Differential Nitrogen Uptake By Aquatic Communities In A Chesapeake Bay Tributary And In The Coastal Alaskan Arctic

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Differential Nitrogen Uptake by Aquatic Communities in a Chesapeake Bay Tributary

and in the Coastal Alaskan Arctic

A Dissertation

Presented to

The Faculty of the School of Marine Science

The College of William & Mary

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

by

Brianna Caitlin Stanley

May 2021
This dissertation is submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

Brianna Caitlin Stanley

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This dissertation is dedicated in loving memory to my nana, Betty Jo Salchunas

I miss you, always
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ABSTRACT

Nitrogen (N) is one of the essential building blocks for all life and is available in the form of dissolved N in aquatic ecosystems. It is important to understand how this N can support primary and secondary production mediated by phytoplankton and bacteria, respectively, as it can affect both microbial loop biogeochemistry and the higher trophic levels of food webs. Nitrogen studies have traditionally focused on dissolved inorganic N (DIN) as a labile N source. Dissolved organic N (DON), while still often considered refractory, has been increasingly recognized as an important N source supporting primary and secondary production. However, the inclusion of DON into uptake studies is still limited. Expanding N research to encompass DON will be important as researchers continue to assess how nutrient cycles respond to a changing climate.

The goal of this dissertation was to expand the understanding of how phytoplankton and bacteria use N by investigating uptake rates of a suite of DIN and DON substrates in two different ecosystems. Research for this dissertation was conducted in the York River, VA and the coastal Alaskan Arctic. In both systems, nutrient uptake rates were measured using $^{13}$C and $^{15}$N stable isotopes for N and carbon (C) substrates. In the York River, N uptake (>0.3 µm size class) was investigated in alternating months during a period of elevated precipitation. Ammonium ($\text{NH}_4^+$) uptake was found to be the greatest, but urea uptake was elevated relative to other substrates in late fall. Rates of $\text{NH}_4^+$ regeneration were lower than measured uptake rates, which indicates that autochthonous production was insufficient and allochthonous sources were needed to meet the N demand. Finally, this study also reported the rates of $\text{NH}_4^+$ release from urea, finding that urea provided minimal $\text{NH}_4^+$, averaging <1% of $\text{NH}_4^+$ needed to support measured $\text{NH}_4^+$ uptake rates.

Further study in the York River used 16S rDNA sequencing to determine if wastewater effluent with different DIN and DON content affected the composition and diversity of the microbial communities in receiving waters. Overall, addition of minimally treated effluent with high DIN lowered microbial diversity, while exposure to more heavily treated effluents resulted in communities that were more similar to the control community without effluent addition.

In the Alaskan Arctic, late season N uptake was investigated through $^{15}$N and $^{13}$C substrate incubation experiments in the Chukchi and Beaufort Seas over two summers for the >0.3 µm size class. During these experiments, urea uptake was often greater than nitrate, but $\text{NH}_4^+$ was taken up at the highest rate. Differing sea-ice conditions were also found to support different rates of $\text{NH}_4^+$ regeneration and C uptake.

Collectively, the results of this dissertation demonstrate that while DIN is the form of N primarily used in coastal and marine ecosystems, DON can be an important nutrient source to aquatic microbial communities. Future studies should aim to incorporate DON substrates as both nutrient cycling and community composition will likely continue to shift as anthropogenic activity alters ecosystems.
Differential Nitrogen Uptake by Aquatic Communities in a Chesapeake Bay Tributary

and in the Coastal Alaskan Arctic
CHAPTER 1

Introduction
Nitrogen in Marine and Coastal Systems

Nitrogen (N) is one of the most complex building blocks of life. It has five stable oxidation states, which means it can undergo many biogeochemical reactions and exist in myriad compounds before it is used as a substrate for building biomass (Gruber 2008). The availability of N nutrients significantly impacts water quality. An excess supply of N can lead to increased phytoplankton production and the resulting eutrophication has serious consequences for water quality, including hypoxia and fish-kills (Kemp et al. 2005; Diaz and Rosenberg 2008; Anderson et al. 2002; Wallace et al. 2014). While N supply is considered as a pollution issue in many coastal systems, it necessary for the growth of phytoplankton that form the base of the food web and many marine systems are N-limited (Gruber 2008). Nitrogen is often transported to marine coastal ecosystems in the form of dissolved N, and the study of how N supports phytoplankton primary production has been narrowly focused on only a few N compounds.

Total dissolved N (TDN) is divided into two pools, dissolved inorganic N (DIN) and dissolved organic N (DON). Most prior research has targeted the DIN compounds of ammonium ($\text{NH}_4^+$), nitrate ($\text{NO}_3^-$), and nitrite ($\text{NO}_2^-$), which have traditionally been considered the most energetically favorable for phytoplankton and bacteria to use (Glibert et al. 2016; Mulholland and Lomas 2008). Research on how these communities use DIN nutrients is widespread and ranges from individual algae culturing studies to long-term monitoring programs. DIN can enter coastal and riverine systems via multiple allochthonous and autochthonous routes, including overland runoff, riverine and
groundwater discharge, atmospheric deposition, excretion, and benthic remineralization (Paerl and Pihler 2008; Gruber and Deutsch 2014).

DON, while often considered refractory, has been receiving increasing recognition as an important N source in addition to DIN. The DON pool contains a variety of N-containing compounds with variable levels of bioavailability, which includes complex humics, nucleic acids, dissolved free amino acids, and urea (Sipler and Bronk 2015). These different forms of DON can originate from multiple sources including fertilizer runoff, wastewater treatment discharge, and bacterial release (Bronk and Steinberg 2008; Sarmento et al. 2013). Many of these sources are common with DIN sources, yet most nutrient regulation efforts have been focused on the reduction of DIN inputs to coastal systems rather than DON (Glibert et al. 2006; EPA 2016). DON availability, however, has received increasing attention, as organic substrates, such as urea, have been tied to the occurrence of harmful algal blooms (HABs) (Glibert et al. 2006). Numerous studies have now shown that components of the DON pool can be used to support phytoplankton growth (Sipler and Bronk 2015; Bronk et al. 2006). Urea uptake has been tied to increased HAB species toxicity and even some bacteria have been found to have the enzymes necessary to degrade the more complex structures in DON that until recently were considered refractory (Anderson et al. 2002; Solomon et al. 2010; McCarren et al. 2010; Kisand et al. 2008; and others). Even with this recognition, the inclusion of DON substrates into marine N cycling research has been limited.
Nitrogen is an essential component in understanding how coastal ecosystems function, yet there are many gaps in our biogeochemical knowledge because of poor spatial coverage for measurements and consideration of only select substrates. As these ecosystems change due to anthropogenic activity, these gaps may be compounded as modelers, resource managers, and research scientists try to predict ecosystem response (Bonan and Doney 2018; Mannino et al. 2018). In addition, although efforts have been made to reduce nutrient pollution in the Chesapeake Bay watershed, this waterbody is still considered degraded with N pollution receiving an F in the 2020 State of the Bay Health Index (Chesapeake Bay Foundation, 2021). Simultaneously, the region is experiencing changes in precipitation patterns that can affect nutrient cycling patterns (Najjar et al. 2010). In polar marine systems, such as the Arctic Ocean, the growing season is elongating as nutrient availability changes with tundra melt and changing ice patterns (Jahn 2018; Wassmann et al. 2011; Overland and Wang 2013; Arrigo and van Dijken 2015; Vincent et al. 2011). It is critical to obtain more data on how the phytoplankton and bacteria that form the base of these aquatic food webs respond to the full spectrum of N availability to better anticipate how coastal and marine communities will respond to our changing climate.

**Chesapeake Bay and Changing Nitrogen Cycling Patterns**

One region where N cycling is likely impacted by global climate change and anthropogenic activity is Chesapeake Bay. Chesapeake Bay is an estuary, supplied by multiple tributaries including the York River. As the biogeochemistry of this estuary is dependent on nutrient-rich freshwater discharge, seasonal and climate-driven changes in
weather patterns may have an impact on the nutrient cycling in Chesapeake Bay and the York River, as water quality changes. In the York River specifically, historical rainfall records show this region averaging 111-121 cm of rainfall per year, yet during 2018 this region saw exceptional rainfall, exceeding the long-term average by 45 cm (Reay 2009; Bukaveckas et al. 2020). This increased rainfall and freshwater discharge resulted in promoting heterotrophic activities and reducing gross primary productivities in the York River’s freshwater tributaries (Bukaveckas et al. 2020). How changes in precipitation patterns affect nutrient availability, particularly the potential influence of understudied DON substrates, is an important topic to consider. This understanding is crucial, as the region has struggled with water quality due to increased N loading in Chesapeake Bay.

Nitrogen pollution has led to formation of harmful algal blooms and eutrophication (Kemp et al. 2005; Diaz and Rosenberg 2008). The York River has experienced seasonal blooms of dinoflagellates *Margalefidinium polykrikoides* and *Alexandrium monilatum*. *A. monilatum* is known to utilize the DON portion of the TDN pool, yet these algae are also thought to be dependent upon salinity conditions, which will change with precipitation pattern variation (Anderson et al. 2012; Juhl 2005). Better understanding the response of dinoflagellates and other estuarine organisms to changes in salinity and all sources of N is critical to maintaining the health of Chesapeake Bay and similar estuaries. Investigating the particular role of DON in this process is of increased importance given the potential changes induced by climate change.
**Effluent DON**

Nitrogen in the coastal environment can originate from autochthonous sources such as cell-mediated release, grazing and remineralization, as well as allochthonous sources such as fertilizer and runoff (Paerl and Piehler 2008). A significant N source in many coastal regions, including Chesapeake Bay, is the anthropogenic discharge of wastewater effluent. Wastewater effluent can contribute to N pollution and stimulate algae growth (Howarth 2004; Carey and Migliaccio 2009). The regulation of effluent discharge into waterways started with the Federal Water Pollution Control Act in 1948, and with subsequent regulations such as the Chesapeake Bay Total Maximum Daily Load, effluent N inputs have been reduced in order to reach the goal of limiting input of total N to the Bay at 185.9 million lbs. of N per year (Carey and Migliaccio 2009; EPA 2010; Fleischer et al. 2005). In Chesapeake Bay, improvements in treatment technology and increased regulatory enforcement has resulted in a decrease in the effluent contribution to total N-loading, from 28% to 16% between 1985 and 2015 (EPA 2016). However, most wastewater resource and recovery facilities (WRRFs), focus reduction strategies on DIN discharge (Pagilla et al. 2008; EPA 2010; Pehlivanoglu-Mantas and Sedlak 2006).

Recent research has demonstrated that while this effluent DON (EDON) is not targeted within WRRFs, the N is still bioavailable to the water’s microbial community (Filippino et al. 2011; Bronk et al. 2010; Funkey et al. 2015; Yao et al. 2019). Effluent DON lability is heavily influenced by WRRF treatment processes. The identifiable labile compounds, like urea and amino acids, can be less than 3% of EDON, making most of the discharged organic N uncharacterized (Pehlivanoglu-Mantas and Sedlak 2006). While research has
tried to clarify the controls of EDON lability by conducting bioassay experiments, study has not been done on how the members of the aquatic microbial community respond to the introduction of this EDON. Limited studies in freshwater systems have focused on DIN in effluent, finding that ammonia oxidizing bacteria correlates with NO$_3^-$ availability near WRRF outfalls (Huo et al. 2017). It is likely that labile EDON might also lead to changes in the aquatic community and may vary as WRRF treatment processes alter the composition of that EDON pool.

*Rapidly Changing Coastal Arctic*

One of the critical areas where the study of N cycling currently has limitations is in the Arctic. The Arctic Ocean is warming at nearly twice the global rate and is expected to exceed 4°C this century, leading to sea-ice free summers and potentially a cascade of biogeochemical changes (Jahn 2018; Wassmann et al. 2011; Simpson et al. 2008; Arrigo et al. 2008). With these environmental changes, including elevated sea surface temperature and a lengthened sea-ice free summer, the net productivity of the Arctic Ocean is expected to increase as the phytoplankton growing season lengthens (Arrigo et al. 2008; Arrigo and van Dijken 2015). Changes in both phytoplankton and bacteria growth will likely have a significant impact on the Arctic food webs and climate feedbacks (Vincent et al. 2011). For instance, increased productivity in this region may lead to the Arctic Ocean acting as a carbon (C) dioxide sink, as C trapped in phytoplankton biomass is exported to depth (Cai et al. 2010).
A sufficient supply of N to fuel this production may be a limiting growth factor. However, the study of biogeochemical processes in this region is spatially and temporally limited (Mannino et al. 2018). What N cycling rates are reported in this region focus primarily on inorganic N sources, such as NO$_3^-$, rather than reduced organic species (Arrigo and van Dijken 2015; Brown et al. 2015; Letscher et al. 2013). Nitrate, typically accumulates in this region in the winter before being drawn down to nearly zero at the start of the summer growing season, creating a N-limited environment (Arrigo et al. 2008; Mills et al. 2018). DON likely plays an underappreciated role in this N-limited environment (Tremblay and Gagnon 2009). Reported rates for DON cycling are sparse (Baer et al. 2017; Letscher et al. 2013) and likely need to be considered in biogeochemical models of this region. This section will expand the understanding of N uptake rates in the Arctic region by considering uptake of both DIN and DON species.

*Dissertation Structure and Objectives*

This dissertation aimed to determine how different N substrates contribute to aquatic N uptake in coastal environments, including a tributary of Chesapeake Bay and the coastal Alaskan Arctic. These two environments provide case studies in how changing environmental conditions may impact N cycling. Additional work in the York River, a tributary of Chesapeake Bay, also demonstrates how differences in anthropogenic wastewater inputs affect the microbial community in coastal systems. Chapters 2 through 4 are the principle chapters that detail the three facets of this research.

*Chapter 2* examines how DIN and DON uptake supports productivity in the York River over an annual cycle. Uptake rates were measured using stable isotopes in
experiments conducted during 2018 and 2019, a period of elevated precipitation and riverine discharge. Four channel surface sites were sampled in alternating months, with N substrates considered including NH$_4^+$, NO$_3^-$, urea, and mixed amino acids. Over a 1-hour period, biomass parameters, nutrient concentrations, and uptake rates were measured for the >0.3 µm size fraction of the aquatic community.

Chapter 3 examines how the introduction of differently-treated wastewater effluent alters the composition and diversity of the microbial community in receiving waters. Wastewater effluent was collected from Hampton Roads Sanitation District, where sequencing batch reactors duplicated four of the common wastewater treatment methods. These effluents were used for five and nine-day incubations with whole water from the York River in both summer and winter, respectively. The changes in the aquatic community as a function of exposure of the different effluent treatments were examined based on an Illumina MiSeq sequencing of 16S rDNA amplicons.

Chapter 4 examines how uptake of DIN and DON substrates supports late-season productivity in the Alaskan coastal Arctic. Over two years in late-summer of 2016 and 2017, N uptake rates were measured using stable isotopes in water collected across the Chukchi and Beaufort Seas. Nitrogen substrates included NH$_4^+$, NO$_3^-$, urea, and mixed amino acids. Over a 24-hour period, biomass parameters, nutrient concentrations, and uptake rates were measured for the >0.3 µm size fraction of the aquatic community.
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CHAPTER 2

Nitrogen Uptake in a Chesapeake Bay Tributary during High Annual Discharge
Abstract

During 2018, the Chesapeake Bay region experienced a period of historically elevated precipitation leading to increased freshwater and nutrient discharge to both the Bay and its major tributaries. This time-period provided a glimpse of the nutrient dynamics this region may experience with periods of intense precipitation due to climate change. Over the course of an annual study of nitrogen (N) cycling in the York River, Virginia, the temporal variability of (1) ambient nutrient concentrations was monitored and (2) uptake rates of ammonium (NH$_4^+$), nitrate (NO$_3^-$), urea, and amino acids were measured in $^{15}$N and $^{13}$C-tracer incubation experiments. In addition, remineralization processes were investigated including, (3) NH$_4^+$ regeneration rates and (4) release rates of NH$_4^+$ from $^{15}$N-urea for the first time. During the June 2018 to July 2019 study, high concentrations of dissolved inorganic N (DIN) were observed with maximum concentrations of 11.05 µmol N L$^{-1}$ for NH$_4^+$ and 26.12 µmol N L$^{-1}$ for NO$_3^-$. Despite higher ambient NO$_3^-$ concentrations than NH$_4^+$, NH$_4^+$ uptake rates were the highest. When concentrations of NH$_4^+$ were less than 1 µmol N L$^{-1}$, however, uptake rates of NO$_3^-$ exceeded those of NH$_4^+$. During late summer and fall, organic N contributed to a large portion of the N taken up, with urea contributing upwards of 35% of total N uptake, comparable to previous work in this region. Rates of NH$_4^+$ regeneration were lower than measured uptake rates, which indicates that autochthonous production was insufficient and allochthonous sources were needed to meet the N demand. Despite the input of DIN, no dinoflagellate blooms formed during the fall of 2018. In the spring 2019, as freshwater discharge levels recovered, uptake of urea-C was observed during a bloom of a *Prorocentrum sp.* Cycling of urea was further investigated by measuring the first reported
rates of NH$_4^+$ release from $^{15}$N-urea, which provided minimal NH$_4^+$, averaging <1% of NH$_4^+$ needed to support measured NH$_4^+$ uptake rates. Extracellular release of urea to NH$_4^+$, however, was found to be as high as 22.3% of gross urea uptake. Urea uptake is clearly a complex process that warrants further investigation. DON substrates should be included along with DIN in future N studies.
Introduction

Nutrients, including nitrogen (N), are essential elements for phytoplankton primary production. In estuaries, such as Chesapeake Bay, N enters the waterways through overland runoff, riverine and groundwater discharge, atmospheric deposition, and benthic remineralization (reviewed in Gruber and Deutsch 2014). The increased N loading in Chesapeake Bay has had serious negative impacts on water quality, particularly promoting formation of harmful algal blooms and coastal eutrophication (reviewed Kemp et al. 2005; Diaz and Rosenberg 2008). This cultural eutrophication may have amplified consequences, as changing climate patterns may lead to periods of intense precipitation that transport high concentrations of nutrients to coastal waters and further supports the formation of algal blooms (Najjar et al. 2010). The recent period of elevated precipitation in 2018 has allowed for study of how Chesapeake Bay estuarine nutrient dynamics may respond to these future conditions. This study focused on one of the major tributaries of the lower Chesapeake Bay, the York River, VA.

The York River (York) is a microtidal estuary that is fed by two freshwater rivers, the Mattaponi and the Pamunkey Rivers (Reay 2009). Nitrogen in the York primarily comes from the watershed through these two rivers (Figure 1), however, nutrients are also delivered from sub-basins lower in the estuary, through overland runoff, atmospheric deposition and groundwater discharge (Reay et al. 2009). Major anthropogenic point sources to the York include the WestRock West Point Paper Mill near the head of the estuary and the York River Treatment Plant, which has an outfall in the lower portion of the estuary (Figure 1). Total dissolved nitrogen (TDN) in the York typically ranges from
22 – 24 umol N L\(^{-1}\) (Reay 2009). Dissolved inorganic N (DIN), consisting of ammonium (NH\(_4^+\)), nitrate (NO\(_3^-\)), and nitrite (NO\(_2^-\)), typically decreases with distance from the headwaters, as the salinity increases. Whereas, the concentration of dissolved organic N (DON) remains relatively constant (Reay 2009). In the York, DON has been reported as being as much as 97% of the TDN pool and depending on season and location, upwards of 50% of that DON may be labile urea or amino acids (Sipler and Bronk 2015).

The uptake of these different sources of N have been sporadically studied in the York over the last few decades. Previous studies from 2007 to 2009 reported that uptake of DIN substrates was greater than DON substrates, with NH\(_4^+\) uptake contributing to upwards of 80% of total uptake (Killberg-Thoreson et al. 2020). The rates of NH\(_4^+\) regeneration was also found to be greater than uptake rates, indicating autochthonous N input as a major N source to support nutrient uptake rates (Killberg-Thoreson et al. 2020).

The York has historically seen seasonal algal blooms that are predicted to increase in the Bay region with changing climate and nutrient conditions (Najjar et al. 2010). Spring diatom blooms are initiated with increased freshwater input that brings nutrients combined with destratification (Marshall 2009). These spring blooms are typically followed by late summer harmful algal blooms (HABs) that form in the lower part of the estuary (Morse et al. 2013; Pease 2016). The cyst forming dinoflagellates *Margalefidinium polykrikoides* and *Alexandrium monilatum* typically bloom in succession in late summer. *M. polykrikoides* has regularly produced blooms in the York since the late 1960s and *A. monilatum* began making an appearance in 2007 (Marshall
The newer addition, *A. monilatum*, is known to have an ideal blooming condition at 25°C and a salinity of 15, and it has been observed that members of the *Alexandrium* genus can thrive on organic nutrient sources such as humics and urea (Juhl 2005; Anderson et al. 2012; Killberg-Thoreson et al. 2020). During an *A. monilatum* bloom in 2007, uptake of urea was found to be 31% of total N uptake, indicating that organic N may be important for sustaining these blooms (Killberg-Thoreson et al. 2020). These HABs have been of great interest, because the local region depends on both tourism (Historic Yorktown, VA) and shellfish operations, which have been estimated to be worth $53.4 M in Virginia (Hudson 2018).

