May 2022

Tackling Chloride Pollution in Southeastern Wisconsin: Halophilic Bacterial Indicators and Policy Alternatives

Elexius Kaitlyn Passante
University of Wisconsin-Milwaukee

TACKLING CHLORIDE POLLUTION IN SOUTHEASTERN WISCONSIN: HALOPHILIC BACTERIAL INDICATORS AND POLICY ALTERNATIVES

by

Elexius (Lexi) Passante

A Thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

in Freshwater Sciences and Technology

at

The University of Wisconsin Milwaukee

May 2022

ABSTRACT

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Elexius (Lexi) Passante

The University of Wisconsin Milwaukee
Under the Supervision of Professor Sandra McLellan

There are few biological indicators for freshwater systems subjected to high chloride levels. Freshwater systems receive many forms of chloride such as road salts (e.g., NaCl, $CaCl_2$, $MgCl_2$), fertilizers (e.g., KCl), and year-round water softener pollution. The goal our study was to investigate *Halomonadaceae* populations as prospective biological indicators of chloride-impacted freshwaters. The bacterial family *Halomonadaceae* are halophiles that generally require the presence of salt to survive, which makes them an attractive candidate in determining chloride impaired areas. Field sediment surveys assessed how salt tolerant and halophilic bacteria abundance corresponded to chloride and conductivity measurements. Colony forming unit (CFU) counts on modified M9 6% NaCl plates (w/v) at urbanized sites compared to the rural sites had highest counts during winter and spring when chloride concentrations were also highest. Select CFUs identified as *Halomonadaceae* through 16S rRNA sequencing were kept as active cultures to determine the NaCl concentration and temperature preference that resulted in the isolates optimal growth. Isolates tested under 5°C (cold) grew optimally in 2% NaCl (w/v), whereas under 18°C (warm), isolates showed optimal growth at 6% NaCl. The majority of isolates had maximum growth in the warmer temperature, however, select isolates grew better in the cold temperature. Culture-independent methods were used and identified *Halomonadaceae* were widespread and permeant members of the microbial community in a Lake Michigan drainage basin. Quantitative polymerase chain reaction (qPCR) specifically targeting *Halomonadaceae* genera demonstrated that abundance varied by site, but overall were present throughout the year. However, community sequencing revealed there were a large relative proportion of unique *Halomonadaceae* populations in winter versus summer. Methods targeting salt tolerant bacteria and specific members of *Halomonadaceae* appears to be a promising approach to assess chloride-impacted areas to better understand the long-term ecological impacts as we continue to salinize freshwater resources. Furthermore, to better raise awareness of our findings and chloride pollution, we created educational outreach materials in the forms of a video series and classroom activities. Lastly, we took an economical approach and proposed a framework in valuing reduction in chloride loading, which could, if implemented, help inform winter road maintenance and stormwater management decisions.

To

my loving family who has shown and taught me perseverance and

my life partner who gave unconditional love, support, and understanding

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ACKNOWLEDGMENTS

I would like to thank Brigid Meyers for always being a helping hand throughout and making this graduate school process feel less alone starting amid a global pandemic. Thank you to Tim Wahl, for his analytical chemistry guidance at the School of Freshwater Sciences analytical laboratory and Angela Schmoldt in the Great Lakes Genomic Center. Thank you to Adelaide Rouget, former post-doc in the McLellan Lab, for teaching me your molecular and bioinformatic-ways. A big thank you to Andrew Laing, undergraduate student from UW-Madison, for your help during summer and winter breaks. I am thankful to the Southeastern Wisconsin Regional Planning Commission, specifically Nicklaus Neureuther, and MMSD for obtainable chloride and real-time conductivity data. Thank you to those at the University of Illinois at Urbana-Champaign Roy J. Carver Biotechnology Center for their speedy sequencing services and Doc | UWM for telling my research story through video. I would like to thank Dr. James Price for taking the time to teach me the ropes of nonmarket valuation methods and inspiring me to look at environmental problems from a different lens. I would like to thank my advisor Dr. Sandra McLellan for taking a chance on me when I proposed the idea of looking for halophiles in freshwater sediment— your support and guidance was appreciated. Thank you to the rest of my thesis committee Dr. Rebecca Klaper and Steve Corsi. Lastly, I would like to thank Dr. Charlie Paradis and Leah Dechant who were collaborators with this research. Support for this project was provided by Milwaukee Metropolitan Sewerage District (MMSD) fellowship, the Naulin Foundation research award, and Cooperative Institute for Great Lakes Research (CIGLR) ECO award.

1. Introduction

1.1 Chloride pollution in a Lake Michigan drainage basin

Located in Southeastern Wisconsin (WI), the Milwaukee River Basin is a large, 882.3 square mile drainage area with over 1.3 million people that consist of streams and rivers that ultimately discharge into Lake Michigan (Milwaukee Riverkeeper, n.d.). This is an especially important area, as stormwater runoff from impervious surfaces carries various types of pollutants from these urbanized spaces. Particularly, chloride is a large contributor to stormwater runoff in snowy climates, like Wisconsin (WI). In 2012, a long-term ion study found that Lake Michigan had the strongest upward trend in specific conductivity and chloride when compared to the rest of the Great Lakes (Chapra et al., 2012). More recently, it was calculated that Lake Michigan is receiving 1 million metric tons of chloride on an annual basis (Dugan et al., 2021). Once chloride enters a freshwater system, it raises the salinity of the water altering the osmotic pressure where freshwater organisms may not be able to tolerate these changes. With chlorides free-flowing capability, it can stay in solution for unknown periods of time interacting with shallow groundwater where it can be temporarily stored (Cooper et al. 2014). Due to this unique characteristic, once chloride is exposed in the environment, there are currently no technologies to successfully remove it.

1.2 Impact on freshwater ecosystems

The Milwaukee Riverkeeper estimates about 54 Lake Michigan fish species use the Milwaukee River Basin as important habitat, as most freshwater fish like Chinook Salmon or Walleye rely on streams and rivers to spawn (Milwaukee Riverkeeper, n.d.). Elevated levels of chloride can alter lower trophic levels in the food web like microbial and planktonic communities, which indirectly impact organisms up the food chain (e.g., fish) (Arnott et al., 2020). Using bacteria in freshwater stream and river sediment could help understand what is happening in freshwater ecosystems impacted by chloride from a bottom-up perspective— especially seasonal surges from road salt runoff. At the base of the food chain, microbial communities are fundamental for biogeochemical processes in aquatic ecosystems which can provide insight from an environmental health perspective which makes them a useful tool as environmental indicators. Environmental conditions largely shape microbial communities, making them a sensitive benchmark for identifying stressful pressures (e.g., chloride pollution). Here, we investigated halophilic (i.e., salt-loving) and salt-tolerant bacteria at our study sites to help develop a biological indicator for chloride-impacted areas. Halophiles may serve as a suitable candidate for specific indicators of chloride pollution due to the different osmotic strategies they have adopted that allow them to thrive in diverse saline conditions.

1.3 Scope of this work

There are currently few attempts in developing biological indicators for freshwater systems that are subjected to high chloride levels What is currently missing in chloride pollution research is the ability to better understand how freshwater ecosystems have responded after years of chloride exposure. Toxicity standards developed by the U.S. Environmental Protection Agency (EPA) for chronic and acute concentrations have not been revised since 1988, stated at 230 and 860 mg/L, respectively (US EPA 1988). These toxicity standards are useful in identifying hotspots in surface waters to understand the state of chloride and its adverse effects to aquatic organisms. However, these standards make it difficult in assessing exactly how impacted a freshwater system is from chloride as each waterbody interacts and handles contaminants at its own capacity (e.g., shallow groundwater interactions). With the use of a biological indicator (i.e., halophilic bacteria), this could provide more insight on how impacted freshwater systems are after years of anthropogenic chloride exposure. This is an observation and potential measurement that toxicity standards miss because it does not show a clear biological response, but rather a biological assumption based on laboratory-controlled conditions.

To further investigate halophilic bacteria in freshwater sediment as biological indicators, we began by identifying prospective study sites in Southeastern WI. As this is the first study to investigate halophilic bacteria in sediment, we decided to expand and cover our efforts with different river and stream settings varied in sediment type and land use, which limited us to a low frequency sampling schedule (e.g., visit sites once or twice a month). This survey technique was decided upon in order to set the foundation for halophilic bacteria. A total of seven stream or river study sites were selected located around the Milwaukee River Basin, with two sites considered rural based on the land use around them. These sampling sites were also strategically chosen close to Milwaukee Metropolitan Sewerage District (MMSD) water quality monitoring sites where surface water samples have been previously collected, as well as real-time conductivity stations from Southeastern Wisconsin Regional Planning Commission (SEWRPC). In addition, we investigated two sites South of the Milwaukee River Basin along the Root River to further expand on setting our foundation for this research by working with UWM's Geoscience Department on a road salt transport study that was being conducted at the time. This collaboration allowed for a high frequency sampling in summer where we could compare and contrast low versus high frequency sampling and conduct site by site comparisons.

We found halophilic bacteria (specifically, the family *Halomonadaceae*) in freshwater sediment year-round across all of our study sites. Isolate 16S rRNA gene sequences demonstrated how closely related the *Halomonadaceae* genera *Halomonas*, *Halovibrio*, and *Salinicola* were from each other regardless of study site and season from which they were cultured out of the sediment. We measured each isolates growth capabilities in laboratory-controlled experiments to test growth dynamics at varying temperature and salt concentrations. The salt concentrations selected were much higher than ecological relevant chloride concentrations observed at our study sites. This was done to highlight the extent in which these halophiles can grow when given their optimal environmental conditions as described in previous literature. Sediment samples collected were analyzed to measure the response of bacteria present by direct plating on non-selective media which also allowed us to captured salt tolerant bacteria and observe notable trends. To target the halophile of interest, *Halomonadaceae*, a quantitative polymerase chain reaction (qPCR) assay was designed and community sequencing was done to compare relative proportions.

This research has contributed knowledge on better understanding how freshwater ecosystems have responded to chloride pollution throughout the year, long after major sources of chloride have entered the system (e.g. road salt runoff) through the presence of halophilic bacteria. More specifically, by 1.) measuring the presence of these halophilic and salt tolerant bacterial populations across all study sites and how they related to one another, 2.) responses of halophilic bacteria collected from study sites growth capabilities in laboratory-controlled experiments in high salt concentrations, 3.) creating an assay that specifically targeted the halophile of interest with suggestions of specific strain indicators from deep sequencing, and 4.) comparing low versus high frequency sampling. This work has ultimately laid the foundation to further investigate halophilic bacteria, *Halomonadaceae*, as biological indicators for chloride-impacted waterways in hopes it could be widely used as an integrated measure of ecosystem health and water quality from the help of future research.

