# Effects of Freshwater Salinization and Associated Base Cations on Bacterial Ecology and Water Quality

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Crop and Soil Environmental Sciences

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November 30, 2020
Blacksburg, Virginia

Keywords: Freshwater salinization, base cations, fecal indicator bacteria, microbiome, microbial ecology, biogeochemistry, water quality

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ACADEMIC ABSTRACT

Anthropogenic freshwater salinization, which is caused by numerous human activities including agriculture, urbanization, and deicing, impacts an estimated 37% of the contiguous drainage area in the United States. High salt concentrations in brackish and marine environments (~1,500 – 60,000 µS $cm^{-1}$) influence aquatic bacteria. Less is known about the effects of freshwater salt concentrations (≤ 1,500 µS $cm^{-1}$) on bacterial ecology, despite the pervasiveness of freshwater salinization. Bacteria perform many fundamental ecosystem processes (e.g. biogeochemical cycling) and serve as indicators of human health risk from exposure to waterborne pathogens. Thus, to understand how salt pollution affects freshwater ecosystems, there is a critical need to understand how freshwater salinization is impacting bacterial ecology. Using a series of controlled mesocosm experiments, my objectives were to determine how (1) survival of fecal indicator bacteria (FIB), (2) the diversity of native freshwater bacterial communities, and (3) bacterial respiration and nutrient uptake rates responded across a freshwater salinity gradient of different salt profiles.

Survival rates ($t_{90}$) of *Escherichia coli*, the EPA recommended freshwater FIB, increased by over 200% as salinity increased from 30 to 1,500 µS $cm^{-1}$. Survival rates were also significantly higher in water with elevated $Mg^{2+}$ relative to other base cations, suggesting that different salt sources and ion profiles can have varied effects in FIB survival. Thus, freshwater salinization could cause accumulating concentrations of FIB even without increased loading, increasing the risk of bacterial impairment. Diversity of

native bacterial communities also varied across a freshwater salinity gradient, with a general increase in species richness as salinity reached 1,500 µS $cm^{-1}$. Community variability (β-diversity) was greatest at intermediate salinities of 125 – 350 µS $cm^{-1}$ and decreased towards the upper and lower extremes (30 and 1,500 µS $cm^{-1}$, respectively). These diversity patterns suggest that osmotic stress is an environmental filter, but filtering strength is lowest at intermediate salinities causing a change from more deterministic to more stochastic assembly mechanisms. Different salt types also produced distinct bacterial community structures. Lastly, bacterial respiration doubled as salinity increased to 350 – 800 µS $cm^{-1}$, revealing a subsidy-stress response of bacterial respiration across a freshwater salinity gradient. Corresponding changes in nitrogen and phosphorus uptake increased N:P ratios in ambient water, especially in mesocosms with elevated $Ca^{2+}$, which could affect nutrient limitation in salinized streams enriched with $Ca^{2+}$. Bacterial community structure based on Bray-Curtis dissimilarity was not correlated to pairwise changes in respiration rates but was linked to net nitrogen and phosphorus uptake after five days.

Collectively, these results establish that freshwater salinization alters bacterial ecology at the individual population, whole community, and ecosystem process scales. Further, different salt types (e.g., $CaCl_2$, $MgCl_2$, NaCl, KCl, sea salt) had varying effects on bacteria at all levels and should be considered when predicting the effects of salinization on freshwater ecosystems. Developing more nuanced salt management plans that consider not only amount, but different types, of salts in freshwaters could help improve our ability to predict human health risk from waterborne pathogens and mitigate/ reduce salinity-induced impacts to freshwater ecosystem processes and services.

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GENERAL AUDIENCE ABSTRACT

Humans rely on streams, rivers, and lakes for many services including transportation, recreation, food, and clean drinking water. Despite our reliance on freshwater ecosystems, human activity has significantly degraded freshwater resources worldwide. Recently, salt pollution caused by human activity on land, known as freshwater salinization, has emerged as a widespread water quality issue. Numerous human activities including agriculture, urbanization, resource extraction, and deicing have increased freshwater salt concentrations in 37% of the United States' contiguous drainage area. Large changes in salinity (*i.e.* from freshwater to oceanic salinities) are known to affect bacteria that perform many important ecological functions, such as nutrient cycling and water purification, while the effects of smaller changes in salinity more typical within the freshwater range are unknown.

I used controlled laboratory experiments to determine how freshwater salinization affects (1) survival rates of *Escherichia coli*, (2) diversity of native bacterial communities, and (3) bacterial nutrient cycling. My results revealed that freshwater salinization can significantly increase how long *E. coli* survive in freshwater. *E. coli* are used to detect the presence of waterborne pathogens and reduce human health risk. Thus, freshwater salinization might reduce the reliability of *E. coli* as an indicator of waterborne pathogens as well as increase concentrations of bacterial that are potentially harmful to human health in freshwater. Additionally, freshwater salinization affected bacterial diversity by altering the ways in which bacterial communities form. In general,

the number of bacterial species present increased as salinity reached the upper freshwater limit, but communities were most variable at intermediate freshwater salt concentrations. These diversity patterns suggest that different salt concentrations can either cause or reduce stress in bacteria, resulting in significantly different bacterial communities. Lastly, moderate increases in freshwater salt concentrations doubled bacterial respiration and nutrient uptake rates. Bacterial respiration influences how energy flows through ecosystems, and freshwater salinization could potentially alter this process. Different salt types also had different effects of bacterial ecology. Collectively, my results establish that freshwater salinization impacts bacteria at the individual, community, and ecosystem levels.

**Acknowledgements**

I sincerely thank my advisors, Drs. Meredith Steele and Brian Badgley, for their support, advice, and patience through this process. Learning from their expertise and experiences helped my growth as a scientist. I am also grateful to my committee members, Drs. Leigh-Anne Krometis, Bryan Brown, and Erin Hotchkiss, for their sound scientific insight and consistent encouragement. Thanks to everyone at the CRC for your help and camaraderie. A special thank you to Lucas Waller, who showed me the ropes in the lab, went out of his way more than once to fix my problems, and kept me company in Myrtle Beach. Thank you to the entire Global Change Center/ Interfaces of Global Change community. Their continued support, guidance, insight, and inspiration without question made me a better scientist and human being. Thank you Bobbie Niederlehner, Julie Burger, and Athena Tilley, who helped with sample analyses. Lastly, I sincerely thank all of my friends and family who supported me over the last several years. I couldn't have done it without you.

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## 1. Introduction

Human activities have degraded freshwater resources worldwide. According to the United States Environmental Protection Agency (US EPA), 53% of all assessed streams and 71% of all assessed lakes and reservoirs in the United States are either threatened or impaired. There are numerous consequences of degraded freshwater ecosystems, including reduced availability of safe drinking water, decreased biodiversity, contaminated food sources, and exposure to waterborne pathogens. Major causes of impairment include pathogens, sediments, nutrients, hypoxia, and contaminants including PCBs and metals (EPA 2016). More recently, salt pollution has been recognized to cause significant degradation of freshwater resources via freshwater salinization; however, salts are not currently regulated as a pollutant by the US EPA (Corsi et al. 2010, Kaushal 2016, Kaushal et al. 2018, Tyree et al. 2016). Thus, relative to other pollutants, freshwater salinization is an emerging water quality issue with many unknowns. For example, marine salt concentrations, which are orders of magnitude higher than freshwaters and are not considered a pollutant, are known to alter many ecosystem processes. Yet, links between freshwater salt concentrations and ecological processes in streams, rivers, and lakes, have yet to be established.

### 1.1 Freshwater Salinization

Salinization refers to the accumulation of total dissolved solids in water and is a natural process occurring over geologic timescales. Natural, or primary, salinization results from accumulation of rain-associated and terrestrially leached salts in surface waters and occurs predominately in arid and semi-arid regions (Williams 2001a). The degree of primary salinization has historically depended on multiple factors including

watershed characteristics (i.e. endorheic or exorheic basins), glacial cycles, and changes in continental water and sea level (Herbert et al. 2015). Temporal variation of salinity associated with primary salinization tends to occur on time scales of $10^4$ years, although shorter scales of $10^3$ years have been observed (Neukom et al. 2014). Natural salinity regimes are ultimately governed by the balance between evaporation, precipitation, and ion contributions from underlying geology (Williams 2001b).

In contrast to primary salinization, anthropogenic or secondary salinization is human-induced and occurs at much shorter timescales of decades or less (Kaushal et al. 2018). Secondary salinization is the result of multiple, often interrelated, anthropogenically-mediated processes. For example, deforestation for agriculture replaces deeply rooted perennial plants with shallow rooted annual crops. This alteration in plant types reduces the depth of soil profile drying by root systems and increases rainfall infiltration. The combination of these processes causes the water table to rise, dissolving and mobilizing soil-stored salts during the process (Clarke et al. 2002, Ruprecht and Schofield 1991, Wood 1924). Deforestation-related hydrologic shifts result in a saline surficial water table that can increase salinity in surface waters through hyporheic exchange and bank recharge (Taylor and Hoxley 2003). A notable example of these processes is in the Blackwood River in south-western Australia. Extensive clearcutting for agriculture resulted in a salinity increase of 500% in less than a century (Williams 2001b). Return flow from irrigation, which also mobilizes salts from soils, has led to secondary salinization in up to 25% of irrigated land world-wide (Martin-Queller et al. 2010). Other hydrologically-mediated effects have also been observed downstream of

dams due to decreased freshwater inflows from upstream and increased evaporation (Murgulet et al. 2016).

In contrast to processes related to hydrologic shifts, direct application of salts to the landscape can also salinize freshwaters. Salinization from urbanization and the related deicing of roadways has become a significant threat to freshwater resources. In 2018, the United States used ~24.5 million metric tons of deicing salts, an almost a 60-fold increase since the 1940s (Corsi et al. 2010, Survey). Significant portions of road salt, which is mostly NaCl and lesser amounts of magnesium, potassium, and glycol-based salts, is flushed into surface waters during precipitation events (Lakoba et al. 2020). Chloride concentrations in streams in the northwestern United States can be up to 100 times higher than in non-urbanized, forested watersheds (Kaushal et al. 2005). Chloride concentrations in southeastern Wisconsin were measured as high as 11,200 mg/L (30,800 µS/cm), which is ~13 and 50 times higher than the EPA acute and chronic criteria, respectively (Corsi et al. 2010). Although deicing comprises most of the salt production and consumption in the United States, other human actions increase salinity of freshwater as well.

Resource extraction is another major source of secondary salinization. World production of minerals has nearly doubled since 1984 to keep up with demands for energy and goods (Canedo-Arguelles et al. 2017, Reichl et al. 2016). Many resource mining operations produce saline tailings that are either left unconfined or poorly managed and leach into surface waters during precipitation events. For example, extracting one ton of potash produces three tons of tailings rich in NaCl and has been linked to surface and groundwater salinization (Canedo-Arguelles et al. 2017, Otero and Soler 2002). Mountaintop removal coal mining has also been linked to increased salinity

in surrounding streams. The weathering of exposed rock from the mining process, some of which is placed directly in headwater streams known as valley fills, results in elevated concentrations of $HCO_3^-$, $SO_4^{2-}$, $Ca^{2+}$, and $Mg^{2+}$ and increased total salinity (Griffith et al. 2012, Nippgen et al. 2017). Fracking also produces brine enriched with NaCl that can be several times saltier than average ocean salinities; affordable methods of brine disposal have not been developed. As a result, fracking wastewater is often injected back into the ground in regions with porous geology (Konkel 2016).

Many of the processes causing freshwater salinization manifest in multiple changes in water chemistry via coupled geochemical processes. For example, accelerated chemical weathering increases salinity, base cation ($Ca^{2+}$, $Mg^{2+}$, $K^+$, $Na^+$), and dissolved inorganic carbon (DIC) concentrations as well as pH and alkalinity (Kaushal et al. 2017). Road salt application and dissolution of construction materials in urban environments also increases specific base cation concentrations ($Na^+$, $Ca^{2+}$), DIC, and alkalinity, even in non-karst watersheds (Kaushal et al. 2014). These coupled processes have been observed across the United States. In the contiguous United States, salinization and alkalization impact an estimated 37% and 90% of drainage basins, respectively (Kaushal et al. 2018). Further, variable sources of salinization produce distinct, source-specific ion profiles creating resulting in highly variable stream ion chemistry across large geographic areas (Griffith 2014). These coupled processes (i.e. salinization, alkalization, increased pH, and increased base cation concentrations), termed “the freshwater salinization syndrome” (Kaushal et al. 2018), pose a significant threat to freshwater resources.

Water chemistry changes resulting from salinization can alter multiple facets of freshwater ecosystems and resources. For example, salinization has reduced the

availability of safe drinking water in certain regions and increases the burden on water resource infrastructure (Kaushal 2016). Increased salt concentrations have also been linked with the displacement of native flora and fauna by more salt tolerant invasive species, altered organic matter decomposition and stream metabolism rates, shifts in biomass production, changes in nutrient cycling pathways and rates, and contaminant attenuation and transport (Adamek et al. 2016, Arce et al. 2014, Berger et al. 2018, Schäfer et al. 2012, Silva et al. 2000).

### 1.2 Salinization and Bacteria

Aquatic bacteria are consequential in the relationship between salinization and maintenance of freshwater ecosystems. Bacteria mediate many basal ecosystem services (e.g. nutrient cycling, organic matter decomposition, contaminant degradation) and are also used as indicators of waterborne disease risk. Most studies of salinity impacts on bacterial ecology examine changes over large salinity gradients from freshwater to saline or hypersaline (specific conductivity (SC) ~ 60,000 $\mu S\ cm^{-1}$) (Poffenbarger et al. 2011, Wang et al. 2018). In contrast, the effects of low concentrations of salts within the freshwater gradient (SC $\leq$ 1,500 $\mu S\ cm^{-1}$) on bacterial ecology, as well as the effects of different salt types, are understudied. Yet, these knowledge gaps directly influence preserving ecosystem health, water quality, and minimizing the occurrence of waterborne gastrointestinal illness (GI), especially in the face of global freshwater salinization.

#### 1.2.1 Fecal Indicator Bacteria

Pathogen contamination of surface waters is a major concern world-wide. Human contact with waterborne pathogens can cause GI and disease with symptoms including diarrhea, nausea, vomiting, fever, abdominal pain, and in severe cases, death. In the

United States alone, 187,092 stream/river miles failed to meet bacterial water quality standards and the associated economic costs from GI were estimated to be $2.9 billion annually (DeFlorio-Barker et al. 2017, DeFlorio-Barker et al. 2018, EPA 2016). Considering the significant human health and economic burden of waterborne disease world-wide, developing reliable methods for detecting waterborne pathogens and reducing illness is needed. The most common method of monitoring for pathogens, which dates back as early as the late 1800s, is the use of fecal indicator bacteria (FIB) (Odonkor and Ampofo 2013). Fecal indicator bacteria are enteric bacteria including enterococci, fecal coliforms, and *Escherichia coli*, which are excreted into the environment through fecal matter from warm blooded animals. However, while FIB are monitored to assess the risk of pathogens, they are nonpathogenic and serve only as a proxy for water contamination. Thus, there are important assumptions regarding characteristics of an ideal indicator, particularly parity of survival between FIB and pathogens (Bonde 1966).

### 1.2.2 Persistence of Fecal Indicator Bacteria in the Natural Environment

Parity of survival between indicators and pathogens in the environment (Bonde 1966) can be impacted by many environmental variables. Over the last several decades numerous studies have aimed at disentangling the complex relationships between environmental factors, persistence of indicators, and their effectiveness at predicting risk of GI in humans post aquatic recreation. For example, it has been known since the early 1900's that enteric bacteria of public health interest decay rapidly once exposed to natural seawater (ZoBell 1936). As such, the utility of *E. coli* to predict GI is known to be significantly lower in marine systems relative to freshwater, suggesting parity is lost at high salt concentrations and reducing the efficacy of *E. coli* as a FIB in marine

environments (Dufour 1984). Since then, numerous studies have identified environmental factors that influence survival of FIB in the environment – including predation, temperature, pH, nutrients, and salinity – and linked FIB decay rates in natural waters to biotic and abiotic factors.

However, few studies have considered the effects of low salt concentrations on FIB. Coliform bacteria were positively correlated with SC between 95 – 110 µS $cm^{-1}$ in a field survey (McFeters and Stuart 1972). Another field survey in southwestern Virginia found significant, positive correlations between individual base cations ($Ca^{2+}$, $Mg^{2+}$, K) and *E. coli* suggesting that low levels of individual ions may increase *E. coli* persistence in freshwater (Badgley et al. 2019). Specific base cations have been shown to interact directly with bacterial cells influencing many bacterial functions and properties including membrane permeability, ATP activity, and ribosomal stability (Anderson et al. 1975, Fiil and Branton 1969, Gesteland 1966, Xie and Yang 2016). Given that the effects of different ions can differ among bacterial species, there is reason to believe that both salt concentration and ionic composition of stream water can alter FIB ecology and accuracy of water quality assumptions as well as natural bacterial community structure and function.

### 1.2.3 Freshwater Bacterial Community Structure

Despite advances in DNA-based methods for characterizing bacterial communities (e.g. next generation sequencing), less is known about how freshwater microbiomes are structured relative to more commonly studied ecosystem types. In general, stream bacterioplankton communities are dominated by Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, and Verrucomicrobia (Crump et al. 1999,

Liu et al. 2012, Mueller-Spitz et al. 2009, Zwart et al. 2002). Within a single stream channel, bacterial diversity tends to decrease with distance downstream, but the effects of tributaries within the watershed has little influence on regional bacterial diversity (Savio et al. 2015). The proportion of typical freshwater bacteria also increases with distance downstream, which could be explained by decreased inputs of terrestrial taxa farther downstream (Savio et al. 2015, Wisnoski et al. 2019). However, an overall consensus on the composition of stream bacterioplankton communities is lacking despite the importance of stream ecosystem functions and services (Savio et al. 2015).

Interactions between bacterial communities and the environment can significantly alter community structure and emergent community properties (Fierer et al. 2007, Lozupone and Knight 2007, Wintermute and Silver 2010). Similar to more thoroughly studied macroorganism biogeography, microorganisms exist in predictable patterns across the globe (Martiny et al. 2006). It has long been hypothesized that patterns of biogeography are driven by selective pressures of the environment (Baas Becking and Nicolai 1934). In benthic stream bacterial communities, for example, stream water pH and nutrient concentrations can significantly impact bacterial diversity patterns (Fierer et al. 2007). Other variables including concentrations of suspended sediments, temperature, and ecosystem size have also been linked to patterns of aquatic bacterial diversity (Bouvier and del Giorgio 2002, Herlemann et al. 2011, Hiorns et al. 1997, Reche et al. 2007, Wise et al. 1997). However, across multiple ecosystem types (terrestrial and aquatic), salinity may be one of the most important variables influencing bacterial diversity globally (Lozupone and Knight 2007). Freshwater salinization has been linked to reduced bacterial diversity in streams (Bier et al. 2015b, Vander Vorste et al. 2019).

However, the effects of low freshwater salt concentrations on community structure and underlying assembly mechanisms are not well known.

### 1.3 Interactions Among Salinization, Aquatic Bacteria, and Ecosystem Function

Once thought as only passively transporting organic carbon and nutrients from land to sea, we now know the importance of inland waters in global carbon and nutrient cycling (Cole et al. 2007, Seitzinger et al. 2006). Large salinity gradients across brackish and marine ecosystems can alter many biogeochemical concentrations, transformations, pathways, and process (Aitkenhead-Peterson et al. 2009, Steele and Aitkenhead-Peterson 2012). Salinity can cause compositional changes in dissolved organic matter (DOM) as well as impact bacterial respiration rates, ultimately influencing carbon cycling and ecosystem metabolism (Curtis and Adams 1995). Increasing salinity causes DOM flocculation as a result of neutralizing negative surface charges, potentially shifting the proportions of dissolved and particulate organic carbon in aquatic systems (Sholkovitz 1976). DOM flocculation can occur at salinities as low as ~3,000 – 4,000 $\mu S\ cm^{-1}$, resulting in alterations to both organic carbon quantity and quality including a reduction of DOM molecular weight (Asmala et al. 2014). Flocculation at lower salinities depends on the salt profile, where sodium-dominated chemistries dissolve carbon, while calcium salts tend to flocculate colloids (Haynes and Naidu 1998). Salinity-induced shifts in DOM molecular weight can in turn impact bacterial organic matter utilization efficiency, with bacterial growth and respiration tending to decrease as DOM molecular weight decreases (Amon and Benner 1996). Multiple studies suggest that bacterial respiration rates are highest at an intermediate salinity and decrease towards either extreme (Bouvier and del Giorgio 2002, Pakulski et al. 1995).

Phosphorus and nitrogen dynamics are also influenced by large salinity gradients. For example, phosphate is released from terrestrial sediments when they are deposited in marine waters, increasing concentrations of dissolved phosphorus in the water column (Jordan et al. 2008). Release of phosphate is often facilitated by high sulfate concentrations in estuarine environments and can influence whether an ecosystem is phosphorus or nitrogen limited (Fisher et al. 1999). Dissimilatory reduction of nitrate to ammonia and total ammonia fluxes and have been shown to be positively correlated to salinity (Koop-Jakobsen and Giblin 2009; Weston et al. 2010). Correlations between salinity and ammonia fluxes can be in part due to ammonium release from sediments under high ion concentrations in brackish or marine systems (Weston et al. 2010). Anaerobic ammonium oxidation is often negatively associated with salinity as well, which can result in decreased N removal in more saline environments (Koop-Jakobsen and Giblin 2009). However, similar to respiration rates, denitrification rates are highest at intermediate salinities and are then reduced by increasing freshwater and marine salt concentrations (Marks et al. 2016, Pakulski et al. 1995). However, the effects of low salt concentrations on freshwater biogeochemical processes, many of which are mediated by bacterial communities (Battin et al. 2016, Cole et al. 2007, Falkowski et al. 2008), are less understood.

Despite significant efforts to characterize geographic patterns of bacterial diversity, the degree to which bacterial community structure is a useful predictor of changes in biogeochemical cycling is still debated. Microbial ecologists still lack a predictive framework for when microbial membership or community structure can predict changes in ecosystem processes (*i.e.*, changes in fluxes or pools of resources) (Hall et al. 2018). The metabolic pathways that drive many microbial processes (e.g. C mineralization) are phylogenetically broad. Thus, changes in bacterial community

structure are unlikely to predict changes in such fluxes or pools or resources because of functional redundancy in the community. For these processes, other community characteristics including biomass or bacterial nutrient ratios may better predict or constrain estimates of process rates (Elser et al. 1995). Conversely, more phylogenetically constrained pathways (e.g. methanogenesis) may be more tightly coupled to changes in community membership and structure (Judd et al. 2016). However, detecting linkages between community structure changes and process responses is often confounded by differences in response times between structure and process. Typically, changes in microbial processes occur faster than changes in community structure (Ruiz-González et al. 2012). This temporal decoupling of community structure and processes can make it difficult to identify functional linkages. Further, as a result of variability of metabolic states and pools of dormant taxa, the detection of a particular taxon does not necessarily mean that taxon is contributing to microbial processes (Lennon et al. 2018, Wisnoski et al. 2019).

Although linking bacterial community structure to microbial processes has been challenging, studies have identified significant correlations. For example, a meta-analysis of 148 studies that analyzed bacterial community structure and process rates found 96 incidents where there was a significant correlation between structure and function (Bier et al. 2015a). Most of these studies focus on phylogenetically narrow processes and were conducted in terrestrial ecosystems. However, bacterial community composition can affect phylogenetically broad processes as well (e.g. organic matter turnover) when the rate limiting step is biological (Schimel and Schaeffer 2012). Yet, our understanding of when and how bacterial community structure affects both phylogenetically narrow and

broad processes is lacking and warrants further research. Determining how bacterial community structure impacts function in freshwater ecosystems can improve our understanding of how anthropogenic global change, like freshwater salinization, might impact freshwater resources.