As an estuarine system dependent on nutrient-rich freshwater discharge, seasonal and climate-driven changes in weather patterns may have an impact on the N cycling in the York as water quality changes. In the York specifically, historical rainfall patterns show this region averages 111-121 cm of rainfall per year (Reay 2009). During 2018, this region saw exceptional levels of rainfall, exceeding the long-term average by 45 cm, due to multiple storm fronts and two hurricane remnants. The Mattaponi and Pamunkey Rivers’ average discharge was 85% and 54% above their long-term averages, respectively, which resulted in an increase in heterotrophic conditions and lowered gross primary production levels in the York’s freshwater tributaries (Bukaveckas et al. 2020). The combined discharge of these tributaries during this period of high precipitation is given in Figure 2, however the current USGS gauge stations in the Mattaponi and Pamunkey Rivers’ are located above approximately 35% of the basin and likely do not
represent the full magnitude of nutrient-rich freshwater inputs to the York during this time period (Reay 2009).

Nitrogen dynamics, particularly the fates of DON, are understudied in the high discharge conditions that the York was exposed to during 2018. The objective of this study was to identify any change in the pattern of DIN and DON concentration and uptake during the periods of high discharge, and to quantify the importance of DON as a N source for biological uptake. To do this, we measured the uptake rates of NH$_4^+$, NO$_3^-$, urea, and amino acids at four sites along the York for one-year from June 2018 to July 2019 when discharge conditions began to recover. In addition, NH$_4^+$ regeneration and NH$_4^+$ release from urea were also measured.

**Materials and Methods**

*Field Sampling*

Field sampling was conducted in the York River Estuary, which is the fifth largest tributary of Chesapeake Bay, located in Virginia (Figure 1; Table S1). The York is a microtidal estuary with a mean tide of 0.7 m at its mouth and a salinity that ranges from polyhaline to freshwater. The salinity of the York fluctuates with interactions of freshwater, saltwater, tidal energy, wind, and seasonal changes in freshwater discharge (Haas et al. 1981; Reay 2009). The York forms at the confluence of two freshwater rivers, the Mattaponi and Pamunkey Rivers, at West Point, VA, which is approximately 48 km from the river’s mouth on Chesapeake Bay. Residence time in the York is dependent on the levels of freshwater discharge from these two rivers, however, it is
estimated to be between 45 and 90 days depending on flow conditions (Shen and Haas 2004).

From June 2018 to July 2019, the York surface waters were sampled every other month at four main channel sites spread across the salinity gradient (Figure 1). Sites were chosen as a part of a larger NSF-funded research project (i.e. the Lower York River Estuary – LYRE project) examining the carbon (C) and N cycles and samplings were timed to correspond with the spring tidal cycle. The LYRE project included sampling of both channel and shoal stations along the York and the naming convention of the LYRE project was used here for ease of comparison. The full site names for the channel stations included in this study are Upper Estuary Channel (Upper), Mid Estuary Channel (Mid), Lower Estuary Channel (Lower), and Mouth Estuary Channel (Mouth). For the rest of this paper, sites will be referred to by the simplified names (i.e. Mouth or Mid). While salinity varied seasonally during this study, the Upper site (N 37.416°, W -76.687°) was generally in the mesohaline portion of the estuary, whereas the Mid (N 37.348°, W -76.616°), Lower (N 37.277°, W -76.548°), and Mouth (N 37.234°, W -76.440°) sites were in the mesohaline to polyhaline portions of the estuary. All sites were sampled for each sampling event of the LYRE project, with the exception of April 2019 when it was not possible to sample the Upper site.

Water sampling was conducted along with measurement temperature and salinity using a YSI 6600 V2 multiparameter sonde during the spring tidal cycle. Surface water was
collected in an acid-washed carboy and immediately transported to the VIMS campus for nutrient measurement and uptake incubation experiments.

Ambient nutrient conditions were measured by collecting filtrate for analysis including, \( \text{NH}_4^+ \), \( \text{NO}_3^-/\text{NO}_2^- \), urea, amino acids (as dissolved primary amines), phosphate (\( \text{PO}_4^{3-} \)), silica (\( \text{SiO}_2 \)), and dissolved organic carbon (DOC)/total dissolved N (TDN). Filtrate was collected after vacuum filtration through a Whatman\textsuperscript{TM} GF-75 filter (nominal pore size 0.3 \( \mu \)m, combusted 2 hours at 450°C). This GF-75 was kept for chlorophyll \( \alpha \) analysis and processed within 24 hours. Samples for DOC/TDN were aliquoted into acid-washed and muffled EPA vials, while remaining nutrient samples were transferred to either polypropylene tubes or salt-conditioned and acid-washed high-density polyethylene (HDPE) bottles. All nutrient samples were immediately frozen at -20°C until analysis within one year of sampling (Dore et al. 1996).

Uptake incubation experiments were conducted using site water pre-screened through a 150 \( \mu \)m Nytex mesh to remove zooplankton and grazers and distributed into 0.5 L acid-washed polyethylene terephthalate glycol (PETG) bottles. PETG bottles were filled to the upper rim for a total incubation volume of 0.6 L. All substrate incubations were conducted in triplicate with additions of \( ^{15} \text{N} \)-labeled ammonium chloride (\( ^{15} \text{NH}_4\text{Cl}; 98.85\% \text{^{15}N} \)), potassium nitrate (\( ^{15} \text{NO}_3^- \); 98%), dual-labeled \( ^{15} \text{N} \)- and \( ^{13} \text{C} \)-urea (98%), or a mixture of \( ^{15} \text{N} \)- and \( ^{13} \text{C} \)-labeled amino acids (97-99%). The samples spiked with \( ^{15} \text{NH}_4\text{Cl} \) and \( ^{15} \text{NO}_3^- \) also received \( ^{13} \text{C} \)-labeled bicarbonate (\( \text{H}^{13}\text{CO}_3^- \)) to estimate primary productivity. Alkalinity calculated from salinity was used to derive ambient bicarbonate
concentrations (Parsons et al. 1984). All isotope labeled materials were purchased from Cambridge Isotope Laboratories, Andover, Massachusetts.

Once the isotope labeled substrates were added, incubation bottles were placed in a walk-in environmental chamber with the temperature set based on the recorded field temperature (Table 1). Full spectrum light was provided using light banks with Sylvania Full Spectrum light bulbs (#F40DSGN50). After a one-hour incubation period to allow for incorporation of added isotopically labeled nutrients, incubations were terminated by vacuum filtration through Whatman™ GF-75 (nominal pore size 0.3 µm, combusted 2 hours at 450°C). Filters were frozen at -20°C for later determination of isotope enrichment. Filtrate collected from the GF-75 filtration was used for any nutrient analysis needed to calculate isotope dilution.

**Chlorophyll a and Phytoplankton Quantification**

Chlorophyll a concentration was measured immediately using a fluorometric method. Filter retained chlorophyll a was extracted with 90% acetone overnight and then analyzed on a Turner Design Model 10-AU fluorometer (Parsons et al. 1984; Arar and Collins 1997) with a limit of detection of 0.025 µg L⁻¹. All chlorophyll a measurements were obtained within 24 hours of collection.

Phytoplankton cells of ecologically and locally relevant species in water samples were identified and enumerated by light microscopy and qPCR assays. These included dinoflagellates, diatoms and raphidophytes (Tables S2 and S3). Four water samples were
collected at each sampling site; three for the molecular assay as described below and one for microscopic analysis. Light microscopy was done on one of the live water samples to examine phytoplankton cell behavior and for identification of dominant cell types. Cell counts of up to 30 different species of phytoplankton were done microscopically on Lugol’s preserved 1 mL aliquots of the live samples using a Sedgewick rafter counting chamber. A select set of dinoflagellate species were quantified by qPCR assays. Briefly, triplicate 100 mL water samples were filtered through 3µm Isopore™ membrane filters (Millipore Corp, Darmstadt, Germany) for DNA extraction using the QIAamp ® Fast Stool Mini Kit (QIAGEN, Inc., Germantown, MD, USA) according to the manufacture’s protocol with modifications as previously described (Wolney et al. 2020). TaqMan® qPCR assays were used to determine the number of cells mL⁻¹ in DNA samples for targeted species as compared to standard curves that were generated based on microscopic cell counts of in vitro cultures maintained at VIMS. The average of the triplicate samples was used as the cell count. *Alexandrium monilatum* was quantified as described in Vandersea et al. (2017) and the qPCR assay for *Margalefidinium polykrikoides* was described in Wolney et al. (2020). Assays for *Prorocentrum cordatum* (previously *P. minimum*) and *Karlodinium veneficum* were described in Pease et al. (2021).

**Nutrient Analysis**

Analysis of individual nutrients (NH₄⁺, NO₃⁻/NO₂⁻, amino acids, urea, PO₄³⁻, and SiO₂), and DOC/TDN were completed at VIMS. Ammonium samples were processed in triplicate using the manual phenol-hypochlorite method on a Shimadzu UV-1800
spectrophotometer (Koroleff 1983), with ammonium sulfate as the primary standard and a detection limit (DL) of 0.05 µmol N L\(^{-1}\). Samples for NO\(_3^-\) and NO\(_2^-\) (combined NO\(_x\)) (DL 0.03 µmol N L\(^{-1}\)), PO\(_4^{3-}\) (DL 0.03 µmol N L\(^{-1}\)), and SiO\(_2\) (DL 0.11 µmol Si L\(^{-1}\)) were measured in duplicate on a Lachat QuickChem 8500 autoanalyzer (Parsons et al. 1984). Primary standards used for NO\(_3^-\), NO\(_2^-\), Si, and PO\(_4^{3-}\) were potassium nitrate, sodium nitrite, potassium phosphate, and sodium silicofluoride, respectively. Urea concentrations were analyzed manually on a Shimadzu UV-1800 spectrophotometer using a modified diacetyl monoxime method (Price and Harrison 1987) with a DL of 0.10 µmol N L\(^{-1}\). Bulk amino acids were measured as dissolved primary amines using the o-phthaldialdehyde method (Parsons et al. 1984, DL 0.025 µmol N L\(^{-1}\)). Measurement of the concentration of primary amines (DPA) and dissolved free amino acids (DFAA) are the same in marine waters where the NH\(_4^+\) concentration is not high (Keil and Kirchman 1991). DOC and TDN concentrations were measured on a Shimadzu TOC-L (Hansell 1993; Sharp et al. 2004) with analytical accuracy ensured using deep-sea reference water samples from the University of Miami (Hansell 2005). DON concentrations were calculated using the difference between TDN and DIN (summed NH\(_4^+\) and NO\(_x\)) with standard deviations calculated using propagation of error (Bronk et al. 2000).

_Nitrogen Uptake and Regeneration_

Filters generated at the termination of uptake incubations were thawed and dried overnight at 40°C before analysis on a Sercon Integra2 Mass Spectrometer. Net uptake rates were calculated according to Dugdale and Georing for N (1967). Assimilation of
$^{13}$C was then calculated according to Slawyk et al. (1977). Filtrate collected at the end of
NH$_4^+$ incubations was used to determine the $^{15}$N atom% enrichment of the remaining
NH$_4^+$ pool using solid-phase extraction (LC-18) columns (Dudek et al. 1986). This
allowed calculation of NH$_4^+$ regeneration rates and to correct uptake rates for NH$_4^+$ due to
isotope dilution (Glibert et al. 1982). Regeneration rates were calculated according to Eq
1.

$$r = \ln \left( \frac{[\text{NH}_4^+]_{\text{atom}\%\text{xs}} - [\text{NH}_4^+]_{\text{atom}\%\text{xs}f}}{[\text{NH}_4^+]_{i} - [\text{NH}_4^+]_{f}} \right) \times \left( \frac{[\text{NH}_4^+]_{f} - [\text{NH}_4^+]_{i}}{\text{Time}} \right)$$

(1)

Where $r$ is the regeneration rate, atom%xs is the excess $^{15}$N isotope dilution measured in
the dissolved NH$_4^+$ pool. The $i$ and $f$ represent the initial and final measurements,
respectively.

**Ammonium Release from Urea**

Filtrate from $^{15}$N-labeled urea incubations were collected to determine if NH$_4^+$ release
from urea was measurable. Phytoplankton and bacteria that have either urease or urea
amidolyase can hydrolyze urea into NH$_4^+$, which can then be incorporated into biomass
or released into the environment, such as during sloppy feeding (reviewed in Solomon et
al. 2010; Bronk and Steinberg 2008). At the end of the incubation with $^{15}$N-labeled urea,
NH$_4^+$ was isolated using the same solid-phase extraction procedure used to measure NH$_4^+$
regeneration (above). The rate of NH$_4^+$ release from urea was estimated from the
difference between gross ($\rho_G$) and net uptake of urea ($\rho$), as previously described for DON (Eq. 2 and 3; Bronk et al. 1994; Bronk and Ward 2000).

$$NH_4^+ \text{ Release from Urea} = \rho_G - \rho \quad (2)$$

$$\rho_G = \frac{([\text{PN}] \times \text{PN atom}\%\text{xs}) + ([\text{NH}_4^+]_f \times \text{NH}_4^+ \text{ atom}\%\text{xs}_f)}{\text{Urea atom}\%\text{xs} \times \text{Time}} \quad (3)$$

Where $\rho_G$ is the gross uptake rate and atom\%\text{xs} is the excess $^{15}$N isotope enrichment measured in the PN and $\text{NH}_4^+$ pools. The $i$ and $f$ represent the initial and final measurements, respectively. The percent of extracellular release (PER) of urea to $\text{NH}_4^+$ was calculated using Eq 4 (based on Bronk and Ward 2000).

$$\text{PER} = \frac{100 \times \text{NH}_4^+ \text{ Release from Urea}}{\text{Urea Uptake} + \text{NH}_4^+ \text{ Release from Urea}} \quad (4)$$

**Statistical Analysis**

The statistical software RStudio was used to conduct analysis of variance (ANOVA) and Tukey’s Honest Significant Difference test to interpret differences in between sampled sites among incubation treatments for bicarbonate uptake rates. Spearman’s rank order correlation was used to compare rates with environmental parameters.
Results

Ambient Physical Conditions

Physical conditions of the York were monitored during the sampling period. As with salinity, surface water temperature varied across the York. The maximum water temperature detected during this experiment was 30.3°C, at the Upper site in July 2019. This was also the month with the greatest average temperature (Table 1). In general, surface water temperature was mostly uniform across the estuary. During December 2018 and April 2019, temperature increased as one moved towards the mouth (Table 1).

Sampling during this study coincided with periods of high precipitation and discharge into the York. These elevated levels of discharge were reflected in the observed variation in ambient physical conditions for the four sampling sites. Across the estuary, the highest salinity was observed in July 2019 (average 14.6) with the lowest average salinity detected during June 2018 (averaged 10.0; Table 1). These differences in salinity correspond to the period of elevated discharge in 2018 and the recovery to more normal discharge conditions in 2019 (Figure 2). Generally, salinity in the York was the lowest at the Upper site, and increased as sampling moved towards the mouth.

Ambient Nutrients

Background ambient nutrients were sampled at the start of each uptake incubation to monitor availability. TDN was on average 23.0 μmol N L⁻¹ in the sample area over the seven sampling periods. The greatest TDN was detected in April 2019 at the Mouth site (52.1 μmol N L⁻¹) and the lowest was also at the Mouth in August 2018 (11.6 μmol N L⁻¹).
Generally, TDN decreased as sampling moved down estuary. The portion of TDN that was DIN ranged from 3 – 84% (average 40%) with high percentages of DIN in winter months. The June 2018 sampling also had high inputs of DIN (Table 1) and included the highest concentrations of NH$_4^+$ measured. Ammonium, decreased as sampling moved downstream, from a peak of 11.05 µmol N L$^{-1}$ at the Upper site to a low of 5.34 µmol N L$^{-1}$ at the mouth. Nitrate was also elevated during this sampling, particularly upstream (Table 1).

Nitrate was detected at all sampling times and in all samples, but had concentrations below 1 µmol N L$^{-1}$ in August 2018 and July 2019 (Table 1). Concentrations of NO$_3^-$ generally decreased moving from the Upper to the Mouth. In February 2019, when NO$_3^-$ concentrations were the highest, it contributed to up to 97% and 81% of the DIN and TDN pools, respectively (Table 1). However, when averaged over the one-year sampling period, NO$_3^-$ averaged 30% of TDN. Nitrite was generally less than 1 µmol N L$^{-1}$, but was elevated in October 2018 when it contributed up to 62% and 36% of DIN and TDN, respectively (Table 1).

DON in the York ranged from 4.3 µmol N L$^{-1}$ to 42.2 µmol N L$^{-1}$, constituting 20 – 97% of the TDN pool (Table 1). Urea concentrations were generally less than 1 µmol N L$^{-1}$ and averaged 10% of DON and 3% of TDN. DPA concentrations were less than 0.35 µmol N L$^{-1}$ and only accounted for 2% of DON and 1% of TDN. While urea was at times
a large portion of the DON pool, on average, 92% of the DON pool remained uncharacterized with undetermined lability.

Phosphate ranged from below detection (BD) to 1.57 µmol P L\(^{-1}\) (Table 1). Silica concentrations remained elevated year-around, with concentrations ranging from 37.4 to 118 µmol Si L\(^{-1}\) (Table 1). Concentrations of SiO\(_2\) decreased from the Upper to the Mouth. DOC concentrations averaged 324 µmol C L\(^{-1}\) but ranged from 188 µmol C L\(^{-1}\) in August 2018 to 593 µmol C L\(^{-1}\) in June 2018 (Table 1).

**Phytoplankton Community**

Chlorophyll \(a\) was extracted from 0.3 µm filters, representing phytoplankton organisms >0.3 µm. Concentrations of chlorophyll \(a\) ranged from 8.52 to 95.52 µg L\(^{-1}\) (Table 1). The greatest chlorophyll \(a\) concentration (95.52 µg L\(^{-1}\)) was detected at the Upper site in July 2019, while the lowest was detected at the Upper site in February 2019, with elevated concentrations in the spring and summer (Table 1).

Phaeopigments, which represent the non-photosynthetic degradation products of chlorophyll, generally varied with chlorophyll \(a\) concentration, being low during winter months. Concentration of phaeopigments ranged from below detection to a high of 6.21 µg L\(^{-1}\), which was measured at the same Upper site and time in July 2019 as when the maximum chlorophyll \(a\) concentration was detected (Table 1). During July 2019, the
phaeopigment concentration was also elevated at the Mid site (>2 µg L⁻¹), when the Lower and Mouth sites were below detection (Table 1).

Cell counts of individual phytoplankton species indicated that no bloom of *A. monilatum* or *M. polykrikoides* formed during our high discharge sampling period (Tables S1, S2, & S3). The only qPCR detected presence of *A. monilatum* (<10 cells mL⁻¹) was in the samples collected separately from the incubation experiments on August 8, 2018 (Tables S1 & S3) at levels less than 10 cells mL⁻¹. *K. veneficum* was detected at generally low levels, with < 100 cells mL⁻¹ (Tables S2 & S3). Dinoflagellates *P. cordatum* and *H. triquetra*, also had typically low cell counts of < 100 cells mL⁻¹ (Tables S2 & S3). On February 14, 2019, *H. triquetra* was detected in the Lower York with cell counts as high as 4,800 cells mL⁻¹. In early April, *H. triquetra* and an unidentified dinoflagellate had high cell counts peaking at 70,900 cells mL⁻¹ in the Mid York (Tables S2 and S3). This unidentified dinoflagellate had a similar structure to *Prorocentrum sp.*, but was larger and was not detected by the qPCR assay. Eukaryotic sequencing in this region found high relative abundances of the dinoflagellate genus *Heterocapsa*, but not *Prorocentrum* (S. Fortin, personal communication). During the April incubation sampling 5 days later on April 9, 2019, counts of both species had subsided, with *H. triquetra* reaching a maximum of 95 cells mL⁻¹ and *P. cordatum*-like counts at a maximum of 4,900 cells mL⁻¹ (Table S3).
Nitrogen Uptake Rates

Ammonium absolute uptake ranged from 0.17 to 13.93 \( \mu \text{mol N L}^{-1} \text{ h}^{-1} \), with an average of 4.30 \( \mu \text{mol N L}^{-1} \text{ h}^{-1} \) (Figure 3). Uptake of \( \text{NH}_4^+ \) was the highest in the spring and summer months, with the greatest rates being in April 2019 and the lowest rates in February 2019. Across estuary, \( \text{NH}_4^+ \) uptake rates did not vary consistently as to whether or not there was an up or down estuary trend. Nitrate absolute uptake ranged from as low as 0.01 \( \mu \text{mol N L}^{-1} \text{ h}^{-1} \) to just over 1 \( \mu \text{mol N L}^{-1} \text{ h}^{-1} \), with an average of 0.29 \( \mu \text{mol N L}^{-1} \text{ h}^{-1} \) across the estuary (Figure 3). Uptake of \( \text{NO}_3^- \) was the greatest in July 2019 and the lowest in June and December 2018.