Beyond our environmental microbiology research, we wanted to better raise awareness of chloride pollution. The general public's stance on chloride pollution, especially road salts, are still not fully understood, which led us to wanting to help bridge this gap through the use of educational outreach materials. To do so, we collaborated with WI Salt Wise, Oconomowoc High School, Waukesha County, and Doc | UWM. Through the Cooperative Institute of Great Lakes Research (CIGLR) ECO program, we received funds to create a series of videos that followed the scientific process of our research findings. In addition, we created an interactive classroom activity called a Data Nugget. Data Nuggets is a free online library, developed in 2011, of teaching lessons covering a wide range of different science topics for educators. Data Nuggets was funded through National Science Foundation (NSF) grants that allowed it to ultimately expand its resources. Data collected from our research was incorporated into the Data Nugget for students to learn how to work with environmental datasets. The videos produced by Doc | UWM are used on social media and chloride awareness purposes that WI Salt Wise conducts. WI Salt Wise is a coalition of organizations with an overarching goal to educate communities on the importance of reducing salt pollution through best management practices. The use of these videos will disseminate through their far-reaching platform but will also be included in the Data Nugget as supplemental materials for the teacher to show the classroom as they followed along with the lesson plan.

Lastly, we incorporated an economical approach by reviewing the extent to which studies have measured the ecological impacts associated with winter deicers (e.g., road salts) with emphasis on current salt alternatives (e.g. brine solutions). Available cost benefit analyses (CBA) and related literature (e.g, cost-savings) were analyzed around this topic. CBA is a systematic assessment method that quantifies in monetary terms all impacts of a policy, including impacts to ecosystem services. The broad purpose of CBA is to help decision-making and to increase social wellbeing. Our findings found there was a lack of complete CBA's that captured the impacts on road salt and brine on aquatic ecosystems. In addition, there were limited findings using nonmarket valuation methods to monetize benefits from reducing chloride loads entering aquatic ecosystems. Here, we proposed a framework for improving the economic valuation of these impacts with two different policy alternatives to better improve water quality CBAs on chloride pollution conducted in the future.

2. Halophilic bacteria in a Lake Michigan drainage basin as potential indicators of chloride-impacted freshwaters

2.1 Abstract

There are few biological indicators for freshwater systems subjected to high chloride levels. Freshwater systems receive many forms of chloride such as road salts (e.g., NaCl, $CaCl_2$, $MgCl_2$), fertilizers (e.g., KCl), and year-round water softener pollution. The goal our study was to investigate *Halomonadaceae* populations as prospective biological indicators of chloride-impacted freshwaters. The bacterial family *Halomonadaceae* are halophiles that generally require the presence of salt to survive, which makes them an attractive candidate in determining chloride impaired areas. Field sediment surveys assessed how salt tolerant and halophilic bacteria abundance corresponded to chloride and conductivity measurements. Colony forming unit (CFU) counts on modified M9 6% NaCl plates (w/v) at urbanized sites compared to the rural sites had highest counts during winter and spring when chloride concentrations were also highest. Select CFUs identified as *Halomonadaceae* through 16S rRNA sequencing were kept as active cultures to determine the NaCl concentration and temperature preference that resulted in the isolates optimal growth. Isolates tested under 5°C (cold) grew optimally in 2% NaCl (w/v), whereas under 18°C (warm), isolates showed optimal growth at 6% NaCl. The majority of isolates had maximum growth in the warmer temperature, however, select isolates grew better in the cold temperature. Culture-independent methods were used and identified *Halomonadaceae* were widespread and permeant members of the microbial community in a Lake Michigan drainage basin. Quantitative polymerase chain reaction (qPCR) specifically targeting *Halomonadaceae* genera demonstrated that abundance varied by site, but overall were present throughout the year. However, community sequencing revealed there were a large relative proportion of unique *Halomonadaceae* populations in winter versus summer. Methods targeting salt tolerant bacteria and specific members of *Halomonadaceae* appears to be a promising approach to assess chloride-impacted areas to better understand the long-term ecological impacts as we continue to salinize freshwater resources.

2.2 Introduction

Anthropogenic sources of chloride entering freshwater systems come in many forms, from fertilizer runoff to year-round water softeners in wastewater treatment plant effluent (Buvaneshwari et al., 2020); (Panno et al., 2002). However, road salts (e.g., $NaCl$, $CaCl_2$, $MgCl_2$) continue to rank as the largest contributor in snowy climates (Kelly et al., 2012). Once in the environment, chloride's conservative ability to flow freely between surface water and groundwater creates the challenge of protecting freshwater resources from permanent salinization (Fay and Shi 2012; Dugan et al. 2021). In 2012, Lake Michigan was observed to have the strongest upward trend in conductivity and chloride concentrations when compared to the other Great Lakes (Chapra et al., 2012). More recently, it was calculated about 1 million metric tons of chloride are entering Lake Michigan on an annual basis (Dugan et al., 2021). Great Lakes drainage basins located in southeastern Wisconsin contain many tributaries that serve as essential habitats for aquatic life, including the fish populations that contribute to a large fishing industry (Great Lakes Fishery Commission, n. d.). Elevated chloride concentrations can be detrimental to lower level trophic organisms like zooplankton and the base of the aquatic food web, bacteria, indirectly impacting organisms up the food chain (Arnott et al., 2020; Hintz & Relyea, 2019). As chloride exposure may alter the microbial community, salt-loving bacteria may be a useful integrated measure when determining chloride-impacted waterways from a long-term monitoring perspective.

Salt-loving bacteria, also known as halophiles, have been found across domains Bacteria, Archaea, and Eukaryota (Edbeib et al. 2016). Halophilic bacteria have two different mechanisms that allow them to live in saline environments. The "salt-in" strategy allows the microorganism to uptake chloride ions to obtain equilibrium with the environment (Siglioccolo et al. 2011; Oren 2013). The second mechanism is known as the "compatible-solute" strategy. To obtain equilibrium with its surroundings, halophiles will pump neutrally charged ions out of the cell to keep up with the high salt environment (Siglioccolo et al. 2011; Oren 2013). Historically, halophiles were classified to be found in high salt environments and cultured out of places with high salt content like the Great Salt Lake in Utah (Frederick 1924). Since then, they have been categorized in many ways, such as facultative halophiles growing in less than 2% NaCl, or obligate growers in solutions greater than 2% NaCl (Flannery 1956). Other assessments like ranking halophiles from no salt, slight (<10% NaCl), moderate, or extreme (> 30% NaCl) salt conditions have been done (Larsen 1986; Edbeib et al. 2016). More recently, a limited number of studies have searched for halophiles in non-traditional environments. Halophilic bacteria have now been observed in freshwater surface and groundwater samples taken from the Rouge River in Michigan (Tiquia et al. 2007). The response of halophiles in soil samples from road salt-impacted watersheds in Baltimore, Maryland, has also been measured (Pecher et al. 2019). Both studies suggest halophile presence in these unorthodox places may be due to the long history of anthropogenic sources of chloride entering the environment. Halophilic bacteria serve as an attractive candidate as a biological indicator for assessing chloride-impacted waters based on their use of salt ions (e.g. chloride) for metabolic processes, unlike salt-tolerant bacteria populations that do not necessarily thrive in high salt environments.

The goal of this study was to evaluate the potential for halophiles, specifically the family *Halomonadaceae*, to serve as biological indicator to assess chloride-impacted freshwater systems. Halophilic isolates were cultured out of the sediments at all study sites. Field surveys demonstrated greater colony forming units (CFU) of salt-tolerant bacteria found at the urban sites compared with rural sites. To measure extreme growth capabilities, each isolate's salt concentration and temperature preference in laboratory-controlled experiments was performed. Through culture-independent methods, we found *Halomonadaceae* populations were widespread and permanent in the sediment year-round. However, community sequencing revealed there were a large relative proportion of unique *Halomonadaceae* populations in winter versus summer. These results suggest certain strains within *Halomonadaceae* may be better indicators of chloride impact for seasonal-type surveys. Using halophiles as potential biological indicators, our research aims to assess chloride pollution in a Lake Michigan Basin to better understand the long-term ecological impacts as we continue to salinize freshwater resources.

2.3 Material and Methods

2.3.1 Milwaukee River Basin study area and low frequency seasonal sampling

A total of seven study sites were chosen around the Milwaukee River Basin located in Southeastern Wisconsin (WI). Sites were chosen based on chloride monitoring data from Milwaukee Metropolitan Sewerage District (MMSD), Milwaukee Riverkeeper, available data from Southeastern Wisconsin Regional Planning Commission, and records from the National Water Quality Monitoring Council. Based on land usage, two of the seven sites were considered rural, whereas five were considered urban (Figure 1) that experience high chloride concentrations throughout the year. Descriptive information was obtained for each site to demonstrate the site and land use variability (Table 1, Table S1).