### 1.4 Dissertation Research Objectives

The overall goal of this dissertation was to investigate effects of freshwater salinization on aquatic bacterial ecology. Links between salinity-induced alterations in bacterial ecology and water quality/ecosystem processes are lacking, but will help to understand and predict the impacts of freshwater salinization on freshwater resources. The specific knowledge gaps and associated objectives are organized into three chapters:

**Chapter 1**: Freshwater salinization increases survival of *Escherichia coli* and risk of bacterial impairment

Knowledge Gaps: There had not been any studies that directly tested the effects of low salt concentrations on *E. coli* survival in a controlled laboratory setting imitating natural freshwater. Further, no studies had assessed the differential effects of different salt types on *E. coli* survival in freshwater. Therefore, there is a critical knowledge gap connecting salt pollution and bacterial impairments in freshwaters. This gap is especially important considering the global spread of freshwater salinization and published field evidence suggesting that freshwater salinization may increase bacterial impairments

Specific Objectives:

1. Quantify changes in *E. coli* survival across a freshwater salinity gradient ($\leq$ 1,500 $\mu S\ cm^{-1}$)

2. Assess the differential effects of different salt types ($CaCl_2$, $MgCl_2$, NaCl, and KCl) on *E. coli* survival

**Chapter 2**: Effects of freshwater salinization on stream bacterial diversity and assembly mechanisms

Knowledge Gaps: Although it is well known that salinity affects bacterial communities, no research had examined these processes across lower salinity gradients in freshwater systems using a controlled laboratory approach. Further, the specific mechanisms by which salinity alters bacterial diversity were unknown but are important in predicting how freshwater salinization may shape patterns of freshwater bacterial diversity.

Specific Objectives:

1. Determine the effects of freshwater salinization and different salt types on bacterial community structure and diversity.
2. Based on observed patterns of bacterial diversity partitioning across a freshwater salinity gradient, infer potential assembly mechanisms that are driving changes in bacterial community structure.

**Chapter 3**: Subsidy-stress response of bacterial respiration and net nutrient uptake to freshwater salinization

Knowledge Gaps: Most of the knowledge regarding salinity and biogeochemical cycles comes from longitudinal studies in estuarine systems across large salinity gradients. Less is known about the effects of small salinity changes on bacterially mediated processes in freshwater. Further, whether or not bacterial community membership can predict changes in biogeochemical processes and rates is not well known. Linking ecosystem processes to

bacterial community structure in changing ecosystems remains a significant hurdle but is important for advancing our ability to predict how ecosystem disturbances like freshwater salinization will affect key ecosystem processes.

Specific Objectives:

1. Measure the effects of different salt types ($CaCl_2$, $MgCl_2$, NaCl, sea salt) across a freshwater salinity gradient on bacterial respiration rates, net nutrient uptake rates, and resource stoichiometry.
2. Identify whether bacterial community structure is linked to changes in respiration or specific nutrient uptake rates.

Collectively across all three chapters, addressing these knowledge gaps and specific objectives elucidates how freshwater salinization affects freshwater bacterial water quality, patterns of freshwater bacterial diversity, and biogeochemical cycling.

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## 2. Freshwater salinization increases survival of *Escherichia coli* and risk of bacterial impairment

## 2.1 Abstract

Elevated levels of *Escherichia coli (E. coli)* are responsible for more designated freshwater stream impairments than any other contaminant in the United States. Although most strains are non-pathogenic, *E. coli* are enteric bacteria and thus, high concentrations in the environment suggest the presence of enteric pathogens. *E. coli* are intentionally used as a sentinel of fecal contamination for freshwaters because previous research indicates that salt concentrations in brackish or marine waters reduce *E. coli* survival, rendering it a less effective indicator of public health risks. Given increasing evidence of freshwater salinization associated with upland anthropogenic land-use, understanding the effects on fecal indicators is critical; however, changes in *E. coli* survival along the freshwater salinity range ($\leq$ 1,500 µS $cm^{-1}$) have not been previously examined. Through a series of controlled mesocosm experiments, I provide direct evidence that salinization causes *E. coli* survival rates in water to increase at conductivities as low as 350 µS $cm^{-1}$ and peaking at 1,500 µS $cm^{-1}$, revealing a subsidy-stress response across the freshwater-marine continuum. Furthermore, specific base cations affect *E. coli* survival differently, with $Mg^{2+}$ increasing *E. coli* survival rates relative to other chloride salts. Further investigation of the mechanisms by which freshwater salinization increases susceptibility to or exacerbates bacterial water quality impairments is recommended. Addressing salinization with nuanced approaches that consider salt sources and chemistry could assist in prioritizing and addressing bacterial water quality management.

## 2.2 Introduction

Freshwater ecosystem management relies upon the detection of *Escherichia coli* as a sentinel for infectious disease risk (Cabelli 1983, Dufour 1984a, b). An estimated 90 million illnesses annually result from human contact with waterborne pathogens in rivers, streams, and oceans at an estimated cost of $2.2 - $3.7 billion (Deflorio-Barker et al. 2018). Currently, elevated *E. coli* concentrations are the leading cause of freshwater stream impairments in the United States, with 178,579 stream kilometers requiring remediation to meet regulatory standards (USEPA 2016a). In naturally saline estuarine and marine environments, salt concentrations (specific conductance, SC ~1,500 – 60,000 $\mu S\ cm^{-1}$) reduce survival rates of *E. coli* and its efficacy as a pathogen indicator (Anderson et al. 1979, Carlucci and Pramer 1960, Hanes and Fragala 1967). However, human activities including deicing, agriculture, urbanization, and resource extraction are increasing salt concentrations in freshwaters worldwide (Canedo-Arguelles et al. 2017, Clarke et al. 2002, Corsi et al. 2010, Kaushal et al. 2005, Martin-Queller et al. 2010, Otero and Soler 2002, Steele and Aitkenhead-Peterson 2012). This phenomenon, known as freshwater salinization, affects an estimated 37% of the contiguous drainage area in the United States (Kaushal et al. 2018). Freshwater salinization can decrease the survival of benthic macroinvertebrates and change macro-biotic communities (Kefford et al. 2003, Szocs et al. 2014, Timpano et al. 2018); however, freshwater salinization had not previously been linked to the survival of *E. coli* or bacterial impairment designations.

Previous studies systematically assessed *E. coli* survival along salinity ranges that encompass brackish and marine concentrations but ignored possible effects of salinization within the freshwater range, which can be orders of magnitude lower in concentration (Anderson et al. 1979, Carlucci and Pramer 1960, Faust et al. 1975, Hanes

and Fragala 1967, Roper and Marshall 2009). Starting in the 1960s, concerns about pathogen exposure at beaches and shellfish waters (i.e. naturally high saline waters) near municipal sewage discharges motivated research on the effects of salinity on *E. coli* survival. These studies concluded that *E. coli* survival decreases rapidly as salinity increases from brackish to marine concentrations. For example, Carlucci and Pramer (1960) found that the percent survival of *E. coli* after 48 hours decreased from 74.5% in brackish to 8.2% in full strength seawater. Anderson et al. (1979) further supported these conclusions, reporting a decrease in percent survival from 53.5% to 2% as salinity increased from 10 to 30 ppt. The parity of survival between *E. coli* and human pathogens, a key assumption for using fecal indicator bacteria (FIB) to track health risks (Bonde 1966), diminishes at high salinities and renders *E. coli* a less effective indicator of waterborne pathogens in marine environments. In a meta-analysis comparing eight marine studies and nine freshwater studies, the strength of correlation between *E. coli* and gastrointestinal illness decreased from 80.4% in freshwater to 52% in marine waters (Dufour 1984a). Consequently, when the EPA updated bacterial water quality standards in 1986, *E. coli* was recommended for use as an indicator in freshwater only, while enterococci were recommended for both marine and freshwaters (USEPA 1986). It is now widely accepted that *E. coli* is salt intolerant; however, effects of different salt concentrations within the freshwater range ($\leq$ 1,500 µS $cm^{-1}$), where *E. coli* is regularly used to determine impairment and health risks, had not been previously investigated.

In addition to salinity effects along the freshwater range, the direct effects of different salt types and specific ions on *E. coli* survival rates are understudied. Positive correlations between concentrations of *E. coli*, total coliforms, and $Ca^{2+}$, $Mg^{2+}$, and $K^{+}$

across multiple watersheds with varying land use and geology were recently reported, indicating that salinization, and specifically certain base cations, may prolong the survival of FIB in freshwater (Badgley et al. 2019). If generalizable, these effects would have important implications for water quality management of different watersheds because the base cation composition of freshwater differs with watershed land-use, as well as the geology and climate. For example, streams draining agricultural land tend to have elevated $K^+$, $Ca^{2+}$, and $Mg^{2+}$ concentrations from fertilizers and liming applications (Fitzpatrick et al. 2007, Kaushal et al. 2018, Tripler et al. 2006). Regions with underlying karst geology tend to enrich water in $Ca^{2+}$ and $Mg^{2+}$ (Han and Liu 2004). Coastal streams have elevated $Na^+$ from sea salt (Soulsby 1995), while urban streams tend to have elevated $Na^+$ and $Ca^{2+}$ concentrations from deicing salts and concrete dissolution, respectively (Corsi et al. 2010, Kaushal et al. 2017, Kaushal et al. 2014). If salt type or specific ions directly and differentially change *E. coli* survival rates, specific regions may be more susceptible to salinization-induced degradation of bacterial water quality. Therefore, identifying how total salts and different base cations impact *E. coli* within the freshwater realm is critical for improving water quality management efforts.

Here, my goal was to determine if freshwater salinization, as well as different salt types driving salinization, affect *E. coli* survival rates. In two laboratory stream water mesocosm experiments, I experimentally manipulated salinity, measured as SC, and salt types to determine if *E. coli* survival was affected by: 1) increasing salinity of two salt profiles with base cations characteristic of freshwater salinization – a mixture of Ca, Mg, and K chloride salts (hereafter Ca:Mg:K) and NaCl – across the freshwater range (≤1,500 $\mu S\ cm^{-1}$), or 2) four different individual chloride salts ($CaCl_2$, $MgCl_2$, NaCl, and KCl)

held at a constant SC. For each, a known amount of cultured *E. coli* was added, and viable concentrations were measured over time using standard methods for *E. coli* detection. I quantified *E. coli* survival rates by determining the amount of time required for *E. coli* concentrations to decrease by 90% ($t_{90}$).

## 2.3 Materials and Methods

### 2.3.1 Mesocosm design and water sampling

Water for laboratory incubations was collected directly in sterilized mesocosms from a stream draining a 100% forested watershed located in the Jefferson National Forest of southwestern Virginia. This source water was selected specifically for the absence of upland anthropogenic influence and naturally low background concentrations of base cations and low SC. Prior to experimentation, the sample stream was characterized by collecting multiple water samples over a four-month period and measuring SC using an Orion Star A223 portable meter (Thermo Fisher Scientific) and concentrations of Ca, Mg, and K using inductively coupled plasma mass spectrometry to confirm the consistency of background SC and concentrations and ratios of base cations (Table 2.1). Mesocosms were constructed from 1-L HDPE bottles for the first experiment because a large number of mesocosms was required (27) to test all treatments and space was limited. For the second experiment, 15-L HDPE buckets were used because fewer mesocosms were required (15) and thus space was not limited. All mesocosms for both experiments were equipped with an aeration system to maintain oxygen levels and homogeneously mix the water throughout the incubation period. Mesocosms were immediately returned to the lab and incubations were started the day of water collection.

**Table 2.1.** Water chemistry at the sample stream in southwestern Virginia (37° 16' 53.8176" N, 80° 27' 56.6028" W).

| Base Cation | Concentration (mg $L^{-1}$) | n (samples) |
|---|---|---|
| $Ca^{2+}$ | 2.91 ± 0.35 | 9 |
| $Mg^{2+}$ | 1.53 ± 0.33 | 9 |
| $K^{+}$ | 1.21 ± 0.27 | 9 |
| **Specific Conductance (µS $cm^{-1}$)** | 31.47 ± 9.96 | 12 |

### 2.3.2 Experimental design

To determine the effects of increasing salt concentrations on *E. coli* survival in freshwater, two salt treatments were tested: 1) NaCl and 2) a mix of Ca, Mg, and K chloride salts at a Ca: Mg: K ratio of 2.4: 1.3: 1, based on ion ratios measured from the source stream (Table 2.1). These salt treatments were chosen because their base cation compositions are characteristic of two major types of salinization: 1) deicing and sea water (Na) and 2) surface mining, agriculture, and accelerated weathering (Ca:Mg:K). Salts for each treatment were added to triplicate 1 L stream water mesocosms to target SCs of 125, 350, 800, and 1,500 µS $cm^{-1}$, which span the range of freshwater SCs commonly observed in the United States (Griffith 2014b). A no-salt reference containing only ambient stream water with a SC of ~30 µS $cm^{-1}$ (Table 2.1) was also tested in triplicate resulting in a total of 27 individual mesocosms (2 salt treatments, 4 SCs, and the reference). Because evaporation during the experiment caused small increases SC (CV ≤ 16%; Fig 1C and Table 1S), the average of the initial and final SC in each mesocosm were used in analyses. However, for clarity, SC treatments are hereafter referred to by the target values (i.e. 125, 350, 800, 1500 µS $cm^{-1}$).

To test the effects of individual base cations on *E. coli* survival, a second set of experiments with four treatments including Ca, Mg, K, Na, and a no-salt control were tested in triplicate using chloride salts. New mesocosms were constructed from 15 L buckets as described previously and each was spiked with a single cation to a constant SC of 350 µS $cm^{-1}$. This strategy was designed to isolate differential effects of individual base cations while keeping SC constant. Evaporation caused SC to increase over time, but average SC did not differ significantly among base cation treatments (Table 3S). A SC of 350 µS $cm^{-1}$ was selected because it is within the critical range of biological effect concentrations of other stream health metrics including benthic macroinvertebrate indices (Bernhardt et al. 2012, Timpano et al. 2015).

### 2.3.3 *E. coli* culturing and inoculation

Methods for *E. coli* culturing, inoculation, and sampling were consistent between the two experiments. *E. coli* K12 was grown from laboratory cultures in LB broth at 37°C for 16h and gently pelleted via centrifugation at 2,000 rpm for 5 min. Pelleted cells were resuspended and washed with stream water then directly added to mesocosms at a final concentration of ~ $10^5$ cells $mL^{-1}$. After *E. coli* inoculation, time series samples were collected directly from mesocosms using a pipette or syringe depending on required water volume and *E. coli* concentrations were estimated using the Colilert Defined Substrate Test reagents in Quanti-Tray/2000 (IDEXX Laboratories, Inc.; Maine, USA) (EPA Method 9223 B-2004 Colilert®). *E. coli* concentrations are reported as most probable number (MPN) 100 $mL^{-1}$. Sampling times were determined based on *E. coli* concentrations from the previous timepoint and mesocosms were sampled seven times

over 16-17 days. SC and pH were monitored throughout the experiment using an Orion Star A223 portable meter (Thermo Fisher Scientific) (Tables 1S and 3S).

### 2.3.4. Data processing and statistical analyses

Decay constants (k) were calculated assuming first order decay kinetics using the equation $C = C_0e^{-kt}$ where C(t) is the $\log_{10}$ transformed *E. coli* concentration at any given sampling time, $C_0$ is the $\log_{10}$ transformed initial *E. coli* concentration, and k is the decay constant. Therefore, k was determined as the slope of $\log_{10}$ transformed *E. coli* concentrations at each sampling time point. The amount of time it takes for 90% of *E. coli* to decay ($t_{90}$) was derived by taking the inverse of k (1/k). Decay rates ($t_{90}$) across the SC gradient for NaCl and Ca:Mg:K treatments were fit to a logarithmic model to determine the relationship between SC and *E. coli* survival. An ANCOVA test was used to determine differences among treatments. Model statistics are in Table 2S. For the constant SC experiment, a one-way ANOVA and Tukey's HSD test were used to determine significant differences in *E. coli* survival ($t_{90}$) among different base cations at a constant SC. Summary Statistics are in Table 2S. Statistical significance is reported at $p < 0.05$ for all pairwise comparisons. Combined $t_{90}$ data from other studies presented in Figure 3 were fit with a Loess regression to highlight the subsidy-stress response across the freshwater-marine continuum. Statistical analyses were performed in R (version 1.2.5042) (R Core Team 2019).

## 2.4 Results

### 2.4.1 Effects of increased salinity of *E. coli* survival rates

I found that higher concentrations of *E. coli* persisted in the water as salinity increased for both the NaCl and Ca:Mg:K treatments, indicating salinity prolonged *E.*

*coli* survival (Figure 2.1, A and B). *E. coli* survival rates, quantified via $t_{90}$, increased logarithmically along the freshwater salinity gradient for both salt treatments. The largest increases in *E. coli* survival rates occurred across the lowest SC range of 15 to 350 µS $cm^{-1}$ (Figure 2.1C and Table 2.2, model statistics in Table 2.3). For NaCl, $t_{90}$ increased from 9.1 ± 1.2 d in reference (minimal salinity) stream water to 24.3 ± 10.0 d at 1,500 µS cm-1, an increase of 167%. The increase was similarly dramatic for the Ca:Mg:K treatment, with a $t_{90}$ of 28.2 ± 9.5 d at 1,500 µS $cm^{-1}$, indicative of an increase of 210% relative to reference stream water. There was no significant difference in the relationship between salinity and $t_{90}$ between the NaCl and Ca:Mg:K treatments (ANCOVA, $F_{1,27}$ = 0.683, p = 0.308). Cumulatively, these results establish that increasing salinity in the freshwater range will prolong the survival of *E. coli* in water regardless of the primary ionic constituents.

### 2.4.2 Effects of salt type on *E. coli* survival rates

*E. coli* survival rates also varied among chloride salts containing different base cations at a constant salinity (ANOVA: $F_{3,8}$ = 5.761, p = 0.0213). Specifically, *E. coli* in mesocosms with $Mg^{2+}$ survived significantly longer than *E. coli* in mesocosms with $Ca^{2+}$ or $Na^{+}$ (Tukey's HSD Test, both p<0.05) (Figure 2.2, A and B: Table 2.3). When exposed to elevated concentrations of $Mg^{2+}$, $t_{90}$ was 14.1 ± 3.4 d, whereas $t_{90}$ was 6.3 ± 2.8 d, 9.5 ± 1.9 d, and 7.1 ± 1.4 d for $Ca^{2+}$, $K^{+}$, and $Na^{+}$, respectively (Table 2.4). These results suggest that during salinization, changes in *E. coli* persistence in natural waters further depend on salt type.

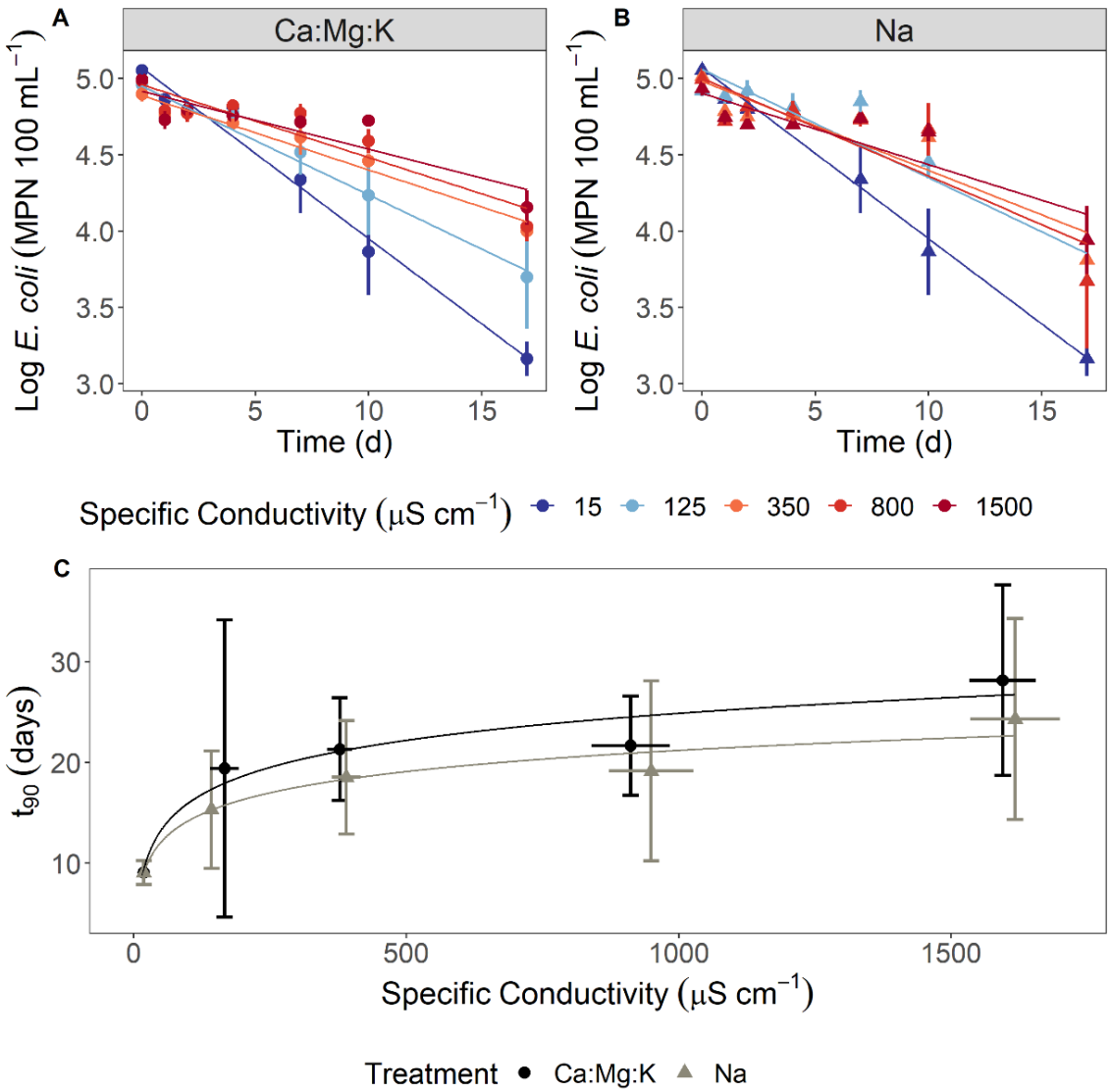


**Figure. 2.1**. A freshwater salinity gradient with two distinct salt compositions increase *E. coli* survival over time. Concentrations of *E. coli* over time exposed to a mixture of Ca, Mg, and K chloride salts (Ca:Mg:K) (A) and NaCl (B). Derived $t_{90}$ values of *E. coli* (C) exposed to both salt treatments across the freshwater conductivity gradient. Data for each treatment were fit via logarithmic regression (Ca:Mg:K – $r^2 = 0.383$, $p = 0.00823$; NaCl – $r^2 = 0.354$, $p = 0.0114$). Error bars represent +/- 1 standard deviation. Horizontal error bars represent the variability of average conductivity values across triplicate mesocosms because of evaporation during the incubation.