Uptake of urea ranged from 0.02 to 1.71 \( \mu \text{mol N L}^{-1} \text{ h}^{-1} \), with an average of 0.40 \( \mu \text{mol N L}^{-1} \text{ h}^{-1} \) across the estuary (Figure 3). Like \( \text{NO}_3^- \), uptake of urea was elevated in July 2019, with rates up to 1.0 \( \mu \text{mol N L}^{-1} \text{ h}^{-1} \). Uptake of amino acids was generally less than uptake of the other substrates with rates ranging from 0.03 to 0.58 \( \mu \text{mol N L}^{-1} \text{ h}^{-1} \) and averaging 0.11 \( \mu \text{mol N L}^{-1} \text{ h}^{-1} \) (Figure 3). The greatest uptake of amino acids was in April 2019 and the lowest in winter.

Uptake of \( \text{NH}_4^+ \) generally constituted >50% of total uptake (Figure 4). While \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) made up the majority of total uptake, the organic substrates contributed up to 39.2% of total uptake during the August sampling (Figure 4). Urea contributed up to 35.4% of total uptake during the late summer and fall, yet decreased to 0.6% of total uptake in
December 2018 (Figure 4). Amino acids averaged only 3% of total uptake and contributed a maximum of 7.8% in October 2018 (Figure 4).

**Carbon Uptake Rates**

As expected, bicarbonate was by far, the dominate source of C, with uptake rates as high as 69.7 µmol C L⁻¹ h⁻¹ when (Figure 5). Bicarbonate uptake rates were combined between ¹⁵N-labeled NH₄⁺ and NO₃⁻ incubations as there was no significant difference between these incubations across sites (ρ = 0.61; Table S4). On average, bicarbonate was responsible for 97% of total measured C uptake and its uptake rate was greatest in the summer of 2018 and during the April spring bloom (Figures 5 and 6; Table S4). Spearman’s rank order correlation found that bicarbonate uptake was positively correlated with temperature, chlorophyll a, urea, and PO₄³⁻, but negatively correlated with NO₃⁻ (ρ <0.05). Bicarbonate uptake did not directly correlate with either NH₄⁺ or NO₃⁻ uptake rates, but was positively correlated with urea-N and amino acid uptake (Spearman’s rank order correlation, ρ <0.05).

Uptake of urea-C was generally not detectable. When it was observed, urea contributed an average of 3% total C uptake. Urea-C uptake contribution was low even during the peak urea-N uptake in August and October 2018, where uptake of urea-C was up to 0.3 µmol C L⁻¹ h⁻¹ or 0.7% of total C uptake (Figure 5). Urea-C uptake was detected at its greatest during April 2019, where urea-C contributed up to 14% of total C uptake, at the Lower site (Figure 6). The uptakes rates of urea-N and urea-C were not proportional to the two to one ratio within the urea compound and varied considerably from 0.05 in April
2019 to 42.8 in August 2018. Urea-C and urea-N uptake were not correlated ($\rho = 0.91$), and urea-C uptake was positively correlated with salinity and negatively correlated with $\text{PO}_4^{3-}$ and urea concentrations ($\rho < 0.05$). Uptake of amino acid carbon (AA-C) was detected at all sites and all samplings, but rates were low, on average 0.07 $\mu$mol C L$^{-1}$ h$^{-1}$, contributing less than 1% of total C uptake (Figures 5 and 6).

Ammonium Regeneration

Rates of $\text{NH}_4^+$ regeneration ranged from below detection in winter to as high as 3.6 $\mu$mol N L$^{-1}$ h$^{-1}$ during June 2018 (Table 2). Rates were the highest in June 2018 and July 2019 and lowest in October 2018 and February 2019. Ratios of $\text{NH}_4^+$ regeneration to $\text{NH}_4^+$ uptake were all less than 1 (Table 2).

Ammonium Release from Urea

The atom% enrichment of $\text{NH}_4^+$ was measured in the filtrate from urea uptake incubations. This allowed the calculation of $\text{NH}_4^+$ release from urea, based on the difference between gross and net urea uptake rates (Figure 7 and Table 2). Ammonium release from urea was found to be much lower than $\text{NH}_4^+$ regeneration (Figure 7). Rates of $\text{NH}_4^+$ release from urea could only provide an average of 0.6% of the $\text{NH}_4^+$ demand each month. The fraction of urea uptake released as $\text{NH}_4^+$ ranged from 0.2 to 22.3%, with the greatest percent extracellular release (PER) being observed during June 2018 and April 2019. Ammonium release from urea was not correlated with $\text{NH}_4^+$ uptake rates, urea-N uptake rates or urea-C uptake rates, but was positively correlated with $\text{NH}_4^+$
concentrations, as well as bicarbonate uptake (Spearman’s rank order correlation, $\rho < 0.05$).

**Discussion**

*Ambient Physical Conditions*

During the sampling period, the York experienced higher than normal discharge due to increased rainfall associated with two spring storm fronts and two fall hurricane remnants. In May 2018, a stationary front brought 21 cm of precipitation over 3 days to the region, leading to elevated discharge. In June a second stormfront brought 24 cm of precipitation to the region. Discharge to the York from the Mattaponi and Pamunkey Rivers remained elevated for much of the 2018 summer as this rainfall traveled through the watershed and the freshwater tributaries to the York (NOAA National Weather Service; [https://www.weather.gov/akq/cli_archive](https://www.weather.gov/akq/cli_archive); Bukaveckas et al. 2020; Figure 2). In the fall of 2018, remnants of hurricanes Florence and Michael brought additional rain to the region. Analysis of these storm events found that the average discharge from the freshwater tributaries of the York was twice the historic average for May to June and September to October (Bukaveckas et al. 2020). By 2019 the freshwater input from these storm events had subsided leading to a return of discharge conditions that were more similar to the historical average (Figure 2).

This influx of freshwater not only brought nutrients to the York, but also lowered the salinity gradient. During this study, salinity never reached above 17, whereas a previous N uptake study found the salinity of the lower York to be between 19 and 24 (Killberg-
Thoreson et al. 2020). Taken together, these two studies provide a more comprehensive picture of the nutrient dynamics in the York under changing climate conditions, where rainfall patterns are expected to be more variable and Chesapeake Bay is predicted to experience periods of intense rainfall as well as drought (Najjar et al. 2010).

This increased discharge was associated with elevated nutrient inputs as well as freshwater (Moyer & Blomquist 2019). After storm events in May 2018, $\text{NH}_4^+$ and $\text{NO}_3^-$ were found to be as high as $11.05 \text{ µmol N L}^{-1}$ and $10.01 \text{ µmol N L}^{-1}$, respectively, during sampling three weeks later in June. As the residence time in the York is at least 45 days, these DIN nutrients were likely due to this system (Shen and Haas 2004). Concentrations of $\text{NO}_3^-$ remained elevated through the fall and into winter months, as two more storm events passed through southern Virginia. Previous studies have reported that increased discharge was associated with higher inputs of total N, total phosphorus and NOx ($\text{NO}_3^- + \text{NO}_2^-$) into the freshwater tributaries of the York (Reay 2009). Regardless of efforts to reduce nutrient inputs into the Chesapeake Bay region, these high rainfall and subsequent discharge events bring nutrients from the watershed into the estuary in high concentrations (Bukaveckas et al. 2020; Najjar et al. 2010). By spring and summer of 2019, when discharge levels subsided, background nutrient concentrations decreased (Table 1).

**Nutrient Dynamics and Limitation**

Coastal estuaries are transition zones, where freshwater from the surrounding watershed meets saline water from the coastal ocean. The York is fed by the Mattaponi and
Pamunkey Rivers, which collect water and nutrients from the surrounding watershed, opening at its mouth to the more saline Chesapeake Bay (Reay 2009). Generally, concentrations of DIN substrates decrease with distance down estuary, towards the mouth of the York (McCarthy et al. 1977; Killberg-Thoreson et al. 2020; Reay 2009). High and variable concentrations of DIN substrates were observed in this current study, which is consistent with previous work in this region (McCarthy et al. 1977; Sin et al. 1999; Killberg-Thoreson et al. 2020). Unlike previous years, the highest seasonal NH$_4^+$ concentrations were seen in the spring during this study, rather than the fall. This may be due to low rates of regeneration as discussed below. While not a primary focus of this study, a maximum in NO$_2^-$ concentrations was also observed in the fall (Table 1). In many coastal environments, NO$_2^-$ is often undetectable because of the coupling between NH$_4^+$ and NO$_2^-$ oxidation in nitrification, however NO$_2^-$ has been historically detected in the York during the warm summer months, and other estuaries during periods of high temperatures and N loading (Killberg-Thoreson et al. 2020; McCarthy et al. 1984; Glibert et al. 2016). Higher temperatures may contribute to a decoupling of these processes and nitrite accumulation has been observed in southern estuaries with summer water temperatures exceeding 20°C (Schaefer and Hollibaugh 2017).

Concentrations of the DON substrates, specifically urea and amino acids, were consistent with what has been reported in previous research (Sipler and Bronk 2015; Killberg-Thoreson et al. 2020; Killberg-Thoreson et al. 2013). Urea and amino acids had ambient concentrations that were generally less than 2 µmol N L$^{-1}$ and showed a conservative pattern while moving down the estuarine gradient. DON still made up a large portion of
the TDN pool, averaging 60% and typically more than 90% of this DON was uncharacterized (Table 1). Uncharacterized DON has unknown liability, but research is increasingly showing that this N and its complex compounds, like humic acids, may be bioavailable and should be considered in studies of microbial N nutrition (Antia et al. 1991; Bronk et al. 2006; Heil 2005).

Temporal variations in nutrient concentrations and nutrient limitation are known to occur in coastal estuaries, including the York. Nutrient limitation in aquatic systems can be inferred, by calculating nutrient ratios. In this study, limitation of DIN was estimated by using the DIN:DIP ratio calculated with the sum of DIN substrate concentrations and \( \text{PO}_4^{3-} \) (DIP). The DIN:DIP ratio was then compared based on the 16:1 relationship as defined by Redfield, however, it is important to note that this relationship does not include organic N and P and can have considerable variability (Redfield 1958; Gruber and Deutsch 2014). The stoichiometry of DIN:DIP ranged from 0.7 to 225.9 (Table 1), with the York generally having lower DIN:DIP conditions during the summer and transitioning to higher DIN:DIP conditions during the winter. This implied pattern of N-limitation in summer/fall and P-limitation in winter has been previously observed (Sin et al. 1999; Killberg-Thorseson et al. 2020). During past periods of peak riverine discharge, the estuary was P-limited, as N input exceeded P inputs (Sin et al. 1999). In our study, DIP inputs appear to be sufficient to keep the York supplied relative to DIN even during periods of high discharge. For instance, the DIN:DIP ratio was between 15.1-22.6 in June 2018 when there were high DIN concentrations (Table 1). A major source of the P-input is the erosion of P-rich soils and subsequent riverine transport and intense precipitation.
conditions likely contribute to P input (Najjar et al. 2010). Measurements in the York tributaries also observed increased discharge of total P (Bukaveckas et al. 2020).

Community Composition and Blooms

Unlike summers during the previous decade, the late summer of 2018 did not see the formation of either *Margalefidinium polykrikoides* or *Alexandrium monilatum* blooms. This change could be due to multiple environmental reasons, but one contributor may have been the lower salinity from the high levels of discharge in the York. During both August and October 2018, salinity was less than 15 in the York, with the exception of the Mouth site in August. Previous research has indicated that ideal conditions for these dinoflagellates to bloom include a salinity of at least 15 for *A. monilatum* (Anderson et al. 2002; Juhl 2005). When uptake incubation experiments were conducted during a 2007 *A. monilatum* bloom, salinities were measured at over 20 (Killberg-Thoreson et al. 2020). It is possible that the lower observed salinities contributed to the lack of blooms in 2018. While neither of these dinoflagellates bloomed in 2018, *M. polykrikoides* and *A. monilatum* both form resting cysts when conditions are unfavorable that sink into the sediments (Walker and Steidinger 1979; Tang and Gobler 2012). In subsequent years, as the York has returned to lower discharge and higher salinity conditions, these dinoflagellates have formed blooms. As HAB occurrence is predicted to increase with rising temperatures and nutrient availability, the change in salinity due to discharge may need to be considered (Najjar et al. 2010; Paerl et al. 2014).
Unique nutrient and N uptake dynamics were observed during April 2019 when background discharge conditions recovered and a spring bloom formed. Prior to April sampling, monitoring of the York’s phytoplankton community composition observed high abundance of the dinoflagellate *Heterocapsa triquetra*, with counts of ~35,000 cells mL\(^{-1}\) on March 28, 2019 measured between the Lower and Mouth sites. By April 2019, *H. triquetra* accounted for only 95 mL\(^{-1}\) at the Mouth site. At the point of this study’s sampling, a dinoflagellate similar to *P. cordatum* was present at elevated levels, with a count of 4,900 mL\(^{-1}\) at the Mouth site, and decreasing further upstream. While more study is needed to clarify the classification of this dinoflagellate, *P. cordatum* has been shown to prefer NH\(_4^+\) as a substrate (Fan et al. 2003). Indeed, this April sampling is when the greatest rates of NH\(_4^+\) uptake were observed (Figure 1). Uptake of NO\(_3^-\) was higher than urea during this sampling (Figure 2), even though previous research has indicated *P. cordatum* has an affinity for urea and that NO\(_3^-\) uptake may be suppressed by urea (Fan et al. 2003; Matantseva et al. 2016). This previous research into NO\(_3^-\) suppression was done with cultured *P. cordatum* and with much higher urea concentrations than our observed <0.5 µmol N L\(^{-1}\) (Table 1).

During this bloom period, DIN was depleted at the Mid site compared to the Lower and Mouth sites (Table 1). For example, concentrations of NO\(_3^-\) were greater than 5 µmol N L\(^{-1}\) at the down estuary sites, whereas the Mid site had a NO\(_3^-\) concentration of less than 0.1 µmol N L\(^{-1}\) (Table 1). At the time of sampling the chlorophyll a concentration was 25.2 µg L\(^{-1}\), whereas on April 4, 2019 they had been reported as 139.4 µg L\(^{-1}\) at that site.
(I. Anderson, personal communication). It may be likely that DIN had been drawn-down by uptake that was not captured by this single April sampling event.

**Nitrogen Uptake Rates**

Most sites in this study showed a N uptake pattern of $\text{NH}_4^+ > \text{NO}_3^- > \text{Urea} > \text{Amino Acids}$, which was also observed by Killberg-Thoreson (2020). However, this N uptake pattern was found to vary somewhat. Ammonium, by far, was taken up at the greatest rate (Figure 3) and the dominance of $\text{NH}_4^+$ as the substrate with the fastest rates has been well established in the literature (Glibert et al. 2016). Uptake of $\text{NH}_4^+$ averaged 74% of total N uptake across all seasons and sites, with a maximum rate of 13.9 µmol N L$^{-1}$ for the >0.3 µm size class during the April 2019 remnants of the spring blooms of *H. triquetra* and *P. cordatum*-like blooms (Figure 3).

High concentrations of $\text{NH}_4^+$ have been previously shown to suppress uptake of other N substrates, including $\text{NO}_3^-$ (Lomas and Glibert 1999). During the June 2018 sampling, $\text{NH}_4^+$ concentrations were greater than 5 µmol N L$^{-1}$ (Table 1), while high concentrations of $\text{NO}_3^-$ were also present (1.21 – 10.01 µmol N L$^{-1}$). During this sampling, $\text{NH}_4^+$ made up more than 90% of total N uptake, whereas $\text{NO}_3^-$ was less than 2% of total N uptake. Indeed, when $\text{NH}_4^+$ concentrations were greater than 2 µmol N L$^{-1}$, uptake of $\text{NO}_3^-$ appeared to be suppressed, as also observed in 2008 in the York by Killberg-Thoreson (2020).
While N uptake was dominated by uptake of inorganic N, organic N sources were also important in the York. In the fall, uptake followed a pattern of $\text{NH}_4^+ > \text{Urea} > \text{NO}_3^- > \text{Amino Acids}$ and urea consisted of upwards of 35% of total N uptake (Figures 2 and 3).

It is important to note that the previous report of $\text{NO}_3^-$ uptake exceeding urea was for the size fraction $>5 \ \mu\text{m}$ and that size fractionation was not done in this study (Killberg-Thoreson et al. 2020). Urea uptake has been previously shown to reach a maximum in the fall in the Chesapeake Bay region (Lomas et al. 2002; Killberg-Thoreson et al. 2020). While not observed in this study, uptake of urea has been shown to exceed $\text{NH}_4^+$ uptake during some dinoflagellate blooms (Mullholland et al. 2018). This study found urea to be a seasonally important source of N that should receive further attention, particularly as the input of this organic nutrient has been increasing in many systems due to anthropogenic activity (Glibert et al. 2006).

**Organic Carbon Uptake Rates**

Uptake of urea-C was primarily seen in elevated rates during the April 2019 sampling when a $\text{P. cordatum}$-like dinoflagellate was present and averaged 8.9% of total C uptake (Figure 5). The urease enzyme, which is encoded in the genome of $\text{P. cordatum}$, hydrolyzes urea to $\text{NH}_4^+$ and bicarbonate (Matantseva et al. 2016; Solomon et al. 2010). The uptake of urea-C has been previously reported in the York in 1974, where measurement with urea-$^{14}\text{C}$ found that 10-50% of urea-C was incorporated into biomass, but community composition was not reported (Webb and Haas 1976). A culturing study of $\text{P. cordatum}$ reported that urea-C only contributed to 1% of total C uptake (Matantseva
et al. 2016). However, in the neighboring tributary to the York, the James River, uptake incubations during a *P. cordatum* bloom in 2003 found that urea-C was taken up at rates as high as 11.8 µmol C L⁻¹ h⁻¹, exceeding the uptake of even bicarbonate (Mulholland et al. 2018). Uptake of organic-C may provide a competitive advantage to mixotrophs over other photoautotrophs (Mulholland et al. 2018). Incubation experiments in the northern part of Chesapeake Bay also found low levels of urea-C uptake that increased during the progression of a natural *P. cordatum* bloom and may have resulted from decreasing C:N ratios as the bloom progressed (Fan and Glibert 2005).

Further research has shown that *P. cordatum* uptake of urea-C is likely uncoupled from uptake of urea-N, because urea-N uptake exceeds urea-C in proportions greater than two to 1 (Matantseva et al. 2018). This may imply that N-supply is limiting cell growth. When urea-C uptake was detectable in this study, it’s proportion to urea-N was highly variable. In summer and fall, urea-N uptake was up 40 times greater than urea-C uptake, further supporting that these processes are uncoupled. It is unclear whether the incorporation of urea-C is due to incorporation after urea hydrolysis to bicarbonate, subsequent release, and then uptake through C-fixation, or if the urea-C is assimilated during internal urea hydrolysis. It has been suggested that urea-C may be incorporated if the carbamate intermediate of urea hydrolysis is phosphorylated by carbamoyl phosphate synthetase and subsequently incorporated into arginine biosynthesis (Krausfeldt et al. 2019). More research is needed in order to understand which conditions lead to urea-C uptake, the controlling mechanisms for urea-C incorporation, and if urea-C uptake provides a competitive advantage for bloom forming mixotrophs.
**N Regeneration**

N-limited systems often depend on NH$_4^+$ regeneration and nutrient recycling to maintain the supply of N needed to support phytoplankton growth (McCarthy et al. 1977; Collos et al. 2003). In Chesapeake Bay, heterotrophic processes with regenerated N often exceed autotrophic uptake (Bronk et al. 1998; Killberg-Thoreson et al. 2020). If the ratio of regeneration to uptake is $\sim 1$, regeneration processes are sufficient to supply the N needed to maintain phototrophic processes. In this study, the regeneration to uptake ratio was always less than one, with uptake rates of NH$_4^+$ far exceeding rates of NH$_4^+$ regeneration. At its lowest, the ratio was just 0.03, with NH$_4^+$ uptake being up to 34 times NH$_4^+$ regeneration (Table 2). In previous seasons in the York reported ratios were less than one during late fall to spring, but during summer months, NH$_4^+$ regeneration rates were as great as 24 times NH$_4^+$ uptake (Killberg-Thoreson et al. 2020). Rates reported here (Table 2) were generally lower than those reported in 2008 and 2009, which were as high as $7.5 \pm 1.1$ µmol N L$^{-1}$ h$^{-1}$ (Killberg-Thoreson et al. 2020). The lower rates reported in 2018 indicated that the phytoplankton and other members of the microbial community needed additional N sources to balance uptake rates and these were likely provided by DIN coming into the estuary from the watershed rather than N recycled within the system. While allochthonous N-based autotrophic production has been observed in Chesapeake Bay in the spring (Bronk et al. 1998), it appears that allochthonous N sources continued to supply the York with DIN from the watershed, rather than the York needing to depend on N recycled within the system during summer and fall.
**Ammonium Release from Urea**

While the internal hydrolysis of urea to NH$_4^+$ is well established, we believe these are the first reported $^{15}$N rates of urea hydrolysis and its extracellular release as NH$_4^+$ as a contributor to N cycling in the aquatic environment. Urea transformation and subsequent extracellular release to DON has been previously reported. In the Atlantic, the PER of $^{15}$N-labeled urea to the DON pool was as high as 26% and comparable to NH$_4^+$ to DON release (Varella et al. 2005). In the current study, rates of NH$_4^+$ release from urea were low compared to NH$_4^+$ regeneration (Figure 7). However, NH$_4^+$ release from urea was, at times, a large contributor to PER. PER was the greatest in June 2018, but was highly variable (Table 2). This variability was also seen in the rates of NH$_4^+$ release from urea, particularly during April 2019 (Table 2). Variability in extracellular release has previously been observed for DON and urea release (Varella et al. 2005; Lomas et al. 2002), and may be due to the small size of these release pools (Lomas et al. 2002). Additionally, intracellular rates of NH$_4^+$ release may be higher than the extracellular rates reported here (Bronk 1999). Further study with cultures may be necessary to elucidate who is releasing NH$_4^+$, its controls, and the role of grazing (as reviewed for DON in Bronk and Steinberg 2008).