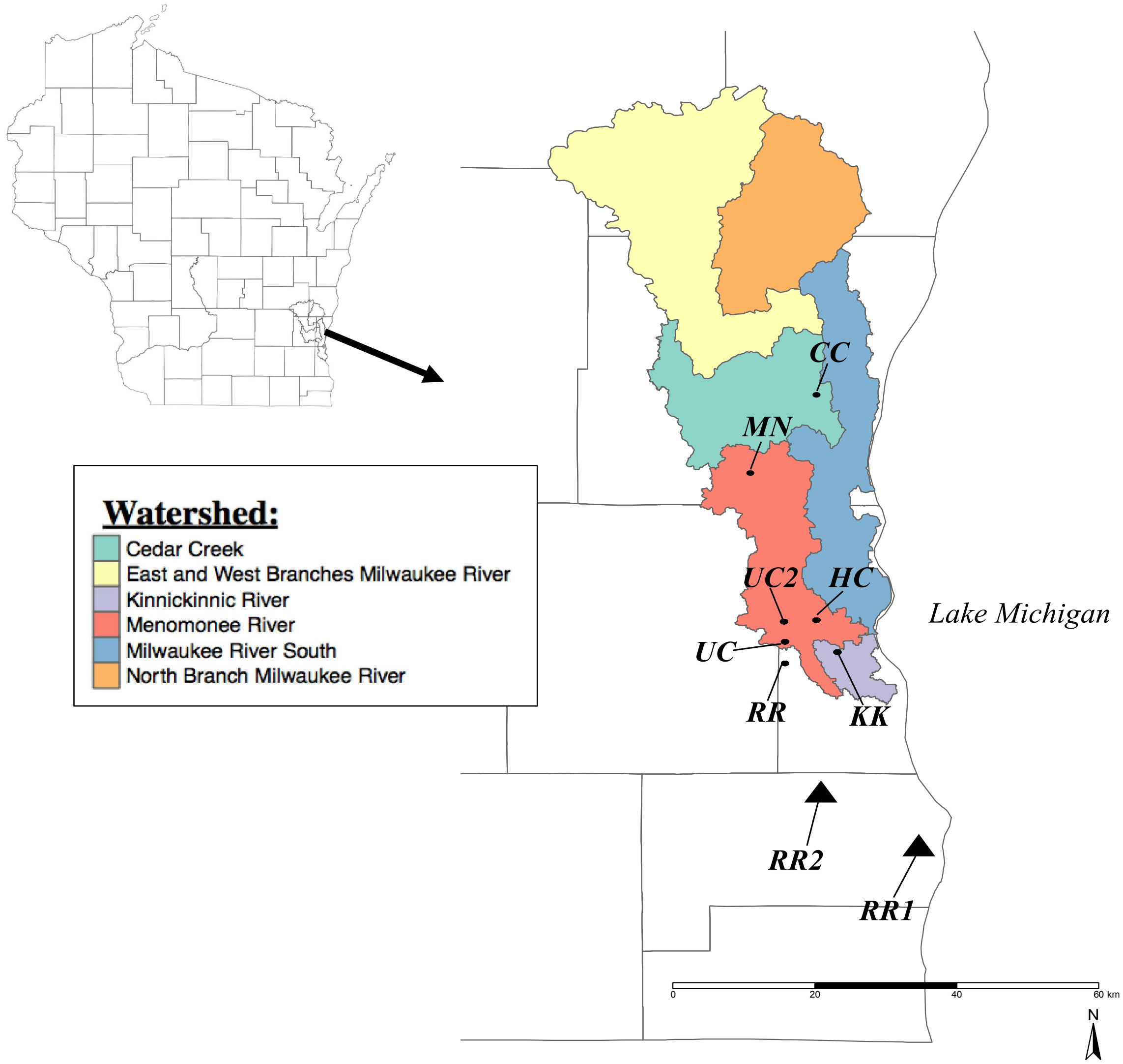

Figure 1. State of WI and the Milwaukee River Basin (shown in color), a Lake Michigan drainage area. Each point is a study site where sediment samples were collected. Milwaukee River Basin study sites are indicated as circles, CC = Cedar Creek, MN = Menomonee, HC = Honey Creek, KK = Kinnickinnic River, RR = Root River, UC = Underwood Creek, and UC2 = downstream Underwood Creek. Triangles are two sites selected along the Root River outside of the Milwaukee River Basin, RR1 = downstream Root River and RR2 = upstream Root River. Mapping done in ArcGIS and RStudio package tmap (ArcGIS Imagery, N.d.; Tennekes 2018).

We collected sediment samples at each site starting in February 2021 and continued each month until September 1st, 2021. Further winter sampling was completed in January and February of 2022. All sites were accessible with waders and sediment samples were grabbed in triplicate with an extendable scooping device from as close to the center of the stream and the top layer of sediment was sampled. Other basic water quality parameters were recorded while at the study sites (e.g., conductivity, pH, and temperature). Chloride concentrations were determined in the surface water grab samples and pore water of the sediment. All samples obtained were immediately placed on ice. Samples were processed in the laboratory immediately and did not exceed a 24-hour window if further dilutions were needed.

2.3.2 Root River study area and high frequency sampling

An additional two sites along the Root River, south of the Milwaukee River Basin, was sampled on a high frequency sampling basis to coincide with a related study that was collecting high frequency chloride data. Samples were processed the same as the Milwaukee River Basin sites samples. The upstream site was considered as a rural, whereas the downstream site was determined to be urban impacted (Figure 1). Descriptive information was obtained for both sites to demonstrate the site and land use variability (Table 1, Table S2). Sediment samples were collected during the summer of July through September 2021 to observe the summer dynamics of the *Halomonadaceae* populations and compare to the low frequency sampling at the Milwaukee River Basin sites.

Table 1. Study site information and maximum chloride concentration observed from MMSD's sampling 2018 – 2020 period for context.

Site	Site abbreviation	Type	Latitude	Longitude	Urban land coverage in 1 km^2 radius around site (%)	Near MMSD monitoring site*	Maximum chloride concentration from 2018 - 2020
Honey Creek	HC	Urban	43.044229	-88.002657	84	HC-03	3/8/2018 **1500 mg/L**
Kinnickinnic River	KK	Urban	43.002465	-87.966301	80.5	RI-33	2/21/2019 **2700 mg/L**

Root River	RR	Urban	42.986476	-88.057485	92.5	RR-02	11/29/2018 **1100 mg/L**
Underwood Creek	UC	Urban	43.015223	-88.057412	100	UC-04	11/7/2019 **1800 mg/L**
Underwood Creek downstream	UC2	Urban	43.042203	-88.058814	100	UC-06	11/7/2019 **770 mg/L**
Cedar Creek	CC	Rural	43.33785	-88.003797	26	CC-01	8/6/2018 **110 mg/L**
Menomonee River	MN	Rural	43.235367	-88.126845	33.5	RI-16	8/6/2018 **190 mg/L**
Root River upstream	RR2	Rural	42.815407	-87.9926012	8	N/A	N/A
Root River downstream	RR1	Urban	42.7466813	-87.820891	85	N/A	N/A

* Data obtained from the National Water Quality Monitoring Council, The Water Quality Portal (WQP)

2.3.3 Chloride and real-time conductivity

In the laboratory, each site's sediment was centrifuged to collect pore water samples. Sediment supernatant and surface water samples were filtered through a 0.2-micron filter. The filtered liquid was then diluted and loaded onto the Ion Chromatography (IC) instrument at the School of Freshwater Sciences Analytical Laboratory. A standard curve was produced to obtain the linear regression slope and intercept. Each site's surface water grab sample was analyzed in with three technical replicates. Due to sediment heterogeneity, sediment pore water eluents were collected in duplicate (i.e., A and B) where technical triplicates of each duplicate were analyzed. Percent relative standard error (RSE) was calculated and with sites that did not exceed beyond 5% RSE. After the first two sampling events, all sites except UC2 did not exceed this threshold, therefore technical triplicates were discontinued. Real-time conductivity data was collected from Southeastern Wisconsin Regional Planning Commission and Milwaukee Metropolitan Sewerage District (MMSD) stations for each sampling site from the year 2021 (Table S3).

2.3.4 Direct plating

To quantify salt tolerant bacteria and isolate out potential halophilic bacteria present in the sediment sample, we developed a direct plating technique. A M9 salts solid media was modified to achieve a 6% NaCl concentration (w/v). This was not halophilic-selective media; therefore, bacteria were expected to be salt-tolerant or halophilic. Sediment samples were weighed out, sterile water added to achieve desired dilution, and then shaken by hand vigorously for two minutes. Dilutions were determined by site and previous plate results. After settling for one minute, a 100-300 µl aliquot of the supernatant was spread plated and incubated at room temperature for 7-8 days. All colonies were counted to determine colony forming units (CFUs) per gram of sediment. In the case where plate counts were too numerous to count (TNTC), and the sample was past the holding time to re-plate, a conservative estimate of 250 colonies was assumed (only applicable for KK 4/6/21, UC2 4/6/21, and UC2 8/31/21 sampling events).

2.3.5 M9 minimal media salt optimization

M9 minimal media adjusted to 6% NaCl (w/v) was decided for direct plating through aliquots of the sediment eluent spread plated on a variety of NaCl solid media (2, 4, 6 and 8%). Although higher salt concentrations were chosen than what may not be observed in the freshwater environment, the concentrations were selected to provide optimal conditions for halophilic organisms potentially present in the sediment. When comparing the growth results, 2 and 4% NaCl M9 media appeared to have the most growth from the urban sites with fungal growth throughout— making it difficult to isolate colonies. However, 6% NaCl M9 solid media appeared to be more selective as it is higher in salt concentration with overall less fungal growth. 8% NaCl M9 solid media plates did not yield enough colonies compared to 6% NaCl. To help control fungal growth and successfully isolate unique colonies, an anti-fungal solution, Nystatin suspension (stock concentration of 10,000 units), was added to the media to achieve a concentration of 0.5 units/mL.

2.3.6 Isolate identification by 16S rRNA gene sequencing and screening with **Halomonas** *specific primers*

Colonies with different morphologies were isolated out for identification from the Milwaukee River Basin sediment samples (supplemental Dataset S1). Presumptive halophilic isolates from direct plating of the Milwaukee River Basin and the Root River sites were identified using full length 16S rRNA sequencing. PCR amplification was conducted using universal 8F and 1492R primers on individual colonies. Product was purified using the QIAGEN QIAquick PCR Purification Kit and ran on 2% agarose gel electrophoresis to verify against the 1kb Plus ladder exACTGene. Amplified DNA was placed at –20°C for temporary storage and Sanger sequenced at the Roy J. Carver Biotechnology Center (University of Illinois at Urbana-Champaign). Each isolate was identified and true halophilic isolates (i.e., the halophilic family *Halomonadaceae*) were either stored at -80C° in 25% glycerol or maintained as active cultures on 2% NaCl M9 solid media for further experimentation.

16S rRNA gene sequences were used to generate phylogenetic trees to profile the populations at each of the study sites. Forward and reverse sequences were trimmed and merged using the program DNAStar SeqMan Ultra package (Version 17.2.1 (61)). The sequences were put into a Newick file for compatibility with the program Interactive Tree of Life (iTOL). The Milwaukee River Basin phylogenetic tree identified five major clades: clade I, II, III, IV, and V. Clades were assessed and grouped by isolates that were greater than 98% identity to each other. In addition, isolates in clades III, IV, and V had (with the exception of isolates #288 and #179) had identities of 97% or greater of the species in *Halomonadaceae*, *Halomonas variabilis*, when compared to the other two clades. *Halomonas variabilis* was formerly known as *Halovibrio variabilis* (Dobson et al. 1993; Dobson and Franzmann 1996).

We further designed primers specific to the major genus cultured out of the sites, Halomonas, within *Halomonadaceae* to screen salt tolerant colonies (see supplemental text 1). From M9 6% NaCl plates from the Root River high frequency summer samples, we screened with these primers to determine the percentage of CFU that were halophilic. Colonies from the direct plating of sediment were screened by taking every accessible colony on the plate.

2.3.7 Salt and temperature profiles of **Halomonadaceae** *isolates*

Laboratory-controlled experiments were conducted on *Halomonadaceae* isolates that were cultured out of the Milwaukee River Basin study sites (i.e., *Halomonas, Halovibrio, and Salinicola*). The isolate's salt and temperature preference were determined by inoculating into 96-well plates at a series of concentrations ranging from 0, 2, 4, 6, 8, and 10% NaCl modified M9 media (w/v). The two different temperatures were selected to represent the stream and river average water temperatures throughout January to May and May to September by using U.S. Geological Survey stream gauges at Honey Creek (Site 04087119) and Menomonee River (Site 04087120). The cold temperature chosen was 5°C whereas 18°C was chosen for the warm temperature. Within the 14-day period, absorbance readings were read every other day using the Synergy H4 Hybrid Reader in the Great Lakes Genomic Center where change in absorbance was measured to determine growth of each halophilic isolate over time.

Each isolate in the beginning of the experiments grew at different rates. Therefore, the cold temperature Day 14 and warm temperature Day 12 datasets were selected as this is where isolate change in growth was observed to have the least variation (Figure S1). At the warm temperature Day 12, growth rates decreased suggesting die off. Whereas cold temperature Day 14 was the most representative of optimal growth. Our results use these two data sets when analyzing temperature preference, as well as each isolates optimal salt concentration.