**Table 2.2.** Mean decay constant (K) and $t_{90}$ values ± 1 standard deviation for conductivity gradient experiments. Average Conductivity is the average of the initial and final conductivity accounting for evaporation-induced increases in salt concentrations that occurred over the course of the experiment. Standard deviation of Average Conductivity represents variability across triplicate mesocosms, not conductivity range. Average pH ± 1 standard deviation is also provided.

| Treatment | Target Conductivity (µs $Cm^{-1}$) | Average Conductivity (µs $Cm^{-1}$) ± 1 Sd | pH | K ($D^{-1}$) | $T_{90}$ (D) |
|---|---|---|---|---|---|
| **Reference** | 15 | 18.4 ± 3.0 | 6.97 ± 0.02 | 0.11 ± 0.02 | 9.1 ± 1.2 |
| **Ca:Mg:K** | 125 | 166.4 ± 26.3 | 6.52 ± 0.16 | 0.07 ± 0.04 | 19.4 ± 14.7 |
| | 350 | 377.9 ± 23.2 | 6.69 ± 0.11 | 0.05 ± 0.01 | 21.3 ± 5.1 |
| | 800 | 912.1 ± 72.1 | 6.46 ± 0.32 | 0.05 ± 0.01 | 21.7 ± 4.9 |
| | 1,500 | 1595 ± 60.6 | 6.75 ± 0.07 | 0.04 ± 0.01 | 28.2 ± 9.5 |
| **Na** | 125 | 142.5 ± 8.9 | 6.58 ± 0.42 | 0.07 ± 0.02 | 15.3 ± 5.8 |
| | 350 | 389.9 ± 26.6 | 6.83 ± 0.14 | 0.06 ± 0.04 | 18.5 ± 5.6 |
| | 800 | 949.7 ± 77.0 | 6.84 ± 0.15 | 0.06 ± 0.04 | 19.1 ± 8.9 |
| | 1,500 | 1618 ± 82.3 | 6.88 ± 0.01 | 0.05 ± 0.02 | 24.3 ± 10.0 |

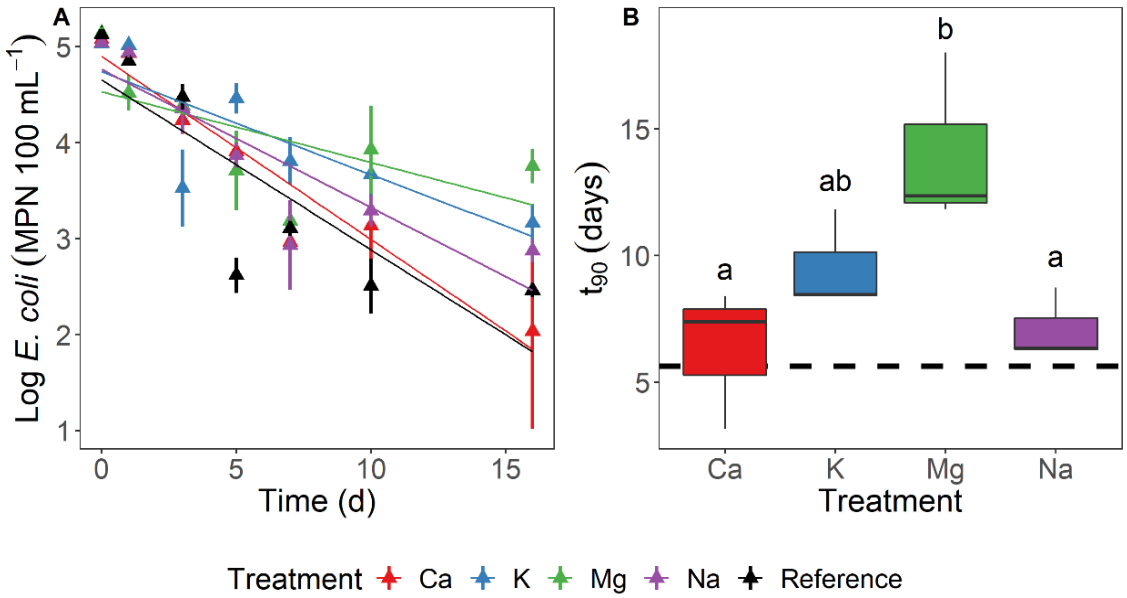


**Figure 2.2**. Individual base cations at the same conductivity influence *E. coli* survival rates. Panel A shows concentrations of *E. coli* over time exposed to individual base cations at a conductivity of 350 µS $cm^{-1}$. Panel B shows derived $t_{90}$ values of *E. coli* for each base cation treatment. The black dotted line in panel B represents the average $t_{90}$ value of E. coli in reference stream water that received no salt treatment (specific conductivity ~ 30 µS $cm^{-1}$). Contrasts are significant at $p<0.05$.

## 2.5. Discussion

### 2.5.1 Revising the salinity-*E. coli* paradigm

In contrast to the prevailing paradigm, I observed increased *E. coli* survival rates along a salinity gradient within the freshwater range (≤ 1,500 µS $cm^{-1}$). Previously, increased salinity was assumed to decrease *E. coli* survival rates in aquatic ecosystems, though this assumption was solely the result of studies targeting the effects of brackish and marine salinities (~15,000 – 75,000 µS $cm^{-1}$) on *E. coli* survival (Anderson et al. 1979, Carlucci and Pramer 1960, Faust et al. 1975, Hanes and Fragala 1967, Roper and Marshall 2009). Observations along a freshwater salinity range reveal a subsidy-stress gradient that suggests that the optimal salinity for *E. coli* survival occurs at concentrations near the transition between freshwater and brackish salinities (Odum et al. 1979) (Fig. 2.3). Based on this concept, my results suggest that increases in dissolved salts within the freshwater range, as seen during freshwater salinization, constitute a usable resource for *E. coli* resulting in higher survival rates. However, based on my projection from combined data in Figure 2.3, once salinity reaches brackish concentrations between 1,500 and 15,000 µS $cm^{-1}$, additional increases in salinity would result in an exponential decrease in *E. coli* survival rates in keeping with previous work in marine waters. The subsidy-stress model is commonly observed in the natural environment but had not been demonstrated between *E. coli* and salinity (Biggs et al. 2002, King and Richardson 2007, Niyogi et al. 2007). The potential implications of this revised understanding are significant for water quality managers tasked with addressing water quality impairments due to elevated levels of indicator bacteria.

**Table 2.3.** Summary Statistics for logarithmic models, ANCOVA, one-way ANOVA, and Tukey's HSD Tests. Significant p-values are bolded.

| Test/ Model | Variable | F/ T | P | Df | $R^2$ | Coefficient (Log Conductivity) | Intercept |
|---|---|---|---|---|---|---|---|
| **Gradient** | | | | | | | |
| Logarithmic model | Ca:Mg:K | 3.11 | **0.0083** | 1,13 | 0.38 | 3.86 | -1.81 |
| | NaCl | 2.94 | **0.011** | 1,13 | 0.35 | 3.03 | 0.26 |
| ANCOVA | Log (conductivity) | 18.25 | **0.0002** | 1 | - | - | - |
| | Salt Treatment | 1.05 | 0.31 | 1 | - | - | - |
| | Residuals | | | 27 | | | |
| **Constant Conductivity** | | | | | | | |
| ANOVA | Salt Treatment | 5.76 | **0.021** | 3,8 | - | - | - |
| Tukey's HSD | K vs Ca | 1.58 | 0.44 | - | - | - | - |
| | Mg vs Ca | 3.78 | **0.023** | - | - | - | - |
| | Na vs Ca | 0.39 | 0.98 | - | - | - | - |
| | Mg vs K | 2.20 | 0.20 | - | - | - | - |
| | Na vs K | -1.19 | 0.65 | - | - | - | - |
| | Na vs Mg | -3.38 | **0.039** | - | - | - | - |

**Table 2.4.** Mean decay constant (K) and $t_{90}$ values +/- 1 standard deviation for the constant conductivity experiment. Average Conductivity represents the average of initial and final conductivity for each mesocosm accounting for evaporation during the incubation. Standard deviation is the variability across triplicate mesocosms.

| Treatment | Average Conductivity ($\mu S\ cm^{-1}$) ± 1 SD | pH | K ($d^{-1}$) | $t_{90}$ (d) |
|---|---|---|---|---|
| **Reference** | 21.6 ± 0.6 | 7.23 ± 0.03 | 0.18 ± 0.01 | 5.7 ± 0.3 |
| **Ca** | 359.4 ± 1.2 | 7.23 ± 0.07 | 0.19 ± 0.11 | 6.3 ± 2.8 |
| **K** | 361.0 ± 1.2 | 7.26 ± 0.07 | 0.11 ± 0.02 | 9.6 ± 2.0 |
| **Mg** | 359.1 ± 0.1 | 7.21 ± 0.02 | 0.07 ± 0.02 | 14.1 ± 3.4 |
| **Na** | 360.0 ± 1.6 | 7.17 ± 0.01 | 0.14 ± 0.03 | 7.1 ± 1.4 |

The largest increase in *E. coli* survival occurred at the lowest SC range (< 350 µS $cm^{-1}$), suggesting small salinity increases can dramatically affect bacterial water quality. Salinization of streams with very low background SC (e.g., headwater streams) are therefore likely the most susceptible to increased FIB impairments. Median and mean SC

of wadeable streams in the United States were found to be 132.7 µS $cm^{-1}$ and 312.4 µS $cm^{-1}$, respectively (Griffith 2014b), suggesting that freshwater salinization might be increasing violations of water quality standards designed to protect public health. In addition to the length of time *E. coli* survives in the stream, loading and residence time are also critical factors affecting the concentration of *E. coli* or pathogens in streams and other water bodies. Typical water residence times for streams and rivers range from 12 – 20 days (Wetzel 1992). In my study, *E. coli* $t_{90}$ values increased from 9.1 ± 1.2 to 28.2 ± 9.5 d as SC increased from 15 to 1,500 µS $cm^{-1}$ and exceeded 20 d at a SC as low as 350 µS $cm^{-1}$. Increasing *E. coli* survival at low freshwater salinities suggests that at SC as low as 350 µS $cm^{-1}$, *E. coli* persistence would exceed water residence times in many streams, resulting in accumulating concentrations over time even with no increases in loading.

In addition to total salinity, increased *E. coli* survival rates from exposure to $Mg^{2+}$ may be important at regional or continental scales. Differences in land cover, usage, and geology result in markedly different compositions of base cations in surface waters (Corsi et al. 2010, Fitzpatrick et al. 2007, Griffith 2014a, Kaushal and Belt 2012, Kaushal et al. 2018, Steele and Aitkenhead-Peterson 2012). While previous research has linked other base cations, including $Ca^{2+}$ and $K^{+}$, to bacterial ecology in freshwater ecosystems, less is known about $Mg^{2+}$ effects in aquatic systems or their microbial communities (Verhougstraete et al. 2015). Yet, my results suggest $Mg^{2+}$ concentrations may be a key constituent of water chemistry that causes freshwaters to be more susceptible to salinity-induced FIB impairments relative to regions with similar increases in other ions. Therefore, the particular upland land-uses present, and their associated salts are critical when predicting the impacts on downstream water quality.

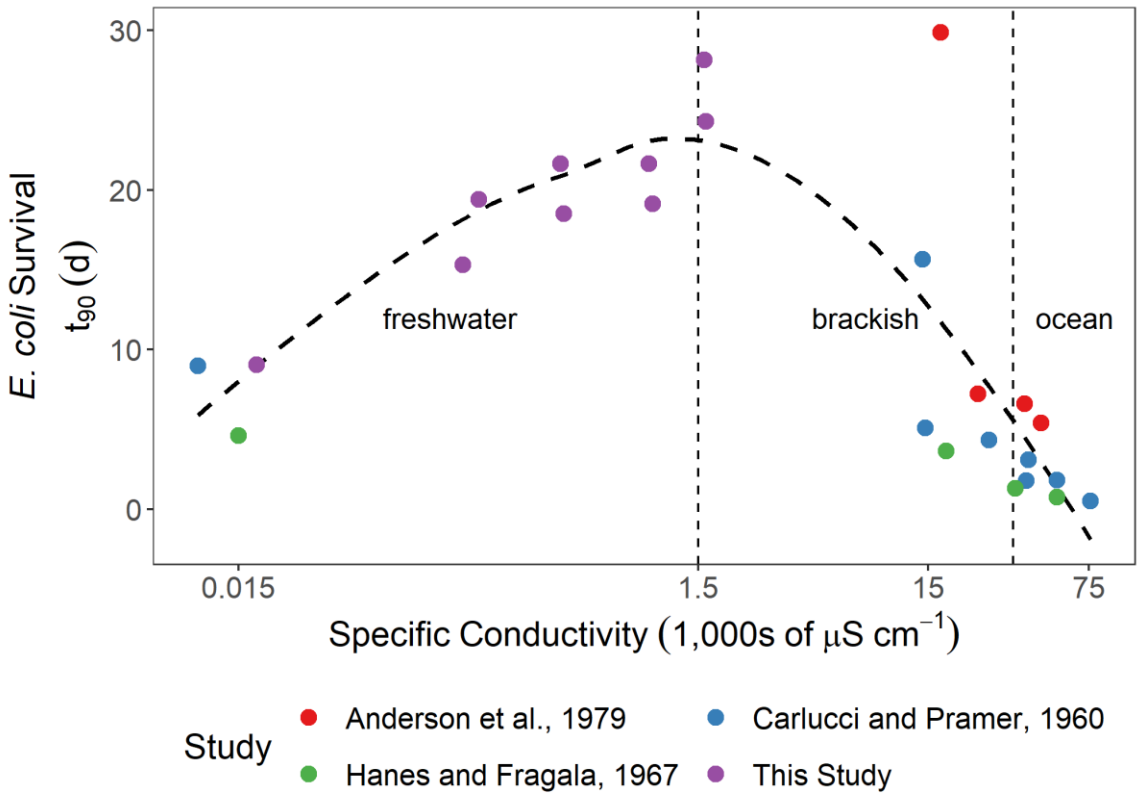


**Figure 2.3.** *E. coli* $t_{90}$ (survival) values across the freshwater-marine continuum showing a subsidy-stress response. Values in brackish and marine waters were compiled from previous studies and converted to $t_{90}$ values when needed. Note that the x-axis is on a log scale, but non-log transformed values are displayed for ease of interpretation. Data are fit to a Loess regression.

### 2.5.2 Potential mechanisms

A subsidy-stress relationship between *E. coli* and salinization is congruent with knowledge of the underlying biological mechanisms of how *E. coli* tolerates salts. The effects of hypo- and hyper-osmotic stress on *E. coli* are well documented and cause cell death, or the "stress" response. For example, under conditions of low osmolarity, several mechanosensitive (MS) channels rapidly release cytoplasmic solutes to reduce turgor pressure and prevent bursting of the cell envelope (Berrier et al. 1992, Levina et al. 1999). However, the effectiveness of these mechanisms depends on the rate of change in salinity. When salinity decreases rapidly, e.g. when enteric microbes are excreted into the

environment and flushed into streams, the protective ability of MS channels decreases, and cells cannot adjust their internal osmolarity fast enough to prevent lysis and death (Bialecka-Fornal et al. 2015). Increased salinity in the freshwater range appears to alleviate osmotic stress, supporting increased survival and the "subsidy" response. However, once salinity reaches brackish or marine concentrations, degradation of cellular energy charge can inactivate nutrient transport across the cell membrane, resulting in a viable but not culturable state (Troussellier et al. 1998). These processes are highly dependent on the transport of $K^+$ and are linked to specific genes, including *rpoS, proP, and proU*, which code for the transport of osmoprotectants such as glycine betaine and glutamate (Csonka 1989, Munro et al. 1989, Rozen and Belkin 2005).

When compared at identical SC levels, $Mg^{2+}$ increased *E. coli* survival rates by as much as seven days relative to other base cations (Fig. 2b). $Mg^{2+}$ is an important micronutrient and cofactor for numerous bacterial cellular processes and $Mg^{2+}$ starvation can permanently impact membrane permeability (Fiil and Branton 1969). Divalent cations stabilize the outer membrane of gram negative bacteria by binding and neutralizing repulsive forces of negatively charged lipopolysaccharide molecules (Raetz et al. 2007). The presence of excess $Mg^{2+}$ can assist in the maintenance of membrane stability in the presence of outer membrane disorganizing agents including weak acids and antibiotics (Leive 1974, Sahalan et al. 2013). Elevated $Mg^{2+}$ concentrations in the environment may therefore increase the ability of *E. coli* to adapt to changes in salinity through maintenance of proper membrane permeability providing an effective barrier to solutes and preventing cell leakage. Additionally, the stringent response in *E. coli*, which is a protective response to stress conditions that diverts resources away from nonessential

processes, is $Mg^{2+}$ dependent and limited at low extracellular $Mg^{2+}$ concentrations (Chochran and Byrne 1974, Shyp et al. 2012). Therefore, it is also possible that elevated $Mg^{2+}$ enables *E. coli* to endure unfavorable environmental conditions like osmotic stress by facilitating the stringent response.

### 2.5.3 Implications for water quality and salt management

Effective use of FIB in water quality monitoring is based upon a paradigm that assumes parity of survival between the indicator (*E. coli*) and human pathogens in the environment (Bonde 1966). This study did not assess the effects of freshwater salinization on survival rates of pathogens but suggests two possible scenarios. If rising salinity also increases survival rates of pathogenic bacteria, then freshwater salinization could be increasing concentrations of pathogens and human health risks. Conversely, if pathogen survival rates decrease or are not impacted by freshwater salinization, then the effectiveness of *E. coli* as an indicator of human health risk would decrease with salinization. Erroneous bacterial impairments resulting from overestimates of public health risks would lead to needless restrictions to stream access and use, as well as potentially wasted mitigation costs. Already, billions of dollars are spent annually in the United States on water infrastructure improvements and nonpoint source pollution mitigation to improve bacterial water quality (USEPA 2016b, Vedachalam and Geddes 2017). Therefore, assessing the effects of freshwater salinization on pathogenic bacteria is critical to fully understanding the relationships between salt use, freshwater salinization, and bacterial water quality.

Development of more nuanced salt management strategies that consider salt type, in addition to concentration alone, could offer additional benefits for improving water

quality. Salt management plans and deicing practices could have particularly significant ramifications for bacterial water quality at northern latitudes. Replacement of traditional Na-based deicing salts with Mg salts such as $MgCl_2$ and Mg-Ca acetate is increasingly widespread as these salts melt ice at lower temperatures. However, increased use of Mg-based deicers may actually exacerbate the effects of salinization on *E. coli* survival, which almost doubled in my study compared to *E. coli* exposed to $Ca^{2+}$ or $Na^{+}$ chloride salts (Figure 2b, Table 3S). Given that less $MgCl_2$ (8-11 $g/m^2$) is required per de-icing application than NaCl (13-68 $g/m^2$), further examination of the potential offset in decreasing overall SC but increasing *E. coli* survival is warranted (Zhang et al. 2009). Assessing the tradeoff of reduced SC vs. increasing *E. coli* survival is particularly true for urban landscapes that tend to experience both salinization and bacterial water quality issues (Kaushal and Belt 2012, Young and Thackston 1999).

**2.6 Conclusion**

Increasing salt concentrations in the freshwater range can increase survival rates of *E. coli*, the EPA-recommended FIB for freshwater. Thus, freshwater salinization may reduce our ability to effectively predict human health risks from exposure to waterborne pathogens. Further, regions prone to elevated $Mg^{2+}$ concentrations may be more susceptible to *E. coli* impairments. Effective salt management strategies could reduce FIB impairments by decreasing survival rates of *E. coli*. Conversely, in the absence of salt management, reductions in FIB loading to surface waters may fail to meet water quality goals if increased FIB survival and subsequent accumulation negate the reduced loads.

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## 3. Effects of freshwater salinization on stream bacterial diversity and assembly mechanisms via environmental filtering

## 3.1 Abstract

Anthropogenic freshwater salinization is an emerging and widespread water quality stressor that increases salt concentrations of freshwater, with specific upland land-uses producing distinct ionic profiles. Globally, salinity is one of the strongest environmental factors structuring bacterial communities; however, impacts on aquatic microbial communities from small salinity shifts within the freshwater range are poorly understood. By manipulating salt concentration and type in controlled mesocosm studies, I identified direct effects of freshwater salinization on bacterial diversity. Salt concentration and type produced distinct bacterial communities. Richness increased at conductivities as low as 350 µS $cm^{-1}$ and evenness increased as conductivity reached 1,500 µS $cm^{-1}$. Community variability (β-diversity) was greatest between 125 – 350 µS $cm^{-1}$ and most similar at the lower and upper extremes suggesting osmotic stress is an environmental filter for freshwater bacterial communities at low salt concentrations (< 125 µS $cm^{-1}$). Meanwhile, modest freshwater salinization (125 – 350 µS $cm^{-1}$) likely reduces osmotic stress shifting community assembly mechanisms from largely deterministic to more stochastic. Communities exposed to $CaCl_2$ were less diverse relative to other chloride salts at the same conductivity, indicating salt types exert differential effects on bacterial diversity. These results reveal that freshwater salinization influences stream bacterial diversity and assembly mechanisms.

## 3.2 Introduction

Identifying environmental factors and mechanisms that influence bacterial diversity is critical for understanding how ecosystems respond to environmental change. Bacterial community structure across habitat types varies in response to environmental gradients such as nutrient concentrations, temperature, pH, and salinity (Crevecoeur et al. 2015, Ikenaga et al. 2010, Lin et al. 2012, Rousk et al. 2010). Frequently, environmental gradients induce subsidy-stress responses, where peak growth rates, process rates, or biological diversity occur at optimal environmental conditions and decrease towards the upper and lower extremes of the gradient (*i.e.*, unimodal curve) (King and Richardson 2007, Odum et al. 1979). Such shifts in species composition can not only alter ecological functions of the community (Falkowski et al. 2008), but also the functions and services of the ecosystems they inhabit (del Giorgio and Bouvier 2002, Eilers et al. 2010, Graham et al. 2016, Palmer et al. 2004, Palmer et al. 2014, Schimel and Gulledge 1998, Wintermute and Silver 2010). In fluvial systems, less is known about how microbiomes are structured than in more commonly studied ecosystem types, despite their importance in biogeochemical cycling at a global scale (Battin et al. 2016, Cole et al. 2007).

Of the many abiotic variables that have been linked to bacterial diversity, salinity may be one of the most important factors globally (Lozupone and Knight 2007). In aquatic ecosystems, large salinity gradients spanning freshwater, marine, and hypersaline habitats (50 to ~60,000 $\mu S\ cm^{-1}$) are known to significantly alter bacterial diversity and cause broad shifts in dominant taxa (Bouvier and del Giorgio 2002, Campbell and Kirchman 2013, Herlemann et al. 2011). However, freshwater salinization is a specific phenomenon that occurs when human activity causes relatively small shifts in salinity within the freshwater range ($\leq$ 1,500 $\mu S\ cm^{-1}$.) This emerging water quality issue is

estimated to impact 37% of the contiguous drainage area in the United States (Kaushal et al. 2018). Different upland land-uses such as agriculture, resource extraction, urbanization, and deicing can result in distinct salt profiles varying in base cation compositions in impacted surface waters (Canedo-Arguelles et al. 2017, Corsi et al. 2010, Fitzpatrick et al. 2007, Kaushal et al. 2017, Kaushal et al. 2014). Recently, fecal indicator bacteria concentrations in streams were linked to freshwater salinization and associated base cations (Badgley et al. 2019, Verhougstraete et al. 2015, Chapter 1), indicating that freshwater salt concentrations can impact bacterial ecology. The effects of mining-induced salinization on bacterial ecology have also been studied, but can be confounded by other mining-associated water quality stressors (e.g. heavy metals, trace elements, alkalinity) (Bier et al. 2015, Vander Vorste et al. 2019). The direct impacts of salts on native bacterial communities within the freshwater range ($\leq$ 1,500 $\mu$S $cm^{-1}$) have yet to be isolated via controlled laboratory experiments.

Metacommunity theory provides important concepts for describing patterns of biological diversity and useful tools to understand how freshwater salinization affects bacterial communities (Brown et al. 2017, Brown and Swan 2010, Leibold et al. 2004, Wisnoski and Lennon 2020). Metacommunity theory considers multiple spatial scales, including α-diversity (diversity of a single community), γ-diversity (region or landscape diversity), and β-diversity (compositional variance among distinct communities) (Jost 2007). Patterns of α-, β-, and γ-diversity can indicate specific species assembly mechanisms. For example, more deterministic assembly often occurs when environmental conditions, or filters, favor specific species adapted for those conditions (Bazzaz 1991, Nobel and Slatyer 1977). Conversely, neutral theory postulates that

differences in communities result from stochastic processes (e.g. probabilistic colonization) (Hubbell 2001) in the absence of strong environmental filters. Determining the relative importance of deterministic vs. stochastic processes can help infer the mechanisms by which different salt concentrations or types modulate bacterial diversity patterns, which ultimately will better predict the effects of global change on ecosystem processes.

My aim in this study was to identify the direct impacts of freshwater salinization, a pervasive water quality issue, on native aquatic bacterial communities. I used controlled laboratory experiments to determine: 1) the effects of a salinity gradient within the freshwater range (< 1,500 $\mu S\ cm^{-1}$) on stream bacterial community structure and diversity, and 2) whether individual chloride salts characteristic of common sources of freshwater salinization exert differential impacts on freshwater bacterial communities. Based on observed changes in bacterial diversity, I inferred how bacterial assembly mechanisms change across the freshwater salinity gradient. Based on my previous work, I hypothesized that small salinity increases within the freshwater range would alleviate osmotic stress, facilitating survival of more taxa and resulting in more diverse bacterial communities. Because base cations are micronutrients involved in varying cellular processes, I also hypothesized that exposure to different salt types would produce distinct bacterial communities. Finally, I explored whether bacterial community assembly mechanisms change across the freshwater salinity gradient.