These low rates of NH$_4^+$ release from urea are likely a minimal contributor to NH$_4^+$ available for uptake and primary production (averaging <1% of NH$_4^+$ uptake demand), yet the PER indicates that the full cycling of urea is not fully captured with just net uptake rates. These rates may also be important for clarifying the role of urea-C uptake or release. This NH$_4^+$ release from urea may also coincide with release of the C in urea,
making it available for urea-C assimilation. A back of the envelope calculation assuming that urea-C is released at half the rate of NH$_4^+$ release from urea (Table 2), reveals that this urea-C could be the source of on average only 11% of the urea-C uptake rates that we observed (Figures 5 and 6). During April 2019, when urea-C uptake was the greatest, any urea-C coming from our NH$_4^+$ release rates would only account for less than 1% of this C-uptake. Our rates of NH$_4^+$ release from urea and urea-C are then also likely uncoupled for this system, but the controls of this relationship are unknown.

**Conclusions**

During this study period the York experienced historically high levels of freshwater discharge due to several storm events. While the storms that passed through the region brought historic rainfall, weather patterns in this region are expected to continue to intensify with climate change (Najjar et al. 2010). Therefore, it is important to understand how nutrient cycling may change as a result. The high period of discharge in 2018 was characterized by low salinity and high concentrations of inorganic N. While NH$_4^+$ uptake continued to be the dominate N substrate, NO$_3^-$ uptake exceeded those for NH$_4^+$, when concentrations of NH$_4^+$ were less than 1 µmol N L$^{-1}$. Organic N also contributed to a large portion of total absolute N uptake seasonally. Urea contributed upwards of 35% of total N uptake during the fall, indicating that organic N sources need to be considered in this region. N substrates, including DON then need further consideration during periods of high discharge, as our rates of NH$_4^+$ regeneration were also lower than what has been observed during past periods of lower discharge. This study also calls for the further study of urea-C, as elevated uptake of urea-C was observed during a dinoflagellate bloom.
in April 2019. Finally, this study reported the first rates of urea release to NH$_4^+$.
While rates were low, they contributed up to 22% of PER. This clearly demonstrates that uptake
of urea is more complex than can be captured with just urea-N uptake measurements.
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Figures and Tables:

Table 1: Ambient concentrations of total dissolved nitrogen (TDN), ammonium (NH$_4^+$), nitrate (NO$_3^-$), nitrite (NO$_2^-$), dissolved organic nitrogen (DON), urea, amino acids measured as dissolved primary amines (DPA), dissolved organic carbon (DOC), phosphate (PO$_4^{3-}$), silicate (Si), chlorophyll $a$ (Chl $a$) and phaeopigments (Phaeo) collected on a 0.3 $\mu$m filter. DIN:DIP is calculated based on the sum of NH$_4^+$, NO$_3^-$, and NO$_2^-$ as DIN and PO$_4^{3-}$ as DIP. BD = below detection. NS = not sampled. Values are given with one unit standard deviation.
| Sampling | Temperature | Salinity | TDN | NH$_4$$^+$ | NO$_3$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^
Table 2: Ammonium (NH\textsubscript{4}\textsuperscript{+}) regeneration (Regen) rates measured from solid-phase extraction of the NH\textsubscript{4}\textsuperscript{+} pool after incubation experiment termination compared to net NH\textsubscript{4}\textsuperscript{+} and urea uptake rates, as well as NH\textsubscript{4}\textsuperscript{+} release from urea. The percent of extracellular release (PER) was also calculated. BD = below detection. NS = not sampled. Values are given with one unit standard deviation.

<table>
<thead>
<tr>
<th>Sampling</th>
<th>NH\textsubscript{4}\textsuperscript{+} Absolute Uptake</th>
<th>NH\textsubscript{4}\textsuperscript{+} Regen</th>
<th>Urea Absolute Uptake</th>
<th>NH\textsubscript{4}\textsuperscript{+} Release from Urea</th>
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<td>0.038 ± 0.011</td>
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<td>NS</td>
<td>NS</td>
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<td>NS</td>
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Figure 1: Sampling locations in the York River Estuary, VA, a tributary of Chesapeake Bay. Four surface sites were sampled along the estuarine gradient in the main channel every two months from June 2018 to July 2019. Yellow star VIMS: Virginia Institute of Marine Science, Blue star YRTP: York River Treatment Plant, Pink star WRPM: WestRock Paper Mill. Map taken from Google© with permission.
Figure 2: Annual discharge combined from the Mattaponi and Pamunkey Rivers into the York River averaged from 1971 to 2017 in dark blue bars. Error bars are one unit of standard deviation. The red line represents the daily discharge from April 2018 to July 2019 during which this experiment occurred. Data is from USGS gauge stations (https://waterdata.usgs.gov/).
Figure 3: Absolute uptake rates (µmol N L⁻¹ hr⁻¹) for four substrates measured bimonthly in the York River Estuary, (A-D). Substrates included ^15^N-labeled ammonium, nitrate, urea, and an algal mixture of amino acids. Sites move down estuary, with York Upper (A) at the head of the York and Mouth (D) near the mouth. All sites were in the main river channel and water was collected from the surface. Error bars are one unit of standard deviation. Note that in panel A (April 2019) the Upper site was not sampled, represented by *.
Figure 4: Percent of total N uptake (µmol N L⁻¹ hr⁻¹) for four substrates measured bimonthly in the York River Estuary, VA across the four sites (A.-D.). Note that in panel A (April 2019) the Upper site was not sampled, represented by *.
Figure 5: Absolute uptake rates (µmol C L\(^{-1}\) hr\(^{-1}\)) for three C substrates measured bimonthly in the York River Estuary, VA across four sites (A.-D.). Substrates included \(^{13}\)C-bicarbonate, urea, and an algal mixture of amino acids. Error bars are one unit of standard deviation. Note that in panel A (April 2019) the Upper site was not sampled, represented by *.
Figure 6: Percent of total C uptake (µmol C L\(^{-1}\) hr\(^{-1}\)) for three C substrates measured bimonthly in the York River Estuary, VA across the four sites (A.-D.). Axis are adjusted to >80% in order to visualize organic-C. Note that in panel A (April 2019) the Upper site was not sampled, represented by *.
A.

- Net Ammonium Uptake
- Net Urea Uptake
- Ammonium Regeneration
- Ammonium Release From Urea

B.

- Net Urea Uptake
- Ammonium Regeneration
- Ammonium Release From Urea
Figure 7: Across estuary monthly average rates of net NH$_4^+$ uptake, net urea uptake, NH$_4^+$ regeneration from NH$_4^+$ uptake incubations, and NH$_4^+$ release from urea uptake incubations (Panel A). Panel B does not include net NH$_4^+$ uptake, allowing for better comparison of release processes. Reported rates of NH$_4^+$ release from urea uptake incubations were estimated based on $^{15}$N-labeled NH$_4^+$ extracted from incubations bottles with $^{15}$N-urea additions and reported as extracellular release rates. Error bars are one unit of standard deviation.
**Supplementary and Tables**

Table S1: Site name and location for sites included both in this paper and in the larger project

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<th>Longitude</th>
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Table S2: Phytoplankton cell counts collected during uptake incubation water sampling.

Counts were obtained using both qPCR and visual counting. BD = below detection. NS = not sampled. Values are given with one unit standard deviation. *Visual analysis completed in lieu of qPCR for *P. cordatum*-like dinoflagellate

<table>
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<th><em>Alexandrium monilatum</em></th>
<th><em>Margalefidinium polykrikoides</em></th>
<th><em>Karlodinium veneficum</em></th>
<th><em>Prorocentrum cordatum</em></th>
<th><em>Heterocapsa triquetra</em></th>
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<td>qPCR cells/ml</td>
<td>qPCR cells/ml</td>
<td>qPCR cells/ml</td>
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<td>Upper</td>
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Table S3: Phytoplankton cell counts collected throughout the York River Estuary, VA by the LYRE project during the time period of uptake incubation experiments. Counts were obtained using both qPCR and visual counting. BD = below detection. NS = not sampled. Values are given with one unit standard deviation. *Visual analysis completed in lieu of qPCR.

<table>
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<th>Alexandrium monilatum</th>
<th>Margalefidinium polykrikoides</th>
<th>Karlodinium veneficum</th>
<th>Prorocentrum cordatum</th>
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Table S4: Absolute uptake rates (μmol C L⁻¹ hr⁻¹) for ¹³C-bicarbonate measured separately for incubations with additions of ¹⁵N-labeled ammonium (NH₄⁺) and nitrate (NO₃⁻). NS = not sampled. Values are given with one unit standard deviation.

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<tr>
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<td>8.2 ± 1.4</td>
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CHAPTER 3

Aquatic Community Composition Shifts with Exposure to Variable Treatment of Wastewater Effluent
ABSTRACT
Nitrogen (N) discharged from water resource and recovery facilities (WRRFs, also termed wastewater treatment plants) is a widespread nutrient point source to receiving waters. Recently, many WRRFs have been upgrading their facilities to improve N removal processes, which may have significant impacts on water quality and primary production in receiving waters. However, there has only been a few studies examining the aquatic microbial community responds to the exposure of variably treated wastewater effluents.

We examined the changes in composition and diversity of estuarine aquatic communities exposed to the WRRF effluents with different composition and concentration of nutrients, including dissolved organic nitrogen (DIN) and dissolved organic nitrogen (DON). Sequencing batch reactors were used to produce four differently treated effluents from nitrification-only (NO) to a complex 5-stage Bardenpho process with biological or chemical phosphate removal (BNCPR). These effluents were used for two incubation experiments to compare the responses of summer and winter communities in the surface water of the York River, a tributary of Chesapeake Bay. The biomass from these experiments was collected for DNA extraction and Illumina MiSeq sequencing of 16S rRNA genes to assess the impact of the differently treated effluents on the aquatic communities. Sequencing analysis clearly showed differential responses of aquatic communities to different effluents. The effluents that had been minimally treated (NO and nitrification-denitrification, NR) had significant impacts on aquatic community, with increased abundance of *Rhodobacteriales* and *Bacillariophyta*. However, the effluents more heavily treated (biological nitrogen and phosphate removal, BNPR and BNCPR) had little impacts on microbial communities that were similar to the control community without
effluent added. Availability of DIN, and specifically nitrate, correlated with the observed changes of community composition. This indicates that the wastewater industry’s current efforts to reduce WRRF DIN discharge may be sufficient enough to mitigate estuarine and coastal eutrophication from these point sources.
Introduction

Since passage of the Federal Water Pollution Control Act of 1948, there has been an increasing demand to regulate wastewater treatment plant-derived nutrients entering receiving waters (Carey and Migliaccio 2009). Reducing nutrients discharged by water resource and recovery facilities (WRRFs, also commonly called wastewater treatment plants) is critical to limit algal blooms and subsequent eutrophication in receiving waterbodies. In the United States, wastewater contributes an estimated 12% of riverine nitrogen (N) pollution (Howarth 2004). This excess N can lead to a cascade of negative environmental effects, including harmful algal blooms, fish kills, and associated economic loss (Kemp et al. 2005; Carey and Migliaccio 2009). In the Chesapeake Bay area, efforts to reduce eutrophication have led the N concentration in effluent to be restricted through the Chesapeake Bay’s Total Maximum Daily Load (TMDL). WRRFs have responded by improving their treatment processes to increase N removal capacity (EPA 2010; Fleischer et al. 2005). This increase in regulatory enforcement combined with improvements in treatment technology have led to a decrease in WRRF effluent contribution to total N-loading in the Bay, from 28% to 16% between 1985 and 2015 (EPA 2016). At present, most regulations and WRRF strategies target dissolved inorganic nitrogen (DIN) in WRRF effluent, but not dissolved organic nitrogen (DON), another important N source (Pehlivanoglu-Mantas and Sedlak 2006; Westgate and Park 2010). DON is not currently regulated even though DON can range from 9-50% of the total N discharge of WRRFs (Pagilla et al. 2008).
DON is a complex mixture of compounds with variable lability. Labile forms include urea and amino acids, whereas refractory DON is a mixture of poorly characterized N and carbon (C)-containing compounds, such as humic acids (Sipler and Bronk 2015). The lability of DON that is discharged with effluent into receiving waters by WRRFs, effluent DON (EDON), is heavily influenced by the wastewater treatment process. Labile compounds like urea and amino acids are converted to ammonium (NH$_4^+$) or consumed in the treatment process, leading to discharge of largely uncharacterized DON of variable lability (Pehlivanoglu-Mantas and Sedlak 2006). This pool can also include N from influent that was not degraded during treatment or compounds created during final disinfection processes (Barker and Stuckey 1999; Parkin and McCarty 1981).

Although EDON is traditionally classified as a refractory nutrient source, recent research has shown that EDON is a usable N source in receiving waters. Natural water incubations with wastewater effluent in the Elizabeth River, VA, a Chesapeake Bay tributary, found that within 2 days up to 96% of EDON was removed by the aquatic community (Filippino et al. 2011). Microbial-produced DON and EDON have been shown to have similar chemical properties and it is likely that EDON also supports microbial growth in receiving water (Sattayatewa et al. 2009; Nam and Amy 2008). In culture studies, it has been shown that the hydrophilic component of EDON can stimulate *Selenastrum capricornutum* growth, whereas the hydrophobic component minimally supported algae growth (Liu et al. 2012). Lability of EDON in receiving waters also appears to be influenced by WRRF influent source and differences in disinfection treatment (Bronk et al. 2010; Funkey et al. 2015; Roberts et al. 2020). Bioavailability of this nutrient source to the aquatic community
may also be location-specific, as the salinity in receiving waters may lead to the release of labile compounds such as ammonium (NH$_4^+$) or dissolved primary amines (Bronk et al. 2010; Funkey et al. 2015).

Most of the research into the bioavailability of effluent has either been species-specific culture experiments or have monitored the change in biomass through chlorophyll $a$ measurement (Yao et al. 2019; Roberts et al. 2020; references above). Very few studies have examined the changes of aquatic community composition and identified the specific taxa potentially utilizing the effluent constituents, even though some harmful algal bloom species of dinoflagellates are known to utilize DON as nutrients (Bronk et al. 2006; Solomon and Glibert 2008; reviewed Anderson et al. 2012; reviewed Dagenais-Bellefeuille and Morse 2013).

One potential way to further clarify how an aquatic community responds to the discharge of receiving waters is by examining the changes in composition and diversity of aquatic community. Current research into microbial and planktonic communities influenced by domestic wastewater outfalls is limited, and much of this composition work has targeted changes in antibiotic-resistance bacteria (Szekeres et al. 2017; Tang et al. 2016), rather than the availability of nutrients. This can be challenging to clarify because of all the material that is included in effluent, including heavy metals and pharmaceuticals. To date, the research on effluent nutrients in receiving waters has been limited to mostly benthic or freshwater communities and to inorganic nutrients, such as nitrate (NO$_3^-$), not EDON (Saarenheimo et al. 2017; Price et al. 2018; Huo et al. 2017; Tao et al. 2017). This area of
research must be expanded to more ecosystems and environmental conditions, as some research has suggested that WRRF discharge might create a localized environment where microbes of specific functional groups becomes more prevalent and may decrease in microbial diversity (Saarenheimo et al. 2017; Tao et al. 2017). As these studies are WRRF outfall specific, it is also difficult to elucidate how changes in community composition may vary based on WRRF treatment processes used at different facilities. This work eliminates this issue, by collecting effluent produced by four different sequencing batch reactors that used the same influent source. By then using this effluent to provide a pulse of nutrients to the same microbial community, the impact of nutrient differences from treatments can be explored.

This study aims to address this knowledge gap and to evaluate the impact of both EDON and DIN from differently treated effluents on the microbial community of a single receiving waterbody. This was accomplished through incubation experiments with effluent from four different wastewater treatments combined with natural whole-water from the mesohaline York River, VA, a tributary of Chesapeake Bay. Changes in the composition and diversity of microbial communities incubated with effluent from the four treatment conditions and two different ambient temperatures were considered to address the research question as follows; 1) Does the introduction of effluent alter the composition and diversity of estuarine microbial communities? 2) Is there any correlation between DIN and EDON concentration and community composition? and 3) Do dominant microbial taxa react differently to effluent additions in summer and winter?
Materials and Methods

Description of Wastewater Effluent from Sequencing Batch Reactors

Treated wastewater effluent was collected from the Hampton Roads Sanitation District (HRSD) Chesapeake-Elizabeth Treatment Plant in Virginia Beach, VA in May 2016 and January 2017. This plant processes 24 million gallons of influent per day, receiving primarily domestic household wastewater, some commercial wastewater, and minimal wastewater from industrial sources (Roberts et al. 2020). At this HRSD plant, four pilot scale sequencing batch reactors (SBRs) used various treatments to process the same raw influent from the HRSD Chesapeake-Elizabeth Treatment Plant (Figure 1). Each SBR was designed to mimic a commonly used wastewater treatment process and were maintained at 25 and 15 °C in May 2016 and January 2017, respectively. The four SBRs consisted of nitrification only (NO), which underwent aerobic nitrification. Since this SBR did not include subsequent denitrification, this treatment method produced the effluent with high NO₃⁻ concentration. Nitrogen Removal (NR) processes consisted of an anoxic and aerobic treatments for nitrification and denitrification to remove DIN. Both the NO and NR treatments did not include phosphorus (P) removal. The last two SBRs treatments underwent a 5-stage Bardenpho treatment cycle. The third treatment, biological nitrogen and phosphorus removal (BNPR) included biologically-mediated P removal. The fourth treatment, biological nitrogen and phosphorus removal with chemical phosphorus removal (BNCPR), included biologically-mediated P removal and the addition of ferric chloride during final aeration to chemically remove P from the effluent. These four treatments were then subdivided and disinfected with one of three disinfection treatments, ND – No Disinfection, UV – Germicidal UV, or CL – Chlorination. As both Roberts et al. 2020 and
an initial principle coordinate analysis found minimal differences between disinfection treatments, only ND treatments were considered for this work (Roberts et al. 2020; Figure S1). More detail on these effluent and disinfection treatment processes may be found in Roberts et al. 2020.

All SBRs operated for a minimum of three 15-day solid residence cycles prior to any sampling each season. At each sampling, the effluent was filtered by vacuum filtration to remove the microbes (or microbiomes) from the SBR treatment processes. Effluent was filtered sequentially first through a Whatman™ GF/F (nominal pore size 0.7 µm, pre-combusted 2 hours at 450°C) and then a Supor membrane filter (nominal pore size 0.2 µm). The effluent filtrate from these filtrations was reserved for nutrient analysis and later incubation experiments. Effluent was transported on ice to the Virginia Institute of Marine Sciences (VIMS) in Gloucester Point, VA where it was kept at -20°C. Estimates of initial DON and DIN content provided by HRSD staff were utilized to determine subsequent incubation additions for each of these treatments.

Bioassay Incubations

Bioassay experiments were conducted in June 2016 (summer) and January 2017 (winter) with surface water of the York River. The York River is the fifth largest tributary of Chesapeake Bay and is supplied by the Mattaponi and Pamunkey Rivers, forming a microtidal partially-mixed estuary (Reay 2009). Site-water was collected at the VIMS pier (37.248022°N, 76.499761°W) during ambient salinity and temperature conditions of 14, 23°C in summer and 19, 9°C in winter, respectively. All site-water was collected from the
surface (<0.5 m) into a 10% HCl washed and site-water rinsed high-density polyethylene (HDPE) carboy after pre-screening large zooplankton with a Nitex mesh. Water was then taken to a VIMS lab where initial samples were collected for natural aquatic community composition, phytoplankton biomass, and wet chemical analyses before bioassay incubations occurred.

Summer and winter site-water incubations were amended with one of the four effluent treatment samples for an estimated 15 µmol N L⁻¹ EDON addition. However, the NO and NR treatments produced effluent with high NO₃⁻ concentrations (Roberts et al. 2020). As a result, the NO and NR treatments included NO₃⁻ additions of over 230 and 80 µmol N L⁻¹, respectively (Table 1). In addition, a site-water "Control" without any effluent addition was allocated for alongside incubation. Incubations in 0.25 L polyethylene terephthalate glycol (PETG) bottles were maintained in a Percival incubator set to mimic in situ temperature and light conditions. Daily samples for chlorophyll a concentrations were collected to monitor the growth response of the phytoplankton community to the pulse of effluent (Roberts et al. 2020). Starting chlorophyll a concentrations were 13.33 ± 2.33 µg Chl a L⁻¹ in summer and 7.82 ± 0.55 µg Chl a L⁻¹ in winter (Table 1). Based on this monitoring, the summer and winter incubation experiments were conducted for five and nine days, respectively.

