomass. This calculation is important as scientists attempt to predict the rise in atmospheric $CO_2$ and the corresponding change in global temperature. Scientists are interested in monitoring the carbon storage and carbon movements in different ecosystems of the biosphere (Clair and Ehrman 1996).

An increase in global temperature could lead to an increase in soil decomposition and nitrogen runoff (Clair and Ehrman 1996;Henriksen and Hessen 1997). This increased export of nitrogen could affect forest, estuarine, and marine ecosystem dynamics.

Terrestrial forest ecosystems are increasingly becoming enriched with nitrogen as a result of human activities. Fertilizers and the burning of fossil fuels introduce excess nitrogen into these ecosystems. Although forests are considered nitrogen limited, excess nitrogen can create conditions of nitrogen saturation. Nitrogen saturation is defined as "the state at which the availability of ammonium and nitrate is in excess of the total combined plant and microbial demand, as manifest by leaching of significant amounts of nitrate from the catchment" (Henriksen and Hessen 1997). In these conditions, nitrogen no longer necessarily limits growth. The degree of nitrogen saturation of forests can be estimated and monitored based on nitrogen budgets by measuring the export of bioavailable nitrogen. Determining the degree of nitrogen saturation of forests is an important component of analyses that consider the effects of anthropogenic nitrogen deposition (Hedin et al. 1995). Organisms that have adapted to nitrogen limitation may

be disrupted by dramatic increases in nitrogen (Tamm 1991). Long-term monitoring of nitrogen budgets "is critical for quantifying the impact of disturbance (anthropogenic or natural) on nutrient cycling or for identifying long-term trends in biogeochemical processes in a landscape" (McDowell and Asbury 1994).

*Potential Changes of Forest Nitrogen Budgets*

Information from Quantitative DON analysis

Including DON as a component of the total dissolved nitrogen lost from forested ecosystems could represent a significant loss of nitrogen from these ecosystems that has been previously ignored. Measuring DON export could reduce estimations of nitrogen retained in the ecosystem. This would lower estimates of carbon stored in forest biomass.

Nelson et al. 1996 demonstrate the importance of measuring the concentrations of different species of nitrogen. Although two different streams exported similar amounts of dissolved nitrogen, the forms of nitrogen differed (Nelson et al. 1996). Information about the type of dissolved nitrogen exported can provide insight into the biogeochemical processes leading to nutrient loss.

Information from Qualitative DON analysis

Analysis of the chemical composition of DON exported from forest ecosystems could provide insight into the biology contributing to the nutrient flux from the ecosystem. The bioavailability of DON and the processes of microbial decomposition which produce DON are two such biological parameters that could be studied (Church and Driscoll 1997).

*Importance of Estuary Nitrogen Budgets*

Estuarine nitrogen budgets have many of the same uses as forest nitrogen budgets. Based on the fluxes (import and export) of nitrogen, primary production can be estimated. This estimation is important because "estuaries and coastal waters account for ~40-50% of global oceanic primary production and resultant carbon and nitrogen flux" (Paerl 1997).

Like forests, estuaries are typically nitrogen limited. They also have been recently subjected to increased nitrogen loading from rivers and the atmosphere. This increase in nutrients can lead to eutrophication of the estuary (Paerl 1997). Eutrophication can lead to an eventual decrease in dissolved oxygen in the estuary. Monitoring the fluxes of nitrogen into the estuary is useful for studying the response of the estuary to nitrogen loading and the degree of eutrophication. Nitrogen budgets can help determine the degree of ecosystem imbalance due to nutrient loading (Treguer and Queguiner 1989).

Nitrogen inputs can also affect the general phytoplankton community structure (Paerl 1997). In particular, harmful algae blooms (HAB) have been linked to specific limiting nutrient conditions in estuaries (Paerl 1997). Berg et al. 1997 suggest that when inorganic nitrogen concentrations are low, harmful algae blooms may be stimulated by dissolved organic nitrogen. Harmful algae may utilize DON more efficiently than unharmful phytoplankton when inorganic nitrogen sources are limited or exhausted (Berg et al. 1997). Monitoring nitrogen budgets can help correlate harmful algae blooms with specific nitrogen concentrations and nutrient conditions.

*Potential Changes of Estuary Nitrogen Budgets*

Information from Quantitative DON analysis

Quantitative analyses of the concentrations of DON exported from terrestrial forests and the concentrations of DON in the estuary itself have implications for the estuarine nitrogen budget.

The previously underestimated or ignored DON input to estuaries from rivers can increase estimates of primary production. Riverine DON can provide nitrogen available for primary production in three ways. DON can be metabolized and remineralized to inorganic nitrogen by bacteria and microorganisms. (Berg 1997) This inorganic nitrogen can be used by phytoplankton in photosynthesis (Berg et al. 1997). Ignoring DON underestimates the amount of inorganic nitrogen available for primary production. Berg et al. also cite that certain phytoplankton have extracellular enzymes that allow them to utilize DON directly as a nutrient for photosynthesis. Finally, Buffam et al. 1996 suggest

that organic matter can be degraded to inorganic nutrients by "abiotic, photochemical degradation."

Measuring the DON concentration of estuary water increases the complexity of primary production estimates and calculations. Phytoplankton uptake of inorganic nitrogen has often been assumed to directly relate to primary production. However, Bronk et al. 1994 estimate that 25 to 41% of inorganic nitrogen taken up by phytoplankton is released as DON. This DON can be remineralized and reused as a nutrient, or it can be lost from the surface water.

Comparing the concentration of DON in rivers to the concentration of nitrogen in the estuary can help determine the relative contribution of riverine fluxes of DON to the estuarine DON.

Information from Qualitative DON analysis

Paerl 1997 recognizes that information about the bioavailability of DON is important to consider in estimates of primary production. A qualitative analysis of DON could provide information about the bioavailability of nitrogen for both phytoplankton uptake and microbial remineralization. The degree of nutrient loading also depends on the bioavailability of the nutrients entering the ecosystem (Davidsson et al. 1997). The chemical composition of DON could also affect its uptake by harmful algae. Qualitative information about DON could be useful in the study of harmful algae blooms.

Qualitative analyses of DON could also help resolve controversies about the sources of estuarine DON. Treguer and Queguiner 1989 conclude that terrestrial inputs into a West European macrotidal estuary are negligible. They cite "*in situ* biological recycling" as the major source of DON (Treguer and Queguiner 1989). Bronk et al. 1994 consider the direct release of DON by phytoplankton to be a major source for the estuary. An analysis of the proteinaceous component of DON could indicate its origin. Certain proteins may be more common in terrestrial ecosystems and distinct from DON produced either directly by phytoplankton exudate or indirectly by microbial decomposition.

## Background

### Description of Sample Sites

*Black Rock Forest*

Black Rock Forest is a private research forest located in the Hudson Highlands on the west bank of the Hudson River. The forest (41N; 74W) is located near Cornwall, NY, about 80km north of New York City (Fig. 5). Elevation ranges from about 200m to about 400m. The forest receives about 90cm of rain annually (NADP).

Samples were collected within the watersheds of two different streams that have their headwaters in the 1500ha forest: Cascade Brook and Black Rock Brook (Fig. 6). Cascade Brook begins around an elevation of 430m. The bedrock of Cascade Brook is impermeable to groundwater. All precipitation entering the watershed either flows out of the watershed or is involved in evapotranspiration. This feature of the watershed is useful for constructing water and nutrient budgets. The watershed is dominated by upland deciduous vegetation. Dominant tree species include Quercus rubra (red oak), and Quercus prinus (chestnut oak). A distinctive feature of Cascade Brook is the Glycerine Hollow wetland. Acer rubrum (red maple), Betula (birch) and Acer saccharum (sugar maple) dominate the wetland. Other trees present in Cascade Brook Watershed include Fagus (beech), Carpinus (musclewood), chestnut oak, and silver maple. Samples were collected at two sites along Cascade Brook. At North Bridge (cas a) sample site (Fig. 7) I collected stream water before it entered the wetland. At the Old West Point Road (cas b) site (Fig. 7 ), I collected stream water after it had passed through Glycerine Hollow.

Black Rock Brook flows out of Aleck Meadow Reservoir. A distinctive feature of Black Rock Brook is a stand of coniferous hemlocks. This hemlock stand begins after Aleck Meadow Reservoir. Two samples were collected from Black Rock Brook. The sample collected at the mouth of Aleck Meadow reservoir (hem a) consisted of water that had not passed through hemlock stand. The second sample (hem b) was collected after the stream had passed through the hemlock stand, just upstream of the Chlorinator plant (Fig. 6). Hemlocks comprise about 40% of the watershed vegetation between sample sites (hem a) and (hem b).

Figure 5. Hudson River Watershed (map from 1999 lecture notes of Jim Simpson W4835 Columbia University)

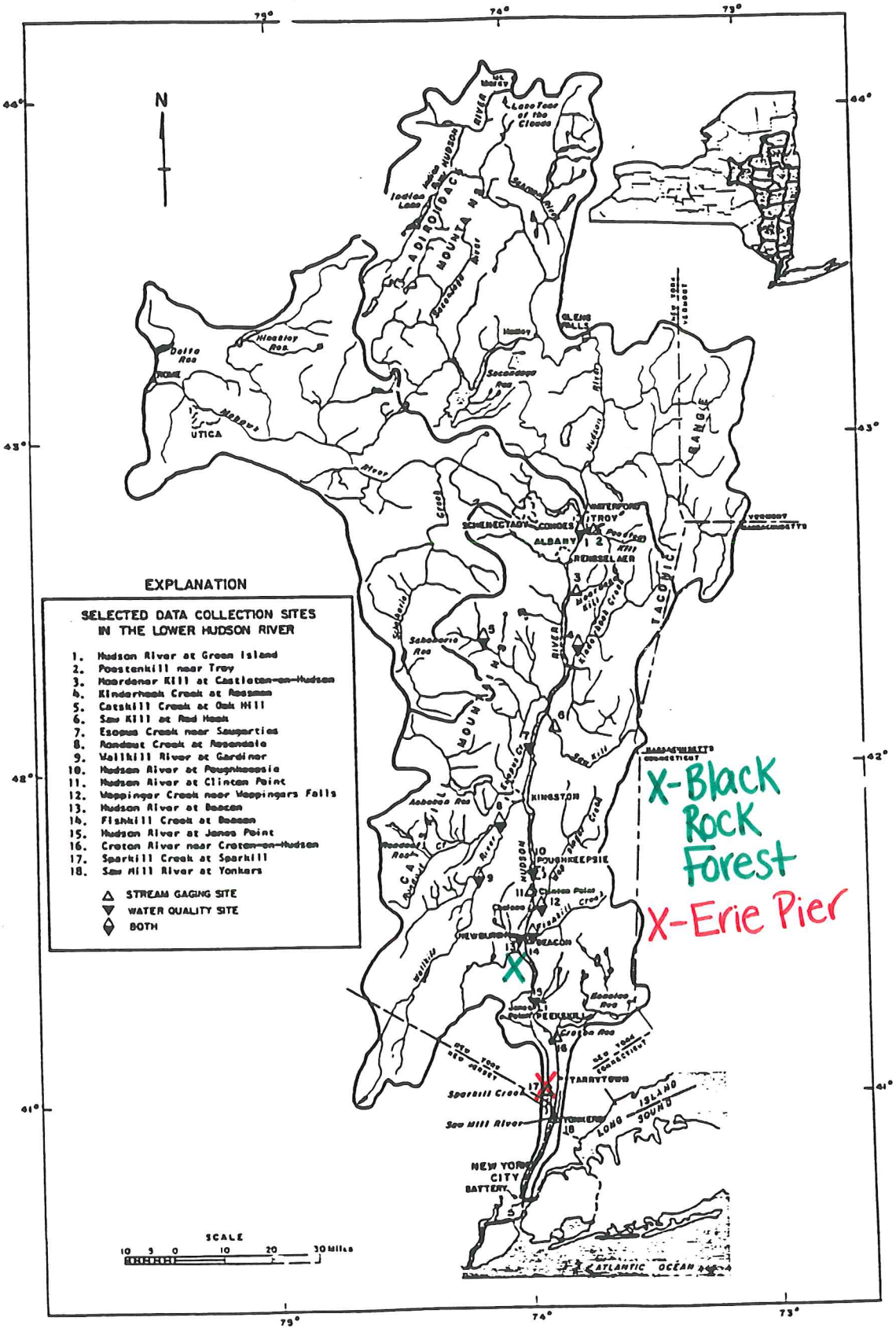


Figure 6. Black Rock Forest (1995 map by F. Shuster and T. Murray, adapted from Karnig 1991)