## 3.3. Methods

### 3.3.1 Methodology Overview

To assess the impacts of freshwater salinization on bacterial diversity, I conducted a series of laboratory stream water mesocosm experiments to directly manipulate salt concentrations and types. I conducted two separate experiments to understand how bacterial diversity is impacted by: 1) a freshwater conductivity gradient (30 to 1,500 µS $cm^{-1}$) using two common freshwater salt profiles, and 2) varying individual salt types characteristic of common salinization sources, while maintaining equal conductivity. I characterized bacterial communities using targeted amplicon sequencing of the 16S rRNA gene. Using a combination of multivariate statistics and metacommunity theory, I assessed bacterial diversity to infer how community assembly mechanisms varied across a freshwater salinity gradient and in response to different salt types.

### 3.3.2 Sample Collection and Mesocosm Design

I collected water for mesocosms between March 2018 and January 2019 from a first order stream in a 100% forested watershed located in the Jefferson National Forest in southwestern Virginia using acid-washed, autoclaved HDPE bottles. Bottles were immediately returned to the lab after collection for mesocosm setup. Mesocosms were constructed from sterilized, HDPE containers equipped with aeration systems to oxygenate and homogenize sample water throughout the experiment. I incubated mesocosms at room temperature for one month.

### 3.3.3 Conductivity Gradient Experimental Design

The primary purpose of the first experiment was to test the effects of conductivity gradients within the freshwater range on bacterial diversity. Because different upland

land-uses produce conductivity shifts with distinct ionic signatures in impacted surface waters (Kaushal et al. 2018), I further tested whether two salt profiles, characteristic of different salinization sources, exert unique effects on bacterial diversity. I used either 1) a mixture of Ca, Mg, and K chloride salts (hereafter referred to as Ca:Mg:K), or 2) NaCl to adjust the conductivity of stream water. Targeted treatment conductivities were 125, 350, 800, and 1,500 µS $cm^{-1}$, with triplicate mesocosms for each salt treatment. Additionally, I included triplicate control mesocosms with no salt additions. Although evaporation caused increased conductivity by the end of the experiment, the coefficient of variation of the average of the initial and final conductivity within a treatment was ≤ 16% and was not enough to overlap adjacent treatments (Table 3.1).

### 3.3.4 Constant Conductivity Experimental Design

The second experiment tested the effects of varying individual chloride salts on bacterial community structure at constant conductivity. I tested four individual chloride salts commonly present in salinized freshwater ($CaCl_2$, $MgCl_2$, KCl, and NaCl). I maintained conductivity at 350 µS $cm^{-1}$, which is a biological effect concentration for other aquatic organisms (Timpano et al. 2018). I also included triplicate reference mesocosms with no salt addition but excluded these reference mesocosms from pairwise comparisons between ion treatments because conductivity could not be adjusted to 350 µS $cm^{-1}$ in reference stream water.

**Table 3.1.** Conductivity (SC) values throughout the gradient experiment. Initial and final conductivity are the average conductivity ± 1 standard deviation of triplicate mesocosms at the beginning and end of the experiment, respectively. Average of initial and final is the average of the initial and final conductivity ± 1 standard deviation of triplicate mesocosms for each treatment and is representative of the average conductivity bacteria were exposed to during the incubation. CV of average is the coefficient of variation of the average of the initial and final conductivity of triplicate mesocosms for each treatment.

| Treatment | Target SC | Initial SC | Final SC | Average of Initial and Final | CV of Average |
|---|---|---|---|---|---|
| **Reference** | 15 | 14.26 ± 0.89 | 22.49 ± 5.98 | 18.34 ± 2.98 | 16.2% |
| **Ca:Mg:K** | 125 | 126.30 ± 1.51 | 192.13 ± 46.50 | 166.37 ± 26.3 | 15.8% |
| | 350 | 351.07 ± 2.95 | 404.73 ± 49.24 | 377.90 ± 23.24 | 6.15% |
| | 800 | 799.53 ± 4.65 | 1024.57 ± 148.20 | 912.05 ± 72.12 | 7.9% |
| | 1,500 | 1497 ± 4.36 | 1698 ± 124.06 | 1595 ± 60.57 | 3.8% |
| **NaCl** | 125 | 125.97 ± 0.15 | 159.07 ± 17.99 | 142.52 ± 8.94 | 6.3% |
| | 350 | 348.87 ± 0.23 | 430.87 ± 53.24 | 389.87 ± 26.59 | 6.8% |
| | 800 | 800.90 ± 2.26 | 1098.47 ± 153.47 | 949.69 ± 76.99 | 8.1% |
| | 1,500 | 1501.33 ± 3.21 | 1734.67 ± 167.68 | 1618 ± 82.27 | 5.1% |

### 3.3.5 DNA extraction and Sequencing

I used targeted 16S rRNA amplicon sequencing to characterize bacterial community structure for both experiments. For all samples, I vacuum-filtered 1 L of water from each mesocosm through a sterile, 0.45 µm cellulose filter and stored filters at -80℃ until DNA extraction. I extracted DNA from filters using the DNeasy PowerWater DNA Isolation Kit (QIAGEN, USA) following manufacturer protocol and stored samples at -20℃ until polymerase chain reaction (PCR). Following the Earth Microbiome Protocol (Gilbert et al. 2014), I amplified the V4 region of the 16S rRNA gene with PCR using 515F-806R primers (Caporaso et al. 2011). After PCR amplification, I purified 16S

rRNA gene amplicons using the QIAquick PCR Purification Kit (QIAGEN, USA). I measured DNA concentrations of purified 16S gene amplicons with a Qubit 2.0 fluorometer (Invitrogen, USA). Based on DNA concentrations, I pooled samples to an equal DNA mass, which I stored at -20℃ until sequencing. I sent samples to the Duke Center for Genomic and Computational Biology (Duke University, Durham, NC USA) for next-generation, 350 base pair, paired-end DNA sequencing on the Illumina MiSeq platform.

### 3.3.6 DNA Sequencing Quality Filtering and Processing

I processed16S rRNA gene amplicon sequences using QIIME2 (Bolyen et al. 2018). To trim low quality regions, I truncated sequences to 250 base pairs. I denoised and dereplicated raw sequence reads using DADA2, allowing a maximum expected error of 2 (Callahan et al. 2016). Additional frequency filtering removed amplicon sequence variants (ASVs) that were present only once in the dataset or occurred in only one sample. ASVs were taxonomically classified using a pre-trained naïve Bayes classifier (Pedregosa et al. 2011) for 16S sequences bound by the 515F/806R primer pair, produced from the Silva database (Silva 132 release; (Quast et al. 2013)) using 99% consensus taxonomy with seven levels. Lastly, I removed any ASVs identified as chloroplast, mitochondria, or those unassigned at the Domain level from the final dataset.

### 3.3.7 Data Analysis of Conductivity Gradient Experiment

I analyzed bacterial community structure based on relative ASV abundances in R (version 1.2.1335) (R Core Team 2019) using the phyloseq (McMurdie and Holmes 2013), vegan (Oksanen et al. 2019), and vegetarian (Charney and Rcord 2012) packages. Prior to analysis, I rarefied samples to the number of total sequences in the sample with

the lowest number of reads (1,075). Differences in bacterial community structure of both salt profiles (Ca:Mg:K and NaCl) across the conductivity gradient were visualized with nonmetric multidimensional scaling (NMDS) using Bray-Curtis Dissimilarity as a secondary matrix. I chose the number of NMDS axes based on the number required to decrease stress below 0.20 (Clarke 1993, Kruskal 1964), which was achieved with 2 in call cases. I tested location effects using a PERMANOVA (Anderson 2001) to determine if bacterial community structures were significantly different 1) between the two salt profiles, and 2) across the conductivity gradient. I excluded reference water with no salt treatment from PERMANOVA of both salt profiles, as it did not constitute a salt treatment. However, I did include reference water in the PERMANOVA of the conductivity gradient because it did represent a unique conductivity treatment.

To better visualize differences in community structure within each salt profile across the conductivity gradient, I solved an NMDS for the Ca:Mg:K and NaCl profiles individually. Additionally, I leveraged a permutational test of multivariate dispersion (PERMDISP2, (Anderson et al. 2006)) to quantify community structure variability among triplicate mesocosms based on multivariate dispersion (average distance to the centroid) in principal coordinates space. Average distance to centroid values were fit with a Loess regression to highlight the trend of dispersion across the conductivity gradient. A Mantel test was conducted to determine the relationship between bacterial community differences (Bray-Curtis dissimilarity) and conductivity.

I assessed differences in patterns of bacterial diversity across the salinization gradient for each salt treatment (Ca:Mg:K and NaCl) by partitioning diversity into separate α, β, and γ components. Numbers equivalents for α-, β-, and γ-diversity were

calculated using diversity orders (q) of 0 for richness, 1 for Shannon Diversity, and 2 for Evenness (Simpson's Diversity). Richness, Shannon Diversity, and evenness were analyzed because independent α and β terms can be derived from Renyi's entropy equation (Charney and Rcord 2012). In this experiment, α-diversity was the ASV richness of an individual mesocosm, γ-diversity was total ASV richness across triplicate mesocosms of a treatment, and β-diversity represented the variability of bacterial communities within a treatment.

### 3.3.8 Threshold Indicator Taxa Analysis - Conductivity Gradient Experiment

To determine which ASVs responded to changes in conductivity, I used a threshold indicator taxa analysis (TITAN, (Baker et al. 2019)). TITAN identifies change points, or thresholds where relative abundance changes in response to conductivity. Prior to analysis, I aggregated ASVs at the family level. TITAN requires a minimum taxon occurrence of three, so I removed all families that were present in fewer than three samples prior to analysis. I used 500 bootstrapping runs to determine if an ASV responded positively or negatively to the conductivity gradient by assessing if the relative abundance of an ASV responded in the same direction as the stressor. High purity taxa indicators responded in the same direction in at least 95% of the bootstrapped runs while high reliability taxa had a distribution significantly different from randomly distributed data in 95% of the bootstrapped runs. Only taxa with $p < 0.05$ are reported.

### 3.3.9 Data Analysis of Constant Conductivity Experiment

I visualized differences in the structure of bacterial communities exposed to individual chloride salts at the same conductivity with NMDS. Prior to analysis, I rarefied samples to the number of total sequences in the sample with the lowest number of reads

(469). I statistically tested if exposure to different individual chloride salts resulted in unique bacterial communities using a PERMANOV. I calculated multivariate dispersion based on mean distance to the centroid using a permutational test of multivariate dispersion (PERMDISP2, (Anderson et al. 2006)). Lastly, I calculated bacterial richness, Shannon diversity, and evenness for each individual chloride salt treatment. Significant differences in diversity metrics were determined with a one-way ANOVA followed by Tukey's HSD test.

## 3.4 Results

### 3.4.1 Bacterial Community Structure Across a Freshwater Salinity Gradient

Both the salinity gradient (Fig. 3.1A; PERMANOVA, $PseudoF_{4,22} = 1.80$, $p = 0.002$) and the Ca:Mg:K and NaCl salt profile treatments (Fig. 3.1B; PERMANOVA, $PseudoF_{1,22} = 3.03$, $p = 0.003$) affected bacterial community composition. Additionally, bacterial communities in each salt-type profile diverged in opposite directions in NMDS space with increasing salinity, indicating that bacterial communities exposed to different salt types became more different as salt concentration increased (Fig 3.1B). The Mantel test indicated a positive correlation between Bray-Curtis Dissimilarity of bacterial composition and pairwise conductivity differences ($p = 0.015$), further supporting the divergence of bacterial community structure along the conductivity gradient. When bacterial community structures in each salt profile were visualized separately, salinity had a pronounced effect on bacterial community composition, again with communities becoming more different with increasing conductivity (Figs 3.2A and 3.2B). Salinity also affected multivariate dispersion, which peaked at 125 $\mu S\ cm^{-1}$ in the Ca:Mg:K treatment and at 350 $\mu S\ cm^{-1}$ in the NaCl treatment, but was lower at both extremes of the gradient

(Figs 3.2C and 3.2D). Although differences in dispersion can affect location differences produced in PERMANOVA, there is clear visual separation in groups (Figs 3.2A and 3.2B) and dispersion differences have almost no effect on PERMANOVA with balanced experimental designs (Anderson and Walsh 2013).

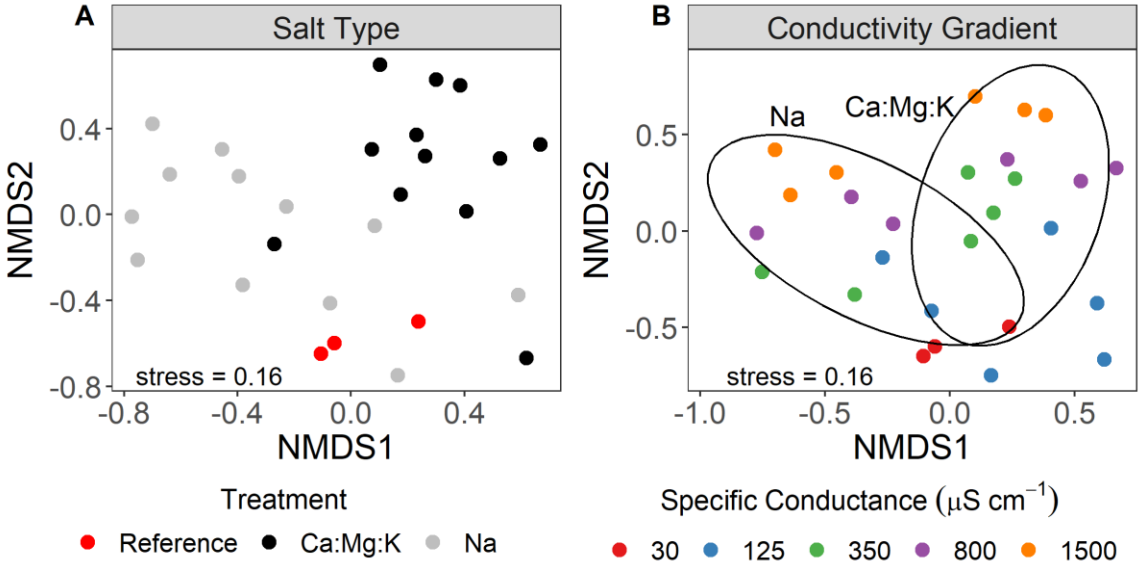


**Figure 3.1.** NMDS plot showing bacterial community dissimilarity based on Bray-Curtis dissimilarity. 1A depicts differences in community structure across a freshwater salinity gradient. 1B shows differences in bacterial community structure based on salt type.

### 3.4.2 Diversity Partitioning Across a Freshwater Salinity Gradient

Investigating diversity using multiple metrics and partitions provides insight into both the characteristics of diversity that are changing, as well as the scales at which those changes are occurring. In these experiments, both the freshwater salinity gradient and salt profile (Ca:Mg:K vs NaCl) affected multiple bacterial diversity metrics and partitions. ASV richness peaked at 350 μS $cm^{-1}$ for α- and γ-diversity (i.e., individual mesocosm and whole-treatment richness, respectively) in both treatments, and either remained high (Ca:Mg:K) or decreased (NaCl) towards the upper range of the gradient.

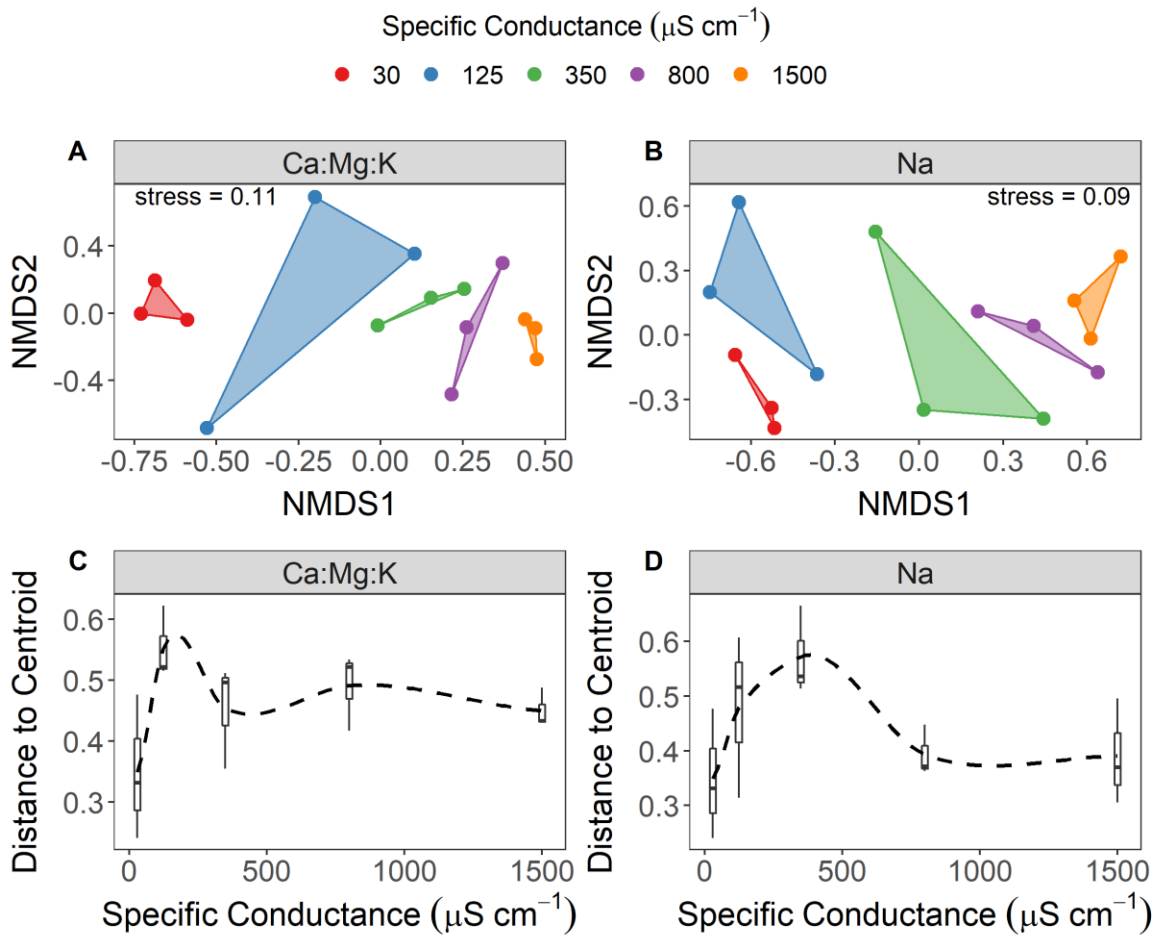


**Figure 3.2.** NMDS plot showing bacterial community structure based on Bray-Curtis dissimilarity and multivariate dispersion for the A and C) Ca:Mg:K profile and B and D) NaCl salt profile.

Based on Shannon diversity, which encompasses both richness and evenness, α- and γ-diversity peaked at 350 μS $cm^{-1}$ in the Ca:Mg:K treatment, but increased consistently across the gradient to peak at 1 500 μS $cm^{-1}$ in the NaCl treatment (Fig. 3.3). Patterns in evenness largely mirrored those of Shannon diversity. Collectively, these results indicate that ASV richness was generally highest at 350 μS $cm^{-1}$ but changes in richness and evenness at higher conductivities were dependent on salt profile. Meanwhile, β-diversity (i.e., within-treatment community variability) consistently showed a unimodal distribution, or subsidy-stress response, peaking between 125 – 350

µS cm$^{-1}$, regardless of salt type or diversity metric. These patterns of β-diversity are consistent with patterns of multivariate dispersion (Figs 3.2C and 3.2D) and indicate that communities within conductivity treatments are least predictable at intermediate conductivities between 125 and 350 µS cm$^{-1}$ in both salt types.

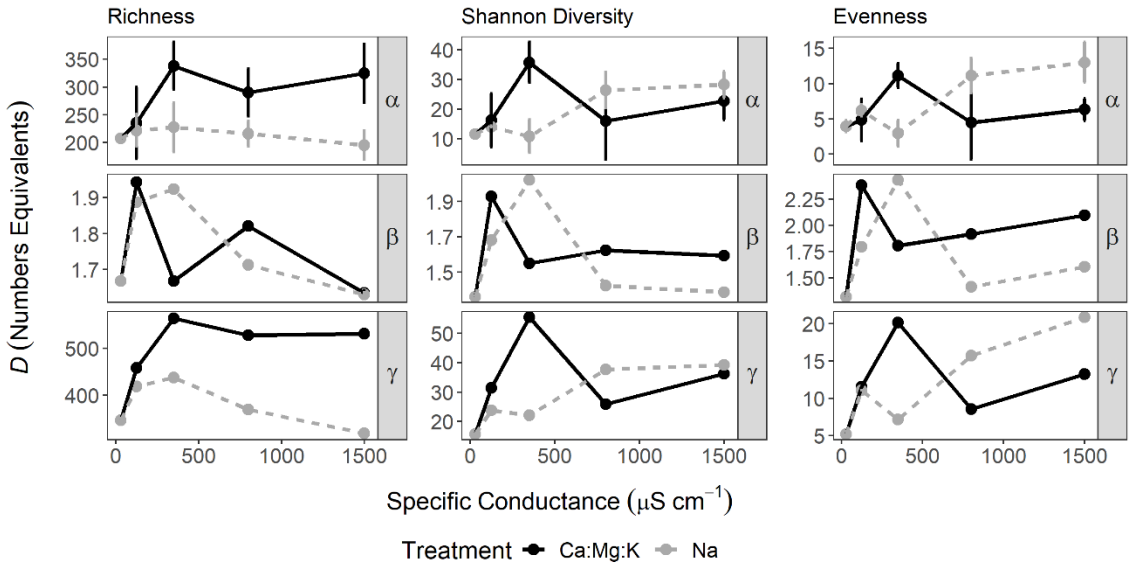


**Figure 3.3.** Alpha, beta, and gamma diversity of bacterial communities exposed to a salinity gradient of two salt profiles. Hill numbers were calculated using q=0 for richness (left column), q=1 for Shannon diversity (middle column), and q=2 for evenness (right column).

### 3.4.3 Indicator Taxa of Freshwater Salinization

TITAN identified seven families that responded consistently to the conductivity gradient (Fig 3.4A). Three families including Opitutaceae, AKYH767, and Bdellovibrionaceae responded negatively with observed change points at 125, 350, and 1,150 µS cm$^{-1}$, respectively. Four families including Spirosomaceae, cvE6, Azospirillaceae, and Caulobacteraceae responded positively with observed change points at 350, 237.5, 237.5, and 125 µS cm$^{-1}$, respectively. The distribution of summed z-weighted probability densities, which peaked at 350 µS cm$^{-1}$ then steadily declined towards 1,500 µS cm$^{-1}$, indicated that most bacterial turnover occurred at lower salt

concentrations (Fig. 3.4B). The distribution of z-weighted probability density was consistent with trends in β-diversity derived from both multivariate dispersion and diversity partitioning (Fig. 3.2C and 3.2D, Fig. 3.3 second row), which peaked at low salt concentrations between 125 and 350 μS $cm^{-1}$ and were lowest at the upper and lower ends of the gradient.

### 3.4.4 Effects of Salt types on Bacterial Communities

Exposure to individual chloride salts at the same conductivity of 350 μS $cm^{-1}$ resulted in different bacterial communities (Fig 3.5A; PERMANOVA, $PseudoF_{4,10}$ = 2.602, $p < 0.001$). There were no significant differences in multivariate dispersion. Different base cations also caused differences in ASV richness, Shannon diversity, and evenness. ASV richness was elevated in all ion treatments relative to reference control water, which was consistent with increased richness at elevated conductivities observed in the gradient experiment (Fig 3.5B). However, ASV richness was higher in the $MgCl_2$ treatment compared to the $CaCl_2$ treatment (Fig 3.5B and Table 3.2; ANOVA: $F_{3,8}$ = 4.579, $p = 0.0379$, Tukey's HSD: $p = 0.027$). Shannon diversity and evenness were both lower in the $CaCl_2$ treatment relative to the $MgCl_2$ ($p = 0.002$ for Shannon diversity and evenness), KCl ($p = 0.006$ and 0.002, respectively), and NaCl ($p = 0.020$ and 0.008, respectively) treatments (Figs 3.5C and 3.5D, Table 3.2). Evenness in the Ca treatment was also lower than the reference stream water (Fig 3.5D).