Treatment bottles in triplicate were randomly sacrificed at each time point for sample collection. Each sample was filtered through Whatman™ GF/F filters (combusted at 450°C for 2 h, nominal pore size 0.7 µm) and filtrate was frozen at -20°C for subsequent nutrient
Biomass was collected with Sterivex™ sterile filters (nominal pore size 0.22 µm) for the initial site water and for each treatment at the end of the incubation periods, and stored at -80°C. In summer, triplicate Sterivex™ samples were collected for each investigated treatment, whereas in winter duplicate samples were collected due to biomass constraints.

**Nutrient Analysis**

Analysis of individual nutrients (NH$_4^+$, NO$_3^-$, NO$_2^-$, urea, amino acids, and phosphate), total dissolved nitrogen (TDN), and dissolved organic carbon (DOC) were completed for the starting treated effluent, ambient York River site-water and for initial and final bioassay time points. Ammonium samples were processed in triplicate using the manual phenol-hypochlorite method with a Shimadzu UV-1800 spectrophotometer (Koroleff 1983). Ammonium sulfate was used as the primary standard and this method had a detection limit (DL) of 0.05 µmol N L$^{-1}$. Nitrate and NO$_2^-$ (combined NO$_x$; DL of 0.03 µmol N L$^{-1}$), and phosphate (PO$_4^{3-}$; 0.03 µmol N L$^{-1}$) were measured in duplicate on a Lachat QuickChem 8500 autoanalyzer (Parsons et al. 1984). Primary standards used for NO$_3^-$, NO$_2^-$, and PO$_4^{3-}$ were potassium nitrate, sodium nitrite, and potassium phosphate, respectively.

Concentrations of urea were measured in duplicate using the diacetyl monoxime method on a Shimadzu UV-1800 spectrophotometer (Price and Harrison 1987) with a DL of 0.10 µmol N L$^{-1}$. Amino acids were measured in bulk as dissolved primary amines (DPA) using the o-phthaldialedehyde method (Parsons et al. 1984), with a DL of 0.025 µmol N L$^{-1}$.

Concentrations of DPA and dissolved free amino acids (DFAA) are the same in marine waters where the NH$_4^+$ concentration is not high (Keil and Kirchman 1991). TDN and
DOC were measured on a Shimadzu 5000A TOC-V/TNM with analytical accuracy ensured using deep-sea reference water samples from the University of Miami (Hansell 1993; Sharp et al. 2004; Hansell 2005). Dissolved organic nitrogen (DON) was calculated using the difference between TDN and DIN (summed NH₄⁺ and NOₓ) with standard deviations including the propagation of error (Bronk et al. 2000). Concentrations filter-retained chlorophyll a (chl a) was extracted with 90% acetone overnight and measured fluorometrically on a Turner Design Model 10-AU fluorometer (Parsons et al. 1984; Arar and Collins 1997) with a DL of 0.025 µg Chl a L⁻¹.

**DNA Extraction and Sequencing**

DNA was extracted from the Sterivex filters with biomass collection using a Qiagen DNeasy PowerSoil Kit (Qiagen) following the manufacture's instruction with some modification. Extracted DNA was quantified with Qbit fluorometric quantification (Thermo Scientific), and each sample was diluted to 1 ng µL⁻¹. These samples were used for PCR amplification of 16S and 18S rRNA genes after 2 µL of diluted DNA was combined with 12.5 µL GoTaq mix, 8.5 µL nucleic acid-free water and 1 µL of each PCR primer (universal 515F and 926R) to target the 16S V4 and V5 regions of the rRNA gene (Parada et al. 2016). The PCR cycle used for these samples included denaturation at 95°C for 3 minutes and 25 annealing cycles at 95°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute. Annealing was followed by elongation at 72°C for 5 minutes. After DNA amplification was confirmed with gel electrophoresis and quantified using a Qbit fluorometric quantification, the DNA was purified using an Agencourt AMPure XP Purification Kit. After another Qbit quantification, samples were then diluted to 0.2 ng
DNA μL⁻¹ for PCR amplification with an Illumina barcode sequence. Samples were sequenced with the Illumina MiSeq platform.

**Sequence Processing**

The sequences from the Illumina MiSeq were processed using the DADA2 pipeline for RStudio (Callahan et al. 2016). Forward-only sequences were trimmed to 240 base pair and a maximum error number of 0 and 2 errors, respectively. Forward and reverse sequences were not merged to allow identification of 18S eukaryotes, but had chimeras removed. The RDP reference database (version 18) was used to match the taxonomy of sequences (Cole et al. 2014). The RStudio packages phyloseq, ggplot2, and vegan were used for both graphical and statistical analyses, including PERMANOVA and Spearman’s rank-order correlation (McMurdie and Holmes 2013).

**Results**

*Effluent Composition, Environmental Parameters, and Bioassay Results*

The different treatments of wastewater influent produced effluent with variable DIN and DON content which created different nutrient conditions for the bioassay experiments (Table 1). Regardless of season, NO and NR incubation treatments had the greatest TDN, NO₃⁻, NO₂⁻, DON and PO₄³⁻ content. BNPR and BNCPR incubation treatments had lower DIN and DON concentrations and lower PO₄³⁻ concentrations, leading to more P-limited conditions (Table 1). While BNPR and BNCPR had lower DIN and DON concentrations, DON contributed a greater percentage of the TDN; 95.5% TDN and 92.7%, respectively (Table 1). These incubation studies found that effluent with high DIN content resulted in
phytoplankton growth (NO and NR), and effluent with lower DIN concentrations and high relative DON content had limited incubation growth (BNPR and BNCPR) (Roberts et al. 2020; Table 1). More details about the chemical differences in effluent treatment and the results of the bioassay studies can be found in Roberts et al. (2020).

**Richness and Diversity**

Indices of richness and \( \alpha \)-diversity indicated that the communities incubated with the effluents of minimal treatments (NO and NR) had lower diversity and richness than the those exposed with the effluents treated BNPR and BNCPR as well as Initial York River and the Control (Table 2). The communities incubated with BNPR and BNCPR had similar diversity to the Initial and Control communities in summer, but less diversity in winter (Table 2). The communities with the NO winter treatment had the lowest richness and \( \alpha \)-diversity. ACE and Inverse Simpson indices were also greater during the summer incubations than winter, indicating more diverse community during the warmer months (Table 2).

Both season and WRRF treatment significantly (PERMANOVA, \( p < 0.05 \)) affected the aquatic community’s \( \beta \)-diversity after effluent exposure. A principal coordinate analysis (PCoA) was conducted to represent the \( \beta \)-diversity of the water communities based on the Bray-Curtis calculated dissimilarities among incubation treatments for each season (Figure 2). The first two coordinates represent 61.6% of the variation in communities for both summer and winter. The Initial York River community (Figure 2) clustered separately from the other treatments, including the final Control sample. This may be a result of bottle
effects during the multi-day incubation period (Venrick et al. 1977). Regardless of season, the final community in the Control treatment is closely associated with the communities incubated with the effluents of BNPR and BNCPR. All of which have more similar nutrient profiles (Table 1). The communities of BNPR and BNCPR were clustered closely together during both seasons. Replicates of the communities with the effluents of the NO and NR treatments were more variable. In both summer and winter, NO and NR communities separated from the other treatments. The NO and NR communities were more tightly coupled in winter, while in summer the replicates spread out (Figure 2) indicating that there was a more variable community shift during that experiment.

The dissimilarity of these effluent treatments was further constrained through a canonical analysis of principal coordinates (CAP) plot (Figure 3) that was created using the Bray-Curtis measure and the treatment environmental conditions, including nutrients and temperature. The first two CAP coordinates represent 52.5% of the dissimilarity between samples, regardless of sampling seasons. The two seasons did separate out, with the temperature vector pulling towards the summer samples. Summer sampling generally had higher initial temperature, DOC and Si, which is reflected in the CAP plot (Figure 3). The vectors representing TDN, NO$_3^-$, NO$_2^-$, PO$_4^{3-}$, urea, and bulk DON were associated with the more minimally treated effluent treatments of NO and NR. Ammonium concentration was slightly associated with summer’s highly treated effluent and Controls, however this association is small, as given by the eigenvalue of the NH$_4^+$ vector (Figure 3).
Community Composition and Environmental Parameters

The 16S rRNA genes include prokaryotes and chloroplast of eukaryotic phytoplankton. The relative abundance of the eukaryotic phytoplankton is represented by the class Chloroplast (Figure 4). It is important to note that these relative abundances may be skewed because phytoplankton contains multiple chloroplasts (Flori et al. 2017). No archaea sequences were obtained. The relative abundances for the classes contributing to more than 0.5% of the community are present in Figure 4. There are some discernable differences between the Initial and Control communities, and between the highly and minimally treated effluent communities. The NO and NR communities have greater relative abundances of Alphaproteobacteria, regardless of season (Figure 4). Spearman’s rank-order correlation of Alphaproteobacteria revealed that this class was positively correlated with NO$_3^-$ and NO$_2^-$ in summer and NO$_3^-$, PO$_4^{3-}$, and DON in winter ($p <0.05$, Figure 7 A&B).

More variability in the community composition with effluent exposure is visible at the family-level in winter than summer (Figure 5). The Control, BNPR, and BNCPR communities appear to have more similar composition than NO and NR communities (Figure 5).

In both seasons, eukaryotic phytoplankton (Chloroplast) were found to have the greatest abundance in the NO and NR communities (Figure 6). Spearman’s rank-order correlation Chloroplast abundance was positively correlated with DIN, NO$_3^-$, and NO$_2^-$ ($p <0.05$, Figure 7 A) in summer and DIN and NO$_2^-$ in winter ($p <0.05$, Figure 7 B). The class
Cyanobacteria was also highly abundant in summer, contributing to upwards of 20% of the relative abundance in the Control, BNPR, and BNPCR communities (Figure 6). This was nearly double the relative abundance of the minimally treated effluent. A similar pattern was observed in winter, but more muted with abundances less than 10% (Figure 6). Spearman’s rank-order correlation indicated that Cyanobacteria relative abundance decreased with increasing DIN availability in both summer and winter (p <0.05, Figure 7 A&B).

The family-level comparison illuminates these differences with better classified taxa. Bacillariophyta was an abundant phytoplankton taxon that was found to vary with effluent treated communities (Figure 8). Bacillariophyta (diatoms) had the greatest abundance in NO and NR communities and was found to positively correlate with DIN regardless of season (p <0.05, Figure 9). Cryptomonadaceae, Chlorophyta, and Streptophyta generally decreased in abundance with effluent exposure (Figure 8). For the order Cyanobacteria, GpIIa was the most abundant family and was also found to decrease in abundance with effluent exposure (Figure 8). Spearman’s rank-order correlation revealed that GpIIa was negatively correlated with DIN in summer and winter (p <0.05, Figure 9 A&B).

Other members of the bacterial community were found to vary with effluent treatment at the family-level (Figure 10). Rhodobacteriales was highly abundant (>30%) in the minimally treated effluent regardless of season (Figure 10). Spearman’s rank-order correlation indicated that Rhodobacteriales was positively correlated with DIN in summer and NO₃⁻ in winter (p <0.05, Figure 11 A&B). Many other taxa decreased in abundance
when exposed to NO and NR effluent, including *Comamondaceae, Methylophilaceae, Rubritaleaceae, and Verrucomicrobiaceae* (Figure 10).

*Labile EDON*

Many of the changes in taxon abundance was tied to changes in DIN concentration rather than EDON and its measured constituents (Figures 7, 9, & 12). To further elucidate a possible role of EDON, the % labile EDON calculated by Roberts et al. from the observed DON drawdown was correlated with every identified family (Roberts et al. 2020; data not shown). No families were found to positively correlate with either DON or the calculated % labile EDON (Figures 13 & 14).

*Discussion*

The changes of community composition have previously been reported when waterways acted as receiving waters for wastewater effluent (Saarenheimo et al. 2017; Price et al. 2018; Huo et al. 2017). Yet, most previous studies have targeted the response of natural receiving waters near WRRF outfall sites. As these WRRF facilities consider upgrades to meet changes in water quality standards, increasing attention has been given to both the lability of effluent and changes in phytoplankton production, but not how composition and diversity of aquatic community may change. By incubating water from the York River with effluent from different methods of wastewater treatment, we were able to better elucidate how the communities of receiving waters respond to the introduction of WRRF effluent.
**Background Nutrient Conditions**

It is important to consider that the whole water collected from the York River was P-limited (Redfield DIN:DIP ratio <16) at the start of the incubations and remained throughout the experiments, even with the addition of effluent (Redfield 1958). With low starting concentrations of PO$_4^{3-}$ at the beginning, winter samples treated with BNPR and BNCPR effluents were completely depleted of PO$_4^{3-}$ by the end of the incubation period (Table 1). This P-limitation may have been a contributing factor to the observed shifts in community composition. In winter, PO$_4^{3-}$ positively correlated with the abundance of *Rhodobacteriaceae*, a member of *Alphaproteobacteria* (Figure 11 B). Phosphate also correlated with DIN in summer and may also be a secondary factor for observed changes due to DIN (Figure 7A, 9A, 12A, and 14A). During this study the York River estuary was potentially P-limited based on comparison of calculated DIN:DIP ratios to Redfield stoichiometry (Redfield 1958). The York River typically undergoes periods of N-limitation during the summer and transitions to P-limitation in the winter (Sin et al. 1999; Killberg-Thorseson et al. 2020; Chapter 2, this dissertation). Since the DIN:DIP ratio indicated the York River community was P-limited, it is impossible to know how the phytoplankton community might have shifted if high DIN containing effluent was added during DIN-limited conditions. DIN:DIP conditions should be taken into further consideration if the results of this study are applied to other aquatic systems with different starting nutrient regimes.
The Role of DIN

As effluent was more heavily treated in the SBRs, the availability of DIN, particularly NO$_3^-$, decreased. Both the minimal NO and NR treatments had incredibly high NO$_3^-$ concentrations, and the additions of NO and NR treated effluents to the York River community yielded NO$_3^-$ concentrations reaching upwards of 260 µmol N L$^{-1}$. It is important to note that York River typically has much lower concentrations of NO$_3^-$, <20 µmol N L$^{-1}$ (Chapter 2, this dissertation), but that inland waters can experience quite high concentrations of NO$_3^-$. The Anacostia River in northern Chesapeake Bay has been reported to experience NO$_3^-$ concentrations in excess of 80 µmol N L$^{-1}$ and culture studies whose results are applied to natural systems often use concentrations in the mM range (Solomon et al. 2019; Collos and Harrison 2014; reviewed Subba Rao 2006).

Increased relative abundance of *Bacillariophyta* (diatoms) was observed with increased NO$_3^-$ conditions and Spearman’s rank-order correlation found that this group was associated with the increase in DIN (Figure 9). Research finds that high NO$_3^-$ conditions typically show an increase in larger phytoplankton cells and a shift to diatoms when Si is available (reviewed Glibert et al. 2016). Roberts et al. 2020 did not qualitatively identify phytoplankton cell counts, but chlorophyll *a* concentration was elevated in the samples amended with NO and NR treated effluents. Reduction of Si concentration was also observed in that study (Roberts et al. 2020).

Spearman’s rank-order correlation analysis showed that NO$_3^-$ was an important factor for observed abundance changes of several taxa and it was one of the CAP plot vectors closely
associated with NO and NR treatments (Figure 3). For instance, *Rhodobacterales*, a member of the class *Alphaproteobacteria*, increased in relative abundance for NO and NR treatments, regardless of season (Figure 10). The *Rhodobacterales* family members are known to metabolize NO$_3^-$ in the pathways of assimilation or denitrification (Luque-Almagro et al. 2011; Wawrik et al. 2012). Changes in *Rhodobacterales* abundance could then be considered as a marker for the lability of different effluents in receiving waters. The availability of DIN from differently treated effluent appears to contribute to the community composition changes that were observed with addition of NO and NR effluents.

*The Role of EDON*

As a nutrient point-source that is relatively easy to trace and monitor, the wastewater industry has rigorously developed new treatments so that WRRFs can meet permit nutrient regulations. In the Chesapeake Bay watershed, this industry has managed to reduce N loading input by 57% and has already met the 2025 N reduction goal of the TMDL (EPA, 2016). Still, there have been many lingering questions for researchers, regulators, and WRRF operators about the role of DON in wastewater effluent and if DON should be targeted to removal (Mulholland et al. 2007). In the last two decades, there have been numerous studies attempting to elucidate the potential impact that EDON may have on the environment and if EDON is of concern for WRRFs (Bronk et al. 2010; Filippino et al. 2011; Roberts et al. 2020; Yao et al. 2019; Sattayatewa et al. 2010; and others). Yet, results of these studies have been widely variable, finding that EDON can be anywhere from 2 – 96% labile (Bronk et al. 2010; Filippino et al. 2011).
This work attempted to further clarify the role of effluent DIN and DON by investigating the changes in community composition in the aquatic microbial community. The four effluent treatments investigated had variable TDN and DIN content, as well as relative contributions of DON to the available TDN. In the NO and NR treatments, $\text{NO}_3^-$ rather than EDON made up the bulk of available N with DON contributing 10-19% of the TDN in these treatments. In BNPR and BNCPR treatments, DON was more prevalent contributing upwards of 96% of the TDN, even though the TDN in these treatments were as little as a tenth of the TDN in NO (Table 1).

Even though BNPR and BNCPR had higher relative percent DON, this EDON wasn’t necessarily more labile. By comparing the DOC:DON ratio, Roberts et al. was also able to infer the lability of the EDON from each treatment. The NO and NR treated effluents had DOC:DON ratios of $<6$, which indicates the EDON was labile and hydrophilic (Liu et al. 2012). BNPR and BNCPR treated effluents, in contrast, had a DOC:DON ratio closer to 16 that is likely more similar to humic acids and hydrophobic in nature (Liu et al. 2012). The NO and NR treatments then had both higher $\text{NO}_3^-$ and labile EDON for the aquatic community, while BNPR and BNCPR primarily contained refractory EDON.

Based on the community composition examined in the bioassay experiments, neither EDON concentration or calculated labile EDON was correlated with any of the observed community composition changes, indicating that the impact of EDON may have on receiving waters are minimal when compared with the high DIN content of some effluents. Additionally, the NO and NR treatments had clear shifts in community composition, while
the communities exposed with the effluents of BNPR and BNCPR treatments did not have a significant difference from the Control community (Figure 2). This indicates that further DON removal from these effluent treatments may not be necessary for further reducing eutrophication in coastal waterways when compared to DIN availability in some effluents.

**Conclusions**

The discharge of WRRF effluent into receiving waters can bring not only nutrients, but can lead to changes in the receiving aquatic community and contribute to cultural eutrophication. Improvements have been made in recent decades to increase the nutrient reduction capacity of WRRFs and minimize the impact of effluent on receiving waters. This study found that more heavily treated effluent resulted in aquatic microbial communities that are more similar to those in natural receiving waters, but these differences are tied DIN, not DON content. WRRFs that choose to upgrade from minimal treatment methods to more advanced methods with N and P removal will not only reduce their discharge of DIN and labile EDON, but will likely result with a minimal impact on the aquatic community in its receiving waters. At this time, this work does not support further reduction of DON in effluent. Upgrading WRRFs to processes similar to BNPR and BNCPR may be sufficient to prevent estuarine and coastal eutrophication associated with these point sources.
Acknowledgments

Thank you to the staff at HRSD for setting up and maintaining the SBRs, as well as their assistance during effluent sampling. Thanks to V. Johnson, L. Killberg-Thoreson, X. Yao, Z. Norton, J. Sedlak, M. Epperson, and S. Scharf for assistance with the bioassay experiments and with sample analysis. Thank you to R. Passie and G. Scott for MiSeq sequencing assistance, and to S. Fortin for assistance with R. This research was supported by NSF-CBET Grant #1511120 to D. Bronk, R. Sipler, and C. Bott, NSF-OCE Grant # 1737258 to B. Song and by the Virginia Institute of Marine Science, Office of Academic Studies which provided a student research grant for sequencing analysis.
References


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EPA. 2010. “Chesapeake Bay TMDL Executive Summary.”