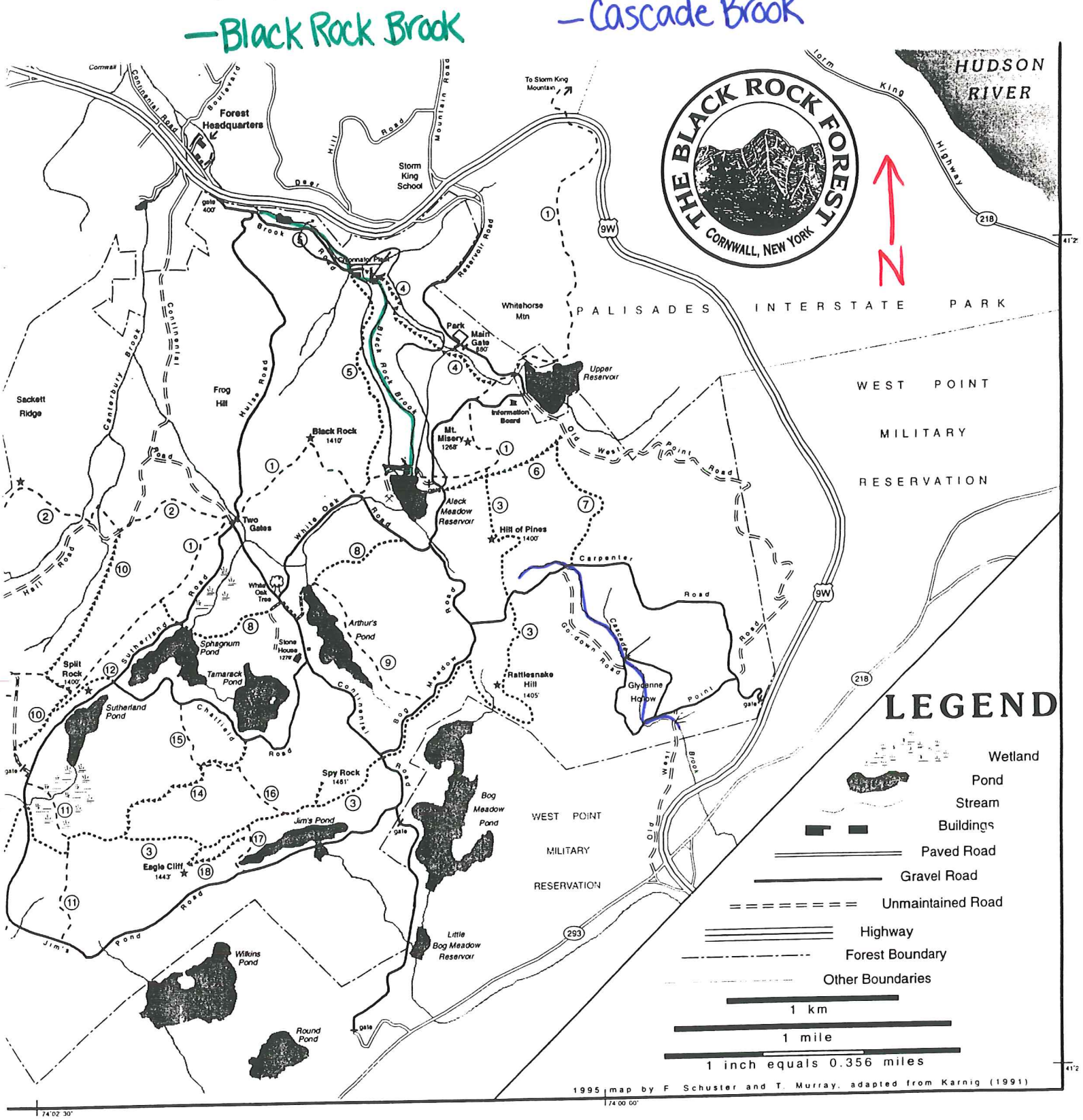
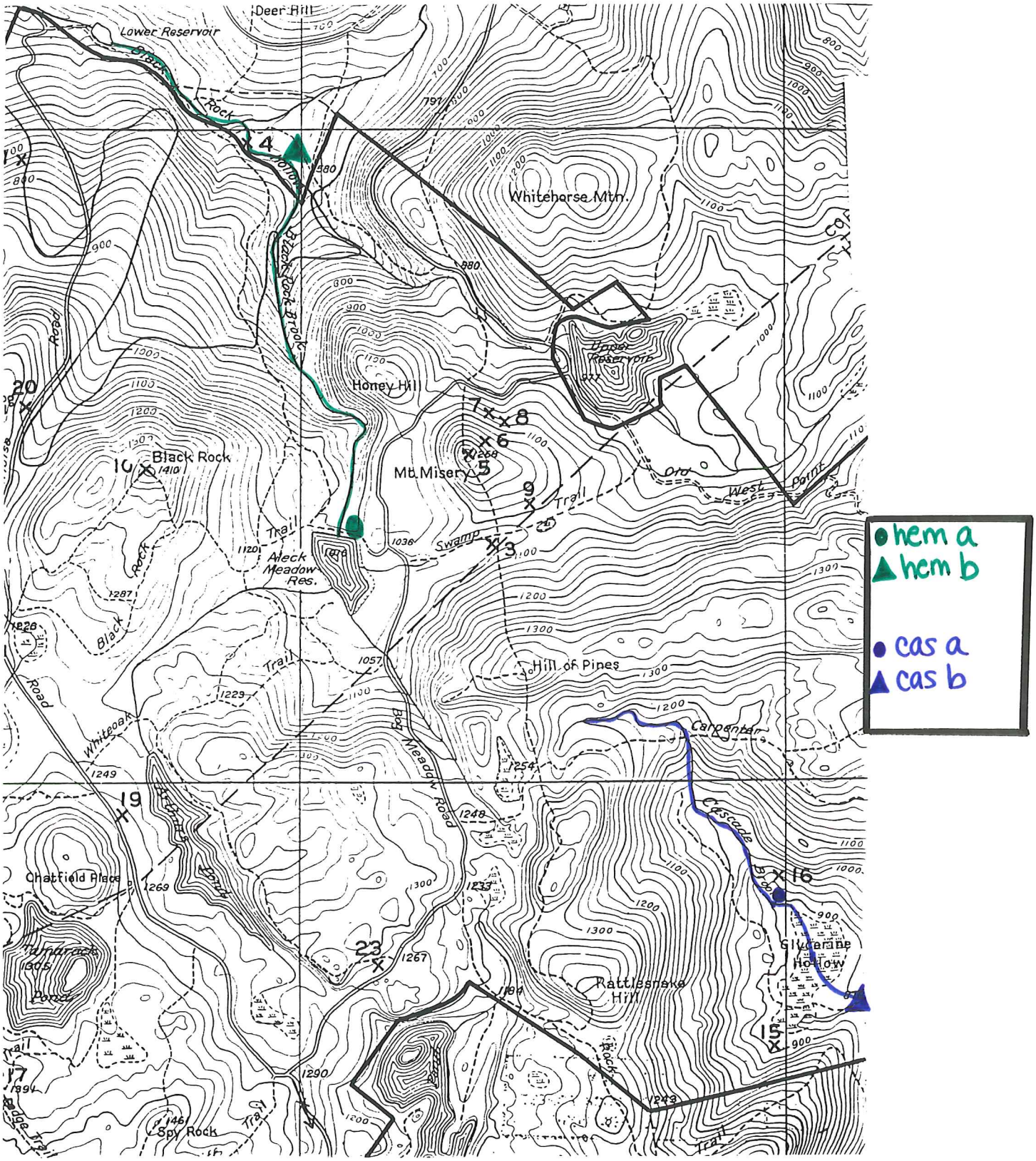


Figure 6. Sample Sites in Black Rock Forest Watersheds



*Hudson River*

The Hudson River begins at Lake Tear O'Clouds in the Adirondack Mountains of upstate New York and flows 500km to Battery Park at the tip of Manhattan (Fig. 5). The river drains an area of about 36,000 $km^2$. For the last 240km, the Hudson River is a tidal river. The estuarine environment begins about 160km north of the Battery in New York City (Information for this paragraph from Hudson River NERR website).

Samples were collected at Erie Pier located in Piermont, NY (Fig. 5). Piermont is located on the west bank of the Hudson River about 6.5km south of Nyack, NY. Erie Pier extends about 1.6km from shore into the middle of the river. The tidal range at Piermont is about 1m. All samples were collected during the flood tide (Information for this paragraph from Hudson River NERR website).

## Methods

### General Notes:

1. Gloves were worn for all procedures to protect containers and samples from contamination.
2. Distilled/Deionized (DI) water was used in all procedural and cleaning steps unless otherwise indicated.
3. Containers were considered "clean" after being washed with 0.1M NaOH and rinsed with DI water three times (NaOH helps degrade many organic compounds).
4. All containers were rinsed three times with DI and three times with sample before being filled with sample.

### Sample Collection

*Black Rock Samples*

Water samples were collected from four sample locations in Black Rock Forest on 2/23/99. Water was collected from the streams using a bucket to fill clean 10L Nalgene containers. Water temperature was recorded at all sites. The 10L samples were filtered through a 0.2μm capsule filter (Gelman sterile mini capsule #12122) using a Masterflex Peristaltic Pump (Cole-Parmer #77250-62) to remove particles and to preserve the sample. The samples were filtered within two hours of collection. 200 mL samples were

collected in Nalgene containers from the 10L of 0.2μm filtrate. These samples were frozen and stored until dissolved nitrogen analyses. The remaining 0.2μm filtrate was stored in clean 10L Nalgene containers in the dark at 4°C until ready for protein concentration by tangential flow filtration (TFF). The 10 L 0.2 μm filtrates were concentrated using TFF within one week of collection.

*Hudson River Samples*

Water samples were collected from Erie Pier on 2/9/99, 2/26/99, 3/8/99, and 3/24/99 using the methods described above. Sampling times were determined by the tides. All samples were collected during the flood tide.

**Total Dissolved Nitrogen and DON Analysis**

Total dissolved nitrogen was determined using ultraviolet (UV) radiation and hydrogen peroxide to oxidize all nitrogen species in the sample to $NO_3$ (Appendix VII). Measurements of $NO_3$ were made before and after this UV oxidation using ion chromatography (Appendix VIII). Initial measurements of $NH_4$ concentrations were also made using spectroscopy (Appendix IX). DON was determined by a subtraction calculation. DON=Total $NO_3$- (initial $NO_3$ + initial $NH_4$).

**Protein Analysis**

The analysis of dissolved protein molecules involved three separate steps: (1) Concentration of dissolved protein by tangential flow filtration, (2) Purification and precipitation in trichloroacetic acid (TCA) of proteins in the crude concentrate, and (3) separation and detection of dissolved proteins by sodium dodecylsulfate-polyacrylamide gel electrophoresis (Tanoue 1995; Appendix V)

**Concentration of Protein by Tangential Flow Filtration (TFF)**

See Appendix I for detailed procedure.

Concentration by TFF retains only particles larger than 10 kilodaltons (kDa) and reduces the initial sample volume from about 10L to about 150 mL. Concentration of this 150mL sample using a Centrivap reduces the sample volume to about 30mL of protein crude

concentrate. Store crude concentrate frozen until purification and precipitation in Trichloroacetic acid (TCA).

**Purification and Precipitation in Trichloroacetic acid (TCA) of Proteins in the Crude Concentrate**

See Appendix II for detailed procedure

Addition of TCA to the crude concentrate precipitates proteins; proteins are insoluble in TCA. Centrifugation and washing of the precipitate with ethanol and ether removes TCA, sodium dodecylsulfate (SDS), and non-protein dissolved organic matter from sample. Sample volume reduces from 30mL to about 0.1mL. After final centrifugation, dry pellet. Before pellet dries completely, add 50μL Novex sample buffer solution. Store at either -20°C or 4°C until ready for use in gel electrophoresis.

**Separation and Analysis of Protein using Sodium dodecylsulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)** (Tanoue 1995)

See Appendix III for detailed procedure.

SDS-PAGE separates individual proteins based on molecular weight. Develop gel according to SilverXpress Silver Staining Protocol (Appendix IV). Take picture of gel using Kodak Digital Science DC 40 camera immediately after development. Store gel wrapped in plastic at 4° C.

**Gel Analysis**

Gels were analyzed with Kodak Digital Science 1D Image Analysis Software 1D Mac version 2.0.2 (S/N: DA-517C79-D91111-139191). Transfer the gel photograph from the digital camera to the computer. Open the program Kodak 1D 2.0.2. Open the gel photograph. Follow these steps within the program:

1. Find lanes
2. Find bands
3. Fit bands
4. Input Lane information- use information for Novex Mark 12 Standard (Appendix V)
5. Show analysis- record molecular weight of sample bands.

## Results

### Quantitative-Total Dissolved Nitrogen and DON Analysis

The values for total dissolved nitrogen, $NO_3$, and DON should be considered preliminary values. Because there is not a direct analytic method to measure DON, the estimates of DON rely on analysis of $NO_3$ by ion chromatography and analysis of $NH_4$ by spectroscopy.

The $NO_3$ concentrations were calculated using calibration curves derived from standard solutions. Because of the wide range of concentrations between the Black Rock Forest samples and the Hudson River samples, different sets of standards and different calibrations were necessary. The precision for all concentrations was +/-0.5μM. "Cas a" and "cas b" were measured using standards ranging from 0.8-3.35μM. "Cas a" was measured using a calibration curve with an $R^2$ value of 0.98. "Cas b" was measured using a calibration curve with an $R^2$ value of 0.975. "Hem a" and "hem b" were measured using standards ranging from 3.35-16.77 μM. This calibration curve had an $R^2$ value of 0.995. The Hudson River samples were measured using standards ranging from 53.7-268μM. The calibration curve had an $R^2$ value of 0.9995. The standards for the Hudson River samples did not have the same salinity as the samples. This could have affected the accuracy of the results.

For each sample, two $NO_3$ measurements were made: initial $NO_3$ concentration and total dissolved nitrogen (after UV oxidation of sample). These two measurements were made using the same calibration curve, and these two measurements were used to calculate DON. For this reason, the relative amounts of DON and $NO_3$ within the sample should have had the least error. Because different calibration curves were used for different samples, comparisons of absolute concentrations between samples were less reliable. However, differences in calculated concentrations due to different calibration curve slopes were within the range of measurement error (0.5μM).

The $NH_4$ concentrations were calculated using an "f-factor" of 4.77 and a blank value of 0.0877 (Appendix VIII). Standards of 10μM, 5μM, and 1.25μM were used. Most sample concentrations were lower than 1.25μM. The standards were not prepared with the same salinity as the Hudson River. Again, this factor could have lead to errors in the concentrations.

The accuracy of the nitrate and ammonium concentrations could be greatly improved by improving the analytic technique. The range of standards should be close to the predicted concentrations and salinities, the calibration curves should have an $R^2$ value of >0.99, and all samples of similar concentrations should be run using the same calibration.

*Black Rock Forest-Cascade Brook*

In Cascade Brook Watershed, the sample above the wetland (cas a) had lower concentrations of all species of dissolved nitrogen than the sample collected after the wetland (cas b). "Cas a" had 2.2μM total nitrogen. Of this total nitrogen, 55% (1.2μM) was DON, 23% (0.5μM) was $NO_3$, and 23% (0.5μM) was $NH_4$ (Fig. 8; Table 2). Cas b had 3.0μM total nitrogen. Of this total nitrogen, 57% (1.7μM) was DON, 27% (0.8μM) was $NO_3$, and 17% (0.5μM) was $NH_4$. The DON concentration increased 140% after water passed through the wetland.

Figure 8. Concentration of Dissolved Nitrogen Species in Cascade Brook Watershed. Sample "cas a" taken above wetland; sample "cas b" taken below wetland.

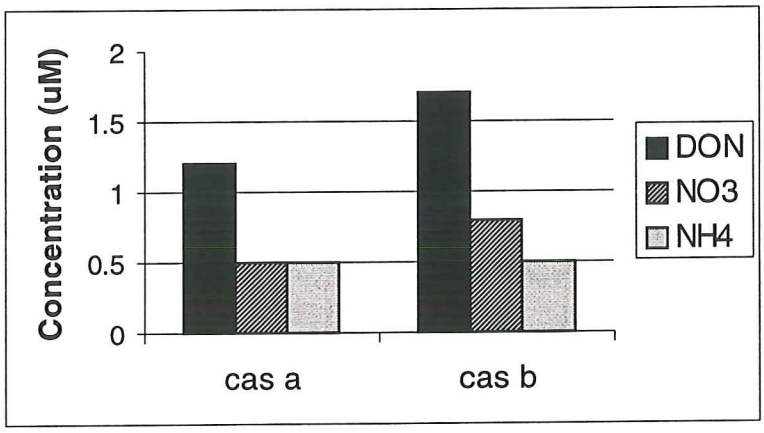


Table 2-Concentrations of Total Dissolved Nitrogen and Dissolved Nitrogen Species in Cascade Brook Watershed. Sample "cas a" taken above wetland; sample "cas b" taken below wetland.

| | Cas a | Cas b |
|---|---|---|
| Total dissolved nitrogen (μM) | 2.2 | 3.0 |
| $NH_4$(μM) | 0.5 | 0.5 |
| $NO_3$(μM) | 0.5 | 0.8 |
| DON (μM) | 1.2 | 1.7 |
| % DON | 55 | 57 |
| % $NO_3$ | 23 | 27 |
| % $NH_4$ | 23 | 17 |

*Black Rock Forest-Black Rock Brook*

In Black Rock Brook watershed the total nitrogen concentrations were similar between samples "hem a" and "hem b." However, the total nitrogen concentrations were about 6μM greater than the samples from Cascade Brook watershed. "Hem a" had 8.1μM total nitrogen. Of this total nitrogen, 46% (3.7μM) was DON, 42% (3.4μM) was $NO_3$, and 12% (1.0μM) was $NH_4$ (Figure 9; Table 3). "Hem b" had 8.0μM total nitrogen. Of this total nitrogen 51% (4.1μM) was DON, 43% (3.4μM) was $NO_3$, and 6% (0.5μM) was $NH_4$.

Figure 9. Concentration of Dissolved Nitrogen Species in Black Rock Brook Watershed. Sample "hem a" taken above hemlock stand; sample "hem b" taken below hemlock stand.

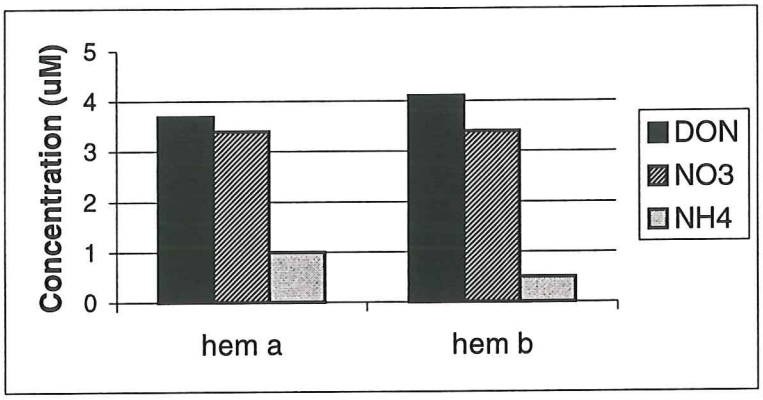



Table 3-Concentrations of Total Dissolved Nitrogen and Dissolved Nitrogen Species in Black Rock Brook Watershed. Sample "hem a" taken above hemlock stand; sample "hem b" taken below hemlock stand.

| | Hem a | Hem b |
|---|---|---|
| Total dissolved nitrogen (μM) | 8.1 | 8.0 |
| $NH_4$(μM) | 1.0 | 0.5 |
| $NO_3$(μM) | 3.4 | 3.4 |
| DON (μM) | 3.7 | 4.1 |
| % DON | 46 | 51 |
| % $NO_3$ | 42 | 43 |
| % $NH_4$ | 12 | 6 |

*Hudson River-Erie Pier*

The total nitrogen concentrations of the four Hudson River samples were higher than the nitrogen concentration found in Black Rock Forest. The dissolved nitrogen speciation in the Hudson River samples varied greatly. Due to analytical error, the 2/26 sample had a negative value for DON (-30μM). I believe this error resulted from an improper labeling of the 2/26 initial $NO_3$ sample and the UV oxidized $NO_3$. Confusing

the initial $NO_3$ and the total dissolved nitrogen concentrations could make such a large negative DON value. Also, the 2/26 "initial" $NO_3$ value is very similar to two other total dissolved nitrogen values; and the 2/26 "total dissolved nitrogen" is very similar to initial $NO_3$ values. Because I cannot confirm the source of this error, I will consider the results of the three other Hudson River samples (2/9, 3/8, and 3/24). However, I do not believe this large negative DON value is due to a large degree of error in the nitrogen measurements.