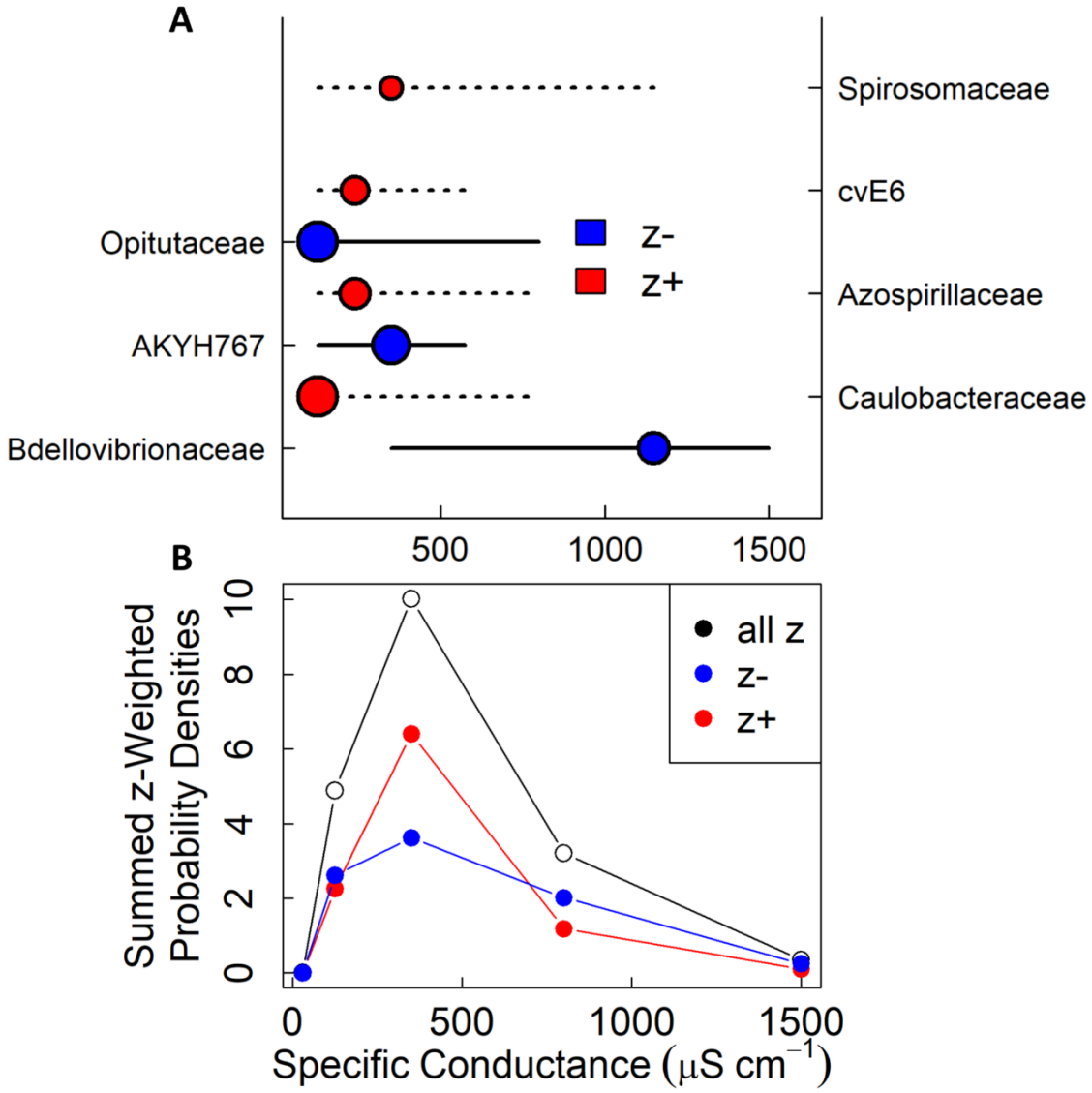


**Figure 3.4.** Threshold indicator taxon analysis showing A) change points for bacterial families that responded either positively (red) or negatively (blue) to a freshwater conductivity gradient and B) the distribution of summed z-weighted probability densities of positive (red), negative (blue), and all (open circles) bacterial family change points.

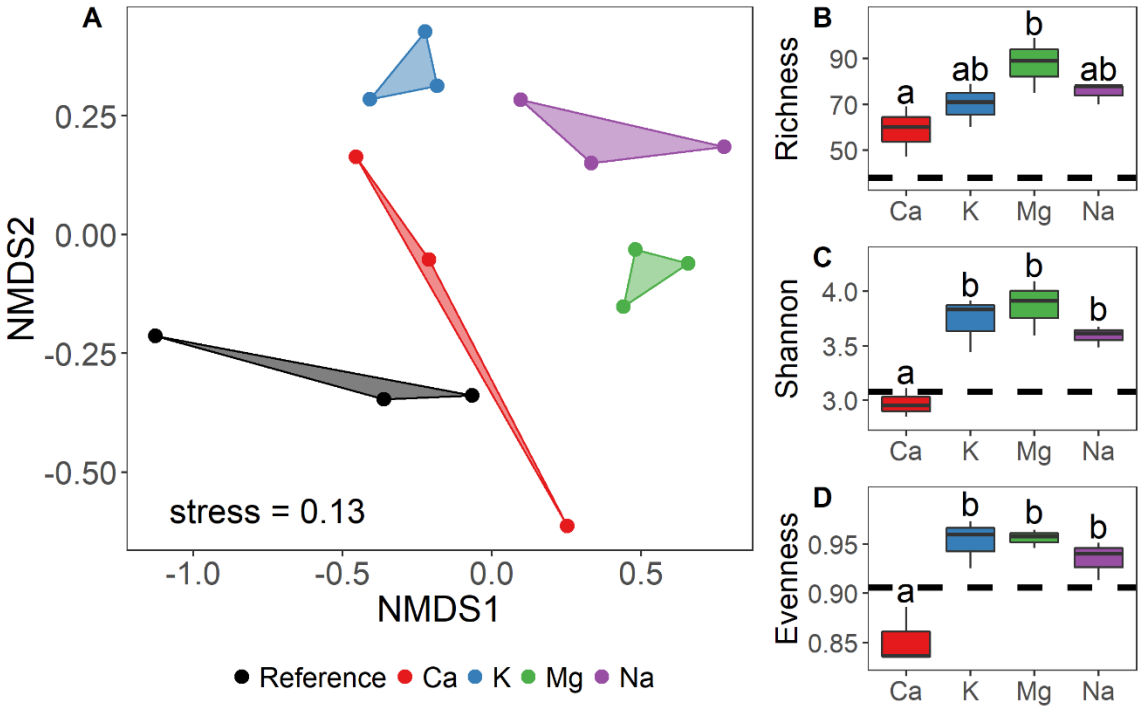


**Figure 3.5.** The effects of individual chloride salts on A) bacterial community structure in NMDS space based on Bray-Curtis dissimilarity, B) ASV richness, C) Shannon Diversity, and D) evenness.

## 3.5 Discussion

### 3.5.1 Freshwater salinization produces distinct bacterial communities

As salinity increased, bacterial communities exposed to both the NaCl and Ca:Mg:K salt profiles consistently diverged based on Bray-Curtis dissimilarity, with the greatest differences observed across the greatest conductivity difference (i.e., 30 and 1,500 µS $cm^{-1}$). A positive correlation between differences in bacterial community structure and differences in conductivity (Mantel test) suggests that compositional shifts in bacterial communities occur continuously across the freshwater range, rather than at a threshold salinity which has been observed across brackish salinity gradients, particularly at mixing zones (Bouvier and del Giorgio 2002, Herlemann et al. 2011). While alkaline mine drainage gradients, which increase salinity as well as heavy metals, have been

shown to increase the dissimilarity of bacterial communities, it was unclear if salinity or other mining-associated stressors were responsible (Bier et al. 2015). My results provide direct evidence that freshwater salinization (< 1,500 µS $cm^{-1}$), in the absence of other water quality stressors, can alter bacterial community structure. Further, as conductivity increased, bacterial communities in mesocosms with different salt compositions also became more different. Such divergence suggests that salt composition, which has been linked to global patterns of bacterial diversity (sea water vs. non-seawater) (Barberán and Casamayor 2010), is important even within relatively small freshwater salinity ranges.

### 3.5.2 β-diversity and Environmental Filtering

Determining assembly mechanisms of microbial communities remains a major goal of microbial ecology. Teasing apart the relative influences of deterministic vs. stochastic processes that shape native bacterial communities is a significant challenge (Stegen et al. 2012). By experimentally manipulating salinity in controlled settings, I isolated the effects of salinization on stream bacterial diversity. Patterns of β-diversity, derived from both multivariate dispersion and diversity partitioning, indicate that stream bacterial communities were most variable across replicates at conductivities between 125 and 350 µS $cm^{-1}$ and most consistent at the upper and lower extremes of the gradient. Further, most bacterial families that responded, either positively or negatively, to the freshwater salinity gradient occurred in the same range of 125 – 350 µS $cm^{-1}$. Collectively, these results suggest that there is a salinity range where bacterial communities change drastically (between 125 - 350 µS $cm^{-1}$), which also happens to be the range that freshwater salinization is occurring.

**Table 3.2.** Summary statistics from ANOVA and Tukey's HSD test for richness, Shannon diversity, and evenness from the constant conductivity experiment. Significant values are bold.

| Test | Diversity Metric | Comparison | F Value | DF | Difference | P Value |
|---|---|---|---|---|---|---|
| **ANOVA** | Richness | - | 4.48 | 4,8 | - | **0.038** |
| | Shannon | - | 12.18 | 4,8 | - | **0.0024** |
| | Evenness | - | 14.73 | 4,8 | - | **0.0013** |
| **Tukey's HSD** | Richness | K vs Ca | - | | 11.3 | 0.52 |
| | | Mg vs Ca | - | | 29.0 | **0.027** |
| | | Na vs Ca | - | | 16.7 | 0.23 |
| | | Mg vs K | - | | 17.7 | 0.20 |
| | | Na vs K | - | | 5.3 | 0.91 |
| | | Na vs Mg | - | | -12.3 | 0.46 |
| | Shannon | K vs Ca | - | | 0.758 | **0.006** |
| | | Mg vs Ca | - | | 0.895 | **0.002** |
| | | Na vs Ca | - | | 0.620 | **0.020** |
| | | Mg vs K | - | | 0.137 | 0.83 |
| | | Na vs K | - | | -0.138 | 0.82 |
| | | Na vs Mg | - | | -0.276 | 0.37 |
| | Evenness | K vs Ca | - | | 0.100 | **0.002** |
| | | Mg vs Ca | - | | 0.104 | **0.002** |
| | | Na vs Ca | - | | 0.083 | **0.008** |
| | | Mg vs K | - | | 0.003 | 0.99 |
| | | Na vs K | - | | -0.018 | 0.75 |
| | | Na vs Mg | - | | -0.021 | 0.65 |

Salt concentrations at the lower extreme of the freshwater salinity gradient (30 µS $cm^{-1}$) appeared to act as an environmental filter. Meanwhile, moderate salinization (350 µS $cm^{-1}$) appeared to reduce filtering (i.e. relieve hypo-osmotic stress). Conversely, high salt concentrations (1,500 µS $cm^{-1}$) appear to again increase filtering by hyper-osmotic stress. However, it is important to note that observed differences in richness and evenness suggest that the upper salinity extreme could have influenced community assembly processes in multiple, potentially interacting ways. Like the lower extreme of the salinity gradient, high salt concentrations may still act as an environmental filter, but for fewer organisms, which resulted in higher diversity yet still confined community membership. Additionally, the upper freshwater salinity range may also have been an optimal environment for a more diverse, but restrictive group of taxa that competitively excluded those less adapted to higher salt concentrations. Overall, alleviating salt-induced environmental filtering across the freshwater salinity gradient resulted in a subsidy-stress response, with an optimal conductivity for most stream bacteria occurring between 125 – 350 µS $cm^{-1}$.

Patterns of bacterial richness were the opposite of what has been observed in streams impacted by mining-induced salinization. In this study, bacterial richness increased with increasing conductivity, particularly in the Ca:Mg:K treatment. However, a negative relationship between salinity and bacterial richness across a similar salinity gradient caused by mining (25 – 1,383 µS $cm^{-1}$) has been observed (Vander Vorste et al. 2019). The extent of upstream mining has also been negatively correlated with bacterial richness (Bier et al. 2015). Both studies assessed the impacts of mining-induced salinity, which is also associated with increased alkalinity, heavy metals, and trace elements.

Thus, alkalinity, heavy metals, or certain trace elements may have a stronger and opposite effect than salts on bacterial richness. However, freshwater salinization from sources that do not contribute additional stressors (e.g. road salts) may actually increase bacterial richness.

A conceptual framework of the impacts of freshwater salt concentrations on bacterial community assembly processes is presented in Figure 3.6. Based on ASV richness, at conductivities < 125 µS $cm^{-1}$ α-, β-, and γ-diversity are low (Figure 3.6, section 1), suggesting that the filtering strength of low salinity (or hypo-osmotic stress) is high and selects for only a small subset of ASVs adapted to low salinities. Low β-diversity under these conditions suggests highly deterministic community assembly processes. Under moderate salinity ranges, 125 – 350 µS $cm^{-1}$, α-, β-, and γ-diversity increase (Figure 3.6, section II), suggesting that the filtering strength of salinity is low. In this scenario, all bacteria in the potential stream microbiome can thrive, but variability in community membership (i.e. β-diversity) could be attributed to demographic stochasticity in the absence of strong abiotic pressure. Under high freshwater salinity ranges of 350 – 1,500 µS $cm^{-1}$, α-diversity remains elevated, γ-diversity decreases slightly, and β-diversity decreases drastically (Figure 3.6, section III). Under these high salinity conditions, many, but not all microbes in the potential microbiome persist (i.e., decreased γ-diversity) in diverse but more confined communities due to combined effects of moderate environmental filtering and competitive exclusion by more salt-adapted taxa. Under these conditions, both deterministic and stochastic processes may be contributing to bacterial diversity.

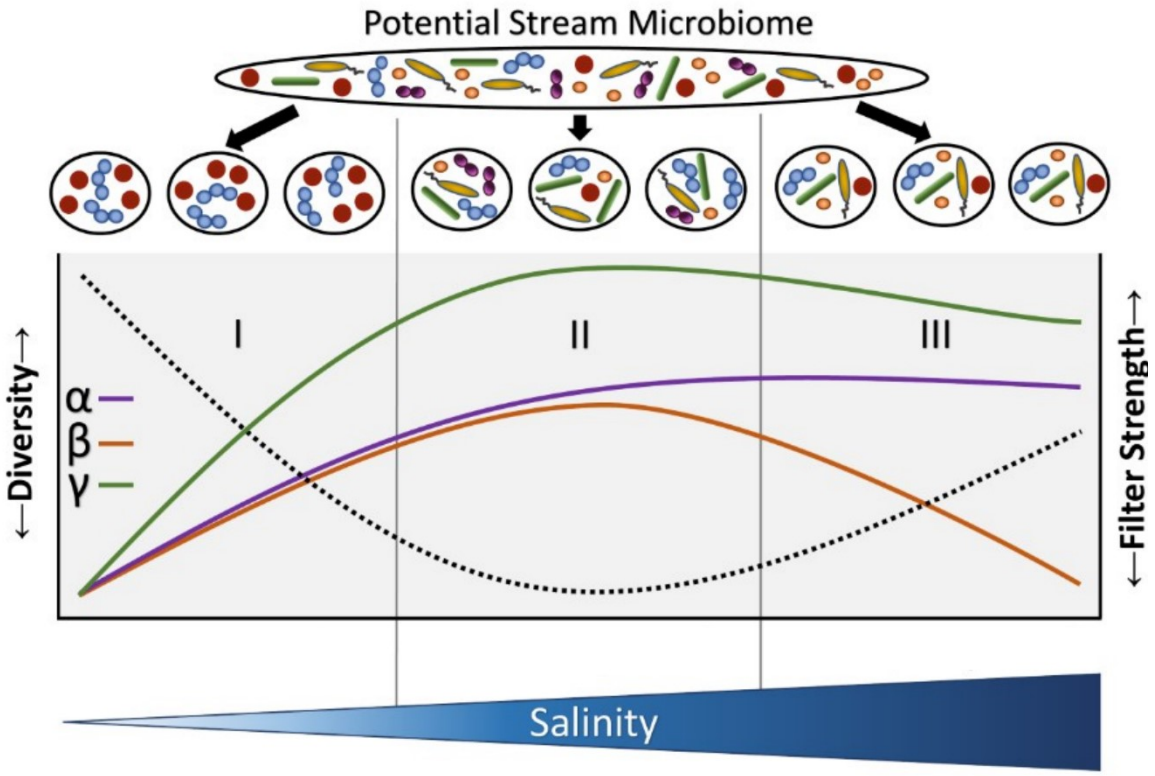


**Figure 3.6.** A conceptual diagram of the effect of salinity on stream microbiomes. Diversity measures are represented by the solid, colored lines and the black dotted line represents the environmental filter strength of salinity. In section I under very low salinity, both richness and beta diversity are low suggesting that the filtering strength of low salinity is high and selects for only a small subset of species adapted to low salinities. In section II under moderate salinity ranges, both richness and beta diversity are high suggesting that the filtering strength of salinity is low. In this scenario, all bacteria in the stream microbiome can potentially thrive, but differences in community membership (i.e. beta diversity) could be attributed to demographic stochasticity in the absence of the filtering effects of salinity. In section III under high salinity ranges, richness is high, but beta diversity is low. In this scenario, many but not all microbes can survive due to a moderate environmental filtering effect of high salinity or competition from more salt-adapted species resulting in diverse but highly similar community membership. The specific salinity ranges vary depending on salt type.

More insight is provided by examining Shannon diversity and ASV evenness, both of which have independent α and β terms. For example, as γ-diversity for ASV richness began to decrease towards the upper range of the conductivity gradient, evenness increased, particularly in the Na treatment. Increased evenness at higher conductivities

suggests that rare ASVs are lost at higher salinities in freshwater and the evenness of relative abundance of remaining ASVs increases. Shannon diversity, which can be interpreted as a 1:1 mix of richness and evenness, highlights the combined impact of salinity on richness and evenness. Based on Shannon β-diversity, it is apparent that the highest variability among bacterial communities of the same treatment occurs at 125-350 $\mu S\ cm^{-1}$. Thus, freshwater salinization appears to modulate the relative importance of stochastic vs. deterministic assembly mechanisms of stream bacterial communities.

Freshwater salinization's effect on bacterial diversity also depended on different salt types that make up the total salinity. For example, α- and γ-richness were significantly higher in the Ca:Mg:K treatment. Trends in α- and γ-evenness were also different, suggesting that these distinct ion compositions affected bacterial communities differently. In the Ca:Mg:K treatment, α- and γ-evenness peaked at 350 $\mu S\ cm^{-1}$, whereas evenness steadily increased across the gradient in the Na treatment. It is possible that the base cations in the Ca:Mg:K treatment were a usable resource while NaCl acted as a stressor. The toxicity of NaCl deicers has been reported for numerous organisms (Corsi et al. 2010) but has not been linked to bacterial communities. Yet, my results suggest that once chronic specific conductivity surpasses 350 $\mu S\ cm^{-1}$, bacterial communities in streams impacted by road salts will have reduced diversity. Such effects may impact functional redundancy and decrease the resiliency of stream microbiomes to additional perturbations (McCann 2000).

### 3.5.3 Indicator Taxa of Freshwater Salinization

Several bacterial families that responded to freshwater salinization may be of ecological significance. For example, some species of Caulobacteraceae, which positively

responded at 125 µS $cm^{-1}$, are denitrifiers (61). *Azospirillium*, which also positively responded at 238 µS $cm^{-1}$, includes well known nitrogen fixers. While largely associated with plant hosts and identified as plant growth promoters (62), aquatic species have been identified (Kwak and Shin 2016). Thus, freshwater salinization may impact freshwater nitrogen cycling at low conductivities < 300 µS $cm^{-1}$, but more research is warranted. The family cvE6, which responded positively at 237.5 µS $cm^{-1}$ and was isolated from a freshwater pond in France (Corsaro et al. 2002), belongs to the Chlamydiae Class. Chlamydiae are obligate intracellular bacteria and are known as parasites of eukaryotes ranging from amoebas to humans (Corsaro and Venditti 2009). The last positive responding family was Spirosomaceae, which responded at 350 µS $cm^{-1}$. Members of this family are known to digest macromolecules including polysaccharides and proteins which may have important implications for carbon cycling and stream metabolism.

Of the three families that negatively responded to conductivity, the ecological role of Bdellovibrionaceae is most widely studied. The majority of Bdellovibrionaceae ASVs belonged to the genus *Bdellovibrio*, which are known bacterial predators (Stolp and Starr 1963). Species of this genus feed on nucleic acids and proteins of the host bacteria, ultimately resulting in host death (Sockett 2009). Thus, at higher conductivities (< 1 150 µS $cm^{-1}$) where Bdellovibrionaceae negatively responded, the pressure of bacterial predation from these species potentially declines and causes diversity and evenness to increase at higher conductivities. Bdellovibrionaceae species could also be scavenging resources made available by cellular lysis of other bacterial cells not adapted to low salinities.

### 3.5.4 Effects of Base Cations

By controlling for salinity, I was able to observe the direct effects of base cations on freshwater bacterial communities. Base cations have previously been correlated to bacterial diversity in field studies, including $Ca^{+2}$, $Mg^{2+}$, $K^{+}$, and $Na^{+}$ in the Noatak River of Alaska (Larouche et al. 2012) and $Ca^{2+}$ in forested, low-order streams with underlying limestone geology in Alabama (Mosher and Findlay 2011). However, these studies are correlative and likely confounded by multicollinearity among environmental variables, particularly ions that tend to maintain consistent ratios (e.g. $Ca^{2+}$ and $Mg^{2+}$), making conclusions regarding effects of individual salt types on community structure difficult to reach. Based on my results, stream water at an identical salinity, but with different base cations, can select for distinct bacterial communities. Additionally, relative to other base cations, bacterial communities in elevated $Ca^{2+}$ had lower ASV richness, Shannon diversity, and evenness. Further, $Ca^{2+}$ negated any increase in diversity resulting from increased conductivity, which is apparent when comparing the $Ca^{+2}$ treatment at 350 µS $cm^{-1}$ to reference stream water (black dotted line in Fig 3.5) with conductivity of 30 µS $cm^{-1}$. Thus, bacterial communities in streams with high $Ca^{+2}$ concentrations (i.e. karst geology or urban streams) might be more resilient to effects of freshwater salinization. $Ca^{2+}$ can influence the stability of bacterial biofilms, which may offer resiliency to stream bacterial communities exposed to increasing solute concentrations (Das et al. 2014, Goode and Allen 2011, Korstgens et al. 2001).

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## 4. Subsidy-stress response of bacterial respiration and nutrient uptake to freshwater salinization

## 4.1 Abstract

Human activity is increasing salt concentrations in freshwaters worldwide. Broad salinity shifts across brackish and marine concentrations change bacterial ecology and biogeochemical cycling, negatively affecting water quality and ecosystem functioning. The effects of increasing freshwater salt concentrations (≤ 1,500 µS $cm^{-1}$), which are orders of magnitute lower, are less understood. Using controlled mesocosms, I characterized the short-term (one to five days) responses of bacterial respiration and net N and P uptake across a freshwater salinity gradient of multiple salt types. After one day, bacterial respiration rates in Mg-, Na- and sea salt-based salinity generally showed a subsidy-stress response, with respiration increasing by over 100% as salinity increased from 30 to 350 – 800 µS $cm^{-1}$. Calcium-based salinity, on the other hand, caused monotonically increasing respiration rates peaking at 1,500 µS $cm^{-1}$. After five days, the mesocosms became hypoxic and an inverse subsidy-stress response was observed with higher respiration rates at the upper or lower ends of the salinity gradient. Ca and Mg salts changed bacterial net nutrient uptake rates, which led to large increases in N:P ratios of water at higher salinities. In addition to altering process rates, salinity and salt type produced distinct bacterial communities after only one day of exposure. Further, after five days, dissimilarity of bacterial community structures based on Bray-curtis dissimilarity were also correlated to pairwise differences in respiration and net nutrient uptake suggesting a link between community structure and function. Collectively, these results establish that short-term exposure to freshwater concentrations of different salts will differentially alter bacterial community membership, process rates, and ecological

stoichiometry. Effects of salinization may also differ in hypoxic conditions characteristic of eutrophic, hypolimnetic waters.