Table 1. Adapted from Roberts et al. 2020. Nutrient concentrations from June 2016 and January 2017 bioassays. Starting, final, and consumed concentrations of total dissolved nitrogen (TDN), ammonium (NH$_4^+$), nitrate (NO$_3^-$), nitrite (NO$_2^-$), dissolved organic nitrogen (DON), urea, dissolved primary amines (DPA), phosphate (PO$_4^{3-}$), silica (Si), and dissolved organic carbon (DOC). Values are the average plus or minus the standard deviation. BD indicates below detection. Negative values indicate production. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>TDN $\mu$mol N L$^{-1}$</th>
<th>NH$_4^+$ $\mu$mol N L$^{-1}$</th>
<th>NO$_3^-$ $\mu$mol N L$^{-1}$</th>
<th>NO$_2^-$ $\mu$mol N L$^{-1}$</th>
<th>DON $\mu$mol N L$^{-1}$</th>
<th>Urea $\mu$mol N L$^{-1}$</th>
<th>DPA $\mu$mol N L$^{-1}$</th>
<th>PO$_4^{3-}$ $\mu$mol P L$^{-1}$</th>
<th>Si $\mu$mol L$^{-1}$</th>
<th>DOC $\mu$mol C L$^{-1}$</th>
<th>Chl a $\mu$g Chl a L$^{-1}$</th>
</tr>
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<tr>
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<td></td>
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<tr>
<td>Cont T0</td>
<td>13 ± 1</td>
<td>0.36 ± 0.03</td>
<td>0.40 ± 0.03</td>
<td>0.15 ± 0.01</td>
<td>0.16 ± 0.02</td>
<td>0.2 ± 0.0</td>
<td>0.22 ± 0.06</td>
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<td>25.6 ± 1.3</td>
<td>329 ± 17</td>
<td>5.20 ± 0.16</td>
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<td>0.24</td>
<td>0.00</td>
<td>0.05</td>
<td>0.02</td>
<td>2.7</td>
<td>-16</td>
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<tr>
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<td>0.39 ± 0.02</td>
<td>42 ± 4</td>
<td>0.5 ± 0.0</td>
<td>0.26 ± 0.03</td>
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<td>-0.04</td>
<td>33</td>
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<tr>
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<td>120.3 ± 1.8</td>
<td>0.38 ± 0.00</td>
<td>14 ± 3</td>
<td>0.6 ± 0.0</td>
<td>0.33 ± 0.01</td>
<td>15.6 ± 0.2</td>
<td>40.7 ± 0.6</td>
<td>351 ± 5</td>
<td>13.33 ± 2.33</td>
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<td>49.1</td>
<td>-0.53</td>
<td>-2</td>
<td>-0.1</td>
<td>-0.05</td>
<td>41</td>
<td>35.0 ± 3</td>
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<td>0.67 ± 0.05</td>
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<td>0.17 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>0.6 ± 0.0</td>
<td>0.31 ± 0.01</td>
<td>0.63 ± 0.01</td>
<td>41.3 ± 0.3</td>
<td>395 ± 20</td>
<td>13.33 ± 2.33</td>
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<td>BD</td>
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<td>BD</td>
<td>0.3 ± 0.0</td>
<td>0.23 ± 0.00</td>
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<td>352 ± 9</td>
<td>5.06 ± 0.61</td>
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<td>0.02</td>
<td>0.3</td>
<td>0.08</td>
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<td>43</td>
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<td>BD</td>
<td>16 ± 2</td>
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<td>0.12 ± 0.07</td>
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<td>38.9 ± 0.6</td>
<td>383 ± 71</td>
<td>4.85 ± 0.47</td>
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<tr>
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<td>0.1</td>
<td>0.19</td>
<td>BD</td>
<td>3</td>
<td>0.1</td>
<td>0.09</td>
<td>0.13</td>
<td>0.3 ± 0.1</td>
<td>-8</td>
<td>-8.48</td>
</tr>
<tr>
<td><strong>JANUARY 2017</strong></td>
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<tr>
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<td>0.79 ± 0.09</td>
<td>0.08 ± 0.01</td>
<td>BD</td>
<td>13 ± 2</td>
<td>0.2 ± 0.0</td>
<td>0.19 ± 0.03</td>
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<td>13.7 ± 0.0</td>
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<td>29 ± 3</td>
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<td>0.23 ± 0.04</td>
<td>7.75 ± 0.14</td>
<td>26.5 ± 0.3</td>
<td>294 ± 4</td>
<td>8.07 ± 0.92</td>
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<td>2.75 ± 0.14</td>
<td>26 ± 5</td>
<td>0.4 ± 0.0</td>
<td>0.76 ± 0.14</td>
<td>0.92 ± 0.03</td>
<td>0.62 ± 0.02</td>
<td>379 ± 25</td>
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<td>23.5 ± 6</td>
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<td>BD</td>
<td>19 ± 0</td>
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<td>BD</td>
<td>20 ± 1</td>
<td>0.7 ± 0.0</td>
<td>0.24 ± 0.03</td>
<td>BD</td>
<td>24.9 ± 0.4</td>
<td>325 ± 4</td>
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<td>0.12</td>
<td>BD</td>
<td>-1</td>
<td>0.0</td>
<td>0.08</td>
<td>0.23</td>
<td>-0.3 ± 0</td>
<td>7</td>
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<td>0.53 ± 0.09</td>
<td>0.21 ± 0.02</td>
<td>BD</td>
<td>19 ± 1</td>
<td>0.7 ± 0.0</td>
<td>0.16 ± 0.00</td>
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<td>21.5 ± 0.3</td>
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<td>9.82 ± 0.87</td>
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<td>BD</td>
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<td>0.04</td>
<td>0.13</td>
<td>-3.4 ± 0</td>
<td>6</td>
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Table 2. Number (No.) of sequencing reads, AVS richness indices (ACE), and ASV diversity indices (Inverse Simpson, Shannon) for each incubation treatment. Values are the average of replicates plus or minus one unit of standard deviation. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. Reads Initial</th>
<th>No. Reads Final</th>
<th>ACE</th>
<th>Inverse Simpson</th>
<th>Shannon</th>
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<tr>
<td>Initial</td>
<td>92748 ± 14148</td>
<td>74024 ± 11836</td>
<td>607 ±  37</td>
<td>67.7 ± 5.3</td>
<td>5.1 ± 0.0</td>
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<tr>
<td>Control</td>
<td>91510 ± 25301</td>
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<td>659 ±  90</td>
<td>53.7 ± 5.7</td>
<td>5.1 ± 0.1</td>
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<tr>
<td>NO</td>
<td>108614 ± 46771</td>
<td>87114 ± 26523</td>
<td>384 ± 192</td>
<td>14.0 ± 7.4</td>
<td>3.8 ± 0.8</td>
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<td>74179 ± 24479</td>
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<td>4.1 ± 0.7</td>
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<td>BNPR</td>
<td>89744 ± 50582</td>
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<td>BNCPR</td>
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<td>726 ± 25</td>
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<td>215 ± 11</td>
<td>19.0 ± 3.7</td>
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<td>413 ± 26</td>
<td>21.8 ± 0.4</td>
<td>4.0 ± 0.0</td>
</tr>
<tr>
<td>BNPR</td>
<td>63941 ± 21618</td>
<td>57382 ± 20314</td>
<td>507 ± 89</td>
<td>35.8 ± 0.1</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>BNCPR</td>
<td>63663 ± 24264</td>
<td>57475 ± 22463</td>
<td>502 ± 68</td>
<td>39.8 ± 0.5</td>
<td>4.6 ± 0.0</td>
</tr>
</tbody>
</table>
Figure 1: Experiment design adapted from the illustration in Roberts et al. 2020. The red and black arrow represents the changing nutrient composition in effluent resulting from the different treatment levels. NO, which had the most minimal treatment, produced effluent with an average TDN consisting of 89% DIN. BNCPR, which had the most complex treatment method, produced effluent with lower TDN consisting of only 3% DIN. DIN percentages are averaged from the effluent composition reported for May 2016 and December 2016 (Roberts et al. 2020).
Figure 2: Principal Coordinate Analysis of effluent treated samples created using Bray-Curtis dissimilarity. Sample treatment, including the initial York River sampling, are indicated by color and season is represented by shape. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
Figure 3: Canonical Analysis of Principal (CAP) coordinates of effluent treated samples created using the Bray-Curtis measure and including treatment nutrient conditions. As with Figure 2, colors indicate sample treatment, along with initial sampling. Season is indicated by shape, with summer a circle and winter a triangle. Arrows represent starting environmental conditions for each treatment that are included in the CAP analysis. Chlorophyll $a$ was not included as each treatment started with the same initial community. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
Figure 4: Class-level community composition with relative abundances for classes constituting more than 0.5% of the community for each treatment. Abundances are from merged replicates and are separated by season. Note that the color legend appears in order of appearance from the top left panel of the bar plot. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
Figure 5: Family-level community composition with relative abundances for classes constituting more than 0.5% of the community for each treatment. Abundances are from merged replicates and are separated by season. Note that the color legend appears in order of appearance from the top left panel of the bar plot. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
Figure 6: Relative sequence abundance of important class members (Figure 4) including Chloroplasts (red) and Cyanobacteria (Teal). Note that colors of Panels A-C correspond with Figure 4. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
Figure 7: Spearman’s rank-order correlation ($\rho<0.05$) between important class members and environmental parameters. Panels A and B. give the correlations for Summer and Winter, respectively. Created with the R Hmisc package. Note that Percent_Labile_EDON is the calculated %EDON consumed as calculated by Roberts et al. 2020. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
Figure 8: Family-level classification of class *Chloroplast* and *Cyanobacteria* (Figure 6) with relative sequence abundances >0.5%. *Bacillariophyta*, *Cryptomonadaceae*, *Chlorophyta*, and *Streptophyta* are members of the class *Chloroplast*. *GpIIa* is a member of *Cyanobacteria*. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
A. Summer

B. Winter
Figure 9: Spearman’s rank correlation ($p<0.05$) between important family members of *Chloroplast* and *Cyanobacteria* identified in Figure 8 with environmental parameters. Panels A and B. give the correlations for Summer and Winter, respectively. Created with the R Hmisc package. Note that Percent_Labile_EDON is the calculated %EDON consumed as calculated by Roberts et al. 2020. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
Figure 10: Family-level classification of classes *Alteromondales, Burkholderiales, Nitrosomondales, Rhodobacterales, SAR11, and Verrucomicrobiales* (Figure 6) with relative sequence abundances >0.5% for at least one treatment. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
A. Summer

B. Winter
Figure 11: Spearmen correlation matrices ($p<0.05$) between family-level classification of classes *Alteromonadales, Burkholderiales, Nitrosomondales, Rhodobacterales, SAR11, and Verrucomicrobiales* (Figure 6 and 11) with relative sequence abundances $>0.5\%$ for at least one treatment and environmental parameters. Panels A and B. give the correlations for Summer and Winter, respectively. Created with the R Hmisc package. Note that Percent_Labile_EDON is the calculated %EDON consumed as calculated by Roberts et al. 2020. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
Figure 12: Family-level classification of ASVs correlated with Percent_Labile_DON with relative sequence abundances >0.5% for at least one treatment. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
A. Summer

B. Winter
Figure 13: Spearman’s rank-order correlation ($p<0.1$) between family-level ASVs correlated with Percent_Labile_DON and environmental parameters. Panels A and B. give the correlations for Summer and Winter, respectively. Note the $p$-value cutoff used here is higher than Figures 7,9 and 12. Created with the R Hmisc package. Note that Percent_Labile_EDON is the calculated %EDON consumed as calculated by Roberts et al. 2020. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal.
Figure S1: Principal Coordinate Analysis of winter effluent treated samples created using Bray-Curtis dissimilarity. Sample treatment, including the initial York River sampling, are indicated by color and disinfection treatment is represented by shape. NO – Nitrification Only, NR – Nitrogen Removal, BNPR – Biological Nitrogen and Phosphate Removal, and BNCPR – Biological Nitrogen and Phosphate Removal with Chemical Removal. For disinfection treatments the treatment methods are described in Roberts et al. 2020 and are given as: ND – No Disinfection, UV – Germicidal Ultraviolet, and CL – Chlorination.
CHAPTER 4

Nitrogen Uptake Across the Western Coastal Alaskan Arctic
ABSTRACT

Phytoplankton productivity in the coastal Arctic is expected to increase as temperatures continue to rise and as the number of open water days grow. With this increased productivity, the coastal shelves of the Arctic Ocean may act as a sink for atmospheric carbon. However, this storage is dependent on a sufficient nitrogen (N) supply and yet the current literature on biogeochemical rates of N uptake in this region is severely limited. Here we report the spatial extent and rate at which the aquatic microbial community utilizes inorganic and organic N substrates in the coastal Arctic. Uptake rates of microbial communities (>0.3 μm) were determined at one site in the Bering Sea and at multiple sites in the Chukchi and Beaufort Seas during late summer in 2016 and 2017 using isotopically-labeled ammonium (NH$_4^+$), nitrate (NO$_3^-$), urea, and mixed algal amino acids; rates of NH$_4^+$ regeneration were also measured. Primary production was estimated using isotopically-labeled bicarbonate. Ammonium was found to be the primary form of N supporting late-season primary production. We also found that uptake rates of urea were often greater than those for NO$_3^-$, and that amino acid uptake was wide-spread and consistent regardless of sampling depth or sea. Differing environmental conditions between 2016 and 2017, including sea ice coverage, were likely responsible for the differences observed in estimated NH$_4^+$ recycling rates and primary production. Obtaining these N uptake rates across the late-season Alaskan Arctic is a critical step in establishing the importance of different N sources supporting late-season primary production as the summer growing season elongates in Arctic coastal waters.
Introduction

As our climate continues to rapidly change researchers have come together to both address these challenges and to help identify critical gaps in our scientific understanding of how the environment functions. Global temperatures continue to rise and it is well established that the Arctic ecosystem has undergone dramatic changes, including an increase in water temperature and a decline in seasonal sea ice coverage (Wassmann et al. 2011; Barber et al. 2009; Stroeve et al. 2012; Overland and Wang 2013; Lindsay and Schweiger 2015; Steele et al. 2008). How these environmental changes will impact the biological activity in this region is a critical area of research, yet the spatial and temporal coverage of relevant measurements is lacking (Mannino et al. 2018). Many of these issues are the result of the logistical challenges of reaching the Arctic.

The Arctic is currently warming at nearly twice the global rate and is expected to see temperatures increasing to 4°C above preindustrial averages during this century (Overland et al. 2019). As the temperature rises, the Arctic Ocean is projected to experience sea ice free summers by 2100, possibly much sooner (Jahn 2018; Overland and Wang 2013). The extent of sea ice during the summer has decreased by over 40% in the last 50 years and the remaining ice has thinned, leading to a prolonged open-water season (Ardyna and Arrigo 2020). The reduction in sea ice coverage has resulted in a lengthened growing season for phytoplankton, leading to both earlier and later bloom formation (reviewed Ardyna and Arrigo 2020). The shift from thick multi-year ice to thinner first-year ice has also been shown to allow increased light penetration, which may further prolong the period of heightened phytoplankton growing conditions during
summer months (Arrigo et al. 2012). With both elevated sea surface temperatures and shrinking ice coverage leading to a longer growing season, the net productivity of the Arctic Ocean basin is expected to increase (Arrigo et al. 2008).

In the coastal Arctic, changes in phytoplankton and bacteria growth can have significant impacts on coastal food webs and climate feedbacks (Vincent et al. 2011). For example, primary production is predicted to increase on the coastal shelf (Cai et al. 2010). This increased level of primary production could cause the Arctic Ocean to be a sink for carbon dioxide (CO$_2$), as carbon (C) trapped as biomass is exported to depth (Cai et al. 2010). Analysis of the Arctic Ocean basin from 1998 to 2012 found a 30% increase of annual net primary production and this increase was associated with an elongated open-water season of ideal growing conditions for phytoplankton (Arrigo and van Dijken 2015). The authors of this study also suggested that nutrient availability was an important driver of increased net primary production (Arrigo and van Dijken 2015). Currently, summer productivity is driven by increased light penetration in ice-free regions and fueled by the buildup of nitrate (NO$_3^-$) during the winter months from high rates of nitrification and transport of nutrient-rich Pacific waters (Arrigo et al. 2008; Baer et al. 2017; Baer et al. 2014; Brown et al. 2015). This accumulated NO$_3^-$ is drawn down to almost zero by the end of the summer (Baer et al. 2017; Mills et al. 2018; Brown et al. 2015). With a depleted supply of NO$_3^-$, there may be insufficient nitrogen (N) to sustain the predicted increase in primary production. In 2012, the Chukchi Sea off of western Alaska saw a decrease in primary production that was tied to reduced availability of inorganic nutrients (Yun et al. 2016; Figure 1).
In the western Alaskan Arctic, dissolved inorganic N (DIN) is primarily delivered through the Bering Strait and into the Chukchi Sea. Pacific Winter Water (PWW) transports cold NO$_3^-$-rich waters onto the Chukchi shelf, where this NO$_3^-$ supply helps fuel the spring bloom (Lin et al. 2019; Arrigo et al. 2017; Brown et al. 2015; Lowry et al. 2015). In the Chukchi Sea, PWW has been reported to have NO$_3^-$ concentrations as high as 12.3 µmol N L$^{-1}$ (Lowry et al. 2015). As the PWW is transported further northward, its NO$_3^-$ supply is drawn down by the plankton and microbial communities. By the time this water mass is advected into the Canada Basin and the Beaufort Sea, this NO$_3^-$ supply is near zero and contributes to low DIN conditions in the region (Codispoti et al. 2005; Brown et al. 2015). During the summer months, instead of NO$_3^-$-rich PWW, warmer and nutrient-poor Alaskan Coastal Water (ACW) is advected across the eastern Chukchi shelf in the process of flushing out PWW (Lowry et al. 2015). This may intensify low summer NO$_3^-$ levels.

While transport of Pacific water masses is an important nutrient source, nutrients in the Arctic are also delivered from other sources. During the warmer summer months, many rivers flow into the Arctic Ocean basin delivering nutrients from inland. Other coastal sources of nutrients are submarine and overland discharge where melting permafrost is an important source of inorganic N (Tank et al. 2012; Letscher et al. 2013). In addition to these nutrient sources, sinks for N may intensify low summer NO$_3^-$ levels. For instance, high summer rates of coupled nitrification-denitrification in coastal sediments may act as a N-sink, preventing further flux of ammonium (NH$_4^+$) and NO$_3^-$ into the overlying
surface waters, further supporting summer DIN-limited conditions (Hardison et al. 2017; Brown et al. 2015).

While many of these studies have focused on the transport and availability of NO$_3^-$ (Mills et al. 2018, Lowry et al. 2015; Yun et al. 2016; Sipler et al. 2017a), other sources of N include N-fixation and the release of dissolved organic N (DON). These sources likely play an underappreciated role in this N-limited environment (Tremblay and Gagnon 2009). In recent years, research has indicated that N-fixation does occur in the Arctic region and that diazotrophs are present in the microbial community (Harding et al. 2018; Sipler et al. 2017a; Blais et al. 2012). N-fixation rates have been detected as high as 0.017 µmol N L$^{-1}$ d$^{-1}$ off of Utqiagvik (formerly known as Barrow), Alaska (Sipler et al. 2017a) and 0.029 µmol N L$^{-1}$ d$^{-1}$ in the Bering Sea (Harding et al. 2018). Uptake of DON substrates, such as urea and amino acids, have also been recognized as regionally important (Connelly et al. 2014; Baer et al. 2017; Alonso-Sáez et al. 2012).

DON can be released through biologically-mediated remineralization and excretion processes (reviewed Sipler and Bronk 2015). In the Arctic, DON can also be transported into the region through allochthonous sources such as riverine output and permafrost melt, including during late summer when this region is depleted of NO$_3^-$ (Tank et al. 2012; Connolly et al. 2020; Holmes et al. 2012; McClelland et al. 2014). Concentrations of directly quantifiable DON compounds in the Arctic are low, with concentrations of urea and amino acids typically less than 1 µmol N L$^{-1}$ (Simpson et al. 2008; Dittmar and Kattner 2003). Sinks of DON compounds include the bacterial remineralization and
subsequent uptake by primary producers and the formation of degradation products from ultra-violet photodegradation (reviewed Sipler and Bronk 2015). In the Arctic, up to 43% of DON originating from terrestrial sources may be removed or transformed as it is transported from rivers over the continental shelves (Tank et al. 2012). This may be due to biological uptake or chemical transformations (Tank et al. 2012). Once offshore, this DON has also been shown to be exported to depth (Dittmar 2004). Finally, biological uptake of both inorganic and organic forms of N is an important sink in this region and the primary focus of this research.

Uptake rates in the Arctic illustrate the importance of different N substrates sustaining primary production. The uptake of NH$_4^+$ is greatest in the summer and is often much greater than NO$_3^-$ (Connelly et al. 2014). Of the DON substrates considered bioavailable, urea is the most frequent subject of study (Sipler and Bronk 2015). Past research in the late summer has found that urea can contribute up to 32% of total N absolute uptake in the waters of Alaska and eastern Canada (Harrison et al. 1985; Baer et al. 2017). In the literature, uptake rates of other DON substrates in the Arctic region are less commonly reported. One of the few studies that measured amino acid (AA) uptake off the Alaskan coast, found that during the summer AAs can contribute up to 67.4% of total N absolute uptake for the small (<3 µm) microbial fraction (Baer et al. 2017). While previous studies and modeling of this changing region have focused on inorganic N supporting future primary production, organic sources clearly need further consideration. Obtaining rates of both DIN and DON are required to generate accurate biogeochemical models desperately needed for both Arctic and global models.
The purpose of this study was to quantify N concentrations and uptake rates of a suite of DIN and DON substrates across the western coastal Arctic. By considering the uptake of both inorganic and organic substrates, this paper further investigates what substrates support late-season productivity in the pelagic Alaskan Arctic. In the summers of 2016 and 2017, we conducted a series of stable isotope tracer studies to determine the spatial extent and rates of NH$_4^+$, NO$_3^-$, urea, and mixed AAs uptake and NH$_4^+$ regeneration. Carbon uptake rates were measured for bicarbonate, urea-C, and amino acid-C.