The 2/9 sample had 50.4μM total nitrogen. Of this total nitrogen, 38% (19μM) was DON, 33% (16.6μM) was $NO_3$, and 29% (14.8μM) was $NH_4$ (Figure 10; Table 4). The 3/8 sample had 51.9μM total nitrogen. Of this total nitrogen 7% (3.4μM) was DON, 73% (37.7μM) was $NO_3$, and 21% (10.8μM) was $NH_4$ (Figure 10; Table 4). The 3/24 sample had 39.3μM total nitrogen. Of this total nitrogen 54% (21.3μM) was DON, 12% (4.7μM) was $NO_3$, and 34% (13.3μM) was $NH_4$ (Figure 10; Table 4).

Figure 10. Concentration of Dissolved Nitrogen Species in Hudson River Estuary (Erie Pier).

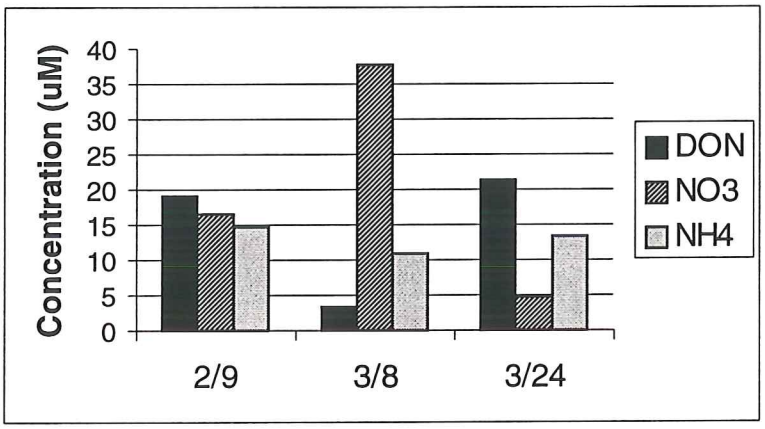


Table 4-Concentrations of Total Dissolved Nitrogen and Dissolved Nitrogen Species in Hudson River Estuary (Erie Pier)

| | 2/9 | 3/8 | 3/24 |
|---|---|---|---|
| Total dissolved nitrogen (μM) | 50.4 | 51.9 | 39.3 |
| $NH_4$(μM) | 14.8 | 10.8 | 13.3 |
| $NO_3$(μM) | 16.6 | 37.7 | 4.7 |
| DON (μM) | 19 | 3.4 | 21.3 |
| % DON | 38 | 7 | 54 |
| % $NO_3$ | 33 | 73 | 12 |
| % $NH_4$ | 29 | 21 | 34 |

**Qualitative-Protein Analysis**

Resolution of protein bands on the gel depends on the amount of protein loaded. Different proteins may be present in different concentrations in a sample. To resolve the maximum number of bands from the samples, different amounts of sample were loaded (Table 5). Lower amounts of sample were run on Gel a (Fig. 11). Gel b had higher amounts of certain samples loaded (Fig. 12).

Table 5. Description of Samples for Protein Analysis

| Sample | Location | Date | Time | Water Temp | Volume filtered by TFF | Volume sample after TCA | Volume sample loaded on gel a |
|---|---|---|---|---|---|---|---|
| Cas a | Above wetland | 2/23/99 | 12:25pm | 1°C | 8.5L | 55μL | 15μL |
| Cas b | Below wetland | 2/23/99 | 11:56am | 1°C | 8.5L | 55μL | 10μL |
| Hem a | Above hemlock | 2/23/99 | 12:51pm | 4°C | 8.6L | 55μL | 8μL |
| Hem b | Below hemlock | 2/23/99 | 1:27pm | 0°C | 8.7L | 55μL | 10μL |
| 2/9 Hudson | Erie Pier | 2/9/99 | 1:25pm | Not recorded | 9.0L | 55μL | 10μL |
| 2/26 Hudson | Erie Pier | 2/26/99 | 3:31pm | 4°C | 8.9L | 55μL | 7μL |

Figure 11. Gel a.

| Lane | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| Sample | Marker | Cas a | Cas b | 2/9/99 Hudson | marker | Hem a | Hem b | 2/26/99 Hudson | marker |
| amount | 5µL | 15µL | 10µL | 10µL | 5µL | 8µL | 10µL | 7µL | 5µL |

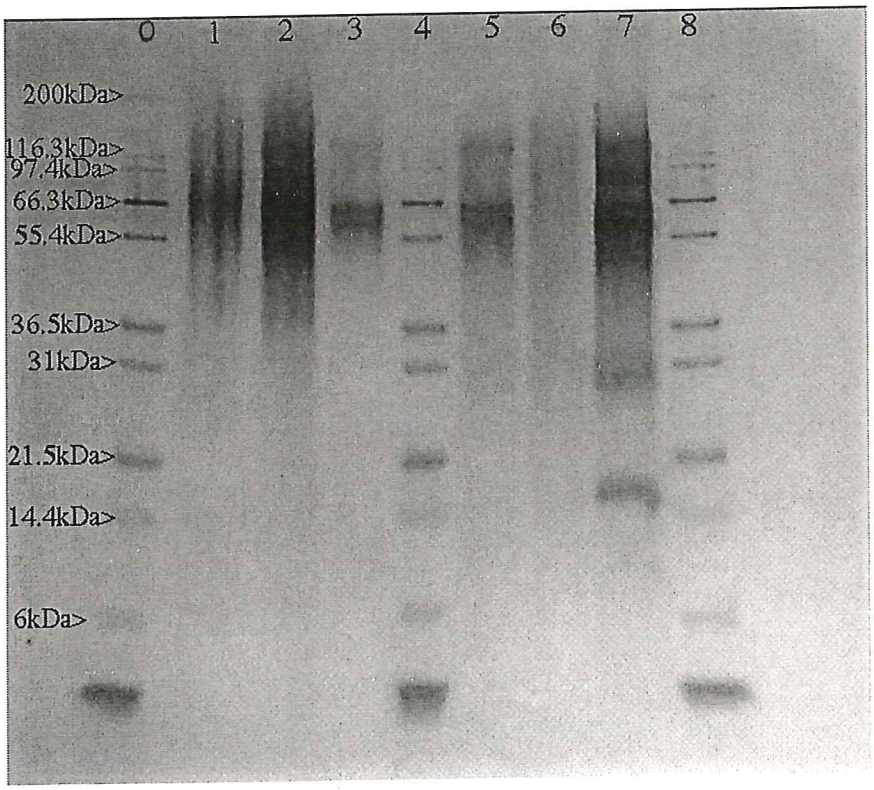
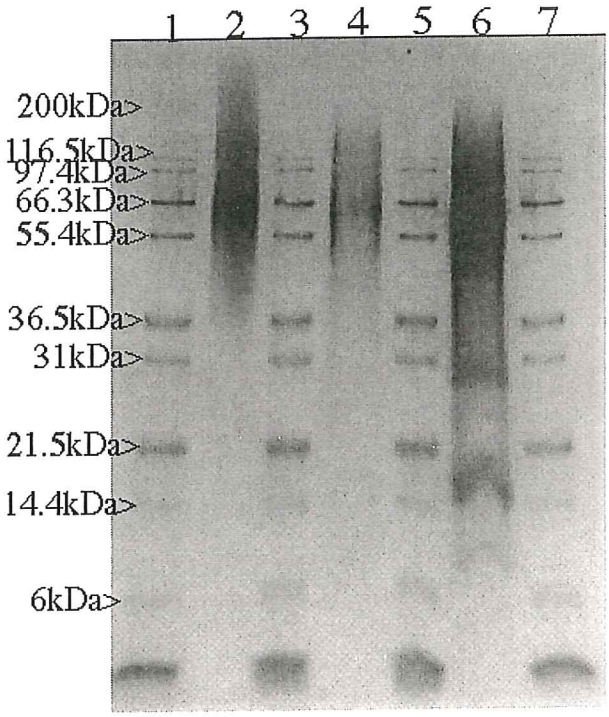


Figure 12. Gel b.

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Sample | marker | Cas b | Marker | Hem a | Marker | 2/26/99 Hudson | Marker |
| amount | 5µL | 15µL | 5µL | 15µL | 5µL | 15µL | 5µL |




The protein's molecular weight is determined by gel electrophoresis based on its movement through the gel. Proteins with lower molecular weights move farther down the gel than high molecular weight proteins. To minimize analytic errors, all samples were run on one gel. Because all samples were run under the same electrophoretic conditions, the differences in the distance traveled on the gel should be due to differences in molecular weight rather than experimental error.

The molecular weights of the samples were determined by the Kodak Digital Science 1D Image Analysis Software and based on the molecular weights of the Novex Mark 12 Standard proteins (Appendix V). Errors in the molecular weight estimations could arise from at least two sources. The first source of error could be the ability of the gel to separate proteins of similar molecular weight. The degree of this type of error can be estimated using the standard. Higher molecular weight proteins (200-50kDa) have less distance between them on the gel than lower molecular weight proteins (50-2kDa).

Table 7- Molecular Weights of Proteins from Black Rock Brook Watershed

| Sample | Location | Band # | Molecular Weight (MW) kDa |
|---|---|---|---|
| Hem a | Above hemlock stand | 1 | 118 |
| | | 2 | 64 |
| | | 3 | 62 |
| | | | |
| Hem b | Below hemlock stand | 0 | No distinct bands |

*Hudson River-Erie Pier*

The Hudson River samples showed similarities and differences compared to the Black Rock Forest samples. The 2/9 sample had three distinct bands estimated at 116kDa, 65kDa, and 62kDa (Figure 15; Table 8). This combination of bands resembled the "hem a" protein signature. The range of the 2/9 protein smear (~150kDa to ~50kDa) also resembled 'hem a." The 2/26 sample had seven bands (118kDa, 85kDa, 64kDa, 59kDa, 29kDa, 17kDa, and 10kDa). The three low molecular weight bands were not found in any other sample. The 10kDa band was only resolved the gel run with high sample concentrations (gel b). The 2/26 sample had three regions of protein smearing: (1) ~150kDa to ~42kDa; (2) ~30kDa to ~25kDa; (3) ~21kDa to ~14kDa.

Figure 15. Electrophoretogram of Hudson River Estuary Samples. (a) 2/9/99 gel a (b) 2/26/99 gel a (c) 2/26/99 gel b.

Table 8-Molecular Weights of Proteins from Hudson River (Erie Pier).

| Sample | Location | Band # | Molecular Weight (MW) kDa |
|---|---|---|---|
| 2/9/99 | Piermont Pier | 1 | 116 |
| | | 2 | 65 |
| | | 3 | 62 |
| | | | |
| 2/26/99 | Piermont Pier | 1 | 118 |
| (gel a) | | 2 | 95 |
| | | 3 | 64 |
| | | 4 | 61 |
| | | 5 | 29 |
| | | 6 | 17 |
| (gel b) | | 7 | 10 |

## Discussion

### Quantitative-Total Dissolved Nitrogen and DON Analysis

*Black Rock Forest*

The quantitative data about dissolved nitrogen in Black Rock Forest correspond to conditions on one winter day. To make conclusions about ecosystem processes and to study potential seasonal variations in these processes, more data must be collected over a longer amount of time. With dissolved nitrogen concentration data for only one day, it is difficult to interpret differences in concentrations. Does an increase in concentration indicate production, accumulation, or export of dissolved nitrogen? Does a decrease in concentration indicate decomposition, denitrification, or nutrient uptake by primary producers? The data collected for this study suggest that transformations of dissolved nitrogen occur within watersheds. This limited amount of data provides a useful introduction to the ecology of the watersheds and hints at the complexity of the fixed nitrogen cycle. These data can also provide suggestions about possible ecosystem processes that affect dissolved nitrogen. With a longer and more detailed study, the dissolved nitrogen concentration data can be used to estimate nitrogen fluxes within the watersheds, and the processes controlling these fluxes can be more accurately described.

The total dissolved nitrogen varied within and between the two watersheds studied in Black Rock Forest. In Cascade Brook, the concentration of total dissolved

nitrogen increased from 2.2μM to 3.0μM as the water passed through the wetland. The wetland seems to produce and export nitrogen. This result is consistent with Rudy 1994 and Nelson et al. 1996 suggestions that anoxic conditions in wetland soils cause nitrogen accumulation. The concentration of DON exported increased from 1.2μM to 1.7μM as water passed through the wetland. This difference in concentration is within the limit of measurement error, and it may not be significant. However, an increase in DON export from wetland soils is predicted by Davidsson et al. 1997. The speciation of total dissolved nitrogen did not change after water passed through the wetland. DON is the dominant species of dissolved nitrogen both above and below the wetland. The high percentage of DON (~50%) to total dissolved nitrogen suggests that the dissolved nitrogen flux within Cascade Brook watershed may be abiotically controlled because DON is less easily used and retained by organisms than the forms of inorganic dissolved nitrogen.

In Black Rock Brook, the concentration of total dissolved nitrogen was not affected by the hemlock stand, and the speciation of nitrogen was slightly affected. After water passed through the hemlock stand the concentration of DON increased from 3.7μM to 4.1μM. The increase in the percentage of DON was accompanied by a decrease in the percentage of $NH_4$ in water that had passed through the hemlock stand. Both the concentration and the percentage of $NO_3$ were unaffected by the hemlock stand. Again, these differences in the concentrations of DON and $NH_4$ may not be significant. However, an increase in DON could be caused either by increased decomposition, or by increased plant growth and root exudation. A decrease in $NH_4$ suggests increased biologic control, possibly from primary production. Perhaps biologic activity (root exudation and primary production) of the coniferous hemlocks influences the water that passes through Black Rock Brook.

The concentration of total dissolved nitrogen was about 5μM higher in Black Rock Brook than in Cascade Brook. Water in Black Rock Brook passes through three lakes, and these lakes could affect the stream chemistry. The "hem a" sample taken before the hemlock stand consisted of water exiting Aleck Meadow Reservoir. If anoxic conditions exist on the bottom of the reservoir, this could lead to an accumulation of dissolved nitrogen and DON in these waters. All three lakes could contribute to the

higher concentrations of dissolved nitrogen in Black Rock Brook. Because the higher concentrations of dissolved nitrogen exist before water passes through the hemlock stand, the cause of this increase must be due to some other feature of the watershed. However, the hemlock stand could influence the speciation of these high levels of dissolved nitrogen.