## 4.2 Introduction

Growing human demand for natural resources has caused substantial alterations and degradation to freshwater ecosystems worldwide (Strayer and Dudgeon 2010). Many imporant ecosystem processes, including biomass production and biogeochemical cycling, are facilitated by microbial communtities (Falkowski et al. 2008). Quantitative linkages between microbial membership, processes, and changes in resource pools are commonly assumed, but rarely tested (Bier et al. 2015a, Rocca et al. 2015). Changes in dominant bacterial metabolic pathways or process rates can alter ecological stoichiometry and have pronounced implications for water quality and ecosystem functions (Friedl and Wuest 2002, Park et al. 2010, Simon et al. 1992, Stockner and Porter 1988, Welti et al. 2017). Bacterial processes, and their rates, change across environmental gradients that are often anthropogenically altered, including flow, nutrient concentrations, temperature, dissolved oxygen, and salinity (Bernhard et al. 2010, Demars et al. 2011, Dodds et al. 2002, Wang et al. 2018, Zhang et al. 2015).

Subsidy-stress responses, where process rates peak at intermediate levels of a particular variable, are commonly observed across environmental gradients (Odum et al. 1979, Simonin et al. 2019). For example, across freshwater-marine salinity gradients (~30 – 60,000 $\mu S\ cm^{-1}$), bacterial respiration and denitrification rates are highest at intermediate, estuarine salinities and decrease towards freshwater and marine extremes (Bouvier and del Giorgio 2002, Langenheder et al. 2003, Pakulski et al. 1995). Most studies on the effects of salinity on biogeochemical cycles focus on estuarine systems that

span large salinity gradients (i.e. freshwater to marine or brackish to marine). Less is known about how bacterial processes vary across freshwater salinity gradients (≤ 1,500 $\mu S\ cm^{-1}$) despite widespread salinization of streams, rivers, and lakes (Kaushal et al. 2018).

Human activities including deicing, agriculture, urbanization, and resource extraction, as well as actions leading to saltwater intrusion, are increasing salt concentrations in freshwaters worldwide (Barlow and Reichard 2010, Corsi et al. 2010, Kaushal et al. 2018, Williams 2001). Different causes of freshwater salinization produce distinct ionic profiles resulting in spatial heterogeneity of base cations in surface waters (Griffith 2014, Kaushal et al. 2018). For example, base cations in deicing salts and seawater are predominately Na (Corsi et al. 2010, Soulsby 1995), fertilizers and liming agents contain Ca, Mg and K (Fitzpatrick et al. 2007), while dissolution of construction materials and alkaline mine drainage add Ca and Mg (Bernhardt et al. 2012, Kaushal et al. 2017). The duration and intensity of salinization disturbances also vary by source and depend on climatic variables such as precipitation and storm intensity (Glasby and Underwood 1996). While salinization from agriculture or accelerated weathering can cause long-lasting, low-intensity disturbances (i.e., a press disturbance) (Kaushal et al. 2005, Williams 2001), flushing of deicing salts or storm surges in coastal regions can cause brief but intense salt disturbances (i.e., pulse disturbances) (Corsi et al. 2010). Varying duration and intensity of disturbances can result in different effects on biological communities and should be considered when assessing the effects of freshwater salinization on bacterial processes (Parkyn and Collier 2004).

Changing salt concentrations in the freshwater range can affect process rates of numerous biological communities. For example, prolonged exposure (two weeks) to slightly elevated Na concentrations (7 ppm, ~ 35 µS $cm^{-1}$), characteristic of a press disturbance, reduced leaf consumption by detritivores in microcosm studies (Tyree et al. 2016). In contrast, organic matter decomposition rates in streams did not vary across a mining-induced salinity gradient dominated by $Ca^{2+}$, $Mg^{2+}$, $HCO_3^-$, and $SO_4^{2-}$ (≤ 1,383 µS $cm^{-1}$) (Vander Vorste et al. 2019). Longer exposure (six weeks) of freshwater to higher Na concentrations (140 ppm, ~ 700 µS $cm^{-1}$) lowered microbial respiration rates by as much as 29% (Tyree et al. 2016). The effects of pulse disturbances of salt on freshwater bacterial communities are poorly understood and often confounded by co-varying changes in other flow-dependent parameters including nutrients and sediments (O'Donnell and Hotchkiss 2019). In addition, the effects of freshwater salinization on bacterial processes in hypoxic waters, which are hotspots of biogeochemical cycling (Klump et al. 2009, Lin et al. 2016), are unknown. Freshwater salinization alters bacterial survival (DeVilbiss et al., *in review*) and diversity (Bier et al. 2015b, Vander Vorste et al. 2019), but quantitative linkages between bacterial community membership and bacterial processes are difficult to establish, especially in changing environments.

Much of what is known about aquatic microbiomes comes from field surveys where relationships to environmental variables are correlative (Bouvier and del Giorgio 2002, Herlemann et al. 2011, Liu et al. 2012). Controlled laboratory studies that identify direct impacts of isolated variables on both bacterial membership and processes are uncommon (Bier et al. 2015a). For example, when abundances of microbial functional genes and rates of the associated microbial processes are measured simultaneously, only

38% of studies report a positive correlation (Rocca et al. 2015). However, in the few studies that did test for relationships between bacterial community structure and processes, 75% of studies reported a significant correlation (Bier et al. 2015a). Identifying linkages between bacterial community structure and processes can improve projections of water quality and ecosystem processes in changing ecosystems.

My objectives were to (1) experimentally test how freshwater salinization (≤ 1,500 µS $cm^{-1}$) from multiple, common salt types will affect bacterial respiration and net nutrient uptake, and (2) identify linkages between bacterial processes and bacterial community structure. Based on my previous work (see Chapter 2), I hypothesized that freshwater salinization would alleviate hypo-osmotic stress, thus causing a subsidy-stress response with higher respiration rates occurring at moderate freshwater salinities (350 – 800 µS $cm^{-1}$). Further, because base cations are micronutrients that support multiple bacterial processes, I hypothesized that different salt types would induce distinct bacterial process responses. Collectively, my results identified how bacterial community structure and processes respond to freshwater salinization. Linking bacterial community structure and function can help predict how aquatic ecosystems respond to changing environmental conditions.

## 4.3 Methods

I designed a mesocosm experiment to quantify the effects of freshwater salinization on bacterial process rates after relatively short exposures of one and five days. I tested the effects of four salt types ($CaCl_2$, $MgCl_2$, NaCl, and artificial sea salt) on bacterial respiration rates (biological oxygen demand; BOD), nitrogen and phosphorus uptake rates, and bacterial community structure across a salinity gradient ranging from

specific conductivities (SC) of 30 to 1,500 µS $cm^{-1}$. I destructively sampled one set of mesocosms after a one-day incubation and the second set of mesocosms after a five-day incubation.

### 4.3.1 Sand inoculation and water collection

To include representative stream communities in mesocosms, I submerged acid-washed sand in nylon bags in a forest headwater stream in Pembroke, VA (37°20'28'' N 80°37'21''W) for two weeks. The site has naturally low nutrient concentrations and SC ranging from ~ 25 – 50 µS $cm^{-1}$ (Cheever and Webster 2014). Acid-washed sand provided a realistic medium for bacterial colonization, but minimized the potential for sediment-bound salts or nutrients to be released into mesocosm water as a result of experimentally altering salinity (Weston et al. 2010). After two weeks, I removed the sand from the stream and gently rinsed it with stream water to remove any natural organic matter. At the same time, I also collected stream water in 20 L acid-washed carboys. Sand and water were immediately returned to the lab for mesocosm setup on the same day.

### 4.3.2 Experimental Design

I used 300 mL biological oxygen demand (BOD) bottles as mesocosms. Using a destructive sampling design, I tested two incubation exposure times: one and five days. Each salt treatment was tested in triplicate and separate sets of bottles were destructively sampled after one and five days (*i.e.*, each sample day had its own complete set of bottles). Each mesocosm received 25 cc of stream-inoculated sand to mimic a natural benthic environment. To prepare individual salt treatments, I spiked 2 L of stream water

with either $CaCl_2$, $MgCl_2$, NaCl, or artificial sea salt in separate acid-washed bottles to target SCs of 125, 350, 800, and 1,500 µS $cm^{-1}$ (four salt types, 4 conductivities, 16 total treatments) measured with an Orion Star A223 conductivity probe (ThermoFisher Scientific, USA) (Fig. 4.1). My primary goal was to determine the potential for freshwater salinization to alter bacterial processes, not measure ambient, *in situ* rates. Therefore, to eliminate any potential nutrient limitations and provide enough carbon to ensure measurable respiration rates, I primed each treatment with carbon (glucose-C), nitrogen ($NH_3NO_3$), and phosphorus ($H_3PO_4$) at the Redfield ratio (106:16:1) at 5 ppm C. After treatments were prepared, I aliquoted 300 mL of each treatment into six separate mesocosms (triplicate sets destructively sampled for both one- and five-day measurements). To determine the relative contribution on uptake rates of the planktonic bacterial community and the episammon bacterial community (i.e., community living on sand substrates), I also included a single, reference triplicate mesocosm set with no sand. Before capping each bottle, I measured initial dissolved oxygen (DO) concentrations using an Orion Star A223 DO probe (ThermoFisher Scientific, USA) and recorded the time of measurement. I incubated bottles in the dark to prevent photosynthetic activity.

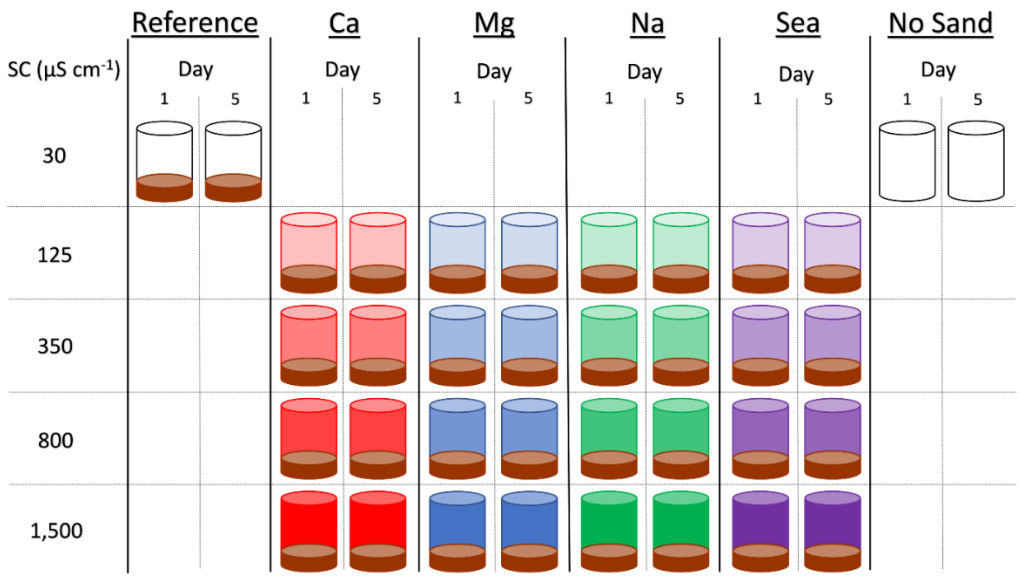


**Figure 4.1.** Experimental design of mesocosms. Each cylindar represents a triplicate set of mesocosms. All mesocosms were spiked with C, N, and P. All mesocosms except the "No Sand" bottles had sand (symbolized by the brown base).

### 4.3.3 Mesocosm Sampling and Nutrient Analyses

After one day, I destructively sampled the first set of mesocosms from each treatment, including reference and water-only mesocosms. For each mesocosm, I measured a final DO concentration and recorded the time of measurement. After DO measurement, I syringe-filtered each sample through a 0.4 µm polycarbonate filter (Whatman®). I stored the filtrate in 60 mL, acid-washed HDPE bottles (Nalgene®) at -20°C and the filter at -80°C until analyses. I followed the same procedure for the other set of mesocosms after five days. For all samples, I measured concentrations of $NO_3$-N, $NH_4$-N, and SRP colorimetrically on a Lachat autoanalyzer (Milwaukee, USA) following manufacture protocol. BOD and nutrient uptake rates in units of either mg $d^{-1}$ (BOD) or µg $d^{-1}$ (nutrients) were calculated as:

$$U_R = [(C_{initial} - C_{final}) * V] / \Delta T \quad (1)$$

Where $U_R$ is the uptake rate, $C_{initial}$ is the initial concentration in mg (DO) or µg (nutrients) $L^{-1}$, $C_{final}$ is the final concentration, V is volume of water in L (0.275 L), and ΔT is elapsed time (initial – final) in d. I calculated the contribution of the episammon community to uptake rates as the difference between mesocosms with and without sediment.

### 4.3.4 Bacterial DNA Sequencing and Quality Filtering

I extracted DNA from the Whatman® filters using the DNeasy PowerWater Kit (QIAGEN, USA) following the manufacturer's protocol and stored DNA extracts at -20°C until amplification. Polymerase chain reaction (PCR) was performed following the Earth Microbiome Project protocol (Gilbert et al. 2014) targeting the V4 region of the 16S rRNA gene using the 515F-806R primer set (Caporaso et al. 2011). I purified the 16S rRNA amplicons using the QIAquick PCR purification kit (QIAGEN, USA) following manufacture protocol and measured amplicon DNA concentrations with a Qubit 4 fluorometer (Invitrogen, USA). I pooled samples to an even DNA mass prior to sequencing. The Duke Center for Genomic and Computational Biology (Durham, NC) performed DNA sequencing on an Illumina MiSeq using 250 base-pair, paired-end reads. 10% PhiX (Illumina) was added to the pooled sample prior to sequencing for quality control.

I processed the V4 16S rRNA gene amplicon sequences using QIIME2 (Bolyen et al. 2018). I used DADA2 (Callahan et al. 2016) to denoise and dereplicate sequences, allowing for a maximum expected error rate of 2. I performed additional frequency-filtering removing any amplicon sequence variants (ASVs) present only once or that appeared in only one sample. I taxonomically classified filtered ASVs using a pre-

trained, naïve Bayes classifier (Pedregosa et al. 2011) bound by the 515-806R primer pair produced by the Silva database (99% consensus taxonomy with seven levels) (Quast et al. 2013). To produce a final dataset, I removed any sequences that classified as Chloroplast, Mitochondria, or were unclassified at the Domain level.

### 4.3.5 Analysis of Bacterial Uptake Rates across a Freshwater Salinity Gradient

I performed all analyses and visualizations in R (version 1.2.1335) (Team 2019). I hypothesized that bacterial process rates would show a subsidy-stress response across a freshwater salinity gradient. Thus, mean BOD and nutrient uptake rates for each salt treatment ($CaCl_2$, $MgCl_2$, NaCl, sea salt) were plotted across the freshwater salinization gradient and fit to a second-order polynomial regression, characteristic of subsidy-stress response curves (Odum et al. 1979). For BOD rates, I calculated observed and modeled maximum and minimum rates, as well as the effect size (Cohen's *d*) based on observed maximum and minimum rates. To determine how changes in BOD and nutrient uptake rates influenced nutrient stoichiometry, I calculate N:P molar ratios (N = sum of $NO_3$-N and $NH_4$-N). At each individual conductivity, I performed a one-way ANOVA to test for significant differences in BOD rates across salt treatments. For significant results, I performed a Tukey's HSD test to identify significant pair-wise differences in BOD rates among salt treatments.

### 4.3.6 Analysis of Bacterial Community Structure

I analyzed bacterial community structure using a combination of diversity metrics (e.g. α- and β-diversity) and multivariate techniques (e.g. ordinations) based on the relative abundances of ASVs using the phyloseq (McMurdie and Holmes 2013) and

vegan (Oksanen et al. 2019) packages in R (version 1.2.1335) (R Core Team 2019). I rarified samples to the sample with the lowest number of reads (9,611) prior to any analyses. To visualize differences in bacterial community structure from both salt-type and concentration, I plotted a principle coordinates (PCoA) ordination using Bray-Curtis dissimilarity as a secondary matrix. One- and five-day samples were plotted and analyzed separately. To test if salt type, salt concentration, or an interaction of both caused differences in bacterial community structure based on Bray-Curtis dissimilarity, I ran a two-way PERMANOVA (Anderson 2001). I also tested for pairwise differences in community structure based on salt type using a pairwise PERMANOVA (Arbizu 2020). I tested for differences in multivariate dispersion using PERMDISP2 (Anderson et al. 2006), which can cause false-positives with PERMANOVA. However, these affects are minimal with balanced experimental designs like ours (Anderson and Walsh 2013). To test for correlations between bacterial community structure and uptake rates for both one- and five-day samples, I performed separate Mantel tests using a bacterial community Bray-Curtis dissimilarity matrix and Euclidean distance matrices based on combined BOD and nutrient uptake rates as well as individual uptake rates. Lastly, I calculated alpha diversity based on Renyi's Entropy using q=0 for richness, q=1 for Shannon diversity, and q=2 for evenness (Jost 2007).

### 4.3.7 Threshold Indicator Taxa Analysis

I used threshold indicator taxa analysis TITAN (Baker et al. 2019) to identify bacterial families that responded positively or negatively to the salinity gradient and the SC range where the response occurred. Prior to analysis, I aggregated ASVs at the family level and eliminated any families that occurred in less than three samples, which is

required for the analysis. Using 500 bootstrapped runs, TITAN identified high purity taxa as families that responded in the same direction across the salinity gradient in 95% of the runs. High reliability taxa had a distribution significantly different from a random distribution in 95% of the bootstrapped runs. Only high purity, high reliability families are reported. I then tested the relationship between the relative abundance of high purity, high reliability families and BOD rates by regressing bacterial family read counts against BOD rates.

## 4.4 Results

### 4.4.1 Biological Oxygen Demand Rates Across Freshwater Salinity Gradients

BOD rates in reference mesocosms with sand were not significantly greater than water-only reference mesocosms, indicating the contribution of the episammon community to uptake rates was minimal. Therefore, reported uptake rates likely reflect the bacterioplankton community, not the episammon community. Additionally, while one-day samples remained oxic (DO range = 4.48 – 7.40 mg $L^{-1}$), high BOD caused the five-day samples to become hypoxic (DO range = 1.20 – 3.31 mg $L^{-1}$) and thus were analyzed separately. However, low DO concentrations provided the opportunity to evaluate how salinization might impact biogeochemical cycling under hypoxia. Because five-day samples were confounded by both time and DO, direct comparisons to one-day samples were not made.

Observed and modeled BOD differed based on salt type and concentration (Fig. 4.2). For one-day samples, maximum BOD occurred at different salinities for different salt treatments, while minimum rates occurred at the lowest salinity (30 µS $cm^{-1}$) for all

salt treatments (Fig. 4.2A, Table 4.1). BOD for Mg, Na, and sea salt showed a subsidy-stress response, while BOD in the Ca treatment increased monotonically as salinity increased. The highest observed BOD occurred in the sea salt treatment at 350 µS $cm^{-1}$, which was 103% higher than the lowest BOD, followed by the Mg treatment at 800 µS $cm^{-1}$ (97% greater), the Na treatment at 800 µS $cm^{-1}$ (85% greater), and lastly the Ca treatment at 1,500 µS $cm^{-1}$ (64% greater) (Table 4.1). Together, these results establish that pulse salinity disturbances affect bacterial BOD, and the effects differ based on salt type.

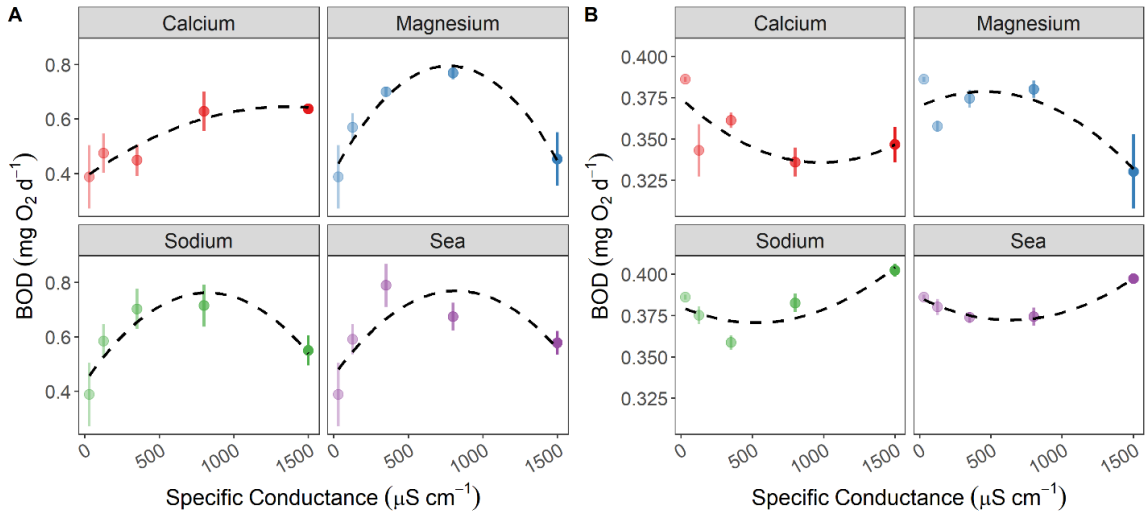


**Figure 4.2.** Biological oxygen demand (BOD) of stream water-colonized sands and surface water incubated across a freshwater salinity gradient of different chloride salts and sea salt after (A) one day under oxic conditions and (B) five days under conditions that reached hypoxia.

**Table 4.1.** Observed and modeled maximum and minimum biological oxygen demand (BOD) rates (mg $O_2$ $d^{-1}$) and corresponding specific conductivities (SC, µS $cm^{-1}$) for each salt type after both one-day and five-day incubations. Data were fit to second order polynomial regressions and model statistics ($r^2$ and p value) are provided as well as percent increase of observed mean minimum to mean maximum BOD rates.

| **Salt** | **Observed Max (± SE)** | | **Observed Min (± SE)** | | **Modeled Max** | | **Modeled Min** | | **Model Fit** | | **Observed % increase** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | SC | BOD | SC | BOD | SC | BOD | SC | BOD | $r^2$ | p value | |
| | | | | | **one-day** | | | | | | |
| **Ca** | 1500 | 0.64 ± 0.019 | 30 | 0.39 ± 0.12 | 1360 | 0.65 | 30 | 0.40 | 0.33 | 0.037 | 64.1% |
| **Mg** | 800 | 0.77 ± 0.023 | 30 | 0.39 ± 0.12 | 770 | 0.80 | 30 | 0.44 | 0.56 | 0.0029 | 97.4% |
| **Na** | 800 | 0.72 ± 0.078 | 30 | 0.39 ± 0.12 | 820 | 0.76 | 30 | 0.46 | 0.33 | 0.035 | 84.6% |
| **Sea** | 350 | 0.79 ± 0.079 | 30 | 0.39 ± 0.12 | 820 | 0.77 | 30 | 0.48 | 0.026 | 0.065 | 102.6% |
| | | | | | **five-day** | | | | | | |
| **Ca** | 30 | 0.39 ± 0.0015 | 800 | 0.34 ± 0.0087 | 30 | 0.37 | 979 | 0.34 | 0.21 | 0.098 | 14.7% |
| **Mg** | 30 | 0.39 ± 0.0015 | 1500 | 0.33 ± 0.022 | 450 | 0.38 | 1500 | 0.33 | 0.37 | 0.024 | 18.2% |
| **Na** | 1500 | 0.40 ± 0.0038 | 350 | 0.36 ± 0.0044 | 1500 | 0.40 | 523 | 0.37 | 0.52 | 0.0050 | 11.1% |
| **Sea** | 1500 | 0.40 ± 0.0028 | 350 | 0.37 ± 0.0032 | 1500 | 0.40 | 636 | 0.37 | 0.66 | 0.00060 | 8.1% |

For the five-day samples that became hypoxic, maximum and minimum rates BOD still occurred at different salinities for different salt types (Fig. 4.2B, Table 4.1). Unlike the one-day samples, maximum BOD occurred at the lowest salinity (30 µS $cm^{-1}$) for the Ca and Mg treatments. Conversely, the highest BOD for Na and sea salt occurred at the highest salinity (1,500 µS $cm^{-1}$). Minimum observed BOD also differed by salt type with the minimum rates in the Ca treatment occurring at 800 µS $cm^{-1}$, minimum rates in the Mg treatment occurring at 1,500 µS $cm^{-1}$, and minimum rates in the Na and sea salt treatments occurring at 350 µS $cm^{-1}$ (Table 4.1). Percent differences of observed maximum and minimum BOD were much lower for the five-day samples than the one-day samples. The greatest observed percent increase was 18% in the Mg treatment and the lowest was 8% in the sea salt treatment. Under longer salt exposure and lower DO concentrations, BOD still differs across freshwater salinity gradient and by salt-type. Trends and effect sizes of BOD after longer exposure and lower DO concentrations also differ from those observed after a one-day exposure.