**Materials and Methods**

*Field Sampling*

To assess the different regions of the Alaskan Arctic, samples were collected from the coastal shelves of the Chukchi and Beaufort Seas over two late-summer seasons (sites given in Figure 1). Field sampling took place September 3 – 23, 2016 and July 25 – August 20, 2017. In 2016 (Year 1), all field sampling occurred aboard the R/V Sikuliaq. During 2017 (Year 2), nearshore sites were accessed aboard the R/V Ukpik and the coastal shelf aboard the R/V Sikuliaq. Due to the sampling equipment available aboard each vessel, water sampling methodology varied across vessels, while uptake incubation procedures were the same.

On the R/V Sikuliaq, a CTD rosette with Niskin bottles was used to collect multi-depth samples when appropriate. While not all depths were sampled at each site, with surface (surf) samples collected in the top 4 m. The depth of the chlorophyll max (chl max) was
determined based on chlorophyll profiles obtained from fluorometers attached to the CTD. Chl max depths averaged 28.0 ± 8.2 m in the Chukchi Sea and 52.4 ± 22.5 m in the Beaufort Sea. Deep refers to the deepest sites sampled. In the Chukchi Sea, these samples were within 6 m of the seafloor, while in the Beaufort Sea deep was at the bottom of the euphotic zone, for example at 200 m of a total 3802 m water column (Sipler and Bronk 2021). CTD data were utilized to monitor hydrographic conditions including temperature, salinity, fluorescence and photosynthetically active radiation (PAR).

Onboard the R/V Ukpik, surface water (1 – 4.3 m) was collected with a low-pressure submersible pump (Johnson Pump model # 16004) through acid-rinsed and salt-conditioned tubing. This pump allowed the rapid collection of large water volumes while minimizing water temperature changes. Site conditions were also monitored using a hand held CTD. Once onboard, site-water from both vessels was filtered for ambient nutrients and used to set up uptake incubations.

Background chlorophyll $a$ and nutrient conditions were measured by collecting filters and filtrate. In Year 1, these filtrations were done sequentially passing site water first through the larger Nucleopore Membrane filter and then through the smaller GF-75 filter. In Year 2, these filtrations were conducted in parallel, which separately passed site water through the Nucleopore Membrane and GF-75 filters. This increased the amount of material collected on the smaller GF-75 filter since site water did not pass first through the Nucleopore Membrane filter. Filters were kept frozen at -20°C for later chlorophyll $a$ analysis. Filtrate after the smaller GF-75 filter was retained for nutrient analyses during
both sampling years. Analyses included, ammonium (NH$_4^+$), NO$_3^-$/nitrite (NO$_2^-$), urea, amino acids (as dissolved primary amines), phosphate (PO$_4^{3-}$), silica (SiO$_2$) and dissolved organic carbon (DOC)/total dissolved N (TDN). Nutrient samples for DOC/TDN were aliquoted into acid-washed and muffled EPA vials, while the remaining nutrient samples were transferred to either polypropylene tubes or acid-washed and salt-conditioned high-density polyethylene (HDPE) bottles.

_Uptake Incubations_

Uptake incubation experiments were conducted using site water pre-screened through a 150 µm Nytex mesh into 1 L acid-washed polyethylene terephthalate glycol (PETG) bottles. PETG bottles were filled to the bottle neck, for a total incubation volume of 1.2 L. All substrate incubations were run in triplicate and were inoculated with additions of $^{15}$N-labeled ammonium chloride ($^{15}$NH$_4$Cl; 98.85% $^{15}$N), potassium nitrate (K$^{15}$NO$_3^-$, 98%), dual-labeled $^{15}$N- and $^{13}$C-urea (98%), or mixed algal amino acids, containing 16 $^{15}$N- and $^{13}$C-labeled amino acids (97-99%). Incubations that were inoculated with $^{15}$NH$_4$Cl and K$^{15}$NO$_3$ also received $^{13}$C-labeled bicarbonate (H$^{13}$CO$_3^-$) to measure primary productivity. All isotopic label was purchased from Cambridge Isotope Laboratories, Andover, Massachusetts. At some sampling sites, a full suite of uptake rates was measured (NH$_4^+$, NO$_3^-$, urea, and mixed amino acids). At other sites, measured uptake rates only included NH$_4^+$ and mixed amino acids (AAs) due to sampling time constraints.

Once the isotopic label was added, incubation bottles were enclosed in mesh screens reflecting the measured PAR at respective sampling depths. These incubation bottles
were then placed in an on-deck flow through incubator that was constructed with clear plexiglass and continuously pulled surface water. An incubation period of 24-hours was chosen to provide sufficient time for incorporation of labeled nutrients and in order to normalize changes in light conditions. During both years, all sample sites received a minimum of 12 hours per day of sunlight. In Year 1, daylight ranged from 12 – 15 hours, whereas in Year 2 day light ranged from 17 – 24 hours.

At the end of this 24-hour incubation period, incubations were terminated by vacuum filtration. Samples were filtered through silver membrane filters (nominal pore size 3.0 µm, Sterlitech Corporation) and Whatman™ GF-75 (nominal pore size 0.3 µm, combusted 2 hours at 450°C). As with ambient nutrients filtrations, these filtrations were sequential in Year 1 and parallel in Year 2. Filters were frozen at -20°C for later determination of isotope enrichment. Filtrate collected from the GF-75 filtration was kept for nutrient analysis needed to calculate isotope dilution, as appropriate.

At the end of field sampling each year, all nutrient, chlorophyll and uptake samples were transported frozen to the Virginia Institute of Marine Science (VIMS) for analysis via freeze-safe by air.

*Chlorophyll a Quantification and Nutrient Analyses*

Chlorophyll *a* concentrations were measured immediately upon return to VIMS using a fluorometric method. Filter retained chlorophyll was extracted with 90% acetone overnight and analyzed on a Turner Design Model 10-AU fluorometer (Parsons et al.
1984; Arar and Collins 1997) with a detection limit (DL) of 0.025 µg L$^{-1}$. While filters were collected using both Nucleopore Membrane filters (nominal pore size 3.0 µm) and GF-75 (nominal pore size 0.3 µm), concentrations reported are for the size fraction >0.3 µm. In Year 1, this size fraction was estimated by combining the chlorophyll $a$ concentrations measured on both filters, which were collected sequentially. Since filtrations were done in parallel in Year 2, chlorophyll $a$ concentrations reported are from the GF-75 filters.

Ammonium samples were processed in triplicate using the manual phenol-hypochlorite method with a Shimadzu UV-1800 spectrophotometer (Koreloff 1983). Ammonium sulfate was used as the primary standard and this method had a DL of 0.05 µmol N L$^{-1}$. Samples for NO$_3^-$ and NO$_2^-$ (combined NO$_x$) (DL of 0.03 µmol N L$^{-1}$), PO$_4^{3-}$ (0.03 µmol N L$^{-1}$), and SiO$_2$ (0.11 µmol Si L$^{-1}$) were measured in duplicate on a Lachat QuickChem 8500 autoanalyzer (Parsons et al. 1984). Primary standards used for NO$_3^-$, NO$_2^-$, SiO$_2$, and PO$_4^{3-}$ were potassium nitrate, sodium nitrite, potassium phosphate, and sodium silicofluoride, respectively. Urea concentrations were analyzed manually on a Shimadzu UV-1800 spectrophotometer using a modified diacetyl monoxime method (Price and Harrison 1987) with a DL of 0.10 µmol N L$^{-1}$. Bulk AAs were measured as dissolved primary amines (DPA) using the $o$-phthalldialedhyde method (Parsons et al., 1984), with a DL of 0.025 µmol N L$^{-1}$. Concentrations of DPA and dissolved free amino acids (DFAA) are the same in marine waters where the NH$_4^+$ concentration is not high (Keil and Kirchman 1991). Any further AA concentration referenced below is to the DPA.
measurement. DOC and TDN concentrations were measured on a Shimadzu 5000A TOC-V/TNM (Hansell 1993; Sharp et al. 2004, with analytical accuracy ensured using deep-sea reference water samples from the University of Miami (Hansell 2005). DON concentrations were calculated using the difference between TDN and DIN (summed NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{x}) with standard deviations including the propagation of error (Bronk et al. 2000).

*Nitrogen and Carbon Uptake and Regeneration*

Collected filters were used to measure \textsuperscript{15}N and \textsuperscript{13}C uptake rates using isotope enrichment and particulate concentrations. These filters were thawed and dried overnight at 40°C before analysis on a Sercon Integra2 Mass Spectrometer. Net uptake rates were calculated according to Dugdale and Georing (1967). Assimilation of \textsuperscript{13}C was calculated according to Slawyk et al. (1977). Bicarbonate concentrations were estimated using salinity and the assumption that the water column was saturated with dissolved oxygen (Parsons et al. 1984).

While filters were collected using both Steriltech silver filters (nominal pore size 3.0 µm) and GF-75 (nominal pore size 0.3 µm), rates reported below are for the size fraction >0.3 µm. In Year 1, this size fraction was estimated by combining the rates measured on both filters, which were collected sequentially. Since filtrations were done in parallel in Year 2, rates from only the GF-75 filters are reported.
Filtrate collected at the end of the NH$_4^+$ incubation was used to determine the $^{15}$N atom% enrichment of the remaining NH$_4^+$ pool by using solid-phase extraction (LC-18) columns (Dudek et al. 1986). This measurement allowed calculation of both NH$_4^+$ uptake rates corrected for isotope dilution and NH$_4^+$ regeneration rates (Glibert et al. 1982). Using the $^{15}$N atom% from the final NH$_4^+$ pool resulted in much higher reported rates of NH$_4^+$ uptake. Rates of NH$_4^+$ regeneration were also calculated using the measured atom % values from the NH$_4^+$ pool according to Eq. 1.

\[
r = \ln \left( \frac{\text{atom}\%_{Xs} - \text{atom}\%_{Xf}}{C_i - C_f} \right) \times \left( \frac{C_f - C_i}{\text{time}} \right)
\]  

Where $r$ is the regeneration rate, atom$\%_{Xs}$ is the excess isotope dilution measured and $C$ is the measured substrate concentration, here being NH$_4^+$. The denotations $i$ and $f$ are for the initial and final measurements, respectively.

Data collected from this field sampling and discussed below are available in full at the National Science Foundation Arctic Data Center (Sipler and Bronk 2021).

**Results**

*Ambient Physical Conditions*

Field sampling for this project occurred during two late-summer periods, September 2016 (Year 1) and August 2017 (Year 2). Due to sampling constraints, slightly different regions of the Alaskan Arctic were sampled over the two periods (Figure 1). During Year

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1, September 3 – 23, 2016, sampling occurred aboard the R/V Sikuliaq and all sites were >30 nautical miles (nm) offshore. Year 2 sampling occurred slightly earlier in the year from July 25 – August 20, 2017 and sampling was done on both the R/V Sikuliaq and the R/V Ukpik. The second year differed not only temporally, but also with sampling locations. Use of the R/V Ukpik allowed nearshore waters (<30 nm) to be sampled in the Chukchi Sea; the Beaufort Sea was not revisited in Year 2 (Figure 1). While limited in coverage (n =1, per year), the Bering Sea was visited during both sampling periods.

The salinity of all sites ranged from 26.50 to 33.50. Depth was the largest factor affecting salinity, with surface depths having the lowest salinity (average 29.1) and the deeper depths having the highest salinity (average 32.2; Figure 2). While salinity increased with depth, it was also found to vary between the two years (Figure 3). Salinity in Year 1 ranged from 26.50 to 33.50 and in Year 2, from 28.45 to 32.67 (Figure 3). These differences are most apparent when the surface waters of the Chukchi Sea are considered. In Year 1, there was considerable and anomalous ice cover in the Chukchi Sea (https://www.weather.gov/afc/ice), due to several storm systems transporting ice into the region and leading to lower salinities and temperatures in the surface waters (averaged 28.2 and 1.0°C, respectively) compared to Year 2 (averaged 30.8 and 6.8°C, respectively). This difference between years were also reflected in the chemical and biological parameters collected, including uptake rates and chlorophyll a concentrations (below).
Although limited in coverage, the Bering Sea was much warmer on average (12°C) than both the Chukchi (2.0°C) and the Beaufort (1.2°C) Seas (Figure 2). In Year 1, water column temperatures in the Chukchi and Beaufort Seas ranged from -1.60 to 4.09 °C, while in the more southern Bering Sea, a maximum temperature of 10.30 °C was detected at the site Off Nome. In Year 2, when we sampled earlier in the year and included shallower inshore waters, temperature varied over a greater range, with water column temperatures of -1.55 to 13.60 °C. Generally, sites in Year 2 were warmer than in Year 1, but temperature decreased with depth regardless of Year (Figure 3; Figure 2).

**Chlorophyll a >0.3 μm**

Chlorophyll a (Chl a) was calculated for the >0.3 μm community size fraction, representing the bulk of the phototrophic aquatic community and corresponding with the uptake rate measurements (>0.3 μm only) included below. Year 1 generally had lower concentrations of Chl a than Year 2 (Figures 3). In Year 1, surface water Chl a in the Chukchi Sea was as high as 0.70 μg L⁻¹, whereas in Year 2, the measured Chl a was as high as 3.06 μg L⁻¹. During both periods the highest detected levels of Chl a were in the shallow region known as Hanna Shoal (Figure 1). Samplings at lower depths found greater Chl a concentration at the chl max than the surface, with a maximum detected concentration in the chl max of 4.34 μg L⁻¹ in a region of Chukchi Sea known as Hanna Shoal during Year 2. As will be touched on below, this same region is also where the greatest concentration of AA was observed. Lower Chl a was generally detected in the Beaufort Sea (average 0.28 μg L⁻¹), than the Chukchi Sea (average 0.94 μg L⁻¹).
*Ambient Nutrients*

Throughout the two sampling years, concentrations of inorganic nutrients were found to be low in the surface waters, regardless of sea, with concentrations less than 1 µmol N L$^{-1}$ for NH$_4^+$, NO$_3^-$, and NO$_2^-$ (Figure 4). Concentrations of NH$_4^+$ were detectable at most sites sampled (n=65 out of 75) and ranged from below detection (Beaufort surface) to 3.65 µmol N L$^{-1}$ in the deep waters of the Chukchi Sea. Ammonium concentrations increased with depth in the Chukchi Sea but were less than 1 µmol N L$^{-1}$ in the Beaufort Sea, regardless of depth (Figure 4). Both NO$_3^-$ and NO$_2^-$ were generally undetectable in the surface waters (Figure 4). Nitrate concentrations increased with depth, with the greatest observed concentration of 14.04 µmol N L$^{-1}$ in the Beaufort chl max. Nitrite was only detectable deeper in the water column in the Chukchi Sea, with a greatest observed concentration of 0.43 µmol N L$^{-1}$. Dissolved inorganic concentrations were depleted in surface waters and higher at depth. Similar patterns were observed for PO$_4^{3-}$, which was detectable at all sites, with concentrations ranging from 0.24 to 2.05 µmol N L$^{-1}$.

Calculated DIN:DIP ratios from inorganic nutrients indicate that the Arctic was DIN-limited regardless of depth, sampling season, or sea (Figure 5).

Concentrations of urea and AAs remained low throughout field sampling (Figure 6). Urea ranged from below detection (where) to 0.8 µmol N L$^{-1}$ in the deep waters of the Chukchi Sea. Available urea was generally greater in the Chukchi Sea than the Beaufort Sea, but was highly variable (Figure 6). Concentrations of AA were generally less than 0.3 µmol
N L⁻¹, with the sole exception the observation of 0.60 μmol N L⁻¹ in the surface waters over Hanna Shoal. DON was 96.8% and 93.6% of TDN in Year 1 and Year 2, respectively. The contribution of DON to the total TDN pool decreased with increased water column depth and was greater in the Beaufort Sea than in the Chukchi Sea. Urea and AAs composed only 3.2 and 2.4% of all total DON, respectively.

*N Uptake Rates – All Seas*

The spatial coverage for N absolute uptake rates varied across each sea, depth and substrate (Figure 7). For example, a single surface water uptake experiment was conducted in the Bering Sea, near Nome, AK during each sampling season (total n=2), whereas the Chukchi surface waters had 28 NH₄⁺ incubations. While coverage is variable, there are clear trends in the uptake of different N species. In the Bering Sea, NH₄⁺ absolute uptake rates were the greatest (Figure 7), followed by urea, NO₃⁻, and AAs.

As in the Bering Sea, uptake of NH₄⁺ was generally the highest in the Arctic Ocean, with rates ranging from below detection to 0.0730 μmol N L⁻¹ h⁻¹ (Figure 7). Measured rates were generally higher in the Chukchi Sea than the Beaufort Sea, and uptake rates of NH₄⁺ also decreased as sampling depth increased. Uptake of NO₃⁻ was much lower than NH₄⁺, with the highest NO₃⁻ uptake rate of just 0.0119 μmol N L⁻¹ h⁻¹ detected in the Chukchi chl max. Rates of organic N substrates were also lower than NH₄⁺, but not necessarily lower than NO₃⁻. Sampling of urea uptake (n=20) was less than NO₃⁻ (n=43), but indicated that at times urea could have rates greater than NO₃⁻. Urea may be an important
source of N to the Arctic ecosystem and was found to contribute upwards of 43% of total observed N uptake (Figure 7). Rates of urea uptake in the Arctic Ocean were the greatest in Beaufort Sea surface waters, with rates as great as 0.0082 μmol N L⁻¹ h⁻¹. No deep rates of urea uptake were obtained. Finally, rates of AA were also low compared to NH₄⁺, ranging from below detection to a maximum detected rate of 0.0076 μmol N L⁻¹ h⁻¹ in the surface waters of Hanna Shoal. However, rates of AA were generally consistent, showing minor variation between depths and seas, when compared to other N substrates. Across all sites, AA uptake averaged 0.0024 μmol N L⁻¹ and in the Hanna Shoal region contributed more than of 43% of total observed N uptake when all substrates were measured.

*N Uptake Rates – Chukchi Sea*

The difference in environmental conditions during Year 1 and Year 2 was likely an important driver of observed N rates. Plots of temperature-salinity including water masses as defined by Pickart et al. (2016; Figure 8) show that different water masses were present during sampling periods. During Year 1, when sea ice was present, most surface water collected was Late Season Melt Water (LSMW). In Year 2, the surface water collected was dominated by Alaskan Coastal Water (ACW). Much of the chl max and deep samples for both years were collected from Chukchi Summer Water (CSW) and Remnant Pacific Winter Water (RPWW).

The presence of these water masses was also likely reflected in the observed N rates (Figure 9). The greatest rates of NH₄⁺ uptake was observed in the surface waters
regardless of water mass, with high rates in both ACW and LSMW (Figure 9). For NO₃⁻, the greatest rates were observed at the surface in Year 2 when ACW was present and deeper when RPWW was present. The rates for urea were also greatest at the surface when ACW was present. For these three N substrates, the presence of ACW was associated with high N uptake rates (Figure 9). As with AA rates separated by sea (above), uptake rates of AA were also consistent across the identified water masses (Figure 9).

C Uptake Rates - All Seas and Chukchi Sea

Rates of HCO₃⁻ uptake ranged from 0.0033 µmol C L⁻¹ to 3.19 µmol C L⁻¹ between the surface and deep waters of the Chukchi Sea. The greatest levels of primary production were observed in the Chukchi Sea. The Beaufort was the least productive with uptake rates of less than 0.2 µmol C L⁻¹ (Figure 10).

Taking into consideration water masses in the Chukchi Sea, the greatest uptake of HCO₃⁻ was observed in the surface when ice-free ACW was present. RPWW also at times had high levels of HCO₃⁻ uptake in deep water, however HCO₃⁻ uptake in this water mass was generally low (Figure 11), reflecting low levels of primary production at depth. On August 12, 2017, a single high HCO₃⁻ rate of 0.9574 µmol C L⁻¹ (Figure 11) was observed in the Chukchi deep waters (water column depth 68 m). Winds up to 29.9 knots were reported in this region the day before and may have led to a mixing event (https://www.ncdc.noaa.gov/cdo-web/quickdata).
Uptake of both urea-C and AA-C were observed, though their uptake rates were much lower than HCO$_3^-$, regardless of sea or water mass (Figure 10 and 11). The contribution of urea-C to total C uptake was minimal, but at times, AA-C could contribute up to 2% of total C uptake.