How does the total dissolved nitrogen concentration of Black Rock Forest compare to other temperate forests? Complete studies of dissolved nitrogen speciation in temperate forests were difficult to find. Often dissolved nitrogen was reported simply as total dissolved inorganic nitrogen and total dissolved organic nitrogen. The Hedin et al. 1995 study of a temperate Chilean forest reported $NO_3$ concentrations of 0.008-0.19μM which accounted for 0.04%-8.9% total dissolved nitrogen and $NH_4$ values of 0.166-1.39 μM which accounted for 0.8%-13% of total dissolved nitrogen. The environmental conditions of the Hedin et al. 1995 forest may not be similar enough to Black Rock Forest to make a valid comparison. Compared to the Hedin et al. 1995 dissolved nitrogen values, Black Rock Forest has similar concentrations of $NH_4$, and higher values of $NO_3$. The percentage contribution of $NH_4$ to total dissolved nitrogen in Black Rock Forest is also similar to the values reported in Hedin et al. 1995, but the contribution of $NO_3$ is much higher in Black Rock Forest. Hedin et al. 1995 consider their experimental forest unaffected and unpolluted by anthropogenic nitrogen. If Black Rock Forest $NH_4$ functions like $NH_4$ in unpolluted forests, this may indicate that Black Rock Forest is not nitrogen-saturated. The dominant contribution of DON to dissolved nitrogen suggests that abiotic factors may significantly affect the fluxes of nitrogen in Black Rock Forest watersheds.

*Hudson River*

The quantitative data about dissolved nitrogen in the Hudson River provides information about conditions of the lower Hudson River estuary during the winter months. To more accurately describe the winter nitrogen conditions, more frequent measurements should be made, and more characteristics of the water should be studied. To make more accurate comparisons of nitrogen concentrations over time, nitrogen concentrations should be correlated with salinity. Because the tidal cycle can influence water chemistry, salinity data could help determine whether changes in nitrogen

concentrations were related to changes in salinity or whether they were related to other factors that influence Hudson River water chemistry. The limited amount of data collected for this study cannot be used to make conclusions about the contribution of DON to estuarine dissolved nitrogen. More frequent measurements of dissolved nitrogen speciation are needed to determine whether the speciation is as variable as these data suggest. Like the data for Black Rock Forest, the Hudson River data collected at Piermont Pier provide an introduction to the dissolved nitrogen cycle of the estuary that hints at its complexity and encourages further study.

The concentrations of total dissolved nitrogen in the Hudson River were greater than the concentrations in Black Rock Forest by factors of between 5 and 25. These larger concentrations result from the fact that the Hudson River has so many sources of nutrient input. The streams draining Black Rock Forest watersheds represent only a few of many tributaries carrying land-derived nutrients into the Hudson River. The Hudson River also has large amounts of nitrogen introduced from sewage.

The concentrations of total dissolved nitrogen were similar for samples taken a month apart, but on the last sample date the concentration decreased by 20%. I would have expected an increase through time because of the corresponding temperature increase. Increased temperature would increase snow melt, runoff, and microbial and photosynthetic activity. All these processes are potential sources of dissolved nitrogen. However, the relationship between photosynthesis, decomposition and nitrogen concentrations are not clear. Increased nutrient uptake during photosynthesis could cause a net nitrogen consumption. Further study about the relative rates of nutrient uptake, photosynthetic DON production, and microbial decomposition is needed to determine the effects of subtle increases in estuary temperature. Perhaps during the time period of this study the temperature increase was not significant enough to affect these processes. A longer study may confirm the correlation between increased temperature and increased DON concentration that Treguer and Queguiner 1989 found.

The speciation of total dissolved nitrogen varied greatly between sample dates. 2/9 had a relatively equal distribution of the three measured nitrogen species ($NO_3$, $NH_4$, and DON). On 3/8 $NO_3$ dominated, and the DON concentration was very low; on 3/24 DON dominated the speciation. I was surprised that $NH_4$ never was the dominant species.

$NH_4$ is the form of nitrogen introduced by sewage. Perhaps freshwater and tidal inputs help dilute the concentration of $NH_4$ and flush the excess from the estuary.

The dramatic changes in speciation translate into changes in the amounts of bioavailable nitrogen and a potentially rapid turnover of DON. These changes could affect the ecosystem as organisms must adjust to use different forms of nitrogen. This result is also significant because of the suggestion that harmful algae are able to use DON when inorganic nitrogen concentrations are low. Monitoring the changes in nitrogen speciation could provide insight into the causes of harmful algae blooms.

## Qualitative-Protein Analysis

*Black Rock Forest*

Like the quantitative analysis of total dissolved nitrogen and DON, the qualitative characteristic proteins of the DON differed within and between the watersheds studied in Black Rock Forest. In Cascade Brook, water that had passed through the wetland had a unique high molecular weight protein. The appearance of this large protein after the wetland suggests production of DON within the wetland. This result is consistent with the quantitative analysis which indicated increased concentrations of DON after the wetland. This high molecular weight protein may result from incomplete or reduced microbial decomposition due to anoxic conditions. The 66kDa band was not present after water passed through the wetland; instead there was a 62kDa band. If this difference in molecular weights is significant, it could be due to degradation of the 66kDa protein. The two different proteins could also be the metabolic products of two different microbial communities.

The hemlock stand appeared to influence the protein signature of the DON released from Black Rock Brook watershed. The water that had passed through the hemlock stand had no distinct protein bands; the three bands that existed before the hemlock stand all appear to have been degraded. This degradation of protein does not necessarily mean that the concentration of DON is decreased. The quantitative data for Black Rock Brook indicates an increase in DON after the hemlock stand. Proteins that are degraded to lower molecular weights can remain dissolved organic nitrogen. This extreme degradation of protein within the hemlock stand could be due to the type of organic material available for decomposition. Coniferous trees also often produce acidic

soils. A lower pH could influence the species assemblage of the microbial community, or it could facilitate decomposition.

The protein signatures of DON released from the two different watersheds appeared to be unique. Both watersheds had proteins around 62kDa, but both watersheds also had unique proteins. Cascade Brook exported the heaviest protein. It would be interesting to determine whether Cascade Brook's high molecular weight protein (150kDa) is common in other anoxic environments. If so, this protein signature could provide an indication of the biogeochemical environment which produced the dissolved nitrogen. It also would be interesting to compare deciduous and coniferous vegetation resistance to microbial decomposition. The qualitative analysis suggests that coniferous vegetation is more effectively degraded. This could be the result of either the chemical composition of the vegetation or the efficiency of the microbial community that degrades each type of vegetation.

In general, the two watersheds studied in Black Rock Forest seem to have opposite effects on the proteinaceous component of DON. Within Cascade Brook watershed there is an accumulation of complex proteins which suggests DON production in the wetland. Within Black Rock Brook there is nearly complete degradation of proteins. The qualitative description of the protein signatures provides insight into the biogeochemistry of the watersheds that enhances the quantitative data. The production of a high molecular weight protein confirmed the production of dissolved nitrogen in Cascade Brook. Although the amount of total dissolved nitrogen remained constant in Black Rock Brook, the qualitative evidence of protein degradation suggests microbial activity within the hemlock stand which may affect the speciation of dissolved nitrogen.

*Hudson River*

The 2/9/99 sample had fewer proteins than the 2/26/99 sample. A difference in water temperature and snow conditions could affect the protein signature of the samples. Runoff from melting snow could carry terrestrial proteins to the river. Warmer water temperatures could also increase primary production and decomposition in the Hudson River surface waters. Photosynthesis is sensitive to both increases in light and temperature. However, respiration is more sensitive to such increases in temperature. These two processes could increase the amount and the complexity of DON in the water.

Due to analytic error the total dissolved nitrogen data for 2/26/99 was not available. Information about the relative concentrations of total dissolved nitrogen could help determine whether the more complex protein signature of 2/26/99 was due to an increased concentration of DON from terrestrial runoff or metabolic processes in the estuary.

Both Hudson River samples had the same pairs of proteins that were found in both watersheds of Black Rock Forest. The 2/9/99 sample looked nearly identical to the "hem a" sample. The commonality of bands ~116kDa and ~64kDa in the Hudson River, Black Rock Brook, and Cascade Brook is striking. Their appearance in all three locations could have several explanations. The proteins in the Hudson River could have terrestrial origins; they could have been transported from forests by streams. It would be interesting to investigate the distribution of these two proteins in freshwater and marine ecosystems. Tanoue 1996 found mostly proteins ranging from 14-66kDa in the open ocean; 48kDa was the most common protein in the ocean. If the 116kDa and 64kDa protein are not found in the open ocean, this could suggest that they are land-derived. These proteins could be used to detect terrestrial DON in estuaries and coastal waters. These two particular proteins also could be common products of decomposition, or they could result from similar biological processes.

The 2/26/99 sample also had unique proteins that were not found in the Black Rock Forest samples. Water that had passed through the wetland in Cascade Brook had the highest molecular weight protein (150kDa), but the Hudson River sample had the lowest molecular weight protein (10kDa). Beside the Black Rock Brook sample below the hemlock stand, the Black Forest samples did not have any proteins lower than 60kDa; the 2/26 sample had four proteins lower than 60kDa. These low molecular weight proteins and the unique 85kDa protein could be land-derived and introduced by a different tributary or by sewage disposal. These proteins also could be excreted by phytoplankton engaged in primary production as Bronk et al. 1994 suggest. The lower molecular weight proteins could be the products of microbial decomposition of typical estuarine organic matter such as phytoplankton. Finally, these unique proteins could be marine-derived and tidally introduced into the estuary.

## Conclusion

This study of dissolved nitrogen in a forest and an estuary provides preliminary answers to questions about the role and importance of DON in these ecosystems. The quantitative analyses of total dissolved nitrogen, DON, $NO_3$, and $NH_4$, suggest that DON can be a significant component of the dissolved nitrogen flux out of forested ecosystems. Measurements of $NO_3$ represent only 20-40% of the total nitrogen lost from the ecosystem. Fluxes of DON should be considered for forest nitrogen budgets. In the Hudson River estuary, the percent contribution of DON to total dissolved nitrogen varied. Ranging from 5% to 70%, DON was a significant component of total dissolved nitrogen that should be considered. The implied rapid turnover of DON and bioavailable forms of dissolved nitrogen suggest complex nitrogen cycling which could affect primary production and possibly harmful algae blooms.

The qualitative analyses of the proteinaceous component of DON demonstrate that proteins can be isolated, analyzed, and characterized. These more qualitative descriptions of DON provide insight into some biogeochemical processes that affect nitrogen concentrations; this information is not apparent from bulk quantitative data. For example, the protein analysis in Cascade Brook watershed sample provided evidence for both production and degradation of DON within the wetland. The protein analysis could also provide information about the conditions and location of DON production. For example, high molecular weight proteins could be an indication of anoxic conditions.

## Recommendations

The dissolved nitrogen results from Black Rock Forest should be compared with results from other temperate forests. This comparison would help determine whether Black Rock Forest is a representative northeastern temperate forest with respect to nitrogen cycles and budgets. If the forest is representative, results from the forest could be applied to make predictions and decisions about other temperate forests. If the forest is not representative, identifying the causes of the nitrogen cycle anomalies would be informative.

The Hudson River estuary is an ecosystem that can be greatly affected by humans. Continued study of nitrogen concentrations and general water chemistry is necessary to determine the anthropogenic effect on the health of this ecosystem. Further insight into

estuarine ecosystem dynamics will provide information about how the ecosystem can respond to anthropogenic pollution.

Long-term studies of total dissolved nitrogen concentrations should be conducted in both ecosystems. More data would provide a more accurate description of the nitrogen cycles and budgets of both ecosystems. Longer studies could identify trends in the dissolved nitrogen speciation and seasonalities of total nitrogen concentrations and nitrogen speciation. Concentrations for all three forms of dissolved nitrogen should be studied rather than simply the concentrations of inorganic vs. organic dissolved nitrogen. Providing concentrations for specific forms of nitrogen provides information about the bioavailability of nitrogen in an ecosystem, about its usefulness as a nutrient, and about the forces controlling its export from the ecosystem. This information can also be used to identify potential conditions of nitrogen saturation in forests.

One of the most useful applications of the qualitative analysis of proteinaceous DON would be to correlate specific proteins with sources of DON. This would provide a more detailed description of forest nitrogen cycles. This would also help resolve the controversy relative importance of DON sources in estuaries.

Nitrogen limitation has been a characteristic of forested and estuarine ecosystems which has defined their structure. With the increasing anthropogenic additions of nitrogen and carbon to these ecosystems, it is important to monitor nitrogen and carbon budgets. These budgets can help determine the impact of humans on the ecosystems, and they can help make predictions about potential changes in ecosystem structure and dynamics.

## Acknowledgments

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**APPENDIX I- Concentration of Protein by Tangential Flow Filtration (TFF)**
(Tanoue 1995)
Method based on R. Sambrotto/ B. Mace; LDEO 10/98
See Appendix VI for more detailed description

Materials:
- 10L Nalgene container with spout
- Millipore Prep/Scale-TFF 2.5 $ft^2$ Cartridge #SK1P026W3 (10kDa regenerated cellulose membrane)
- Cole-Parmer Masterflex Peristaltic Pump #77250-62
- Masterflex silicone Tygon tubing (Cole-Parmer #96420-36)
- Immersion heater
- 250mL glass beaker
- Centrivap
- Distilled/Deionized Water (DI)
- 0.1 M NaOH (Fisher #S-613) (40ml 10N NaOH/ 4L DI)
- 2% Sodium dodecylsulfate (SDS) (Fisher # BP166) (10gSDS/ 500mL DI)
- $NH_4HCO_3$ (Fisher #A643)
- Desalting Buffer (35mM $NH_4HCO_3$ +0.01%SDS) (2.767g $NH_4HCO_3$+5mL 2%SDS/ 1L DI)

Approximate time for procedure:

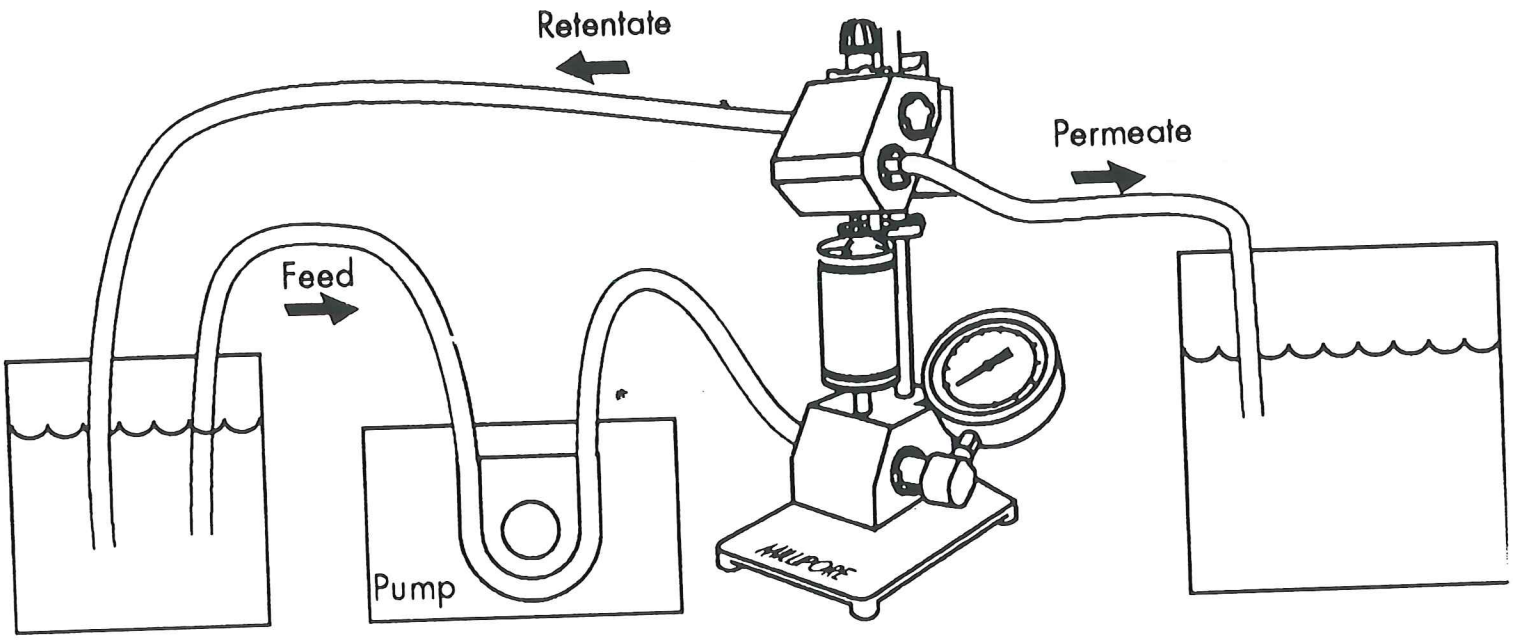
| | |
|---|---|
| Part I and II- | 0.5hr. |
| Part III- | 2hr. |
| Part IV- | 1.5hr |
| Total- | 4hrs. |

Part I: Setup
1. see Fig. 15 for detailed description of filter and flow

Part II: Preparing the tangential flow system
1. The filter should be stored at 4 °C filled with a 0.1M NaOH solution
2. To remove all NaOH from the filter, flush with 8L DI heated to 45 °C
   **NOTE: NaOH will denature proteins and destroy sample**

Part III: Sample Concentration
1. Add SDS to the prefiltered filtrate to a final concentration of 0.01% SDS. Mark initial sample volume on container so exact volume can be determined later and used for concentration estimates.
   **NOTE: Collect 75mL of sample for total DON analysis before adding SDS.**
2. Direct the retentate tubing into the sample reservoir. Pump the sample through the filter at speed 3 and at 14psi back pressure.
3. Pump until retentate volume is as small as possible with the following procedure. When the back pressure drops below 5psi, stop pump. Drain all retentate into tubing, and shut off drain valve from the sample reservoir. Hold tubing upright as you turn the pump back on. Pump until retentate no longer enters reservoir. Drain this retentate into a clean 250 mL glass beaker (about 30-50mL)
4. Add 250 mL desalting buffer to the sample reservoir and repeat retentate collection.