BOD also differed among salt types at the same salinity, but rates were mediated by length of exposure and hypoxia. For one-day incubations, differences in BOD between salt types occurred only at 350 µS $cm^{-1}$ where sea salt was significantly higher than Ca ($p = 0.01$; Fig. 4.3B). However, for the five-day incubations with longer exposure and under hypoxic conditions, differences in BOD among salt types were observed at higher salinities, 800 and 1,500 µS $cm^{-1}$. At 800 µS $cm^{-1}$, BOD for Ca was lower than Mg, Na, and sea salt ($p = 0.005$, $p = 0.004$, $p = 0.012$, respectively; Fig. 4.4C). At 1,500 µS $cm^{-1}$, BOD in the Mg treatment was lower than Na and sea treatments ($p = 0.016$, $p = 0.023$,

respectively; Fig. 4.4D). Thus, under different exposure time and oxygen conditions, different salt types have varying effects on BOD.

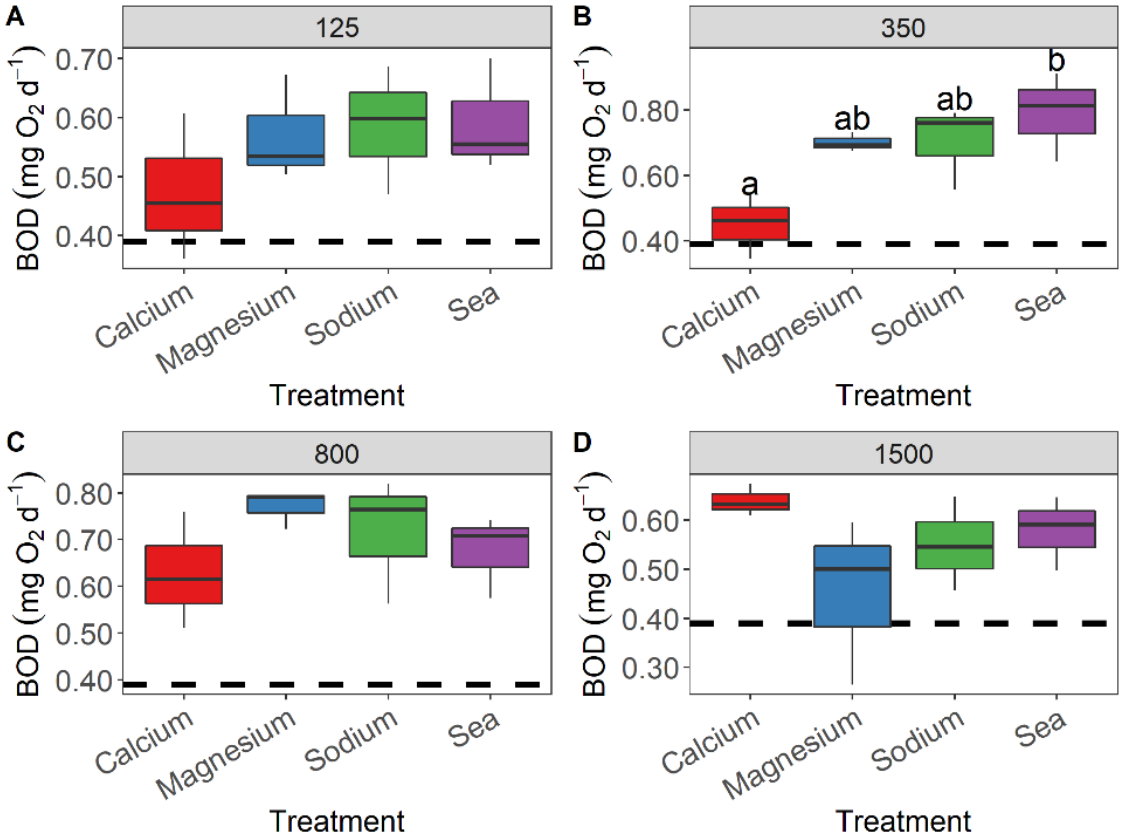


**Figure 4.3.** One-day biological oxygen demand (BOD) of stream water-colonized sands and surface water incubated under oxic conditions exposed to individual chloride salts at specific conductivities of (A) 125 µS $cm^{-1}$, (B) 350 µS $cm^{-1}$, (C) 800 µS $cm^{-1}$, and (D) 1,500 µS $cm^{-1}$. The black dotted line represents the average BOD of reference stream water with no salt additions at a conductivity of 30 µS $cm^{-1}$. Comparisons are significant at $p<0.05$. Only panels with letters had significant differences among treatments.

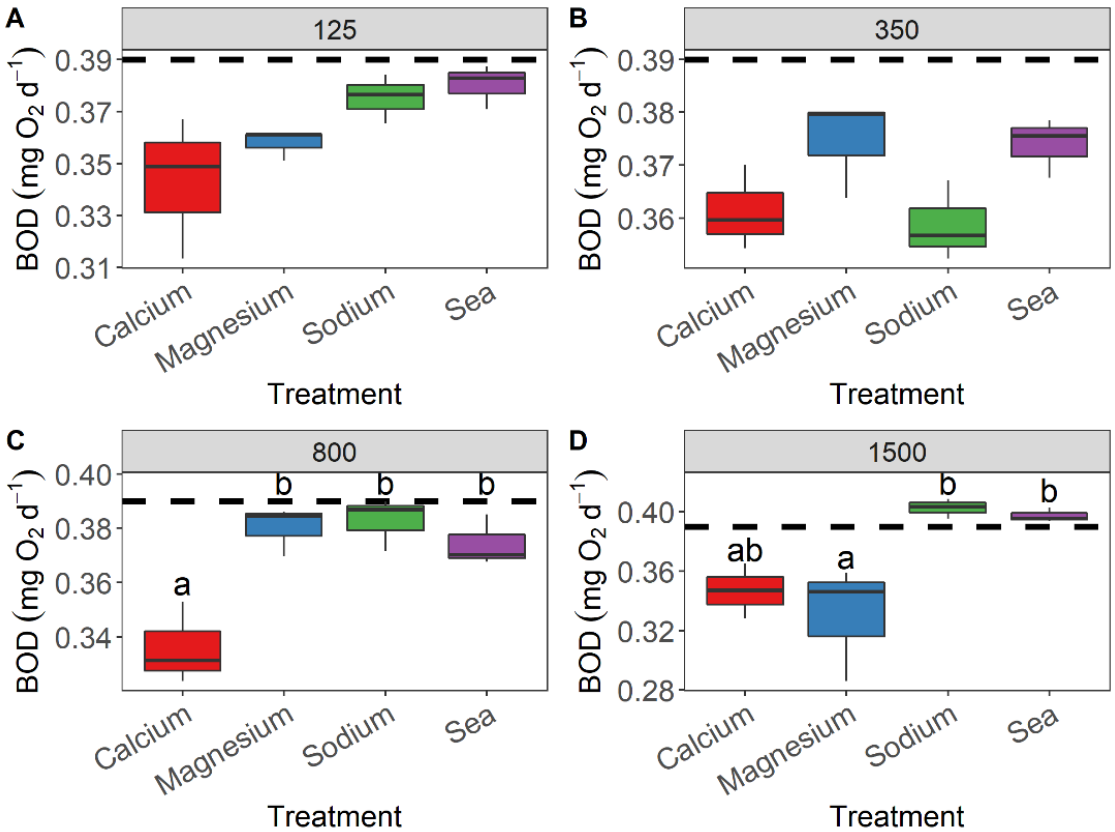


**Figure 4.4.** Five-day BOD of stream water-colonized sands and surface water incubated under hypoxic conditions exposed to individual chloride salts at specific conductivities of (A) 125 µS $cm^{-1}$, (B) 350 µS $cm^{-1}$, (C) 800 µS $cm^{-1}$, and (D) 1,500 µS $cm^{-1}$. The black dotted line represents the average BOD of reference stream water with no salt additions at a conductivity of 30 µS $cm^{-1}$. Comparisons are significant at $p<0.05$. Only panels with letters had significant differences among treatments.

### 4.4.2 Effects of Salt Type and Concentration on Bacterial Nutrient Uptake Rates

Net nutrient uptake trends were similar to BOD and either positively or negatively related to BOD rates. For one-day incubations, uptake of $NH_4$, $NO_3$, and SRP were linearly related to BOD ($r^2 = 0.21$, $p < 0.001$; $r^2 = 0.14$, $p = 0.002$; $r^2 = 0.095$, $p = 0.010$, respectively). The highest $NH_4$-N uptake rates were observed at the lowest and highest salinities, displaying an inverse subsidy-stress response opposite than that of BOD (Fig. 4.5, top row). $NH_4$-N uptake in the Ca treatment was opposite BOD trends, decreasing consistently as SC increased. For all salt types and salinity levels, $NH_4$-N uptake rates

were positive, indicating $NH_4$ removal from the water. Trends of $NO_3$-N uptake across the salinity gradient were the same as BOD trends. However, uptake rates were both positive and negative depending on salinity, indicating that salinity influenced whether $NO_3$ was removed or added, respectively, from the water. In general, $NO_3$ uptake rates were negative at the highest and lowest salinity and positive at intermediate salinity levels (Fig. 4.5, second row). Net SRP uptake rates were positive for all salt treatments across the salinity gradient, indicating that SRP was removed from the water. SRP uptake trends were similar to $NH_4$-N, with the highest rates occurring at the upper and lower extremes of the salinity gradient. While SRP uptake trends were the opposite of BOD trends for Mg, Na, and sea salt, they were the same for Ca, with SRP uptake rates increasing monotonically with increasing salinity (Fig. 4.5, third row). As a result, N:P ratios in the water of Ca treatments increased drastically from 273 ± 48 to 1137 ± 268 (315% increase) across the salinity gradient while relatively minimal differences were observed for Mg, Na, and Sea (Fig. 4.5, bottom row).

Similar to day-one samples, net $NH_4$-N uptake in day-five samples was positive for all salt treatments across the salinity gradient (Fig. 4.6, top row). While net $NO_3$-N uptake steadily declined from positive to negative rates across the salinity gradient in the Ca treatment, net $NO_3$-N uptake was positive and showed little variation across the salinity gradient for Mg, Na, and sea salt treatments (Fig. 4.6, second row). Net SRP uptake rates were similar in Ca and Mg treatments, consistently increasing from negative to positive as salinity increased. Trends in net SRP uptake for Na and sea salt treatments were also similar, showing relatively little change and remaining negative across the entire salinity gradient (Fig. 4.6, third row). While mesocosm water N:P ratios were

consistently lower in five-day samples relative to one-day samples, drastic increases were observed for both Ca and Mg as salinity increased (136% and 86% increase, respectively) (Fig. 4.6, bottom row). However, only $NO_3$ and SRP net uptake rates were linearly related to BOD ($r^2 = 0.23$, $p < 0.001$; $r^2 = 0.38$, $p < 0.001$, respectively).

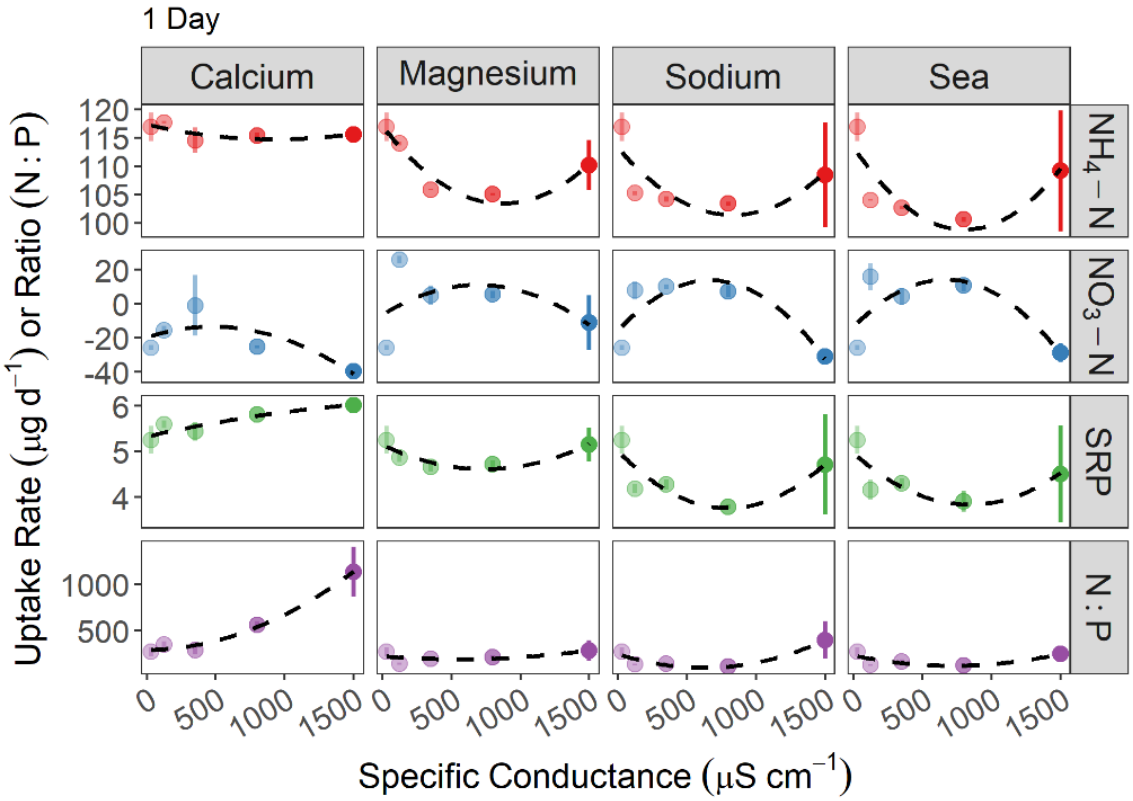


**Figure 4.5.** Net nutrient uptake rates for $NH_4$-N, $NO_3$-N, and SRP and the resulting N:P ratio across a freshwater salinity gradient of different chloride salts after one day under oxic conditions.

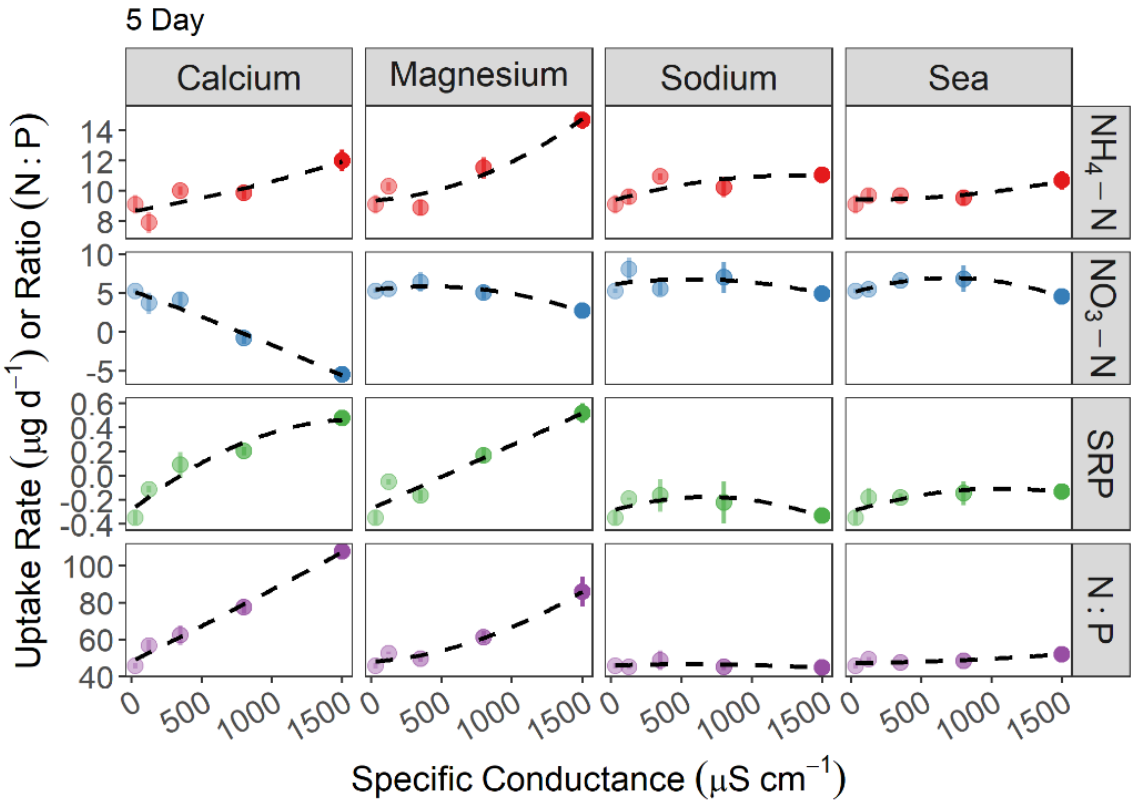


**Figure 4.6.** Net nutrient uptake rates for $NH_4$-N, $NO_3$-N, and SRP and the resulting N:P ratio across a freshwater salinity gradient of different chloride salts after five days under conditions that reached hypoxia.

### 4.4.3 Effect of Salinity and Salt Type on Bacterial Community Structure

Salinity and salt type produced distinct bacterial communities in both one- and five-day samples (Fig. 4.7) (all $p < 0.05$, statistical summary in Table 4.2). After one day, bacterial community structure was different between all salt-type pairwise comparisons except Na vs. sea salt (Table 4.3, Fig. 4.7A). After one day, bacterial communities were also different between different salinities with the exception of 30 µS $cm^{-1}$ vs. 125 µS $cm^{-1}$, 30 µS $cm^{-1}$ vs. 350 µS $cm^{-1}$, and 125 µS $cm^{-1}$vs. 350 µS $cm^{-1}$ (Table 4.4). Five-day samples also differed based on salt type with the exception of Na vs. sea salt. For both one- and five-day samples, bacterial community structure in Na and sea salt treatments were not different. Salinity also produced distinct bacterial communities after day five,

but the trends were not as consistent as after one day. Specifically, I observed no significant differences in community structure between 30 µS $cm^{-1}$ vs. 125 µS $cm^{-1}$, 30 µS $cm^{-1}$ vs. 800 µS $cm^{-1}$, 125 µS $cm^{-1}$ vs. 800 µS $cm^{-1}$, and 350 vs 800 µS $cm^{-1}$ (Table 4.4).

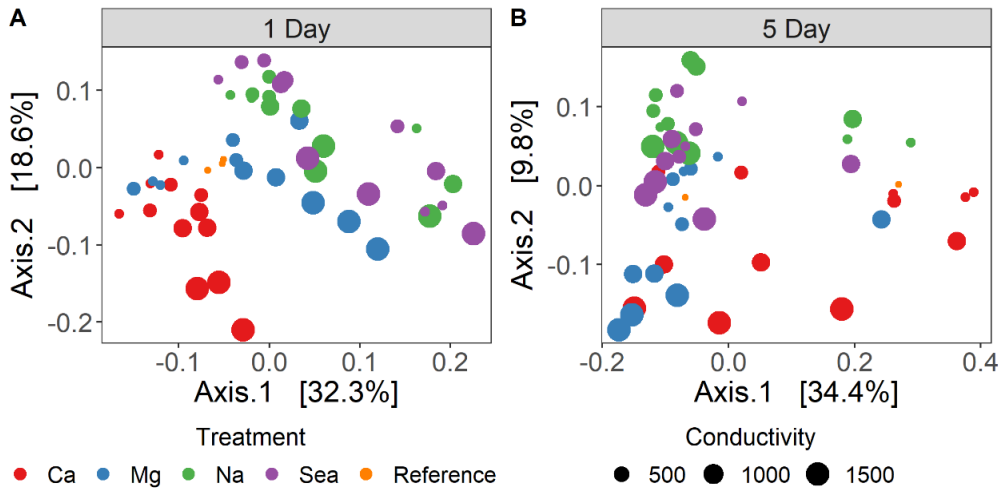


**Figure 4.7.** Principle Coordinates ordinations based on Bray-Curtis dissimilarity of bacterial communities in stream water exposed to a freshwater salinity gradient of different chloride salts after A) one day exposure in oxic conditions and B) a five day exposure under conditions that went hypoxic (right panel).

**Table 4.2.** Statistical summary of two-way PERMANOVA analyses for bacterial community structure based on Bray-Curtis dissimilarity for one-day and five-day samples.

| Variable | DF | Pseudo-F | $r^2$ | p-value |
|---|---|---|---|---|
| | | **One-day** | | |
| **Salt Type** | 4 | 5.74 | 0.28 | < 0.001 |
| **Salt Concentration** | 1 | 9.87 | 0.12 | < 0.001 |
| **Interaction** | 3 | 1.92 | 0.071 | 0.009 |
| **Residual** | 42 | - | 0.52 | - |
| | | **Five-day** | | |
| **Salt Type** | 4 | 3.58 | 0.21 | < 0.001 |
| **Salt Concentration** | 1 | 4.59 | 0.069 | < 0.001 |
| **Interaction** | 3 | 1.92 | 0.087 | 0.013 |
| **Residual** | 42 | - | 0.63 | - |

**Table 4.3.** Pairwise PERMANOVA statistical summary for salt-type contrasts. For all contrasts, salt-treatment DF = 1 and residual DF = 13.

| Contrast | Pseudo F | $r^2$ | P value |
|---|---|---|---|
| | **One-day** | | |
| **Ca vs. Mg** | 4.23 | 0.16 | **0.002** |
| **Ca vs. Na** | 10.77 | 0.33 | **< 0.001** |
| **Ca vs. Sea** | 10.57 | 0.32 | **< 0.001** |
| **Mg vs. Na** | 3.56 | 0.14 | **0.002** |
| **Mg vs. Sea** | 3.87 | 0.15 | **0.002** |
| **Na vs. Sea** | 0.84 | 0.037 | 0.498 |
| | **Five-day** | | |
| **Ca vs. Mg** | 4.45 | 0.17 | **0.004** |
| **Ca vs. Na** | 4.03 | 0.15 | **0.005** |
| **Ca vs. Sea** | 4.98 | 0.18 | **<0.001** |
| **Mg vs. Na** | 3.72 | 0.14 | **<0.001** |
| **Mg vs. Sea** | 3.03 | 0.12 | **<0.001** |
| **Na vs. Sea** | 1.91 | 0.080 | 0.075 |

**Table 4.4.** Pairwise PERMANOVA statistical summary for conductivity contrasts. For all contrasts, conductivity-treatment DF = 1 and residual DF = 13.

| Contrast | Pseudo F | $r^2$ | P value |
|---|---|---|---|
| | **One-day** | | |
| **30 vs. 125** | 1.13 | 0.080 | 0.272 |
| **30 vs. 350** | 1.52 | 0.10 | 0.14 |
| **30 vs. 800** | 2.15 | 0.14 | **0.035** |
| **30 vs. 1,500** | 2.87 | 0.18 | **0.015** |
| **125 vs. 350** | 0.90 | 0.039 | 0.448 |
| **125 vs. 800** | 2.41 | 0.099 | **0.03** |
| **125 vs. 1,500** | 4.75 | 0.18 | **0.003** |
| **350 vs. 800** | 2.16 | 0.060 | **0.036** |
| **350 vs. 1,500** | 6.95 | 0.24 | **< 0.001** |
| **800 vs. 1,500** | 4.04 | 0.16 | **< 0.001** |
| | **Five-day** | | |
| **30 vs. 125** | 0.68 | 0.050 | 0.644 |
| **30 vs. 350** | 1.64 | 0.11 | **0.039** |
| **30 vs. 800** | 1.15 | 0.081 | 0.322 |
| **30 vs. 1,500** | 2.19 | 0.14 | **0.045** |
| **125 vs. 350** | 2.60 | 0.11 | **0.023** |
| **125 vs. 800** | 1.48 | 0.063 | 0.151 |
| **125 vs. 1,500** | 4.17 | 0.16 | **0.004** |
| **350 vs. 800** | 1.54 | 0.065 | 0.11 |
| **350 vs. 1,500** | 2.96 | 0.12 | **< 0.001** |
| **800 vs. 1,500** | 2.26 | 0.093 | **0.036** |

### 4.4.4 Indicator Taxa of Freshwater Salinization and their Relationships to BOD and Nutrient Uptake Rates

TITAN identified a total of 8 salinity indicator bacterial families (two positive, six negative) for one-day samples (Fig. 4.8A) and 15 indicator bacterial families (two positive, 13 negative) for five-day samples (Fig. 4.9A). For one-day samples, the relative abundance of two indicator families, Microbacteriaceae and Spirosomaceae, were negatively correlated with BOD in the Ca treatment only (Fig. 4.8B and C). For five-day samples, the relative abundance of two indicator families, Flavobacteriaceae and Aeromonadaceae, were positive correlated with BOD in the Ca and Na treatments, respectively (Fig. 4.9C). One indicator family, Archangiaceae, was negatively correlated with BOD in the Na treatment. Collectively, out of 293 families identified via targeted 16S gene amplicon sequencing, 13 unique families responded to the salinity gradient and only five were linearly related to BOD.