2.3.8 DNA extractions of sediment samples

To extract bacterial DNA from the sediment samples collected, 0.2 grams was weighed out in a microfuge tube in triplicate and placed in a -80°C freezer for temporary storage. Samples were extracted using the FastDNA Spin Kit for Soil kit and the product was read on the NanoDrop spectrophotometer and placed in the freezer at –20°C for further analysis.

2.3.9 Microbial community analysis and v4 region quantitative polymerase chain reaction (qPCR) assay

A subset of 113 out of 361 DNA extracted sediment samples from the Milwaukee River Basin study sites were chosen and analyzed on the Illumnia MiSeq Desktop Sequencer in the Great Lakes Genomics Center with a mock community and negative template control. Of these samples, most site triplicates were selected to determine the variability between the sediment grab samples, as well as a variety of sampling dates to understand potential seasonal changes. The samples were normalized using the Qubit 2.0 Fluorometer before loaded onto the MiSeq using v4 region primers. Raw data was analyzed using the Dada2 pipeline in RStudio (https://github.com/benjjneb/dada2; Callahan et al. 2016) where bacterial relative abundance, prevalence, and variance of each sample was determined. We found the sites had a similar community structure, with the top ASVs dominate across the sites (Figure S2 and Table S5). The genus *Halomonas* was the only *Halomonadaceae* member detected in the sediment with a total of 12 unique ASVs sequenced (Table S6).

A qPCR assay was designed to target the v4 region of *Halomonadaceae* using isolates cultured out of the study site sediment and the ASVs from the microbial community sequencing. We designed the primers (forward and reverse) and probe (FAM/MGB-NFQ) within the bacterial 16S rRNA gene v4 region to produce a 70 bp amplicon. The forward primer (F467) (5'-GGAACTGTCARGCTAGAG-3') was created to have one degenerate base pair. The reverse (R537) and probe (P510) were designed as antisense (5'-CGATCTCTACGCATTTCA-3' and 5'-CGGGAATTCTACCTTCCTCTCCTGC-3' respectively). The primers and probe were checked in the Ribosomal Database Project (RDP--Release 11) (Table S7). An annealing temperature of 62°C yielded optimal results, where all negative controls closely related to *Halomonadaceae* tested (n = 14) were undetected and sensitivity was not compromised. Validation of the assay was done running all *Halomonadaceae* isolates collected from study sites (n = 93) and negative controls selected that were closely related to the family. All *Halomonadaceae* isolates were detected (supplemental Text 2).

2.3.10 Statistical analysis

Two sample t-tests were performed on the sediment supernatant and surface water samples as well as Root River upstream and downstream CFUs to determine if their means were equal or not to one another. A Shapiro-Wilk normality test determined the use of a non-parametric Wilcoxon t-test for these analyses. Two-way Analysis of Variance (ANOVA) were conducted to test the variance of CFU values across Milwaukee River Basin study site type (i.e., urban and rural) and seasons (i.e., winter, spring, and summer). To determine which sites and seasons were significantly different from one another, a Tukey Post-Hoc multiple comparison of means test was performed at a 95% confidence interval.

2.4 Results

2.4.1 Chloride concentrations and quantification of salt-tolerant and halophilic bacteria in sediment

We measured chloride concentrations in sediment pore water and overlying surface water at the study sites over winter, spring, and summer sampling events. Pore water exhibited significantly higher concentrations compared to matched water samples (Figure 2); non-parametric Wilcoxon paired t-test (p

< 0.0001). This suggests sediment-dwelling organisms in aquatic environments are experiencing higher levels than what is measured from the top of the water column. Chloride concentrations and conductivity show that the rural sites remain constant throughout season, whereas the urban sites experienced a wide range of chloride concentrations, especially in the sediment during the winter season.

CFUs counted on 6% NaCl M9 media contained a variety of salt-tolerant and halophilic bacteria (e.g., *Halomonadaceae*). We found urban sites had the highest CFU counts in winter and spring months compared to our rural sites (Figure 3), with minimal CFUs observed at the rural sites compared to urban sites collectively. However, the study site UC2 exhibited a different trend compared to the rest of the urban sites where it had highest CFUs in summer, and we also observed an anomaly at our rural site, CC, with unusually high CFUs during the summer as well (Figure S3). A two-way ANOVA on the CFUs per gram of sediment across site type (i.e., urban or rural) and season found there was a significant difference in CFUs by site type (p-value < 0.0001), but not across all the seasons. A Tukey Post-Hoc test determined that urban sites were significantly different from rural sites in winter and spring (p-values of 0.024 and 0.005 respectively). However, these statistical results indicated during the summer season CFUs at the urban and rural were not significant from one another (p-value = 0.796).

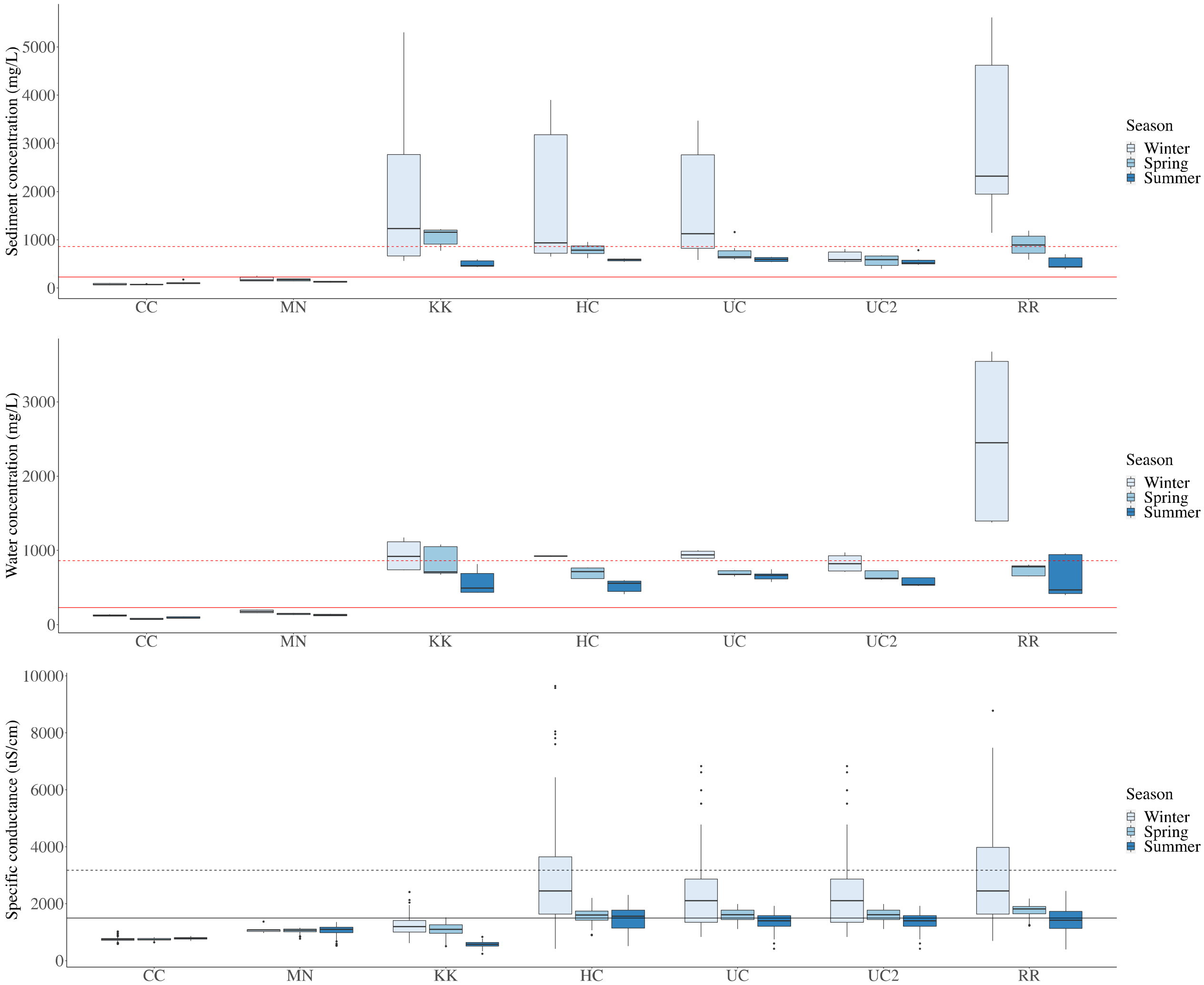

Figure 2. Field data collection of chloride and real-time conductivity from the Milwaukee River Basin study sites by season. Color represents season (Winter = January, February, and March; Spring = April and May; Summer = June, July, August, September). The top and middle graph shows chloride concentrations for sediment pore water and surface water samples. The bottom graph is real-time conductivity collected from MMSD and Southeastern Regional Planning Commission stations that were near the sampling site. Solid red line represents chronic toxicity standard (230 mg/L Cl^-) and dashed red line represents acute toxicity standard (860 mg/L Cl^-) (US EPA 1988). Solid black line represents estimated U.S. EPA chronic and dashed black line represents the acute toxicity standard calculated from the regression relationship between specific conductance and chloride (Corsi et al. 2010).

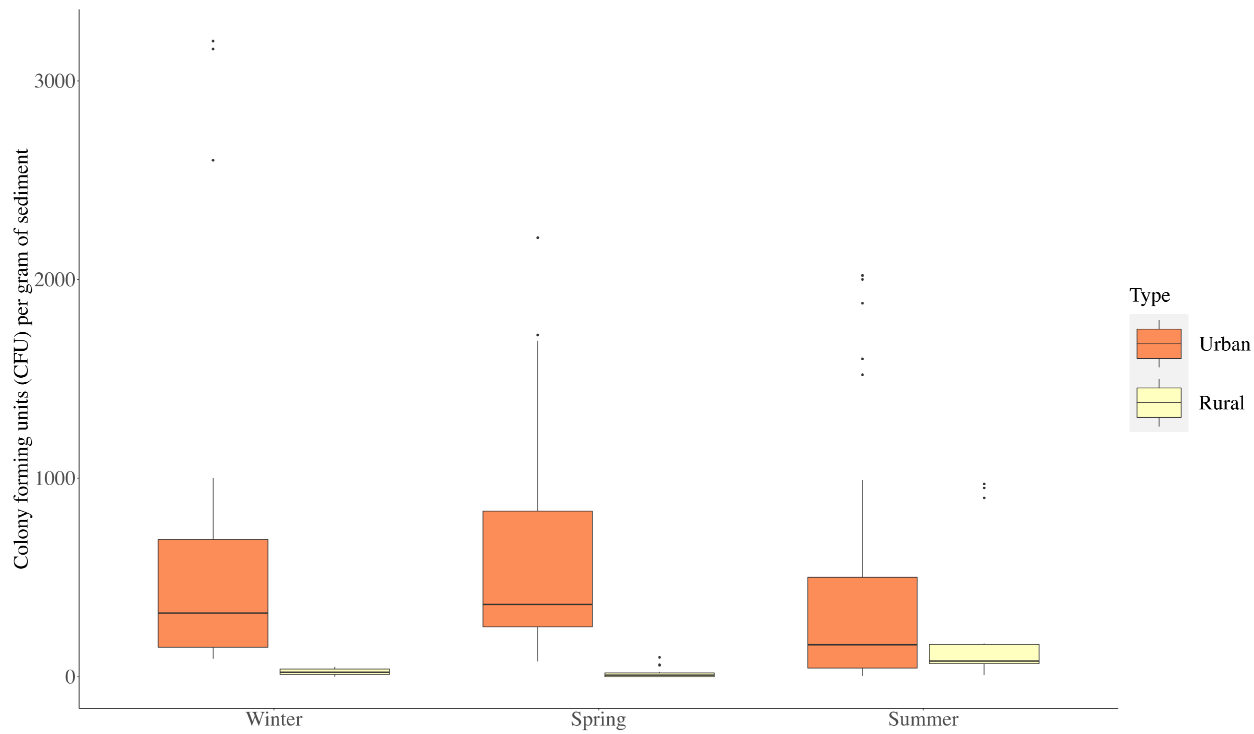

Figure 3. Colony forming units (CFU) per gram of sediment across season from direct plating on 6% NaCl M9 media. Color Dark Orange represents site type, urban (HC, KK, UC, UC2, and RR, n = 118) and yellow represents rural (CC and MN, n = 48). Tukey Post-Hoc results are indicated by letters indicating statistical significance (a, b, or ab).