Figure 16. Diagram of Tangential Flow Filtration System.



5. Add 250 mL desalting buffer to the sample reservoir and repeat retentate collection. After this final desalting step, disconnect the filter and drain the filter into the retentate beaker. The combined retentate will total ~120-150 mL.
6. Distribute the retentate evenly into six 50mL sterile centrifuge tubes taking care not to exceed 25 mL in any one tube.
7. With the caps off, place in Centrivap. Concentrate in Centrivap for 10 hrs. at 45°C, and the remaining time without added heat. Remove tubes from Centrivap just before sample dries out (1-3mL sample remaining).
8. Combine sample volumes into single 50mL centrifuge tube (20-30mL). Use the desalting buffer to rinse sample from each tube.
9. Store crude concentrate frozen until purification and precipitation in TCA

Part IV: Cleaning the Tangential Flow System

1. Always flush before cleaning. Flush filter with 8L distilled or DI water heated to 45°C. Direct the retentate to waste. Three times during flush cycle, increase 2 back pressure to 14 psi for 10 seconds.
2. At a pump speed of 2, pump 4L 0.1M NaOH through the filter. The solution should be kept at 45°C. Direct the retentate back into the NaOH reservoir. Cycle the NaOH solution through the filter for 1hr.
3. The column should be stored at 4°C filled with the NaOH solution.

**APPENDIX II- Purification and Precipitation of Proteins in the Crude Concentrate**
(Tanoue 1995)
Method Based on R. Sambrotto/ B. Mace 4/98
See Appendix IV for more detailed description

Materials: 50mL and 15mL centrifuge tubes
Microfuge tubes
Centrifuge (Fisher ZK380)
Sonicator
100% Trichloroacetic acid (TCA) (Sigma# 490-10)
100% -20•C Ethanol
100% -20•C Diethyl ether
NuPAGE LDS sample buffer (4x) (Novex #NP0007)

Approximate time for procedure: Allow at least 6 hours.

**Procedural Note: Handling of sample is critical. Keep sample at 4 °C at all times. Keep samples on ice and keep centrifuge at 4 °C**

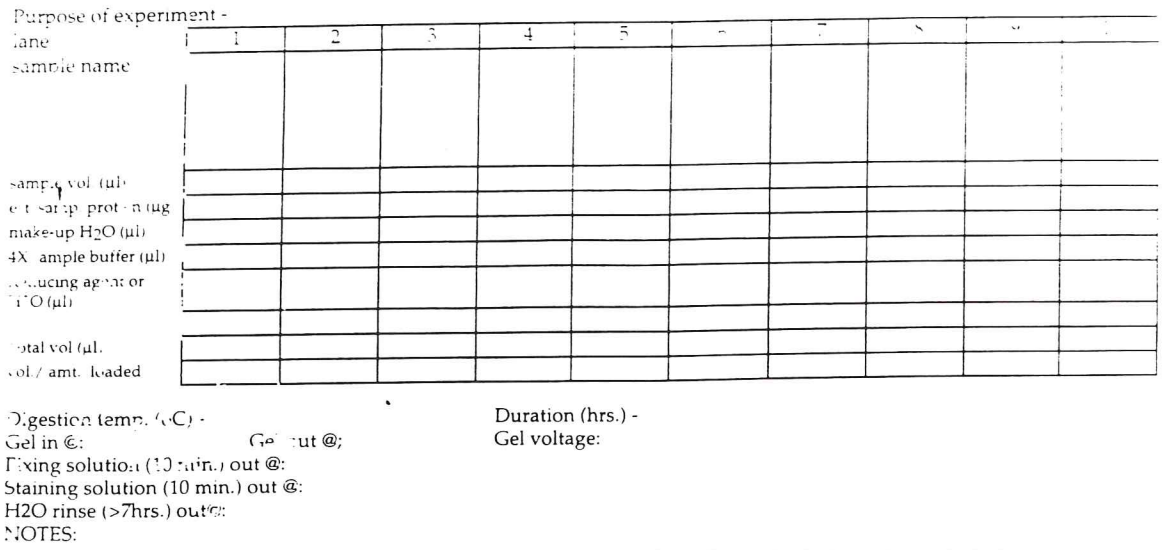
1. Add TCA to the crude concentrate (20-30mL) to a final concentration of 5%. Let solution stand at 4 °C for at least 12 hours.
2. Centrifuge solution to remove TCA soluble material. This and subsequent centrifugations were performed for 60min. at 7000rpm for 50mL and 15 mL tubes 30 min at 12,000 rpm for microfuge tubes at 4 °C unless otherwise noted. For 50mL and 15mL tubes, use centrifuge sleeve. Use minimal brake on centrifuge to minimize pellet disruption.
3. The solution separates into three layers: pellet at the bottom, intermediate layer of supernatant, and a thin layer of low-density material on surface of supernatant. An appreciable amount of protein was found to be associated with the low-density materials on the surface of the supernatant. **Note: 1 hr. is sufficient time for centrifugation although pellet may not be visible. A clear supernatant indicates sufficient centrifugation. Especially if pellet is not apparent, take care to note the orientation of the tube and the expected location of pellet.**
4. Using a glass pipette, remove low-density top layer and save in a microfuge tube. Remove the intermediate supernatant layer and discard. Combine the low-density layer and the pellet in a 15mL centrifuge tube. Use the low density layer rinse all pellet from the original centrifuge tube. **NOTE: Sample recovery is critical. Be careful when removing supernatant. Try to retain all pellet. If the pellet is not visible, assume the bottom 1mL of solution contains pellet. Pipette carefully and rinse pipette and centrifuge tubes well.**
5. Add TCA to a final volume of 5%. Resuspend by vigorous homogenization with sonication for 3 seconds. Keep sample on ice during sonication. Carefully control sonicator to reduce sample loss.
6. Centrifuge solution. Retain top low-density layer and pellet. Discard intermediate supernatant layer. This sample can usually fit into a microfuge tube

To remove residual TCA, excess SDS, and non-protein dissolved organic material from the TCA insoluble fraction:

7. Add ice cold ethanol to 50%. Use ethanol to rinse pellet from 15mL tube and to rinse pipette. Resuspend by vigorous homogenization with sonication for 3 seconds.

8. Centrifuge solution. The ethanol wash eliminated the low-density fraction. The ethanol insoluble fraction formed a pellet at the bottom of the tube after centrifugation. Remove and discard the ethanol soluble materials. Save only the pellet. The pellet can remain in the same tube.
9. Add ice cold diethyl ether to 50%. Resuspend by vigorous homogenization with sonication for 3 seconds.
10. Centrifuge solution. Remove and discard diethyl ether soluble materials. Retain only the pellet in the microfuge tube.
11. Repeat diethyl ether wash.
12. After final diethyl ether wash, air dry pellet ($N_2$ gas can facilitate drying). **Note: Do not allow pellet to completely dry out. The pellet must be able to be dissolved in buffer solution.**
13. Redissolve nearly-dried pellet in 50µL NuPAGE sample buffer solution. Store at either 4 °C .

Figure 17. SDS-PAGE Sample Record



Purpose of experiment -

| lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| sample name | | | | | | | | | | |
| sample vol (µl) | | | | | | | | | | |
| est sample protein (ug | | | | | | | | | | |
| make-up $H_2O$ (µl) | | | | | | | | | | |
| 4X sample buffer (µl) | | | | | | | | | | |
| reducing agent or $H_2O$ (µl) | | | | | | | | | | |
| total vol (µl. | | | | | | | | | | |
| vol / amt. loaded | | | | | | | | | | |

Digestion temp. (°C) - Duration (hrs.) -
Gel in @: Gel out @; Gel voltage:
Fixing solution (10 min.) out @:
Staining solution (10 min.) out @:
H2O rinse (>7hrs.) out @:
NOTES:

Part II: Gel Setup

1. Dilute the 20X NuPage running buffer to 1X by adding 50mL buffer to 950mL DI. Set aside 200mL of 1X running buffer.
2. Use precast NuPAGE gels. **NOTE: Gels are toxic. Use extreme care when handling gels. All waste should be deposited in hazardous waste container.** Open pouch and drain liquid. Rinse cassette with DI. Peel off tape from bottom of cassette. Pull comb out of cassette. Drain liquid from sample wells. Use a disposable pipette to rinse sample wells with 1X running buffer. Invert gel to remove buffer and repeat rinse two more times.
3. Orient the gel so that the notched "well" side of the cassette faces the buffer core (inward). If only one gel is used, use the square plastic buffer dam to replace the second gel cassette. Align the cassettes so that the top edges of the cassettes are f lush with the top edge of the cassette holder.
4. Record gel number, and mark lane orientation on outside of cassette.
5. Add 500µL NuPage antioxidant to 200mL 1X running buffer. Fill the buffer core with this solution. The cassettes should make a impermeable seal, and the core should not leak buffer. This buffer should fill the core and it must cover the sample wells.
6. Insert the gel cassettes into the Mini-Vertical gel system gel box. Fill the outer chamber with the remaining 800mL 1X running buffer.
7. To keep gel cool, surround gel box with ice. **NOTE: Gel box must be level.**

Part III: Running Gel

1. To load the sample, use special Novex gel loading tips
2. Because gel lanes tend to "smile" (curve outward) do not use last lane on either side.
3. Load 5µL of Novex Mark 12 protein standard marker diluted 1:5 in NuPAGE LDS sample buffer into first, middle, and last lanes.
4. Load samples. Samples should correspond to recorded lane number

5. Attach gel cover. Internal chamber buffer must cover sample wells. Plug in electrodes to Fisher Biotech electrophoresis system and run gel at 200 volts.
6. Run gel until blue tracer reaches bottom of gel (about 30 minutes)
7. Allow cassettes to cool before opening.
8. To open cassettes, use gel knife to break all seals around edge of gel. Notch gel to indicate proper lane orientation.
9. Deposit gel in first solution of staining procedure.

Part IV: Developing Gel

1. Follow Novex SilverXpress Silver Staining Protocol for Tricine and NuPAGE BIS-TRIS gels (Appendix IV).
2. Take picture of gel using Kodak Digital science DC40 camera immediately after development
3. Wrap gel in plastic and store at 4°C.

# SilverXpress Silver Staining Protocols*

Note: For samples reduced with DTT, use Tricine Procedure.

** Protocols refer to times required for **1.0mm** mini-gels. For 1.5mm mini-gels, double all times. For detailed instructions and troubleshooting guide, please refer to the Instruction Booklet.*

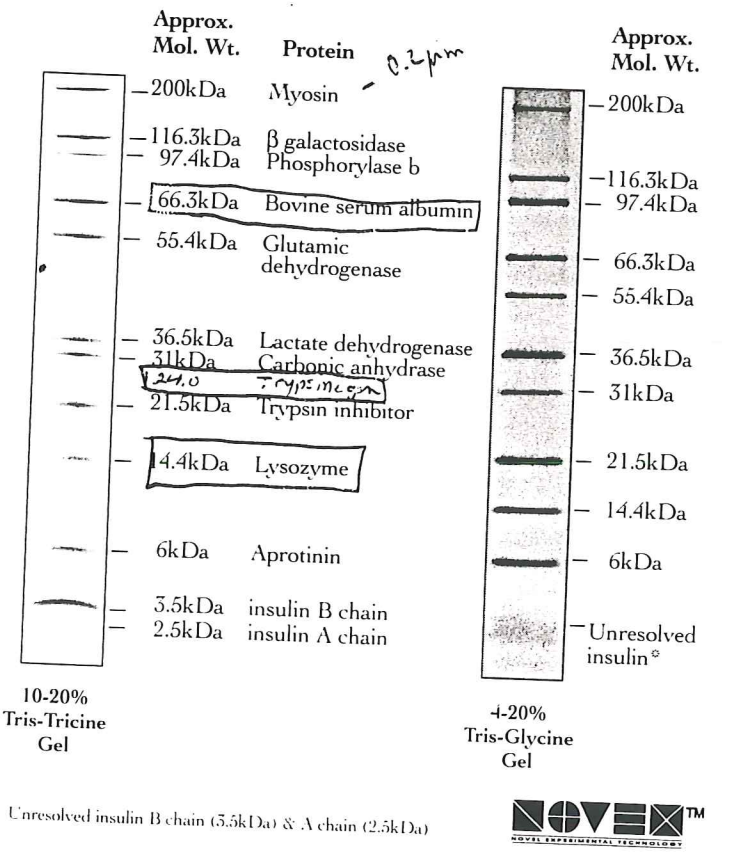
| | TRIS-GLYCINE/ NUPAGE TRIS-ACETATE GELS | TRICINE and NuPAGE BIS-TRIS GELS |
|---|---|---|
| **FIXING** | • 90ml Ultra Pure Water<br>• 100ml Methanol<br>• 20ml Acetic Acid<br>} Final Volume:** 200ml<br><br>Volume / Time / Completed<br>Fix: 200ml / 10 min. ❑ | • 90ml Ultra Pure Water<br>• 100ml Methanol<br>• 20ml Acetic Acid<br>} Final Volume:** 200ml<br><br>Volume / Time / Completed<br>Fix: 200ml / 10 min. ❑ |
| **SENSITIZING** | • 105ml Ultra Pure Water<br>• 100ml Methanol<br>• 5ml Sensitizer<br>} Final Volume:** 200ml<br><br>Volume / Time / Completed<br>1st Sens: 100ml / 10 min. ❑<br>2nd Sens: 100ml / 10 min. ❑ | • 105ml Ultra Pure Water<br>• 100ml Methanol<br>• 5ml Sensitizer<br>} Final Volume:** 200ml<br><br>Volume / Time / Completed<br>1st Sens: 100ml / 30 min. ❑<br>2nd Sens: 100ml / 30 min. ❑ |
| **WASH** | • 400ml Ultra Pure Water<br><br>Volume / Time / Completed<br>1st Wash: 200ml / 5 min. ❑<br>2nd Wash: 200ml / 5 min. ❑ | • 400ml Ultra Pure Water<br><br>Volume / Tricine / NuPAGE<br>1st Wash: 200ml / 5 min. / 10 min. ❑<br>2nd Wash: 200ml / 5 min. / 10 min. ❑ |
| **STAINING** | • 5ml Stainer A<br>• 5ml Stainer B<br>• 90ml Ultra Pure Water<br>} Final Volume: 100ml<br><br>Volume / Time / Completed<br>Stain: 100ml / 15 min. ❑ | • 5ml Stainer A<br>• 5ml Stainer B<br>• 90ml Ultra Pure Water<br>} Final Volume: 100ml<br><br>Volume / Time / Completed<br>Stain: 100ml / 15 min. ❑ |
| **WASH** | • 400ml Ultra Pure Water<br><br>Volume / Time / Completed<br>1st Wash: 200ml / 5 min. ❑<br>2nd Wash: 200ml / 5 min. ❑ | • 400ml Ultra Pure Water<br><br>Volume / Time / Completed<br>1st Wash: 200ml / 5 min. ❑<br>2nd Wash: 200ml / 5 min. ❑ |
| **DEVELOPING** | • 5ml Developer<br>• 95ml Ultra Pure Water<br>Final Volume: 100ml<br><br>Volume / Time / Completed<br>Develop: 100ml / 3–15 min. ❑ | • 5ml Developer<br>• 95ml Ultra Pure Water<br>} Final Volume: 100ml<br><br>Volume / Time / Completed<br>Develop: 100ml / 3–15 min. ❑ |
| **STOPPING** | • 5ml Stopper<br>Add directly to developing solution.<br><br>Volume / Time / Completed<br>Stop: 5ml / 10 min. ❑ | • 5ml Stopper<br>Add directly to developing solution.<br><br>Volume / Time / Completed<br>Stop: 5ml / 10 min. ❑ |
| **WASH** | • 600ml Ultra Pure Water<br><br>Volume / Time / Completed<br>1st Wash: 200ml / 10 min. ❑<br>2nd Wash: 200ml / 10 min. ❑<br>3rd Wash: 200ml / 10 min. ❑ | • 600ml Ultra Pure Water<br><br>Volume / Time / Completed<br>1st Wash: 200ml / 10 min. ❑<br>2nd Wash: 200ml / 10 min. ❑<br>3rd Wash: 200ml / 10 min. ❑ |

**NOTE: The final volumes of solutions containing methanol and water reflect a volume shrinkage which occurs when these reagents are mixed. Do not adjust volumes of components or final volume.