Based on whole-community structure differences (Bray-Curtis dissimilarity), bacterial community structure was only correlated to net nutrient uptake rates (Euclidean distance matrix based on BOD + $NH_4$ + $NO_3$ + SRP) for five-day samples (Mantel statistic = 0.33, p = 0080), not one-day samples. To better understand which individual nutrient uptake rates were most strongly correlated to bacterial community structure for five-day samples, I performed Mantel tests for each individual nutrient. Of the different nutrient uptake rates measured, whole-community structure was correlated with net $NO_3$ uptake (Mantel statistic = 0.33, p = 0.0010) and net SRP uptake (Mantel statistic = 0.18, p = 0.039) (Table 4.5). However, bacterial community structure was not correlated with BOD for either one or five day samples. Additionally, based on linear regression, net

$NH_4$ uptake were negatively correlated to all alpha diversity measures (richness ($r^2$ = 0.33, $p < 0.001$), Shannon diversity ($r^2$ = 0.33, $p < 0.001$), evenness, $r^2$ = 0.35, $p < 0.001$) and multivariate dispersion ($r^2$ = 0.31, $p < 0.001$) (Fig. 4.10).

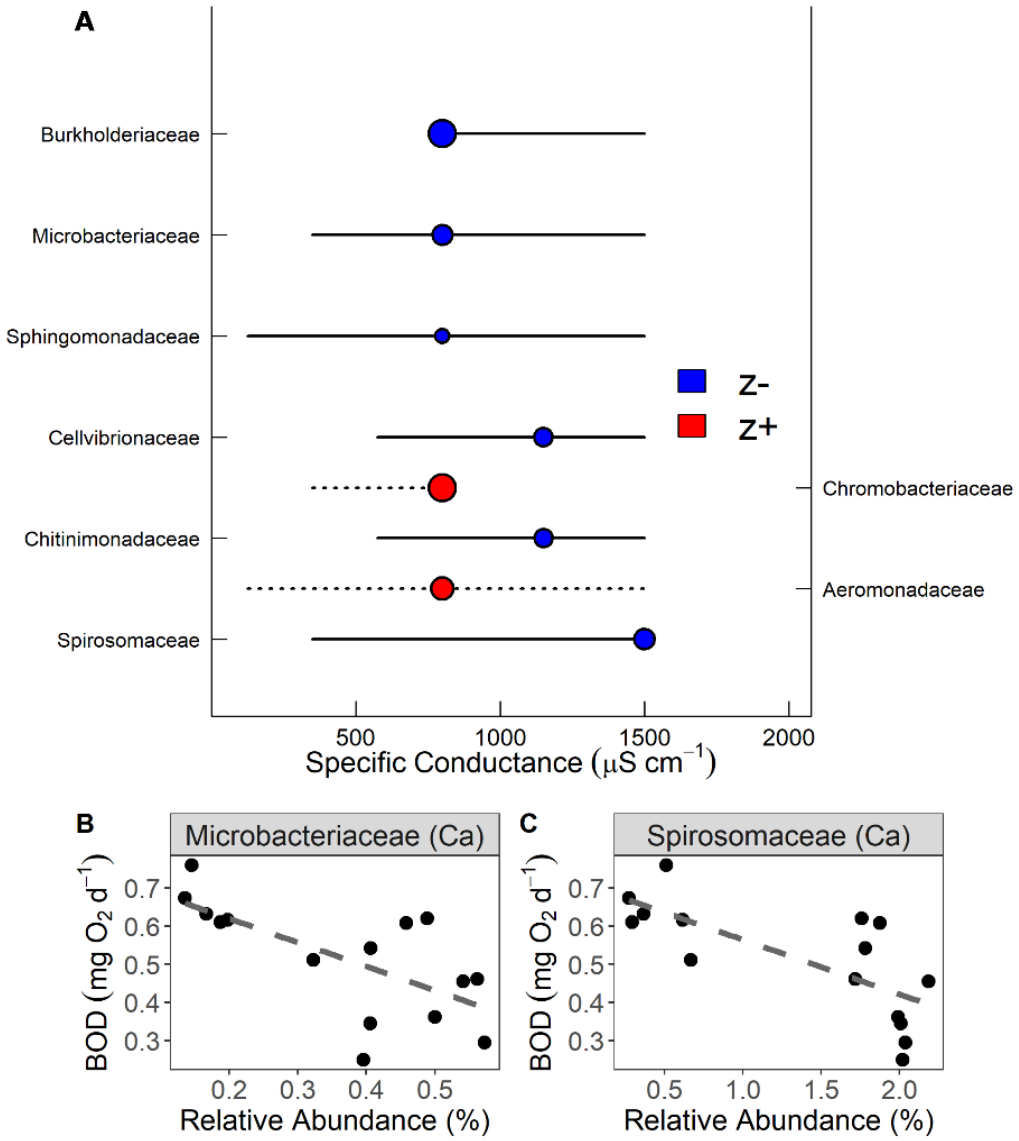


**Figure 4.8.** Threshold Indicator Taxa Analysis for day-1 samples showing A) change points for pure and reliable taxa, B) the relationship between Microbacteriacea and biological oxygen demand (BOD) and C) the relationship between Spirosomaceae and BOD, which were the only two pure and reliable taxa that had significant linear relationships with BOD ($p < 0.05$). The salt treatment in which the significant linear relationship between BOD and Indicator Taxa abundance was observed is shown in the figure header (B&C).

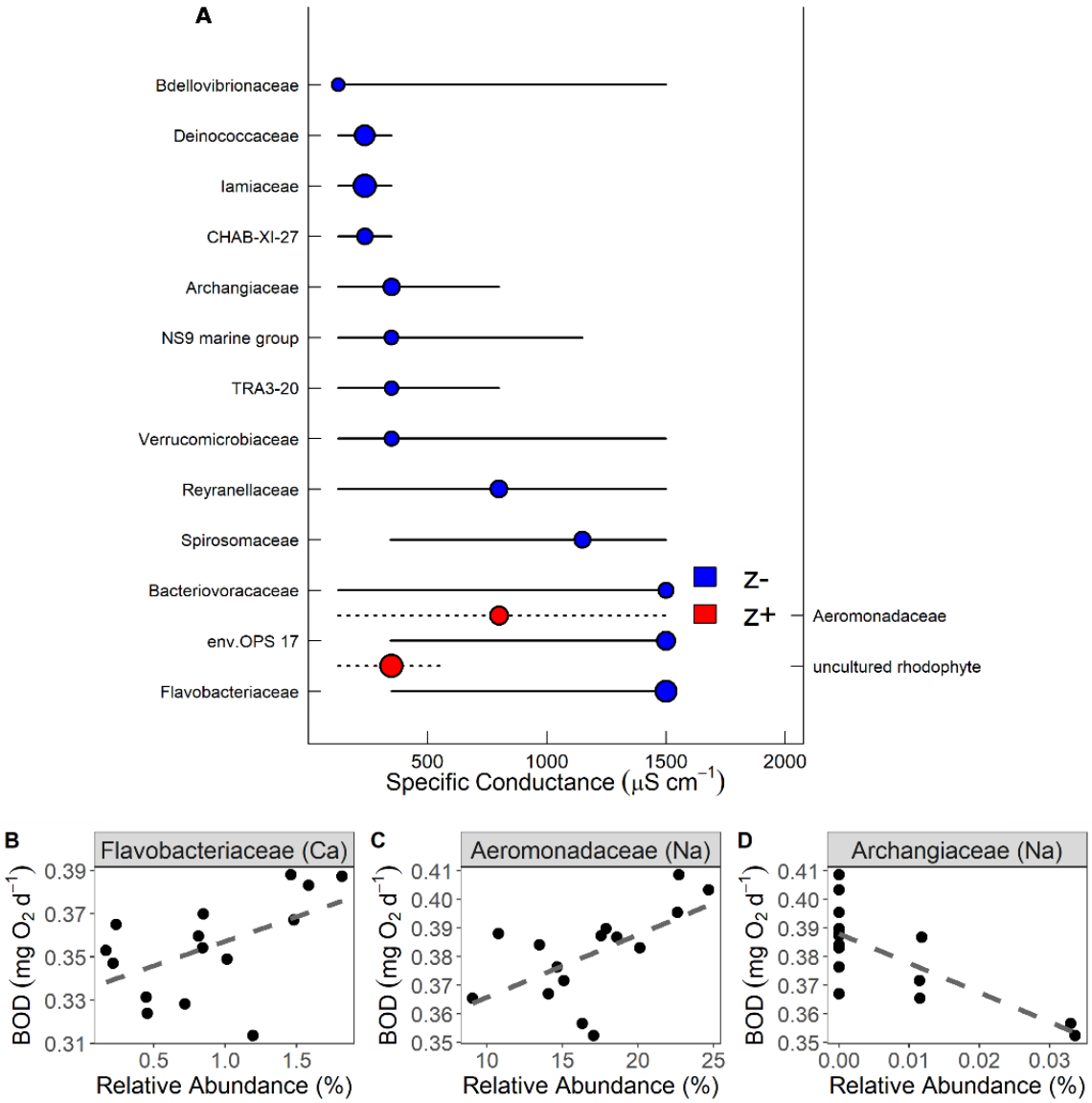


**Figure 4.9.** Threshold Indicator Taxa Analysis for day-5 samples showing A) change points for pure and reliable taxa and B, C, & D) relationship between indicator taxa and biological oxygen demand (BOD) for pure and reliable taxa that had significant linear relationships with BOD ($p < 0.05$). The salt treatment in which the significant linear relationship between BOD and Indicator Taxa abundance was observed is shown in the figure heads (B, C, &D).

**Table 4.5.** Summary statistics from Mantel tests between bacterial community structure and net nutrient uptake. The community structure distance matrix was based on Bray-Curtis dissimilarity. Net nutrient uptake distance matrices were based on Euclidean distance. The specific uptake rates tested are listed in the far left column, "Uptake Rate".

| Uptake Rate | Mantel Statistic | p-value |
|---|---|---|
| **One-day** | | |
| **BOD + $NH_4$ + $NO_3$ + SRP** | 0.042 | 0.23 |
| **BOD** | -0.026 | 0.52 |
| **$NH_4$** | 0.071 | 0.12 |
| **$NO_3$** | 0.039 | 0.19 |
| **SRP** | 0.074 | 0.12 |
| **Five-day** | | |
| **BOD + $NH_4$ + $NO_3$ + SRP** | 0.33 | **0.0080** |
| **BOD** | 0.19 | 0.057 |
| **$NH_4$** | 0.18 | 0.063 |
| **$NO_3$** | 0.33 | **0.0010** |
| **SRP** | 0.18 | **0.039** |

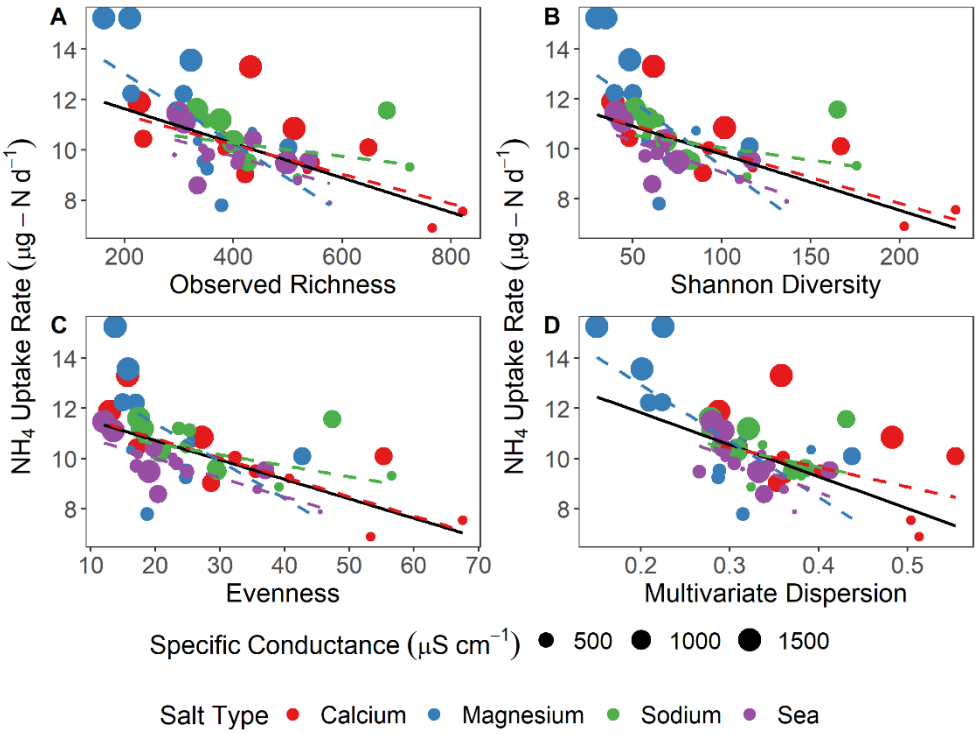


**Figure 4.10.** Relationship between five-day net $NH_4$ uptake rates and A) bacterial richness ($r^2 = 0.33$, $p < 0.001$), B) Shannon diversity ($r^2 = 0.33$, $p < 0.001$), C) evenness ($r^2 = 0.35$, $p < 0.001$), and D) multivariate dispersion ($r^2 = 0.31$, $p < 0.001$). Black regression lines are the linear fit of all salt types while colored, dashed lines are regression lines for individual salt types.

## 4.5 Discussion

### 4.5.1 Potential Impacts of Salinization on Freshwater Biogeochemical Cycles

A key finding of this work was that short term (one-day) exposure to moderate freshwater salt concentrations (350 – 800 µS $cm^{-1}$) doubled bacterial respiration in stream water primed with glucose. Maximum rates occurred at different salinities for different salt types (Fig 4.2A, Table 4.1). Inland freshwaters contribute significantly to global carbon and nutrient cycling (Cole et al. 2007, Raymond et al. 2016). Dissolved salts, particularly at brackish and marine concentrations, can interact directly with natural dissolved organic matter (DOM) altering molecular weight, composition, lability, and downstream fluxes (Amon and Benner 1996, Sholkovitz 1976, Uher et al. 2001, Yamashita et al. 2008); however, the effects of freshwater salinization on bacterially-mediated carbon dynamics were less understood. The increased respiration observed here suggest that in systems with minimal *in situ* primary production (e.g. headwater streams with predominately allochthonous carbon sources), moderate levels of freshwater salinization could create more heterotrophic ecosystems. Inland waters are already significant sources of $CO_2$ to the atmosphere, contributing 2.1 Pg C $yr^{-1}$ (Butman and Raymond 2011, Raymond et al. 2013). Freshwater salinization could further increase $CO_2$ production in and evasion from inland freshwaters. Furthermore, increased respiration under freshwater salinization is also likely to impact the quantity, quality, and relative proportions of different C pools (*i.e.,* dissolved, colloidal, particulate) exported downstream by selectively degrading more labile compounds (Benstead et al. 2009, Rosemond et al. 2014). Stream biotic communities adapt to utilize resources transported from upstream (Vannote et al. 1980). For example, bacterial community structure shifts

in response to altered DOM quality in aquatic systems (Osterholz et al. 2016). Thus, shifts in the quantity or quality of DOM exported downstream, resulting from freshwater salinization, could have cascading effects on downstream biotic communities and energy flow (Creed et al. 2018).

Freshwater salinization could also have important implications for carbon dynamics in lentic ecosystems. Hypoxia is a major concern for aquatic systems that are susceptible to stratification resulting in fish kills (Breitburg 2002) and altered nutrient cycling pathways (Testa and Kemp 2012), particularly under the stress of climate change (Hamidi et al. 2015, Justić et al. 1996). I observed that longer exposure (five days) to freshwater salt concentrations under lower DO concentrations also affected bacterial respiration rates (Fig. 4.2B, Table 4.1). Thus, changing salt concentrations might further exacerbate hypoxic conditions by facilitating increased bacterial respiration and DO consumption; however, my results suggest that the salt concentrations at which such an effect might occur are dependent on salt type. For example, the highest respiration rates for NaCl and sea salt treatments (which is predominately Na) occurred at the highest salinity of 1,500 $\mu S\ cm^{-1}$ while respiration rates generally declined with increasing salinity from $CaCl_2$ and $MgCl_2$. While increased concentrations of Na-based salts could exacerbate hypoxic conditions, increasing concentrations of Mg- and Ca-based salts could actually reduce DO consumption. Taken broadly, this work illustrates that considering both salinity and salt type, in addition to nutrient loading, could help mitigate the effects of hypoxia in lakes, reservoirs, and ponds.

### 4.5.2 Freshwater Salinization Alters Resource Stoichiometry

A second key finding of this work is that salinization differentially altered bacterial net nutrient uptake, resulting in large changes in resource stoichiometry under specific conditions. Resource stoichiometry considers how changes in pools of multiple chemical elements (primarily C, N, and P) influence and are influenced by biota (Welti et al. 2017). Collectively, respiration and net nutrient uptake had only minor effect on N:P ratios across a freshwater salinity gradient after one day (Fig. 4.5). However, salinization from $CaCl_2$ caused increasing net SRP uptake with increasing salinity. At high concentrations, $Ca^{2+}$ can co-precipitate with phosphate ions forming hydroxyapatite (Simmons 2010) which could explain the increase in SRP removal at higher $CaCl_2$ concentrations. N:P ratios also increased drastically with increasing $MgCl_2$ concentrations after five days, which could be explained by struvite precipitation (Rivadeneyra et al. 1992). Regardless of the underlying mechanism, N:P ratios increased by over 300% across a freshwater salinity gradient (Fig. 4.5, bottom row). Changes in N:P ratios as small as 70% impact biogeochemical processes (Rosemond et al. 2014), food web ecology (Glibert et al. 2011), and ecosystem metabolism (Williamson et al. 2016). For example, the relative abundance of species in algal communities shifts across an N:P gradient affecting food quality for algal consumers and other higher trophic levels (Stelzer and Lamberti 2001).

Hypoxic benthic environments might also be affected changes in N:P ratios. Hypolimnetic waters can be significant sources of P regeneration, especially under low DO conditions, causing a positive feedback cycle that prolongs low-oxygen conditions (Conley et al. 2009, Lin et al. 2018). However, changes in bacterial process rates under

elevated concentrations of $Ca^{2+}$ or $Mg^{2+}$ could reduce dissolved P concentrations via hydroxyapatite or struvite precipitation, potentially reducing the effects of sedimentary P regeneration on hypoxia. N:P ratios also alter microbial assemblages. For example, internal P loading can increase N:P ratios to N limiting conditions which facilitates harmful blooms of nitrogen-fixing cyanobacteria in certain lakes (Watson et al. 2016). However, freshwater salinization could potentially reduce blooms of harmful bacteria that thrive under low N:P ratios by scavenging and removing dissolved P from the water column. Generally, N:P ratios of <20:1 indicate N limitation while N:P ratios >50:1 indicate phosphorus limitation (Guildford and Hecky 2000). Thus, a change in N:P ratios of 150%, half of the observed % increase of N:P in this study, can alter nutrient limitations and microbial assemblages that are affected by nutrient stoichiometry.

### 4.5.3 Relationships Between Bacterial Community Structure and Process Rates

A third key finding of this work was that salinity and salt type also altered bacterial community structure in stream water primed with glucose, even after one day of exposure to low salt concentrations; however, there was no link between bacterial community structure and respiration rates after either one- or five-day exposures (Fig. 4.7, Tables 4.2, 4.3, 4.4, and 4.5). Organic carbon mineralization is a phylogenetically broad process that can be carried out by most bacterial groups. So, although bacterial communities responded to freshwater salinization, functional redundancy among carbon mineralizing taxa likely decoupled changes from community structure and respiration rates (Schimel and Schaeffer 2012). Though after five days of salt exposure the overall community structure was not related, the bacterial family *Aeromonadaceae* was positively correlated to respiration rates in the Na treatment and accounted for up to 25%

relative abundance (Fig. 4.9C). Genera in the family *Aeromonadaceae* are known to be mesohalophilic facultative anaerobes, require Na for growth, and are often found in anoxic sediments (Huys 2014). Therefore, *Aeromonadaceae* could be linked to salinization-induced patterns of bacterial respiration because of its apparent increased fitness in higher freshwater salinities; however, it is possible *Aeromonadaceae* positively responded to low oxygen conditions, instead of driving oxygen consumption. It is important to note that community properties like biomass or elemental ratios might better predict the effects of freshwater salinization on phylogenetically broad processes like organic carbon mineralization (Elser et al. 1995, Manzoni et al. 2008)

Unlike respiration rates, net $NH_4$ and SRP uptake rates were correlated to community structure after five day incubations that included exposure to hypoxia (Table 4.5). Certain $NH_4$ transformation pathways are more phylogenetically narrow than carbon mineralization. For example, ammonia oxidizing bacteria (AOB) are generally limited to the genera *Nitrosomonas*, *Nitrospira*, and *Nitrosococcus* (Isobe et al. 2011). Thus, it is more likely that shifts in the relative abundance of AOB would correlate to changes in net $NH_4$ uptake rates; however, no correlation between AOB genera and net $NH_4$ uptake rates were observed. Net $NH_4$ uptake was also negatively correlated with alpha and beta diversity, indicating that less diverse communities have higher $NH_4$ processing rates (Fig. 4.10). The glucose addition also likely caused a bloom of a few dominant taxa that reduced alpha diversity, which could also explain the correlation between $NH_4$ uptake rates and alpha diversity. Bacterial diversity tended to decrease with increasing salinity suggesting that diversity patterns may help predict $NH_4$ fluxes across salinization gradients. Phosphorus utilization as well as N:P ratios can also influence the relative

abundance of bacterial species, as microbes with the ability to fix atmospheric $N_2$ tend to thrive under lower N:P ratios (Stahl-Delbanco 2003). Changes in N:P ratios, which could result from $Ca^{2+}$ or $Mg^{2+}$ based freshwater salinization, might actually be driving changes in bacterial community structure, as opposed to the bacterial community driving changes in nutrient stoichiometry.

### 4.5.4 Integrating Freshwater Salinization and Biogeochemistry Frameworks

Current conceptual frameworks for stream ecology emphasize the importance of stream flow as a master variable. For example, the Pulse-shunt concept and River Network Saturation concept both posit that increasing flow reduces the capacity of stream networks to process and remove nutrients, ultimately increasing downstream fluxes (Knapp et al. 2020, Raymond et al. 2016, Wollheim et al. 2018). Salt loading and transport are also strongly influenced by flow. Depending on land-use and season, high-flow events can either increase (*i.e.*, flushing behavior) or decrease (*i.e.*, diluting behavior) salt concentrations (Corsi et al. 2010, Wymore et al. 2019). Flushing events that increase salinity could have an antagonistic effect on downstream nutrient fluxes by stimulating bacterial respiration and net nutrient uptake, creating hotspots or hot moments of biogeochemical cycling (Vidon et al. 2010). These hotspots or hot moments could also depend on salt type, as exposure to salt types produced significantly different respiration rates, even at the same salinity (Figs. 4.3 and 4.4). Hypoxic and anoxic zones are also hotspots, particularly in lentic ecosystems and could be exacerbated by salinization (McClain et al. 2003). On the other hand, dilution events that decrease salinity could increase downstream export of nutrients by decreasing the rates of bacterial processes. Thus, incorporating salinity and major ion types could improve estimates of downstream

nutrient fluxes and our understanding of hypoxia formation by accounting for salt-dependent changes in bacterial process rates.

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