2.4.2 Identification of **Halomonadaceae** *populations in study site sediment*

Members of a true halophilic family, *Halomonadaceae,* were commonly found across all study sites (*Halomonas,* with rare occurrences of *Halovibrio, and Salinicola*), which were identified by full length 16S rRNA gene sequencing of isolated colonies on 6% NaCl M9 plates. A phylogenetic tree assessed the diversity of the isolates collected across the study sites, which were categorized into five major clades (I to V). Isolates obtained from different sites were highly similar (up to 100% identity) and were within the same clade. For example, some isolates from the rural sites fall within the same clade of isolates at the urban sites suggesting that these organisms are highly related even though they were found in differently impacted environments. In addition, winter isolates collected in January and February of 2022 shared similarities with those cultured out of the sediment in spring and summer.

Out of all the *Halomonadaceae* isolates collected from the Milwaukee River Basin study site direct plating samples (n = 93), *Halomonas* was the dominant genus (98%) when compared to its closely related family members *Halovibrio* (1%) and *Salinicola* (1%). *Halomonadaceae* isolates cultured out of the sediment across each study site show variability between seasons, with HC and UC highest during the winter months (Table 2). However, it is important to note *Halomonadaceae* isolates are present throughout all seasons at all sites, except for the urban site UC2, where no *Halomonadaceae* isolates were obtained in the winter months. Salt tolerant bacteria (non-*Halomonadaceae*) isolates were also identified, with genera *Glutamicibacter, Paracoccus, Bacillus, Psychrobacter, and Shewanella* the most frequently isolated when sequenced for identification, with the highest proportion of salt tolerant bacteria collected during winter months at both urban and rural sites.

Table 2. Number of isolates collected at each study site by season and categorized by *Halomonadaceae* or non-*Halomonadaceae* (n = 93). Salt-tolerant bacterial isolates that were not *Halomonadaceae* were classified as non-*Halomonadaceae*. Note: Not all salt-tolerant isolates were sent for sequencing, but rather verified as *Halomonadaceae* or not through our screening primers.

Site:	$\mathbf{HC}_{\mathbf{urban}}$	$\mathbf{KK}_{\mathbf{urban}}$	$\mathbf{RR}_{\mathbf{urban}}$	$\mathbf{UC}_{\mathbf{urban}}$	$\mathbf{UC2}_{\mathbf{urban}}$	$\mathbf{CC}_{\mathbf{rural}}$	$\mathbf{MN}_{\mathbf{rural}}$
Halomonadaceae							
Winter	14	0	3	12**	0	2	1
Spring	6	7	7	8	6	3	5
Summer	2	3*	3	1	3	3	3
Non-Halomonadaceae							
Winter	25	23	25	16	6	12	7
Spring	3	5	3	4	6	4	4
Summer	4	3	1	2	3	1	1

*Contains one *Salinicola* isolate
**Contains one *Halovibrio* isolate

2.4.3 Optimal salt concentration for growth and temperature preference of **Halomonadaceae** *isolates*

We tested our *Halomonadaceae* isolates in laboratory-controlled conditions for optimal salt concentration and growth. Most isolates grew best in high salt conditions in the warmer temperature, but in the cold temperature optimal growth was observed in lower salt concentrations of 2%, and select isolates preferred

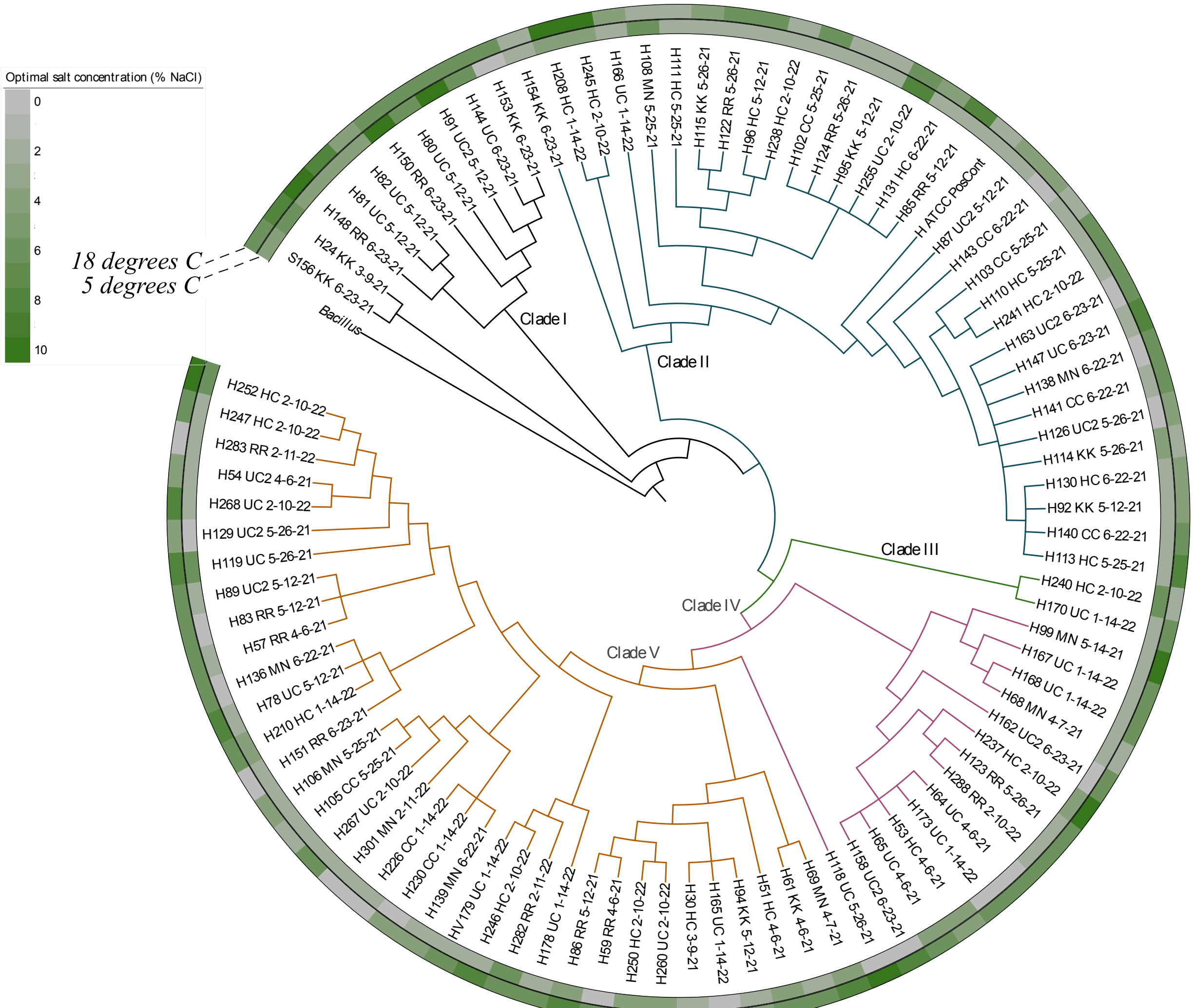

Figure 4. 16S rRNA gene phylogenetic tree of isolates collected from study site sediments. Their associated optimal salt concentration for growth at the warm (outer ring) and cold temperature (inner ring). Salt preference was determined by finding the isolate's maximum absorbance value. Isolate name identification is either H for *Halomonas,* HV for *Halovibrio*, or S for *Salinicola* and the site abbreviation and date they were sampled. The color gradient indicates salt (% NaCl (w/v)) preference. Branch color indicates the different clades: I, II, III, IV, and V). This tree does not include isolate H243.

0% NaCl (Figure 4). We noted differences among the clades. Isolates within clade I showed a similar salt preference between the two temperatures. Whereas clades II, III, IV, and V isolates grew optimally in low salt concentrations in the cold and high salt concentrations in warm temperature. Overall, isolates with similar salt preference patterns under warm and cold conditions spanned the phylogenetic tree.

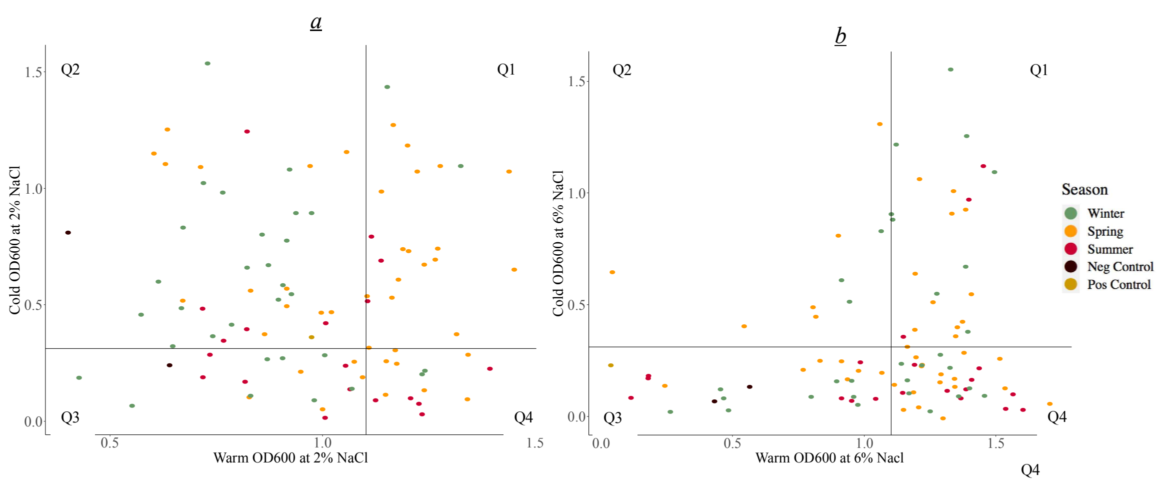

Figure 5. Isolate growth under (a) 2% and (b) 6% NaCl conditions in a warm or cold temperature categorized by season. Each data point represents an isolates OD600 absorbance value at cold and warm from 2% NaCl and 6% NaCl with season in which it was isolated represented by color. A positive control (ATCC *Halomonas* strain GFAJ-1), and negative controls known for salt tolerance (*Shewanella* and *Bacillus*) are represented by black and light brown color. The x and y axis intercept line represents the OD600 median of the whole dataset combined. Four quadrants were assigned as Q1 (+, +): Grows well in both temperatures, Q2 (-, +): Only grows well in cold temperature, Q3 (-, -): Does not grow well in either temperature, Q4 (+, -): Only grows well in warm temperature.