For Technical Service, Call 1-800-55-NOVEX (1-800-556-6839) or E-mail: nvxtech@novex.com

APPENDIX V- Novex Mark12 Protein Standard

# Mark12™ Wide Range Protein Standard




*Unresolved insulin B chain (3.5kDa) & A chain (2.5kDa)

NOVEX™ NOVEL EXPERIMENTAL TECHNOLOGY

## APPENDIX VI

Tanoue, Eiichiro. 1995. Detection of dissolved protein molecules in oceanic waters. Marine Chemistry. **51:** 239-252.

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Marine Chemistry 51 (1995) 239–252

MARINE CHEMISTRY

# Detection of dissolved protein molecules in oceanic waters

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**Abstract**

A method for the extraction and detection of dissolved protein molecules in oceanic waters is described. The procedure involves three separate steps: (1) crude concentration of dissolved protein from seawater by tangential flow ultrafiltration, (2) further concentration and purification of dissolved protein by precipitation with trichloroacetic acid, and (3) separation and detection of dissolved proteins by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Dissolved proteins were successfully extracted with this technique; a relatively limited number of protein molecules (less than 30) were separated and visualized on gels as major bands during the analysis of water from stations located from the subarctic to the tropical Pacific. The majority of proteins had molecular masses ranging from 14 to 66 kilodaltons (kDa). Electrophoretic patterns of dissolved proteins changed both horizontally and vertically in seawater, but some protein molecules were found in all the samples examined. The major proteins detected were relatively pure and were present at high levels. The accumulation of appreciable amounts of a relatively limited number of proteins leads to the hypothesis that particular proteins that make up the majority of the dissolved protein components in seawater are derived from specific sources and contribute quantitatively to the oceanic pool of organic nitrogen.

## 1. Introduction

Amino acids in seawater have been analyzed in terms of dissolved free amino acids (DFAA) and dissolved combined amino acids (DCAA) after hydrolysis of macromolecules. Dissolved combined amino acids (DCAA) represent the largest well-defined molecular forms of dissolved organic matter (DOM; Coffin, 1989; Keil and Kirchman, 1991); they are derived from marine organisms (e.g. Lee and Wakeham, 1988). Most amino acids in living organisms are present as constituents of proteins (Billen, 1984) and proteins account for more than about 50% of the organic matter (Romankevich, 1984) and 85% of the organic nitrogen (Billen, 1984) of marine organisms. Amino acids in cellular constituents of organisms that have been transferred to the pool of DOM might be expected in the form of protein. However, studies of the amino acid composition of the DCAA have yielded limited source-related information because most proteins in living organisms have a similar amino acid composition (e.g. Strickland, 1965; Degens, 1970). To date, neither the chemical forms nor the sources of DCAA have been clarified and virtually nothing is known of the nature of the proteins in the dissolved phase in seawater. The need to clarify the source and the fate of the DCAA is obvious if we are to understand biogeochemical processes related to proteins, as well as DOM, in the sea.

One of reasons that descriptions of proteins at the molecular level have been limited is the methodological difficulty associated with relevant analysis. The extraction of protein molecules from seawater is difficult because of their low levels, with concomitant high levels of inorganic salts, as well as their "sticky" nature (Kirchman et al., 1989; Taylor et al., 1994). Our analytical abilities are also insufficient because analyses at the molecular level typically involve degradation (e.g. via hydrolysis, oxidation or pyrolysis) for the release of components that can then be quantified (Farrington, 1992). The application of sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to particulate organic matter (POM) revealed the occurrence of protein molecules in the particulate phase throughtout the water column (Tanoue, 1992). The observation that discrete species of protein molecules are present in the particulate phase, even in intermediate and deep waters, encouraged our attempts to detect protein molecules in the dissolved phase in oceanic waters. The present report describes methods for extraction of dissolved proteins from seawater and preliminary molecular characteristics of dissolved proteins in the oceanic water column.


*SSDI* 0304-4203(95)00061-5



## 2. Materials and methods

### 2.1. Materials

Samples were taken from various depths at three stations, i.e. *A* (45°10.3′N, 165°34.4′E; water depth, 5934 m), *B* (24°35.0′N, 170°0.1′E; water depth, 5966 m), and $D'_2$ (00°0.2′S, 158°59.1′W; water depth, 4786 m), from 19 October to 17 November 1993. The stations were located from subarctic to tropical regions of the Pacific Ocean and samples were collected during a cruise of the R/V *Hakuho-maru* (cruise KH-93-4). Seawater samples were collected with a diaphragm-type air-driven pump (over 200 m depth), and with a Niskin-type bottle, mounted on a CTDO-Rosette Multiple sampler (less than 200 m depth). Each sample (ca. 60 l) was filtered through a GF/F glass fiber filter (Whatman, Maidstone, UK), immediate after sampling. The filtrate was further filtered through a tangential flow ultrafiltration system (PELICON™, Millipore, Bedford, MA) with a filter with 0.1 μm pores (filter material: low protein-binding polyvinylidene difluoride; DURAPORE®, Millipore) at station *A*. The ultrafiltration through the second filter was omitted at stations *B* and $D'_2$. The filtrates were subjected to concentration of the dissolved protein.

Aged seawater was used for analytical recovery experiments. A sample of seawater from the deep layer of the northwest Pacific, which had been filtered through a GF/F filter on board ship, was used after storage in a glass bottle in the laboratory for approximately 4 yr at room temperature. The following standard proteins (Sigma, St. Louis, MO), whose molecular masses are given in kilodaltons (kDa), were used for recovery experiments: bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

### 2.2. Methods

The procedure involves three separate steps: (1) crude concentration of dissolved protein from seawater with the tangential flow ultrafiltration systems (MINITAN™ and PELICON™, Millipore), (2) further concentration and purification of the dissolved protein by precipitation with trichloroacetic acid, and (3) separation and detection of the dissolved protein by SDS-PAGE.

#### 2.2.1. Crude concentration of dissolved protein

The ultrafiltration systems MINITAN™ (filter area, 240 $cm^2$), in case of the samples of less than 10 l, and PELICON™ (filter area, 4650 $cm^2$) in case of samples of more than 10 l, were used with a filter with a nominal molecular mass cut-off of 10 kDa (10,000 NMMCO filter; filter material, low protein-binding regenerated cellulose), unless otherwise noted. The system was precleaned with 0.1 M NaOH and washed with 20 l of deionized water (18 MΩ) for MINITAN™ and 100 l of deionized water (18 MΩ) for PELICON™ according to the manufacturer's instruction. Before use of each new ultrafiltration membrane and tubing, a 3.5% solution of NaCl (5 l for MINITAN™ and 20 l for PELICON™) was concentrated in the same manner as the seawater sample to serve as a control. We confirmed that no protein molecules were present as contaminants in the ultrafiltration system and reagents by SDS PAGE, and then the system was used for concentrating dissolved proteins in seawater.

For detection of dissolved proteins in natural seawater, the filtrate (c. 20 l; the DURAPORE® filtrate at station *A* and the GF/F filtrate at stations *B* and $D'_2$) in a flexible polyethylene bag (retentate reservoir, capacity 20 l) was concentrated by ultrafiltration (PELICON™) after addition of SDS (final concentration, 0.01%, *w/v*). At the end of the concentration step, the retentate (fraction with a molecular mass of more than 10 kDa) was transferred to a silanized glass-cylinder (capacity, 500 ml) and the concentration was continued. The retentate was concentrated to approximately 150–200 ml, approximately equivalent to the dead volume of the system. Then 250 ml of a solution of 35 mM ammonium bicarbonate buffer (pH 7.8) that contained 0.01% SDS (referred to as the desalting buffer hereafter) were added to the retentate. Concentration of the retentate was continued. This step was repeated three times to desalt the retentate. After retrieval of the desalted retentate, the system was washed by the circulation of 250 ml of desalting buffer to retrieve all the retentate from the system. The combined solutions (approximately 450 ml; the desalted retentate and the washing solution) were further concentrated by ultrafiltration (MINITAN™). At the end of the concentration, the retentate in the system (50–70 ml), approximately equivalent to the dead volume of the system, was desalted by three additions of 70 ml of the desalting buffer. After retrieval of the desalted retentate, the system was washed by circulation of the desalting buffer. The combined solutions were further concentrated in a Speed Vac concentrator (model SC210A; Savant, Farmingdale, NY) with charcoal and molecular sieve traps attached before the pump. The residue was redissolved in the desalting buffer to yield the crude concentrate. The volume of the crude concentrate was usually less than 10 ml, but sometimes, in particular in the case of samples from intermediate and deep waters at station *B*, it exceeded 30 ml because of the large amount of residue after drying. Crude concentrates were stored frozen (−30°C) until use.

For experiments to examine the recovery of standard proteins, aged seawater (2–5 l) with known amounts of standard proteins added was concentrated by the MINITAN™. The procedure was the same as described above, except for the preliminary recovery experiments. In the preliminary recovery experiments, the combined retentate obtained with the MINITAN™ was further concentrated with a vacuum-operated, disposable ultrafiltration unit with a 10,000 NMMCO filter (filter material; low protein-binding regenerated cellulose, IMMERSIBLE-CX™; Millipore), instead of with the Speed Vac concentrator. The combined solution from the MINITAN™ was concentrated to 1–2 ml and was further desalted by three additions of 2 ml of the desalting buffer. After retrieval of 1–2 ml of the concentrate, the filter was washed twice with each time 1 ml of desalting buffer. The combined solution was lyophilized. The lyophilized sample was not subjected to further purification and was directly redissolved in the sample buffer for SDS-PAGE which contained TRIS-HCl (62.5 mM, pH 6.8), SDS (2%), 2-mercaptoethanol (5%, *v/v*) and urea (8 M) (Tanoue, 1992).

### 2.2.2. *Precipitation in trichloroacetic acid (TCA) of proteins in the crude concentrate*

An aliquot (1–5 ml) of the crude concentrate, after addition of trichloroacetic acid (100% TCA; Sigma) to a final concentration of 5% (*v/v*), was allowed to stand in a refrigerator (4°C) for at least 12 h. The solution was centrifuged to remove TCA-soluble material. This and subsequent centrifugations were performed at 14,000 × g, for 30 min at 4°C, unless otherwise noted. In the case of the samples from station *A*, the supernatant was discarded after centrifugation. In the case of the samples from stations *B* and $D'_2$, the solution of crude concentrate sometimes separated into three layers after centrifugation, namely, a pellet at the bottom of the centrifuge tube, an intermediate layer, and a layer of low-density materials on the surface of the supernatant. An appreciable amount of protein was eventually found to be associated with the low-density materials on the surface of the supernatant (see below). The intermediate layer of the supernatant was carefully removed and discarded in the case of samples from stations *B* and $D'_2$. The pellet and the low-density fraction were resuspended by vigorous homogenization with sonication (usually 3–5 s) in a solution of 5% TCA (*v/v*). The homogenate was centrifuged again and the intermediate layer was again carefully removed and discarded.

The TCA-insoluble fraction was washed to remove residual TCA, excess SDS, and non-protein



dissolved organic materials. First, the TCA-insoluble fraction was resuspended by vigorous homogenization with sonication (usually 3–5 s) in ice-cold ethanol. The mixture was centrifuged and ethanol-soluble materials were carefully removed and discarded. The washing with ethanol (usually once) eliminated the low-density fraction, and the TCA- and ethanol-insoluble fraction formed a pellet at the bottom of the centrifuge tube upon centrifugation. After repeated resuspension of the pellet in cold (−20°C) diethyl ether and centrifugation (usually two or three times), the final pellet was air-dried.

The dried pellet was redissolved in a sample buffer solution of TRIS-HCl (62.5 mM, pH 6.8), SDS (2%, *w/v*), 2-mercaptoethanol (5%, *v/v*) and glycerol (10%, *v/v*) and heated at 100°C for 3 min. After centrifugation at 2000 × g for 5 min at room temperature, the supernatant was adjusted to pH 6.8 and analyzed by SDS-PAGE.

### 2.2.3. *SDS-PAGE*

SDS-PAGE, staining and destaining (with Coomassie brilliant blue-R250; CBB-R staining method) were performed following the method of Laemmli (1970) as previously described in detail (Tanoue, 1992), using ready-made continuous-gradient gels (5–20%, PAGEL™, NPG-520 type; Atto, Tokyo). Silver staining was also performed according to the manufacturer's instructions using a silver-staining kit (2D-SILVER STAIN-II™; Daiichi, Tokyo) based on the method of Oakley et al. (1980). The standard proteins that were used in the recovery experiments were also used as reference proteins for SDS-PAGE. Quantitation of each standard protein added in the recovery experiments was performed with a densitometer (model AE-6900; Atto, Tokyo). Known amounts of each standard protein were subject to electrophoresis on the same gel as experimental samples for reference.

In the present study, complete denaturation and reduction of any disulfide bonds in dissolved proteins were accomplished by heating with SDS and 2-mercaptoethanol. During heating, the original configuration of the protein molecules in the dissolved phase was destroyed. The proteins and/or their subunits characterized by SDS-PAGE are defined collectively as proteins in the present study.

## 3. Results and discussion

Polyacrylamide gel electrophoresis is a convenient, versatile and well-established technique for the separation and detection of protein molecules (e.g. Andrew, 1986; Tanoue, 1991). However, for the application of the technique to detection of dissolved proteins, dissolved proteins have to be concentrated as much as $10^5$–$10^6$ times; i.e. for the preparation of a sample for electrophoresis of about 1 μl, about 1 l of seawater is needed. To achieve this, studies have been undertaken to improve methods for the concentration and desalting of dissolved proteins from seawater. I have examined the concentration and desalting by repeated lyophilization and dialysis, by the use of a water-absorbing gel, by adsorption onto blotting membranes and onto affinity gel, and by precipitation using organic solvents. However, these methods were unsatisfactory (data not shown) for the present goals.

### 3.1. *Methodological examinations using standard proteins*

In the preliminary recovery experiments, standard proteins added to aged seawater were not or extremely poorly recovered in the absence of desalting or desalting with deionized water at the end of the concentration steps (data not shown; see below). Only when a solution of ammonium bicarbonate, that contained 0.01% SDS, was used as the desalting buffer solution the standard proteins were recovered. The effect of concentration of ammonium bicarbonate in the desalting buffer solution on the recovery of standard proteins was examined (Table 1). A solution of 35 mM ammonium bicarbonate gave the highest recovery of six standard proteins; the recovery decreased with higher and lower concentrations of ammonium bicarbonate. Even when 35 mM ammonium bicarbonate was used, standard proteins that were added at low level (total amount, 50 μg/l) were not recovered (Table 1, footnote b).