*Ammonium Regeneration*

Rates of NH$_4^+$ regeneration varied from below detection to as high as 0.0843 µmol N L$^{-1}$ h$^{-1}$. Measured rates were highly variable, but were generally higher in the more productive Bering and Chukchi Seas (Figure 12). N recycling of NH$_4^+$ can help supply the N needed to support phytoplankton growth. If the ratio of NH$_4^+$ regeneration to NH$_4^+$ uptake is ~1, regeneration processes are sufficient to supply the N needed to maintain phototrophic processes. The average ratio of NH$_4^+$ regeneration to uptake increased with depth, indicating more NH$_4^+$ release and remineralization with ratios much greater than 1 (Figure 12). When separated by water masses, the greatest rates of NH$_4^+$ regeneration were observed in the LSMW and RPWW, with rates of 0.0843 µmol N L$^{-1}$ and 0.0641 µmol N L$^{-1}$, respectively (Figure 13). CSW and RPWW both averaged regeneration to uptake ratios much greater than 1. As discussed above, in Year 1 LSMW predominated surface waters, while ACW predominated surface waters in Year 2. In these surface waters and water masses, the ratio of NH$_4^+$ regeneration to NH$_4^+$ uptake was greater than 1 in LSMW (average 1.66) and less than 1 in ACW (average 0.63).
Discussion

As the Arctic Ocean warms and the seasons change to include prolonged ice-free summers, one of the critical components needed to create accurate biogeochemical models are rate measurements of biological processes, such as N cycling (Jahn 2018; Mannino et al. 2018). The primary goals of this research were to both increase our understanding of N cycling in the Alaskan Arctic, while also increasing the spatial coverage of N uptake rates during the important late-summer season. In addition to expanding the spatial coverage of these critical biogeochemical rates, this study provides a unique comparison of a late-summer ice-present and ice-free Chukchi Sea. During Year 1, sea ice was present in the eastern Chukchi Sea, resulting in fresher and colder surface waters as this ice melted (Figure 3). This late season meltwater (LSMW) mass was a site of both high rates of NH$_4^+$ regeneration, but also NH$_4^+$ uptake (discussed further below). Previous work with winter and spring ice also found high NH$_4^+$ regeneration rates at the sea ice and water column horizon, which could supply nutrients to the underlying water column community (Baer et al. 2015). The melting of sea ice has also been shown to provide pulses of dissolved organic matter to the surface water community that may then be remineralized to support both primary production and heterotrophic bacterial growth (Niemi et al. 2014; Underwood et al. 2019). The differences between this late-summer ice season and the ice-free Year 2 may be helpful in understanding how prolonged ice-free conditions in the future Arctic may change as LSMW had lower levels of bicarbonate uptake than ACW, indicating that the ice-free conditions in Year 2 had greater levels of primary production (Figure 10).
Ambient Nutrients

Ammonium was detectable for almost all sites sampled during both sampling years even though NH$_4^+$ is often depleted in ocean surface waters due to the tight coupling of NH$_4^+$ uptake and regeneration. The NH$_4^+$ concentrations also increased as sampling moved to depth (Figure 4). This accumulation of NH$_4^+$ in the summer deep waters has been suggested by previous work to be due to a decoupling of N biogeochemical processes of ammonification and nitrification, leading to an accumulation of NH$_4^+$ when nitrification rates are lower (Brown et al. 2015).

Elevated levels of NO$_3^-$ were also observed in the deep water of the Chukchi Sea (Figure 4), where PWW has been attributed as an important source of N for phytoplankton growth (Lowry et al. 2015). Without measurement of nitrification, this elevated NO$_3^-$ cannot solely be attributed to the transport of PWW through this region. Previously reported rates of nitrification in the Chukchi Sea found that rates are low during late-summer (Baer et al. 2017; Christman et al. 2011). Nitrification is also a likely contributor to the accumulation of NO$_2^-$ observed in the Chukchi deep samples (Figure 4). Nitrite is often undetectable because of the coupling between NH$_4^+$ and NO$_2^-$ oxidation in nitrification, however, in other environments, higher temperatures are thought to contribute to a decoupling of these processes (Schaefer and Hollibaugh 2017; Killberg-Thoreson et al. 2020). A previous investigation of the temperature sensitivity of Arctic aquatic nitrification was unable to quantify the first nitrification step to NO$_2^-$ because NO$_2^-$ concentrations remained low (Baer et al. 2014). Nitrite though has been previously
detected in the deep waters of this region and to accumulate in NO$_3^-$ incubation experiments (Brown et al. 2015; Mills et al. 2018).

Concentrations of labile DON compounds, urea and AAs, were generally $< 1$ µmol N L$^{-1}$, as have been reported in previous studies in the region (Baer et al. 2017; Simpson et al. 2008; Connelly et al. 2014; Dittmar and Kattner 2003). Regardless of low background concentrations, urea and AA are an important additional labile source of N in this region. Concentrations of AAs stayed constant throughout the sampling period, which suggests that there is high free AA regeneration in the system (Baer et al. 2017).

During both sampling years, AAs were, at times, the only individually measured N substrates that were detectable. When detectable, these compounds contributed upwards of 6% of the available DON. These substrates may be delivered to offshore waters from the coast or from internal cycling process (Letscher et al. 2013). The vast majority of the DON pool was uncharacterized, but the availability and composition of this DON pool is also likely to change as more terrigenous material is transported as the land warms and tundra thaws (Sipler et al. 2017b). These understudied nutrients also deserve further research attention because of the N-limited conditions in this region (Tremblay and Gagnon 2009). In both years, regardless of depth or sea, the calculated DIN:DIP ratios indicated the Chukchi and Beaufort were DIN-limited when compared to the 16:1 relationship as defined by Redfield (Figure 5; Redfield 1958). The highest DIN:DIP ratio measured during this study was 8.8 in one of the chl max sites in the Beaufort Sea.
Uptake Rates

The N substrate taken up at the greatest rate throughout this study period was NH$_4^+$ (Figure 7). While many studies have found NO$_3^-$ to be the predominant substrate in summer, these NH$_4^+$ rates are consistent with other recent studies in the region (Connelly et al. 2014; Baer et al. 2017). Baer et al. (2017) suggested that this preference of detectable NH$_4^+$, with low rates of NO$_3^-$ uptake, may be due to an inherent metabolic difference in the community present in the western Arctic. This could also be a result of a shift in the aquatic community composition to smaller cells that have lower N requirements during periods of N-limitation (Mills et al. 2018; Lee et al. 2013).

As opposed to NO$_3^-$, urea was the substrate with the next highest average N uptake rates in the Arctic surface waters. Urea uptake in the Arctic is predominately of urea-N, not urea-C. Uptake of urea-C was barely detectable (Figure 10) and previous research in this region only found urea-C to be detectable during late summer, when this study was conducted (Baer et al. 2017). Past research in late summer has also found urea to be an important N source (Harrison et al. 1985; Baer et al. 2017). Whereas Baer found that urea could contribute up to 32% of total N absolute uptake, the current study found that urea could contribute up to 43%. While this study’s contribution of urea was higher, the contribution of AAs was found to be lower (Baer et al. 2017). With organic N substrate contributions to the total measured N uptake nearing half, the contribution of organic N sources clearly needs to be considered when creating biochemochemical models of this changing region.
**Ammonium Regeneration**

An NH$_4^+$ regeneration to uptake ratio $\geq$1 indicates that the rate of NH$_4^+$ release is sufficient to supply the demand for NH$_4^+$. Previous studies in this region in August report an NH$_4^+$ regeneration to uptake ratio $>$ 1 (Baer et al. 2017). During our sampling, however, the ratio was not always $>$1, indicating the NH$_4^+$ regeneration may not be sufficient to NH$_4^+$ demand. The greatest observed rates of NH$_4^+$ regeneration was in the RPWW and LSMW water masses (Figure 13), which both had NH$_4^+$ regeneration to uptake ratios $>$ 1. The RPWW water mass was present near the deep of the Chukchi Sea water column, indicating that the NH$_4^+$ regeneration may be due to either surface material sinking and being remineralized in the water column or from NH$_4^+$ being released from sediment processes that were not a part of this study. These biogeochemical processes may include remineralization or dissimilatory NO$_3^-$ reduction to NH$_4^+$. Sedimentary flux rates for NH$_4^+$ are sparse, but research indicate that these rates in the Chukchi Sea shelf can be as high as 31.3 $\mu$mol N m$^{-2}$ h$^{-1}$, and that these flux into the overlying waters can support coupling between NH$_4^+$ uptake and regeneration (Hardison et al. 2017; Souza et al. 2014; Devol et al. 1997).

In the surface, during ice-covered conditions of Year 1, LSMW was present in the Chukchi Sea surface waters. High rates of NH$_4^+$ regeneration was observed in the LSMW (Figure 13). These rates may have been fueled by any organic matter released by the melting ice (Niemi et al. 2014; Underwood et al. 2019). During Year 2, without sea-ice cover, the predominate surface water mass was the nutrient poor ACW which also had the greatest levels of bicarbonate uptake (Lowry et al. 2015; Figure 11). In the ACW, the
NH$_4^+$ regeneration to uptake ratio was <1 and there was not sufficient NH$_4^+$ recycling to meet the NH$_4^+$ demand (Figures 13 and 14). The ACW water mass was the same water mass that the greatest rates of urea uptake were observed, indicating that more research into DON substrates are needed to understand if and how DON can fulfill this N demand.

**Conclusions**

One of the primary impediments to the creation of accurate biogeochemical models is our lack of direct rate measurements for key biological processes of *in situ* aquatic communities. Today we depend heavily on potential rates provided by culture experiments and the extrapolation from relative few data points and nutrient species. The goal of this research was to enhance our understanding of N cycling within the western Alaskan Arctic through a broader spatial assessment of uptake rates of both DIN and DON. We targeted periods of late season production to assess the roles of these various species and the rate of regeneration to determine what role nutrient recycling plays in supporting late season production. Over two research field periods, with different environmental conditions, we found that uptake of inorganic substrates, particularly NH$_4^+$, is predominant. Uptake of organic substrates, like urea, can exceed NO$_3^-$, but this occurrence is patchy. Amino acids are taken up consistently by the aquatic community, regardless of depth, sea, or our sampling periods. More research is clearly needed into organic N uptake in this region. Sampling conditions in 2016 (Year 1) and 2017 (Year 2), presented the opportunity to observe how the presence of ice-coverage impact N cycling. Regeneration rates obtained with the resulting LSMW water mass indicate that regeneration can be high enough to meet the N demand during sea ice coverage. The
implications of this difference between ice presence must be further explored as the Arctic shifts into ice-free summers.
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Figures

Figure 1. Sample sites in the Alaskan Coast and Arctic Ocean. Sites sampled during Year 1 are given in yellow and sites sampled during Year 2 are given in light blue. Seas are indicated by site shape and the cities of Nome and Utqiagvik (formerly known as Barrow) are indicated with a red star. Map obtained through Google© and mapped using R.
Figure 2. Ambient environmental conditions in sampled seas. Reported chlorophyll $a$ concentrations are from filter extracted chlorophyll representing $\text{>0.3 \, \mu m}$ of the phototrophic community. Sites sampled are indicated by $n=\, x$ indicates the mean, brackets show 1 standard deviation, and circles indicate outliers.
Figure 3. Ambient environmental conditions in the Chukchi Sea separated by sampling years. Reported chlorophyll \( a \) concentrations are from filter extracted chlorophyll representing >0.3 \( \mu m \) of the phototrophic community. Sites sampled are indicated by \( n=\), \( \times \) indicates the mean, brackets show 1 standard deviation, and circles indicate outliers.
Figure 4. Ambient inorganic nutrients: A. ammonium (NH$_4^+$), B. nitrate (NO$_3^-$), C. nitrite (NO$_2^-$), and D. phosphate (PO$_4^{3-}$). Sites sampled are indicated by n=, x indicates the mean, brackets show 1 standard deviation, and circles indicate outliers. Note the variability in y-axis across panels.
Figure 5. Ratio of dissolved inorganic nitrogen (DIN) to dissolved inorganic phosphorus (DIP), calculated based on the sum of DIN species ($\text{NH}_4^+$, $\text{NO}_3^-$, $\text{NO}_2^-$) and phosphate. Sites sampled are indicated by $n=\ldots$, $\times$ indicates the mean, brackets show 1 standard deviation, and circles indicate outliers.
Figure 6. Ambient organic nutrients: A. urea, B. amino acids as dissolved primary amines (DPA), and C. ratio of dissolved organic carbon (DOC) to dissolved organic nitrogen (DON). Sites sampled are indicated by n=, X indicates the mean, brackets show 1 standard deviation, and circles indicate outliers. Note the variability of y-axis across panels.
A. 

NH₄⁺ ρ (nmol N L⁻¹ h⁻¹)

B. 

NO₃⁻ ρ (nmol N L⁻¹ h⁻¹)

C. 

Urea ρ (nmol N L⁻¹ h⁻¹)

D. 

AA ρ (nmol N L⁻¹ h⁻¹)
Figure 7. Absolute (ρ) uptake rates for nitrogen substrates A. NH$_4^+$, B. NO$_3^-$, C. urea and D. amino acids (AA). Reported rates are for total community uptake with Year 1 being sequential filtrations (>0.3 µm and 0.3-3.0 µm fractions combined) and Year 2 being from parallel filtrations. Any Year 1 site, where filters for 0.3 - 3.0 µm without 3.0 µm size fraction that were not processed due to low biomass levels, were left out of Figure 7. These are included in the online repository. Sites sampled are indicated by n=, x indicates the mean, brackets show 1 standard deviation, and circles indicate outliers. The y-axis for Panel A. is four times that of Panels B-D and the red-dashed line illustrates the y-axis change.
Figure 8. Temperature – Salinity plot of sites sampled in the Chukchi Sea. Sampling depth is given by color and sampling year by shape. The size of data points is proportional to the ammonium regeneration to uptake ratio measured at the site. Lines indicate the water mass present in each site. Water masses are as defined in Pickart et al. (2016).
Figure 9. Absolute ($\rho$) uptake rates for N substrates in the Chukchi Sea based on the water mass they were sampled from. Substrates are: A. ammonium ($\text{NH}_4^+$), B. nitrate ($\text{NO}_3^-$), C. urea and D. amino acids (AA). Reported rates are for total community uptake (>0.3 $\mu$m). The y-axis for Panel A. is four times that of Panels B-D and the red-dashed line illustrate the y-axis change. Water masses abbreviations are Alaskan Coastal Water (ACW), Chukchi Summer Water (CSW), Early Season Melt Water (ESMW), Later Season Melt Water (LSMW), and Remnant Pacific Winter Water (RPWW). Sites sampled are indicated by $n=,$ X indicates the mean, brackets show 1 standard deviation, and circles indicate outliers.
Figure 10. Absolute ($\rho$) uptake rates for carbon substrates A. bicarbonate (HCO$_3^-$), B. urea-C, and C. amino acids (AA-C). Reported rates are for total community uptake with Year 1 being sequential filtrations (>0.3 µm and 0.3-3.0 µm fractions combined) and Year 2 being from parallel filtrations. Any Year 1 site, where filters for 0.3 - 3.0 µm without 3.0 µm size fraction that were not processed due to low biomass levels, were left out of Figure 6. Sites sampled are indicated by n=, x indicates the mean, brackets show 1 standard deviation, and circles indicate outliers. The notice the y-axis change between Panels A. and B-C.
Figure 11. Absolute ($\rho$) uptake rates for carbon substrates A. bicarbonate (HCO$_3^-$), B. urea-C, and C. amino acids (AA-C). Reported rates are for total community uptake (>0.3 µm) with rates separated by the water masses present. Water mass abbreviations: Alaskan Coastal Water (ACW), Chukchi Summer Water (CSW), Early Season Melt Water (ESMW), Later Season Melt Water (LSMW), and Remnant Pacific Winter Water (RPWW). Sites sampled are indicated by n=, X indicates the mean, brackets show 1 standard deviation, and circles indicate outliers.
**A. NH₄⁺ Regeneration Rate (nmol N L⁻¹ h⁻¹)**

- Bering
  - n=2, Surf
- Chukchi
  - n=27, Surf
  - n=20, Chl Max
  - n=6, Deep
- Beaufort
  - n=8, Surf
  - n=5, Chl Max
  - n=1, Deep

**B. Regeneration Rate/Uptake Rate**

- Bering
  - n=2, Surf
- Chukchi
  - n=27, Surf
  - n=13, Chl Max
  - n=5, Deep
- Beaufort
  - n=5, Surf
  - n=2, Chl Max
Figure 12. Ammonium (NH$_4^+$) cycling represented via: A. NH$_4^+$ regeneration (regen) rates calculated based on isotope dilution measurements and B. NH$_4^+$ regeneration ratio with NH$_4^+$ uptake rates ($\rho$). S Sites sampled are indicated by n=, X indicates the mean, brackets show 1 standard deviation, and circles indicate outliers. Ratios are only reported for sites where there was enough biomass to obtain both uptake and regeneration rates. NH$_4^+$ regeneration (regen) rates were calculated via isotope dilution measurements.
Figure 13. Ammonium (NH$_4^+$) cycling in the Chukchi Sea represented through A. NH$_4^+$ regeneration (regen) rates calculated based on isotope dilution measurements and B. NH$_4^+$ regeneration ratio with NH$_4^+$ uptake rates ($\rho$). Ratios are only reported for sites where there was enough biomass to obtain both uptake and regeneration rates. Water masses abbreviations are Alaskan Coastal Water (ACW), Chukchi Summer Water (CSW), Early Season Melt Water (ESMW), Later Season Melt Water (LSMW), and Remnant Pacific Winter Water (RPWW). NH$_4^+$ regeneration (regen) rates are calculated based on isotope dilution measurements. Sites sampled are indicated by n=, X indicates the mean, brackets show 1 standard deviation, and circles indicate outliers.
Figure 14: Primary production as measured by bicarbonate uptake and the ammonium regeneration to uptake rate ratio for sites sampled in the Chukchi Sea. Sites water masses are both shape and color-coded. Ratios are only reported for sites where there was enough biomass to obtain both uptake and regeneration rates. Water masses abbreviations are Alaskan Coastal Water (ACW), Chukchi Summer Water (CSW), Early Season Melt Water (ESMW), Later Season Melt Water (LSMW), and Remnant Pacific Winter Water (RPWW). NH$_4^+$ regeneration (rejen) rates are calculated based on isotope dilution measurements.
Figure 15. Ammonium regeneration and ammonium absolute uptake ($\rho$) in the Chukchi Sea separated by sampling year. Panels A-C represent the sampled depths: Surface, Chl Max and Deep, respectively.
CHAPTER 5

Conclusion
Nitrogen (N) cycling forms part of the base for primary and secondary production in aquatic ecosystems, but the sources and the types of N used varies depending on microbial community composition and environmental conditions. The primary goal of this dissertation was to expand the available data on N uptakes to include a wider range of N substrates by considering both DIN and DON in two ecosystems. While DIN was still the form of N taken up at the greatest rates in these studies, uptake rates organic substrates indicate that, at times DON can be an important form of N. However, more research is needed to further clarify the environmental controls of DON uptake.

In Chapter 2 of this dissertation, N uptake was investigated during a period of elevated precipitation. The York River Estuary experienced elevated rainfall in 2018 resulting in higher discharge and elevated background nutrients in the estuary (Bukaveckas et al. 2020). Our study during this period found that DIN had the greatest uptake rates, but during certain periods DON uptake could be considerable. During late summer and fall, urea was found to contribute upwards of 35% of total N uptake (Chapter 3, Figure 3). Rates of NH$_4^+$ regeneration were also lower than measured uptake rates, which indicated that autochthonous production was insufficient to meet the N demand that our rates indicated. Previous regeneration rates in this region obtained during a period of lower precipitation, found that this process was sufficient to support N uptake (Killberg-Thorseson et al. 2020). Finally, this chapter reports NH$_4^+$ release rates from urea for the first time. While this form of NH$_4^+$ release is low compared to NH$_4^+$ uptake, we found that upwards of 22% of the urea-N was released as NH$_4^+$. 

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Chapter 3 of this dissertation further explored N use in the York River Estuary by investigating how the water microbial community responds to the exposure of an anthropogenic N source, wastewater effluent. Four wastewater effluents from different treatments resulted in various conditions of DIN, DON, and phosphate. The greater degree of treatment, the lower the fraction of DIN (NH$_4^+$ and NO$_3^-$). The incubation experiments showed that the water communities with high DIN had substantial changes in composition and diversity while the communities exposed to more heavily treated effluents with lower DIN content had similar composition to the control samples with the York River’s ambient microbial community without effluent amendment (Chapter 4; Figure 2). Changes in abundances of multiple community members were correlated with the availability of DIN in the effluent treatments. However, microbial taxa responding to exposure to effluent DON (EDON) were difficult to ascertain. At this time, this work does not support further reduction of DON in effluent.

Finally, in Chapter 4 of this dissertation, the uptake rates of DIN and DON were measured across the western Alaskan Arctic region. We found that similar to the York River, the DIN substrate NH$_4^+$ had the greatest uptake rates across both the Chukchi and Beaufort Seas. However, DON uptake could be considerable, as urea uptake rates were often greater than those for nitrate (NO$_3^-$) and consisted of up to 43% of total N uptake (Chapter 2, Figure 6). This study also took place over two years with differing sea-ice conditions. During periods of no ice-coverage we found that NH$_4^+$ regeneration rates were not enough to support the measured N uptake rates, which may have implications
for N cycling as this region continues to warm and experience a prolonged ice-free summer (Jahn 2018; Overland and Wang 2013).

Overall, it was found that, as with historical research, DIN is still the predominately utilized substrate (Glibert et al. 2016; Mulholland and Lomas 2008). However, DON should not be ignored from future research as under certain conditions uptake of DON substrates can be quite high, particularly for urea (this work; Lomas et al. 2002; Solomon et al. 2010). Future studies must work at incorporating more DON substrates into their experimental design.
References


VITA

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