We further explored the growth dynamics under different salt concentrations (2% or 6% NaCl) and warm and cold temperatures in relation to the season from which they were collected. A wide range of growth among winter, spring, and summer isolates was observed in both temperatures in 2% NaCl (Figure 5a), whereas in 6% NaCl several of the isolates no longer grew well in the cold temperatures (loss of isolates in Q2), and those that remained were primary isolated in winter and spring (Figure 5b.) Further, a high number of isolates grew optimally at 6% salt concentrations in warm temperatures compared to 2% salt concentrations, showing an enrichment in Q4 (Figure 5a compared to 5b), with this ability evenly distributed among winter, spring, summer isolates. This might suggest that high salt is required for growth in warmer temperatures for these isolates, regardless of the season from which they were isolated.

2.4.4 Culture-independent detection of **Halomonadaceae**

The deep sequencing of microbial communities in sediment provided an alternative survey of halophiles not dependent of the culture method (i.e., plating on M9 6% NaCl media). The genus *Halomonas* was the only *Halomonadaceae* member detected in the sediment with a total of 12 unique ASVs sequenced. We aligned the v4 ASVs to the full length 16S rRNA gene sequences of isolates collected from the study sites to determine their closest match. We found ASVs 237 and 414 had the highest equal percent identities for all clade I *Halomonas* isolates (Table S6). The majority of clade II and III isolates were most closely aligned with ASV144, whereas most isolates in clades IV, and V had maximum percent identity of ASV414.

Unique *Halomonadaceae* ASVs were distributed across sites in the sediment (Table 3). Clade II ASV matches (ASV144 and 237) were detected repeatedly in the winter, and no longer observed in the summer. ASV414 was the only ASV detected in a spring sample. The unknown ASVs show that our culture-dependent method did not capture all of the *Halomonadaceae* population diversity in the sediment. Most importantly, these findings show there were a large relative proportion of the *Halomonadaceae* populations in winter versus summer.

Since the 6% NaCl plating method nonspecifically recovered both salt tolerant and halophilic bacteria, we used qPCR that targeted *Halomonadaceae* to quantify the number of 16S rRNA gene copies per gram of sediment. There results demonstrated *Halomonadaceae* populations were present in the sediment across all the study sites year-round (Figure S4) but did not correlate to the CFU counts that captured salt-tolerant bacteria. These results suggest that *Halomonadaceae* were not easily cultured out of the sediment with the media type used, but our qPCR assay could pinpoint their populations that otherwise would have been missed. Importantly, these results demonstrate our direct plating method capturing salt-tolerant bacteria does not serve as a proxy for assessing *Halomonadaceae* populations present in the sediment but does reflect a response of salt-tolerant bacteria.

Table 3. Deep sequencing results of samples that had *Halomonadaceae* reads (a total of 113 samples sequenced). ASVs were assigned with the clades they were most closely related to; date represents when the sediment sample was collected from the study site.

ASV	**Clade percent identity**	**V4 taxonomic assignment**	**HC urban**[2] 12/3/20	**KK urban** 3/9/21	**RR urban** 3/17/21	**KK urban** 5/26/21	**HC urban**[2] 6/22/21	**RR2 upstream**[2] 7/26/21	**RR2 upstream**[2] 8/11/21
144	II, III	*Halomonas* NA	**7457**	**38**	**848**	0	0	0	0
237	I, II, IV	*Halomonas* NA	**5360**	0	**393**	0	0	0	0
414	I, IV, V	*Halomonas* NA	**2747**	0	**109**	**56**	0	0	0
3199	Unknown[1]	*Halomonas* NA	**269**	0	**20**	0	0	0	0
4267	Unknown[1]	*Halomonas* NA	**187**	0	**19**	0	0	0	0
6131	Unknown[1]	Unclassified *Halomonadaceae*	0	0	0	0	0	**39**	**73**
6669	IV, V	*Halomonas taeanensis*	0	**38**	0	0	0	0	0
9901	Unknown[1]	*Halomonas* NA	**40**	0	0	0	0	0	0
10913	Unknown[1]	Unclassified *Halomonadaceae*	0	0	0	0	**25**	0	0
11015	Unknown[1]	*Halomonas* NA	**31**	0	0	0	0	0	0
14757	Unknown[1]	Unclassified *Halomonadaceae*	0	0	0	0	0	0	**14**
14865	Unknown[1]	*Halomonas* NA	**13**	0	0	0	0	0	0

[1] Means the ASV did not match up with any of the 16S isolates collected from the study sites.

[2] Replicate sample reads were summed together.

2.4.5 Root River high frequency sampling and **Halomonas** *summer dynamics*

Similar to the Milwaukee River Basin sites, our summer high frequency sampling at upstream and downstream Root River sites found salt-tolerant bacteria and *Halomonadaceae* populations present in the sediment during the summer season. CFU counts from the upstream site were greatest at the end of July compared to other sampling periods, whereas CFU counts from the downstream site were greatest in August 2021 compared to other sampling periods (Figure 6). Despite these observational differences, a non-parametric Wilcoxon t-test found that RR1 downstream and RR2 upstream CFUs were not significantly different from one another (p-value = 0.5358). Furthermore, chloride concentrations during these summer months at the upstream site exhibited much higher concentrations compared to the downstream site that rarely exceeded 200 mg/L Cl^-. These higher chloride concentrations at the upstream site on average exceed the EPAs chronic toxicity standard of 230 mg/L, but never went beyond the acute toxicity standard of 860 mg/L which was observed at all of our Milwaukie River Basin urban sites.

To directly assess how many salt-tolerant bacteria were *Halomonadaceae*, colonies were screened using primers targeting the dominant genus, *Halomonas*. Our estimate of *Halomonas* CFUs per gram of sediment for upstream and downstream sites were small in comparison to the total salt-tolerant CFU counts with on average only making up 16% of the total CFU counts, demonstrating the majority of isolates growing on the non-selective M9 6% NaCl media were not halophilic microorganisms (Figure 5). Select PCR positive colonies were sequenced and found to be highly similar to the Milwaukee River Basin isolates when placed in the phylogenetic tree (Figure S5), with 75% of the Root River isolates in clade II.

We also performed qPCR on sediment samples to demonstrate that was consistent *Halomonadaceae* populations at both upstream and downstream Root River sites. Similar to the Milwaukee River Basin sites, we were not able to observe a quantitative response of *Halomonadaceae* in the sediment with instantaneous (i.e., grab samples) measurements of chloride concentration.

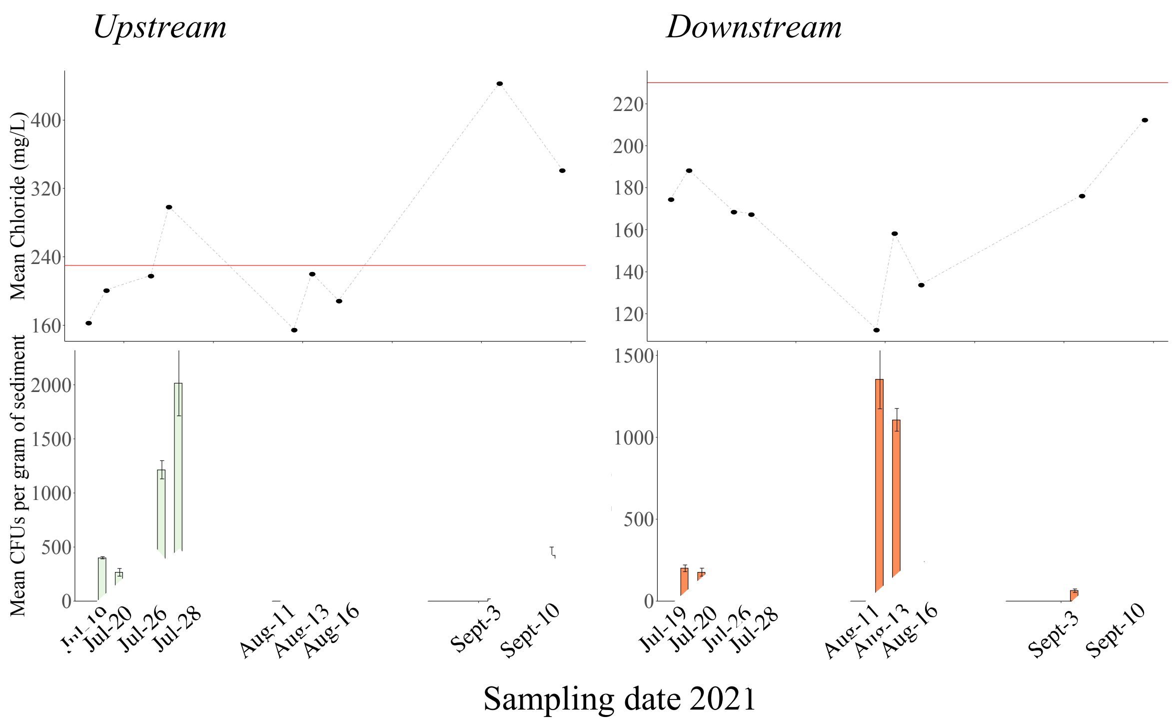

Figure 6. RR2 upstream (left) and RR1 downstream (right) sites surface water chloride concentration and CFUs from July, August, and September 2021 sampling events with respective standard deviation bars. Total CFUs counted are represented by the light green and dark orange bars, with the smaller bars representing positive colonies for *Halomonadaceae* in that sediment sample (RR2 positive colonies in total: n = 36, RR1 positive colonies: n = 19). Red line represents chronic toxicity standard (US EPA 1988).

Unlike the low frequency sampling results at our Milwaukee River Basin study sites, the high frequency sampling was able to capture more variability in the sediment population across sampling dates (Figure 7). A single sampling day for each month at our Milwaukee River Basin sites shows large changes between the two points of CFUs and qPCR sediment data. Our high frequency sampling at upstream and downstream Root River was able to capture a better representation of how salt-tolerant and *Halomonadaceae* populations fluctuate in just a matter of days, establishing the variability within the system. With high frequency sampling, the comparison between the qPCR and CFU data further shows the discrepancies in how they do not coincide with one another— but rather they potentially could be used as two separate indicators for assessing chloride-impacted waterways.