Two reasons for this failure were considered: (1) higher levels of ammonium bicarbonate yielded a large quantity of inorganic residue after lyophilization and, as a result, high levels of salts in the electrophoretic sample interfered with electrophoresis; and (2) a large amount of inorganic residue

Table 1
Effects of the concentration of ammonium bicarbonate buffer (containing 0.01% SDS) [a] on recovery (%) of six standard proteins. The total amount of standard proteins was 210 μg/l (each standard protein was present at 32–39 μg/l) [b]. A solution that consisted of the concentrate and the wash was further concentrated on a vacuum-operated ultrafiltration unit and the resulting concentrate, after lyophilization, was subjected to SDS-PAGE

| | Bovine serum albumin (66 kDa) | Egg abumin (45 kDa) | Glyceraldehyde-3-phosphate dehydrogenase (36 kDa) | Trypsinogen (24 kDa) | β-lactoglobulin (18.4 kDa) | Lysozyme (14.3 kDa) |
|---|---|---|---|---|---|---|
| 7 mM | 9 | 21 | 11 | 15 | 20 | 16 |
| 35 mM | 19 | 41 | 16 | 30 | 27 | 26 |
| 70 mM | 11 | 10 | 5 | 30 | 11 | nd [c] |
| 200 mM | 8 | 5 | 4 | 23 | 9 | 5 |

[a] Concentrations of ammonium bicarbonate were examined upto 700 mM. However, almost no standard proteins were recovered or electrophoresis was hampered by high levels of the salt.
[b] In the case of total amounts of less than 50 μg/l, no standard proteins were recovered.
[c] Not determined because of unsatisfactory resolution after SDS PAGE.

necessitated the preparation of a relatively large sample volume for electrophoresis (usually 300 μl–1.5 ml) and, as a result, it was difficult to prepare a sample that was concentrated enough for the detection of small amounts of standard proteins since the detection by CBB-R required approximately 1 μg of protein per band on the gel (Andrew, 1986). The silver-staining method, which is 50–100 times more sensitive than staining with CBB-R, could not be used because of a high background at this stage. When the same procedure as that used to obtain the results in Table 1 was applied to seawater, faint bands of natural protein molecules were detected. However, electrophoretograms were unsatisfactory because large quantities of inorganic residue interfered with the electrophoresis (data not shown). Thus, further desalting and purification of proteins from 1–5 ml of the crude concentrate were necessary.

Application of small-scale dialysis, immobilized metal-ion affinity chromatography and use of a pressure-driven disposable ultrafiltration unit (MOLCUT-II™; Nihon Millipore, Tokyo) did not give better results than those shown in Table 1 (data not shown). Only precipitation with TCA worked satisfactorily for the preparation of protein samples for SDS-PAGE. Thus, the recovery of the standard proteins throughout the procedure, including the preparation of the crude concentrate and the precipitation with TCA, was examined (Table 2). Six standard proteins were added to seawater at a low level (5 μg/l of each protein) and were recovered with a yield of 21–56%, with an average of 33%. Recoveries differed among the six proteins and were rather low. However, removal of the inorganic residue enabled us to prepare a sample for electrophoresis that was concentrated enough to allow detection of small amounts of proteins in seawater. Removal of non-protein, dissolved organic materials in the crude concentrate by TCA precipitation produced a low background on the gel after electrophoresis and allowed the use of the highly sensitive silver-staining method. In the present study, naturally occurring protein molecules were detected under the conditions used to obtain the results in Table 2.

Table 2
Recoveries (%) of standard proteins by the procedure that was used for detection of dissolved proteins in seawater. The total amount of standard proteins was 30 μg/l (each standard protein was present at 5 μg/l)

| Bovine serum albumin (66 kDa) | Egg albumin (45 kDa) | Glyceraldehyde-3-phosphate dehydrogenase (36 kDa) | Trypsinogen (24 kDa) | β-lactoglobulin (18.4 kDa) | Lysozyme (14.3 kDa) | Average |
|---|---|---|---|---|---|---|
| 56 ± 1 | 21 ± 0 | 26 ± 2 | 22 ± 2 | 26 ± 2 | 47 ± 7 | 33 ± 13 |

Results are means of three measurements ± 1SD.



### 3.2. Examination of techniques for analysis of dissolved proteins in seawater

The behavior of dissolved proteins during their extraction from seawater might be expected to depend on the nature of the specific protein. Therefore, an examination of methods was made using the proteins dissolved in seawater.

#### 3.2.1. Effects of SDS and concentration of TCA on precipitation of dissolved proteins in the crude concentrate

During the treatment with TCA for removal of TCA-soluble materials from the crude concentrate, a layer of low-density material was found above the supernatant after centrifugation, as mentioned above. This low-density fraction was significant in the case of samples from the intermediate and deep waters at station *B*. thus, the TCA-insoluble precipitate (pellet) and the TCA-insoluble low-density fraction of the sample from a depth of 462 m at station *B* were treated separately with different concentrations of TCA (5–20%; Fig. 1). The respective electrophoretic patterns and intensities of staining of proteins in each lane with different concentrations of TCA from 5 to 20% did not differ between the pellets and the low-density fractions. TCA at 5% was sufficient to precipitate the dissolved proteins from the crude concentrate. The results showed that appreciable amounts of protein were included with the low-density material. The layer of low-density material disappeared after one wash with ethanol, indicating that lipids were the main constituents of the low-density fraction.

Standard proteins were not recovered or were recovered with an extremely low yield without the addition of desalting buffer containing SDS, as mentioned above. The crude concentrates were prepared with and without addition of SDS (Fig. 2). The concentrations were started simultaneously from the same filtrate using two sets of the same ultrafiltration system under identical conditions, except that the concentrate without added SDS was neither desalted with the desalting buffer nor dried in the Speed Vac concentrator; thus concentrate was subjected directly to precipitation with TCA. No protein bands were clearly visible after PAGE of the samples prepared without SDS when we analyzed concentrates of the fractions from the 0.1 μm filter (Fig. 2A) and th fractions from the filter of more than 10,00 NMMCO (Fig. 2B), using samples from depths of 15 and 20 m at station *A*. As an example, results electrophoresis of samples from a depth of 5 m station *A* with and without SDS are shown in Fi 2B, with the same amount of original seawater use in each case. Despite the fact that crude concentra tion was started from the same filtrate from a DURAPORE® filter, clear bands of protein are visible onl in the case of the sample with SDS, and no bands a visible for the sample without SDS. The GF/ filtrate at depths of 20, 2000 and 4500 m at statio $D'_2$ gave the same results (Fig. 2C).

SDS, an anionic long-chain amphiphile, binds lipids as well as to proteins with high affini (Helenius and Simons, 1975). In the present stud SDS bound to proteins and lipids in the solub phase. SDS may also interact with polar and apol dissolved organic materials in seawater. After add tion of SDS to the filtrate, SDS might form vario mixed micelles, e.g. micelles of protein–SDS–lipi protein–SDS–other dissolved constituents, etc., du ing preparation of the crude concentrate. Since t micellar mass of SDS in 0.5 M NaCl solution is kDa (Helenius and Simons, 1975), it is possible th mixed micelles of SDS with not only proteins b also non-protein organic constituents of DOM fail to pass through the ultrafiltration filter of 10,0 NMMCO and were concentrated during the cru concentration step. Monomers and various mix micelles of SDS might prevent the selective adsor tion of dissolved proteins to the ultrafiltration me brane and to the inside of the tubing of the ultrafilt tion system during the crude concentration; th dissolved proteins in the samples with SDS we recovered while those in samples without SDS we not.

By the addition of SDS, however, dissolved pr teins were distributed into the pellet and the lo density fraction during TCA precipitation (Fig. Similar patterns after PAGE between the pellet fra tion, which was lacking in lipid materials, and t low-density fraction, which was rich in lipid mate als, indicated that the formation of mixed micell was unspecific in terms of partitioning proteins such micelles. Thus, the molecular composition dissolved protein was unbiased by the precipitat

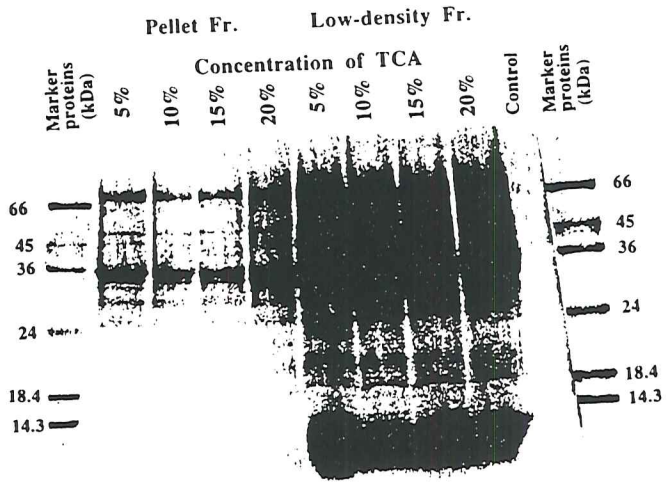



Fig. 1. Effects of different concentrations of TCA on precipitation of dissolved proteins in the crude concentrate. The pellet and the low-density fractions were separately fractionated by electrophoresis. Proteins on the gel were stained with CBB-R. The amount of sample loaded in each lane on the gel was equivalent to 1 l of the original seawater from a depth of 462 m at station *B* and was equivalent to 1 l of the original 3.5% solution of NaCl in the control. Each marker protein in the left- and right-hand lanes was loaded at 1 and 2 μg, respectively.

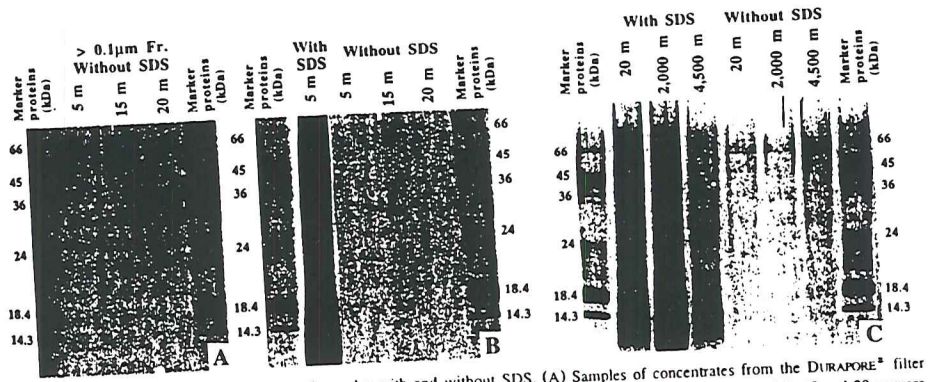



Fig. 2. Comparisons of electrophoretograms of samples with and without SDS. (A) Samples of concentrates from the DURAPORE® filter (pore size, 0.1 μm) without SDS from depths of 5, 15 and 20 m at station *A*. Amounts of samples from depths of 5, 15 and 20 m were equivalent to 71, 49 and 48 ml of the original seawater, respectively. (B) Samples with SDS from a depth of 5 m (second lane from the left) and without SDS from depths of 5, 15 and 20 m at station *A*. The amount of sample from a depth of 5 m was equivalent to 50 ml of the original seawater, and those from 15 and 20 m were equivalent to 27 and 24 ml of the original seawater, respectively. (C) Samples with and without SDS from depths of 20, 2000 and 4500 m at station $D'_2$. The amount of sample loaded on the gel was equivalent to 100 ml of the original seawater in each case. Proteins were visuallized by the silver-staining method. Each marker protein was loaded at 25 ng in the left-hand lane and at 50 ng in the right-hand lane in A, B and C, respectively.



with TCA. However, smaller amounts of naturally occurring proteins in the crude concentrate might lead to significant losses during this step, since the removal of the intermediate layer after centrifugation was difficult to achieve without disrupting the top layer of the supernatant. Even if the intermediate layer was removed very carefully, it might also have contained mixed micelles with incorporated proteins because mixed micelles could not be completely separated by centrifugation. The method for purification of the dissolved proteins from the crude concentrate should be improved in terms of removal of inorganic materials and non-protein DOM, if we are to achieve adequate resolution on electrophoretograms for quantitative and qualitative estimations of dissolved proteins.

*3.2.2. Biological activity during the preparation of the crude concentrate, effects of naturally occurring bacteria and possible contamination by protein from the ultrafiltration membrane*

The crude concentrate of dissolved proteins was prepared at room temperature and no poison, preservative or protease inhibitors were added to the sample. To examine the influence of biological activity during the crude concentration step, a comparison of electrophoretograms was made between samples prepared with and without the addition of sodium azide ($NaN_3$; final concentration, 3 mM) to the GF/F filtrate (Fig. 3A). The GF/F filtrate (c. 20 l) of surface water (75 m depth) at station *B* was divided into two aliquots. Each aliquot was subjected to the crude concentration step in the same manner. The electrophoretic patterns showed little difference (Fig. 3A). This result is consistent with the fact that SDS is a strong denaturing detergent and has strong bactericidal activity (e.g. Andrew, 1986) The electrophoretic patterns of dissolved proteins in the present study were not affected by any biological activity during the procedure for preparation of the crude concentrate.

A GF/F filter did not accomplish the complete removal of naturally occurring bacteria and other microorganisms. Giovannoni et al. (1990) concentrated oceanic picoplankton with at least 37% efficiency by tangential flow filtration with a DURA-

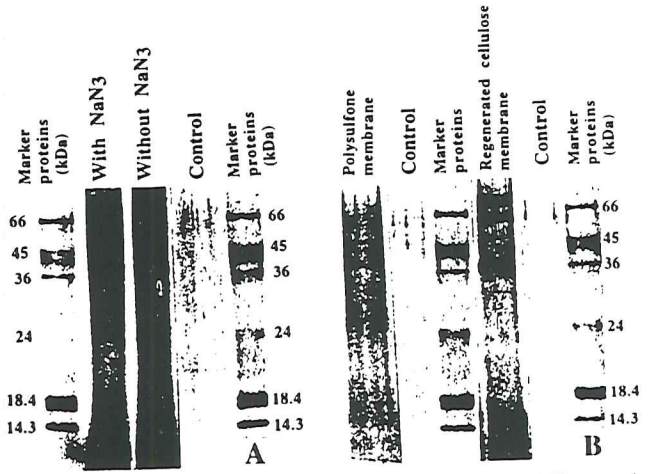



Fig. 3. Electrophoretograms of samples with and without addition of sodium azide from a depth of 75 m at station *B*. (A) Amounts of samples loaded on the gel were equivalent to 100 ml of the original seawater. (B) Electrophoretograms of samples treated with polysulfone and regenerated cellulose membranes. Amounts of samples treated with polysulfone and regenerated cellulose membranes were equivalent to 900 and 1 ml of the original seawater, respectively. Proteins on the gel were visualized by the silver staining method. Amounts of samples in controls (A and B) were equivalent to 100 ml of the original 3.5% solution of NaCl. Each marker protein was loaded at 25 ng in A and in the right-hand lane in B, and at 50 ng in the third lane from the left in B, respectively.

PORE® filter with 0.1 μm pores. Therefore, the naturally occurring bacterial population must have been concentrated in the crude concentrate in the present study. Bacterial counts in the crude concentrate with SDS were not monitored because it was a cloudy slurry, but bacteria were found in the concentrate without SDS. The bacterial count, as determined by epifluorescence microscopy, of concentrates without SDS from depths of 20, 2000 and 4500 m at Station $D_2'$ (Fig. 2C) were equivalent to $1.5 \times 10^5$, $1.6 \times 10^3$ and $2.3 \times 10^4$ cells/ml of the original seawater, respectively (T. Nagata, pers. commun., 1994). The bacterial count for each lane at depths of 20, 2000 and 4500 m was $1.5 \times 10^7$, $1.6 \times 10^5$ and $2.3 \times 10^6$ cells, respectively. Despite the fact that bacteria were detected in the samples, no clear bands of protein were visible on the gel. Although bacteria in the crude concentrate with SDS were not enumerated, there is no reason to postulate that bacterial growth was stimulated in the sample with SDS during the crude concentration, as shown in Fig. 3A; moreover, SDS has a strong bactericidal activity.

A simple estimate shows that the amount of protein in about $10^7$ bacterial cells, the maximum number of bacteria loaded on the gel, is not enough for detection, even if a highly sensitive silver-staining method would be employed (approximately 2–10 ng per band; Pohl, 1990). The amount of each protein component from the bacterial population at a depth of 20 m was calculated to be 0.3 ng, if we assume that the amount of organic matter in a bacterial cell is twice that of carbon (20 fg C/cell; Cho and Azam, 1988), that 50% of organic matter is protein (Romankevich, 1984), and that the protein is divided into equal amounts of 1000 different components (cf. O'Farrel, 1975). This is at least one order of magnitude lower than the detection limit of the analysis. It is, thus, concluded that the proteins detected in the present study were not derived directly from the bacterial population in the GF/F filtrate.

Faint and streaky bands around the site of marker protein with molecular mass of 66 kDa were found in the samples without SDS, in particular in the sample from 4500 m (Fig. 2C). It is unlikely that the bands were derived directly from bacterial proteins because SDS-PAGE of entire organisms yielded smeared electrophoretograms (see below). The silver-staining method is very sensitive but less specific to protein than the CBB-R staining method, and silver stains both DNA and polysaccharides as well as proteins (e.g. Andrew, 1986). Artifactual bands corresponding to molecular masses from 50 to 68 kDa are often observed on silver-stained gels (cf. Merril, 1990; Tanoue, 1991). The bands found in the lane of the sample from 4500 m were considered not to be true proteins extracted from seawater.

Although control experiments confirmed that no extraneous proteins were detectable by SDS-PAGE throughout the procedure, a polysulfone ultrafiltration membrane (10,000 NMMCO filter) that was made of artificial material, instead of regenerated cellulose, was used in the crude concentration step to examine the possible contamination by protein in the ultrafiltration membrane since regenerated cellulose is made from natural materials. Fig. 3B shows that major proteins were also detected when the polysulfone membrane was used for the crude concentration. However, the yield of the dissolved proteins in seawater was extremely low.

### 3.2.3. *Distribution and molecular characteristics of dissolved proteins in oceanic water columns*

Electrophoretograms of dissolved proteins in seawater at station *A* are shown in Fig. 4. At station *A*, located in a subarctic region, proteins were observed with a wide range of molecular masses. Proteins from 14 to 66 kDa were separated by electrophoresis, but proteins were present also in fractions of molecular masses greater than 66 kDa and less than 14.3 kDa. Fewer than 30 major bands were clearly visible in the samples from depths of 8–200 m. The patterns from each depth were very similar. For example, the higher and lower limits of molecular masses detected in each lane on the gel were approximately the same. The detected protein species were quite similar to each other throughout the samples of surface water examined. Bands of proteins of 48, 40, 37 and 34 kDa, estimated from a Ferguson plot of $R_f$ vs. $\log_{10}$ molecular mass (Neville, 1971), were commonly observed as the major bands. A protein with an apparent molecular mass of approximately 48 kDa was the most prominent on the gel. The apparent molecular masses of dissolved proteins estimated from the Ferguson plot showed that the major dissolved proteins tended to migrate more rapidly than the marker proteins. Among samples of dissolved proteins, proteins in the sample from a depth of 45 m appeared to migrate more rapidly.



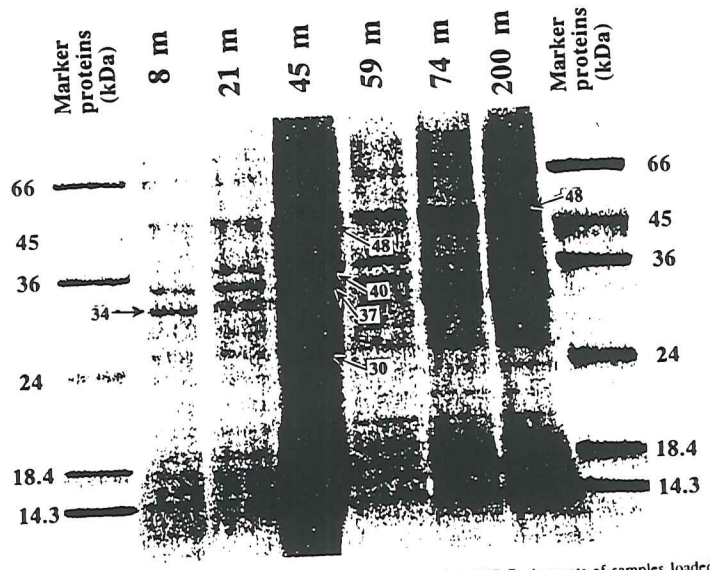


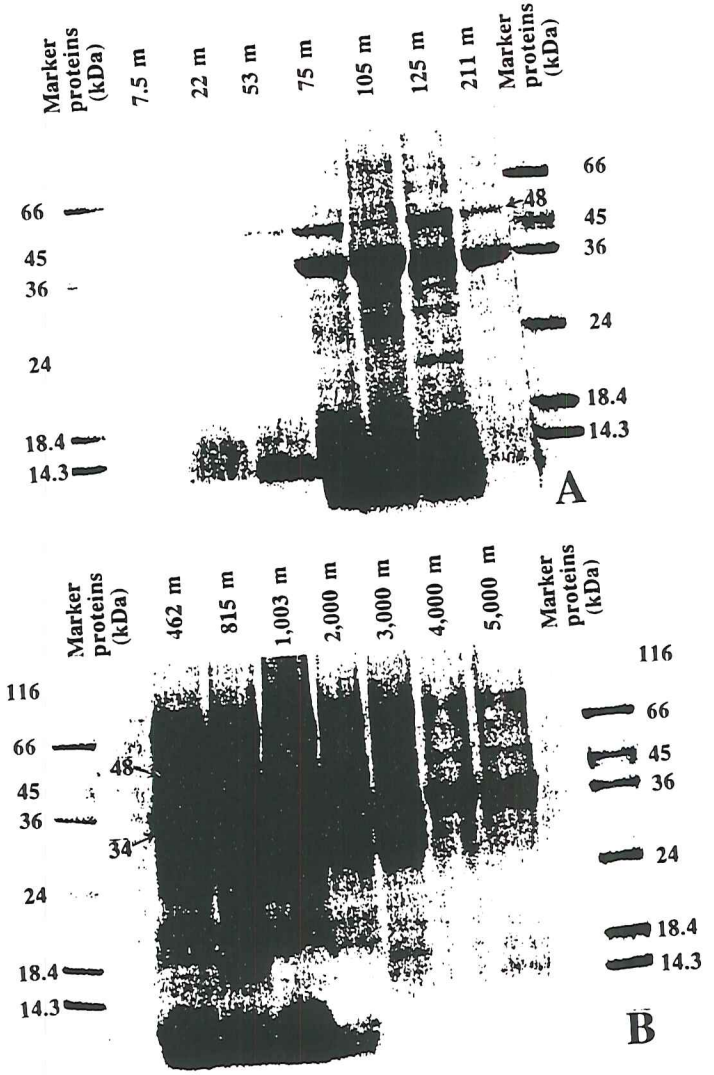

Fig. 4. Depth profiles of dissolved proteins at station *A*. Proteins were stained with CBB-R. Amounts of samples loaded on the gel were equivalent to 1 l of the original seawater in each case. Each marker protein in the left- and right-hand lanes was loaded on the gel at 1 and 2 μg, respectively. Arrows represent only bands that have been confirmed as single protein bands by N-terminal amino acid sequence analysis and the 48 kDa protein was identified as porin P homologue (Tanoue et al., 1995).

The electrophoretic mobility of a protein is dependent upon a number of experimental variables (e.g. polymerization of the gel, the buffer, the temperature during PAGE, the pH and ionic strength of the sample, etc.; e.g. Andrew, 1986). In the present case, experimental conditions other than ionic strength of the sample were identical. The ionic strength of individual samples of dissolved proteins might vary as a result of the extent of purification. The higher the ionic strength the greater the electric conductivity and the greater the amount of heat generated; the electric resistance decreases with rising temperature and the current at constant voltage will rise during the electrophoresis, and give a higher rate of migration. Thus, the electrophoretic mobility of a given lane depends on the degree of purity of a sample.

At station *B*, located in a subtropical area, the patterns after PAGE were different from those at station *A* (Fig. 5). A relatively limited number of bands was visualized by staining with CBB-R 250 above a depth of 53 m (Fig. 5A). The intensities of staining were low at the surface and increased with

Fig. 5. Depth profiles of dissolved proteins from the surface through the deep waters at station *B*. (A) Amounts of samples from depths of 7.5, 22, 53, 75, 105, 125 and 211 m were equivalent to 1, 1.6, 1.5, 0.5, 0.78, 0.76 and 0.74 l of the original seawater, respectively. (B) Amounts of samples loaded on the gel were equivalent to 250 ml of the original seawater. Proteins were stained with CBB-R. Each marker protein was loaded at 1 and 2 μg in the left- and right-hand lanes, respectively, in A and B. In B, β-galactosidase with molecular mass of 116 kDa was loaded as marker protein at 1 and 2 μg in the left- and right-hand second lanes, respectively. Arrows represent only bands that have been confirmed as single protein bands by N-terminal amino acid sequence analysis and the 48-kDa protein was identified as porin P homologue (Tanoue et al., 1995).

Between depths of 75 and 211 m, the number ualized bands increased. Proteins with apparent ular masses of approximately 48 and 37 kDa were clearly visible as the major proteins. Proteins of low molecular mass were also concentrated at the electrophoretic front in the gel. For samples below a






depth of 211 m (Fig. 5B), the gel was heavily stained, indicating that high levels of protein in the dissolved phase were present in the intermediate waters. However, the electrophoretograms of those samples were smeared. Inadequate resolution of electrophoretograms leads to errors in estimations of molecular mass. Thus, no Ferguson plot was constructed and molecular masses of proteins were tentatively determined by a simple comparison between locations of dissolved proteins and those of marker proteins on the same gel for station *B*. Proteins of approximately 66 and 34 kDa were predominant at depths from 462 to 2000 m. The 66 kDa protein was not evident in the surface waters (Fig. 5A) but was found in the intermediate and deep waters. The 48 kDa protein, found in the surface water as the major component, was also found in the intermediate waters. The two samples from greater depths, 4000 and 5000 m, had fewer and lower levels of protein visualized as bands on the gel. The 37 kDa protein was the dominant protein below 4000 m and the 48 kDa protein was also found in the deep waters.

The formation of the low-density material was significant in the intermediate and deep waters at station *B*. The proteins in the low-density fractions gave streaky bands (Fig. 1), of the type usually due to insoluble particles in a sample for electrophoresis. The interference in SDS-PAGE due to inadequate removal of low-density materials resulted in smeared electrophoretograms at station *B*. But the formation of a large quantity of the low-density fraction might be responsible for a high yield of dissolved proteins at station *B*. For example, the amount of sample that was equivalent to 1 ml of the original seawater from a depth of 1003 m at station *B* was sufficient for detection of the major proteins if the high-sensitivity silver-staining method was applied (Fig. 3B). Discarding the low-density fraction gave a better electrophoretogram at station *A* (Fig. 4). However, the yield of dissolved protein might have been low at station *A*.

Previous application of SDS-PAGE to particulate proteins in surface and subsurface samples demonstrated a large number of proteins with a wide range of molecular masses, each at low levels and each overlapping others as "background" proteins on the gel (Tanoue, 1992). Even a single bacterium species, e.g. *Escherichia coli*, contains 1100 different protein components; a one-dimensional PAGE technique such as the one employed here is inadequate for the separation of total proteins in biological systems more complex than a bacteriophage (O'Farrel, 1975) and for separation of proteins in marine particulate matter and microorganisms (Tanoue, 1991). However, the electrophoretograms of dissolved proteins were quite different from those of proteins in the particulate phase or in entire organisms. "Background" proteins were not present at significant levels in the dissolved phase. In addition to the direct methodological evidence (Figs. 2 and 3), the electrophoretograms of dissolved proteins also indicated that the major proteins detected in the present study were proteins that were not derived directly from living bacteria and could be assigned to "DOM".

The overall electrophoretic patterns of the dissolved proteins differed between stations *A* and *B* and also differed through the water column at station *B*. Some proteins appeared to be common to all the samples examined. Although the inadequate degree of purity of the electrophoretic samples did not allow us to identify proteins of interest from the patterns on gels, the 48 kDa protein was commonly observed and was identifiable as one of the major proteins in samples from stations *A* and *B*. The N-terminal amino acid sequences indicated that the 48 kDa proteins in the samples from 45 and 200 m at station *A* (Fig. 4) and from 211 and 462 m at station *B* (Fig. 5) were the same protein. The protein appeared to be a homologue of a membrane pore-forming protein, known as porin P, of the Gram-negative bacterium *Pseudomonas aeruginosa* (Tanoue et al., 1995). The success of eight analyses in Figs. 4 and 5, without exception, of N-terminal amino acid sequences suggests that bands visualized on the gel by staining with CBB-R were not false bands but represented true proteins (Tanoue et al., 1995).

Methodological improvements are required for quantitative estimates of dissolved proteins, as mentioned above. However, a first-order approximation of the abundance of dissolved protein is possible at this time. The lower limit of detection of the CBB-R staining method corresponds, in general, to about 1 $\mu$g of protein per band in the literature (e.g. Andrew, 1986) and the intensity of staining is linear over a range from 0–10 $\mu$g of protein per band on the gel (e.g. Tanoue, 1992). The concentrations of the major

proteins may exceed 0.1 $\mu$g/l of the original seawater, if we assume any given protein on the gel below approximately 0.1 $\mu$g was not visualized as a single band by the comparison of the intensities of staining of dissolved proteins and marker proteins on the same gel in Figs. 4 and 5. The successful analysis of N-terminal amino acid sequences also indicates that the majority of proteins extracted from seawater in the present study were relatively pure and present at high levels. Levels of total dissolved protein in the surface water at station *A* were tentatively estimated by densitometry to be within a range of 12–38 $\mu$g protein/l (equivalent to 0.14–0.43 $\mu$mol N/l) from the relationship between the total staining intensity and total amounts of standard marker proteins on the same gel.

These values for concentration of protein in seawater account for roughly 30% of DCAA, if we assume that the nitrogen content of dissolved protein is 16% and the level of DCAA ranges between 0.5 and 1.5 $\mu$mol N/l in oceanic waters (Sharp, 1983). This value also accounts for from 2 to 12% of the total dissolved organic nitrogen (DON) at station *A*, where levels of DON ranged from 2.7 to 9.1 $\mu$mol N/l (M. Yanada, pers. commun., 1994). This first-order approximation suggests that dissolved protein makes a major contribution to the oceanic pool of organic nitrogen. From the separation of dissolved proteins by SDS-PAGE, a first-order approximation is also possible for individual proteins. The 48 kDa protein was found throughout the water column, even at a depth of 5000 m at station *B*, and the level of this protein was more than 0.1 $\mu$g/l in each case. If this protein occurs throughout the entire ocean, the total mass of the protein can be calculated to be more than $10^{14}$ g. This result suggests that this single protein molecule is equivalent to (or more) than the total living biomass of zooplankton or living bacteria in the sea! (Cauwet, 1978).

Marine organisms, from phage to mammals, produce a variety of proteins. Protein molecules from marine organisms may be transferred to the pool of inanimate organic matter in the dissolved phase. Therefore, hundreds of thousands of different proteins may be present in the dissolved phase. However, a limited number of proteins was visualized as major bands on the gel. The abundance of dissolved proteins was relatively high in intermediate waters at station *B* and is also not correlated with that of chlorophyll *a* in some locations (Tanoue et al., submitted). These results imply, perhaps, that the dissolved proteins detected in the present study might not be linked directly to primary production and that proteins produced in the euphotic layer via primary production are decomposed and do not accumulate in the water column. Evidence for the accumulation of an appreciable amount of a relatively small number of protein molecules in the water column leads to the hypothesis that very specific proteins make up the bulk of the pool of dissolved protein and that these proteins are derived from specific sources. A description of the molecular inventory, sources and pathways by which dissolved proteins are accumulated should provide more realistic information about production of the pool of DOM, as well as the dynamics of proteins, in the sea.

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