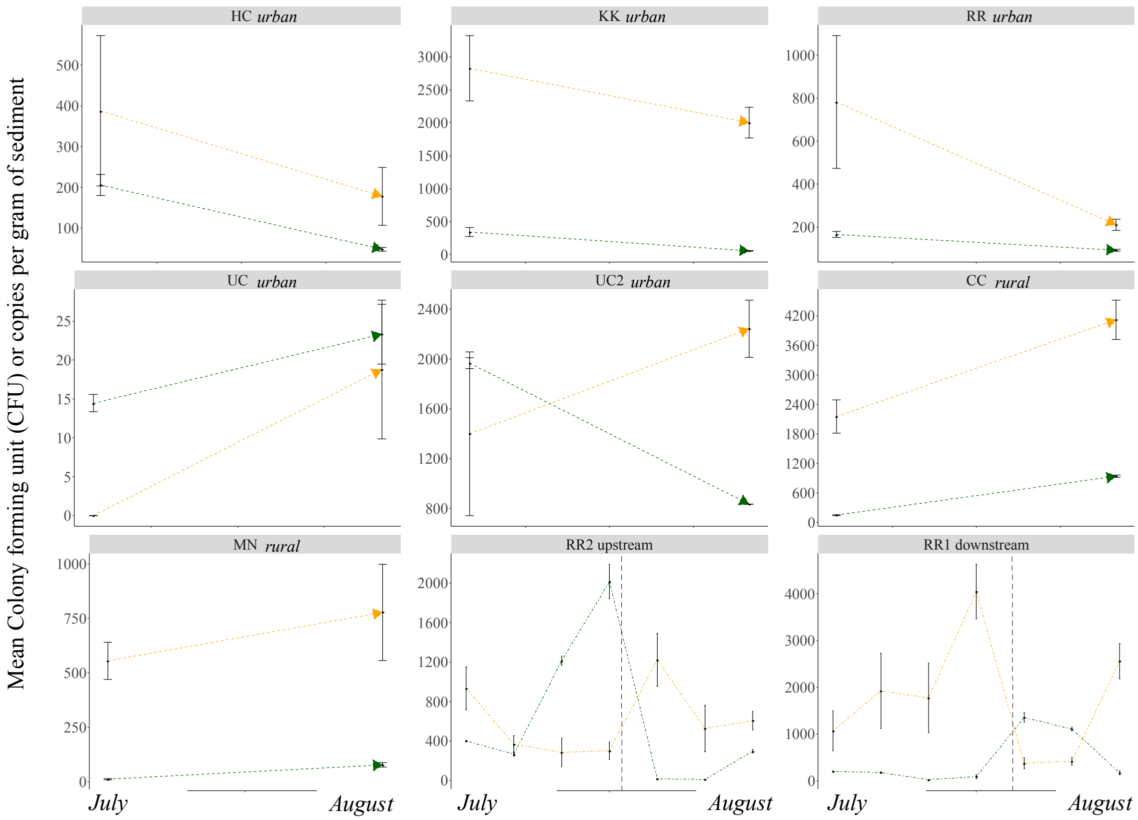

Figure 7. Comparison of low frequency Milwaukee River Basin study sites and Root River high frequency CFU and qPCR copies per gram of sediment for July and August/September 1st 2021 sampling events. Data points represent the mean value for that sampling day. CFUs are followed with a green dashed arrow and qPCR copies in yellow.

2.5 Discussion

2.5.1 Addressing chloride pollution through biological indicators

Despite a recent emphasis on assessing chloride levels in freshwater systems, there is a need to understand how freshwater ecosystems have responded to chloride exposure. Acute and chronic chloride toxicity standards (860 and 230 mg/L, respectively) set by the EPA serve as a benchmark when assessing waterbodies for chloride (US EPA 1998). These toxicity standards have been useful to understand the big picture of where chloride hotspots exist, especially in snowy climates. A regional survey conducted in Southeastern WI had found that 55% of the study sites exceeded acute toxicity and 100% exceeded the chronic toxicity standard (Corsi et al., 2010). However, levels fluctuate in surface waters during runoff events which make it difficult to capture. Toxicity standards may not be applicable to all freshwater systems, as each ecosystem has a different capacity in handling contaminants. These standards were determined through laboratory-controlled toxicity assays, which do not necessarily represent the many potentially confounding variables and interactions in the environment.

If a biological indicator such as halophilic bacteria in sediment along with abiotic measurements is used, this could provide a more integrated assessment. Using biological indicators to assess chloride-impacted waters is not a new idea, where the use of benthic invertebrates based on pollutant "tolerant" or "non-tolerant" classifications have been explored to create biological indices associated with chloride (Williams et al., 2000). However, long-term *Halomonadaceae* monitoring could provide a simplified measure to assess how impacted freshwater systems are from anthropogenic chloride as halophiles generally thrive when salt is available in their environments. This potential measurement is directly linked to a biological response, unlike toxicity standards that are based on a biological assumption in laboratory-controlled conditions.

There are also many other complications with chloride concentration measurements. At our Milwaukee River basin study sites, we were able to find significant differences between the sediment pore water and surface water grab sample chloride concentrations (Figure 2). These results suggest that sediment-dwelling freshwater organisms are experiencing higher chloride concentrations than what many surface water quality assessments miss. Surface water grab samples are likely an under-representation of chloride concentrations of what benthic organisms may be experiencing. This is an important concept to understand as many aquatic organisms spend at least some part of their lifecycle in the sediment.

2.5.2 Salt tolerant bacteria and **Halomonadaceae** *members are widespread and established in freshwater sediment*

By isolating a true halophilic family, *Halomonadaceae* at all study sites, we identified five distinct clades that were widespread within the Southeastern WI region regardless of the season they were isolated out of the sediment. Our direct plating method on 6% NaCl (w/v) was not selective for halophilic bacteria but was able to give an estimate of microorganisms that could grow and/or withstand high salt concentrations and collect present *Halomonadaceae* CFUs for isolation. Interestingly, CFUs were present throughout the year at the urban sites, with minimal counts at the rural sites with the exception during the summer months. This anomaly at the rural sites could be explained by other water quality influences such as fertilizer runoff (e.g., KCl) from rainfall events in the summer months (Kelly et al. 2012). Overall, CFUs were collectively higher at the urban sites versus the rural sites. This suggests that measuring salt tolerant and potential halophilic bacteria may be useful as a non-specific assessment. However, we do acknowledge that sediment type was not characterized, and this could play an important role with the survival of *Halomonadaceae*, and competition with other sediment bacterial populations like salt tolerant bacteria that could be well established especially in urban river/stream settings.

2.5.3 Isolate variability of optimal salt and temperature preference

The laboratory-controlled experiments showed the extreme nature of these halophilic bacteria that were isolated out of freshwater sediment in Southeastern WI by observing their growth capabilities. The family *Halomonadaceae* has traditionally been noted as a moderate halophile (Haba et al., 2014), where genera like *Halomonas* has been reported to optimally grow in the 2 to 6% NaCl range (Wang et al., 2014). Our laboratory results found similar findings in that most isolates grew best in the warm temperature under a salt concentration of 6% NaCl (w/v) (36,000 mg/L Cl^-). At the cold temperature, the majority of isolated shifted to an optimal salt concentration of 2% NaCl (w/v) (12,000 mg/L Cl^-). To put their optimal salt concentrations into perspective, oceans on average contain about 3.5% salt, estimated around 20,000 mg/L of chloride (USGS, n.d.). Although we never observed the study sites at extreme chloride levels like the concentrations used in these experiments, these results confirmed optimal growth under relatively high salt conditions was dependent on temperature. For example, under low salt concentration (2% NaCl) in the cold, many winter and spring isolates grew well (Figure 5a, Q2). However, isolate behavior shifted in the warm temperature where higher salt was required for optimal growth and few isolates no longer showed their optimal growth in 2% NaCl (Figure 5b, Q2 and Q4). These behaviors suggest that salt is preferred but not necessary for survival in freshwater systems, which may explain why *Halomonadaceae* populations are sustained throughout the seasons even when chloride concentrations are the lowest in the summer months.

2.5.4 **Halomonas** ***strains as seasonal indicators***

The direct detection of *Halomonadaceae* in sediment using qPCR demonstrated how widespread and established the populations were at each study site. It is likely that the low frequency sampling at the Milwaukee River Basin sites was not adequate to assess if halophile abundance was coupled to changing chloride concentrations. The populations at the urban sites was consistently present but more variable than populations observed at both rural sites. This may be due to how quickly the environment changes in urban waterways, from rapidly changing flow, contaminants, pH and temperature; basic water quality parameters where bacterial communities are susceptible (Wang et al. 2021; Lindström et al. 2005). These flashy environments also shift bacterial communities, where *Halomonadaceae* may be outcompeted by more resistant microorganisms. Unlike our qPCR assay that was able to target these baseline populations, our sequencing was only able to detect a select number of unique *Halomonadaceae* reads in urban site samples. Interestingly, all of the sites detected with high abundance of *Halomonadaceae* were observed in the winter months rather than summer months (Table 3). This could be due to less microbial activity in the winter, which allowed us to detect a higher relative abundance of *Halomonadaceae* in the total community compared to the summer where other members of the sediment community flourished. This also could be likely a result to higher salt concentrations during the winter in which *Halomonadaceae* isolates can thrive.

Through these culture-independent methods, we found that members within the major genus *Halomonas* could be useful for specific seasonal indicators in future survey work. When we aligned the unique v4 ASVs with our isolate sequences, we found the culture-dependent method did not capture the wide diversity of *Halomonadaceae* in the sediment. The majority of *Halomonadaceae* isolates matched ASV144, 237, and 414, which were all detected in the winter samples. ASV414 was the only ASV found in a spring sample (Table 3). The qPCR assay designed in this study took a generalized approach and was designed around the highly similar v4 region of the isolates. It will be of interest to design additional assays to specifically target strains of interest that may shift with season. A general survey could be useful to understand baseline populations that exist in freshwater sediment, followed by more specific assays targeting different strains. To measure the impact from road salt pollution during the winter at a stream or river, specific assays should be designed around isolate sequences in Q2 (Figure 3a) and ASV144 and ASV237 as they were observed only in the winter months. If interested in measuring spring chloride interactions, Q2 isolates and those corresponding to ASV414 could be an option.

2.5.5 *Root River high frequency sampling summer dynamic comparisons*

The Root River high frequency summer data collection substantiated that *Halomonadaceae* populations in sediment were beyond our study sites in the Milwaukee River Basin and were closely related, particularly to clade II isolates. It has been noted especially in the summer season, freshwater river systems experience chloride peaks in surface waters from the upwelling of chloride temporarily stored in shallow groundwaters (Kincaid and Findlay 2009). Here, we see chloride concentrations at both sites rapidly change across sampling date through high frequency sampling (Figure 6), which was not observed at our Milwaukee River Basin study sites during the summer under low frequency sampling (Figure 2). In addition, the Root River summer sampling results demonstrate the short-term variability in the bacterial community along the same river body with changing water quality (e.g., discharge rates, temperature, etc.) and summer precipitation, which will be an important consideration when developing bacterial indicators for ecological assessments.

Summer CFU counts at RR2 upstream and RR1 downstream were only comparable to summer CFUs observed at the urban site UC2 (Figure S4). However, instantaneous chloride concentrations collected by grab samples at the upstream and downstream Root River sites never exhibited high levels that were observed at UC2. Our urban site UC2 had many urban influences in which chloride concentrations on average were well above the chloride chronic toxicity standard. In addition, the comparison of low versus high frequency sampling shows the variability in *Halomonadaceae* and bacterial communities in general (Figure 7). This suggests that additional sampling in the Milwaukee River Basin study sites may be able to better capture a stronger response to changing instantaneous chloride concentrations or real-time conductivity. Overall, there was no correlation between recovery of cultured salt-tolerant bacteria and *Halomonadaceae*, demonstrating that these two approaches target different responses of the bacterial community to environmental conditions.

2.6 Conclusions and final remarks

Our field sediment surveys found the greatest bacterial CFUs at the Milwaukee River Basin urban sites compared to the rural sites in winter and spring seasons when chloride and conductivity were also the highest. CFUs did not coincide with *Halomonadaceae* populations, but rather these could be two separate field measurements in assessing chloride-impacted freshwaters. A true halophilic family, *Halomonadaceae*, are present in our study site freshwater sediment year-round in a Lake Michigan drainage basin located in Southeastern WI. Isolate 16S rRNA gene sequences demonstrated how closely related they were to each other regardless of study site and season.

Salt and temperature preference were similar for the majority of isolates, with only clade I showing high salt preferences in cold temperatures (Figure 4). These laboratory results indicated that the concentration of salt needed for optimal growth for the majority of *Halomonadaceae* isolates was temperature dependent. These findings may explain why we see populations in the environment even under low chloride concentrations as freshwater *Halomonadaceae* may not need high salt conditions to survive, as is traditionally believed. Our qPCR-based sediment survey demonstrated where *Halomonadaceae* has accumulated in the sediments and is the first to document baseline populations in Southeastern WI. Community sequencing provided insights on the diversity of *Halomonadaceae* that was detected in high abundance in select winter versus summer samples (Table 3). Our work has laid the foundation for developing a biological indicator to assess chloride-impacted waters. Instantaneous measurements change rapidly (e.g., chloride), but halophilic bacteria may give a better integrative picture of longer-term conditions.

This research is the first of its kind to investigate halophilic bacteria, specifically *Halomonadaceae* populations, in freshwater sediment. Due to this, we were limited in our ability to obtain frequent samples at all of the Milwaukee River Basin study sites with how spread apart each site was from one another. With a smaller sample size, we were not able to truly capture the bacterial community fluctuations that can occur quickly in the environment. We suspect that if our sampling schedule was more frequent, we may have been able to observe a clearer potential pattern with chloride concentrations and/or conductivity. We also acknowledge that sediment type was not characterized, and this could play an important role with the survival of *Halomonadaceae*, and competition with other sediment bacterial populations. We especially see this is in our deep sequencing results in which *Halomonadaceae* ASVs were a very small proportion to the top 13 non-halophilic ASVs in the sediment samples across all study sites. Future research should continue to take steps forward in investigating halophilic bacteria in sediment to better understand the long-term impacts of chloride pollution on aquatic ecosystems. This is especially important as each freshwater system is unique in handling contaminants, which could help guide development and decisions of Total Maximum Daily Loads (TMDLs) for chloride in the future.

There are a few next steps that should be done to further examined halophilic bacteria, like *Halomonadaceae*, that could serve as a biological indicator and predictor of ecosystem health and water quality. First, creating a specific *Halomonas* strain assay for winter surveys should be designed to look at population fluctuations with road salt inputs on a high frequency sampling basis. With an emphasis on no more than three stream or river study sites, this will allow for more sample collection and therefore a stronger field collection dataset. In terms of direct plating of the sediment, it would be beneficial to look into selective-type media for halophiles, instead of a non-selective media that also captured salt-bacteria that was used in our research. The last major research question would be investigating organisms one level up the food web, such as benthic invertebrates as they interact frequently with bacterial communities. Ecological assessments, like Index of Biotic Integrity (IBIs), could be done to make comparisons with the halophilic bacteria populations observed in the environment. This type of work could support this idea of using halophilic bacteria not only as biological indicators of chloride-impacted waters but also give insight of ecosystem health from the long-term exposure to chloride pollution.

** This section of the thesis is currently in the process of submittal for publication, with co-author collaboration from the University of Wisconsin Milwaukee's Geoscience Department. Thank you to Dr. Charlie Paradis and Leah Dechant, a Graduate Research Assistant, for their help and review of the manuscript.

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4. Appendices

Appendix A

Descriptive study site information

Table S1. Descriptive Milwaukee River Basin study site data. Location and land use percentages for all seven study sites within 1 km^2 area. Land use data was collected using the 2011 National Land Cover Database base map tool.

Site	Type	Latitiude	Longitude	Area (km2)	Land use type	Coverage(%)
Honey Creek (HC)	Urban	43.044229	-88.0026887	1	Developed, Open Space	14
					Developed, Low Intensity	49.28
					Developed, Medium Intensity	16.16
					Developed, High Intensity	4.49
					Deciduous Forest	8.98
					Mixed Forest	1.08
					Grassland/Herbaceous	1.17
					Woody Wetlands	4.85
Kinnickinnic River (KK)	Urban	43.002465	-87.966301	1	Developed, Open Space	3.06
					Developed, Low Intensity	14.31
					Developed, Medium Intensity	30.15
					Developed, High Intensity	48.51
					Deciduous Forest	0.27
					Mixed Forest	0.63
					Grassland/Herbaceous	1.8
					Woody Wetlands	1.26
Root River (RR)	Urban	42.986476	-88.057485	1	Developed, Open Space	31.02
					Developed, Low Intensity	42.38
					Developed, Medium Intensity	17.31
					Developed, High Intensity	1.8

					Deciduous Forest	1.26
					Mixed Forest	1.17
					Grassland/Herbaceous	1.71
					Pasture/Hay	2.61
					Woody Wetlands	0.72
Underwood Creek (UC)	Urban	43.015223	-88.057412	1	Developed, Open Space	30.22
					Developed, Low Intensity	26.17
					Developed, Medium Intensity	34.17
					Developed, High Intensity	9.44
Underwood Creek 2 (UC2)	Urban	43.042203	-88.058814	1	Developed, Open Space	37.41
					Developed, Low Intensity	35.16
					Developed, Medium Intensity	17.09
					Developed, High Intensity	10.34
Cedar Creek (CC)	Rural	43.33785	-88.003797	1	Developed, Open Space	14.48
					Developed, Low Intensity	11.42
					Deciduous Forest	9.53
					Mixed Forest	0.81
					Shrub/Scrub	2.07
					Grassland/Herbaceous	2.52
					Pasture/Hay	6.12
					Cultivated Crops	28.78
					Woody Wetlands	24.28
Menomonee River	Rural	43.235038	-88.119613	1	Developed, Open Space	16.25
					Developed, Low Intensity	9.07
					Developed, Medium Intensity	4.58
					Developed, High Intensity	3.68

Deciduous Forest	13.73
Mixed Forest	4.94
Grassland/Herbaceous	0.09
Pasture/Hay	11.67
Cultivated Crops	24.06
Woody Wetlands	11.94

Table S2. Descriptive upstream and downstream Root River study site data. Location and land use percentages for study sites within 1 km^2 area. Land use data was collected using the 2011 National Land Cover Database base map tool.

Site	Type	Latitiude	Longitude	Area (km2)	Land use type	Coverage(%)
Root River upstream (RR2)	Rural	42.815407	-87.9926012	1	Open Water	0.27
					Developed, Open Space	3.15
					Developed, Low Intensity	4.14
					Developed, Medium Intensity	0.63
					Developed, High Intensity	0.09
					Deciduous Forest	21.8
					Mixed Forest	1.17
					Shrub/Scrub	0.72
					Grassland/Herbaceous	3.33
					Pasture/Hay	14.05
					Cultivated Crops	35.86
					Woody Wetlands	13.42
					Emergent Herbaceous Wetlands	1.35
Root River downstream (RR1)	Urban	42.7466813	-87.820891	1	Open Water	4.94
					Developed, Open Space	56.96
					Developed, Low Intensity	9.34
					Developed, Medium Intensity	17.79

Developed, High Intensity	0.9
Barren Land (Rock/Sand/Clay)	0.18
Deciduous Forest	4.58
Mixed Forest	1.17
Woody Wetlands	4.13

Real time conductivity data stations

Table S3. List of all real-time conductivity stations used obtained from Milwaukee Metropolitan Sewerage District (MMSD) and Southeastern Wisconsin Regional Planning Commission (SEWRPC).

Agency	Station ID	Site	Time frame with data available	Number of readings per day
SEWRPC	52 Cedar Creek	Cedar Creek (CC)	December 2020 - July 2021	288
MMSD	MS1130	Menomonee River (MN)	December 2020 - September 2021	24
SEWRPC	53 Honey Creek Wawautosa	Honey Creek (HC)	December 2020 - September 2021	288
MMSD	MS1104	Kinnickinnic River (KK)	December 2020 - September 2021	24
SEWRPC	Root River at Grange	Root River (RR)	December 2020 - September 2021	288
SEWRPC	Underwood near 115th street	Underwood Creek and Underwood Creek Downstream (UC and UC2)	January 2021 - August 2021	288
SEWRPC	25 Root River Canal (z6-08204)	Root River Upstream (RR2)	July 2021 - September 2021	288
SEWRPC	59 Root River at Horlick (z6-04959)	Root River Downstream (RR1)	July 2021 - September 2021	288

Supplemental text I

Screening primers to identify Halomonadaceae colonies

The Root River high frequency sediment samples were screened to determine which CFUs were halophilic. Halophilic isolate sequences from the Milwaukee River Basin were used to determine a conserved region for custom primers to amplify the dominant *Halomonadaceae* genera, *Halomonas*, found across all study sites. Sequences were aligned using the package MegAlign Pro (Version 17.2.1 (63)) whereas primers were designed in SeqBuilder Pro (Version 17.2.1 (62)) in DNAStar (Madison, Wisconsin, USA). The primers 406F (5'- ATACCCATTAGGAAAGACATC-3') and 530R (3'-TTATCAAGCCACCTACGC-5') were selected and checked through the Ribosomal Database Project (RDP--Release 11) Sequence Analysis Tool database, Probe Match, feature where the primers targeted within the family *Halomonadaceae*. All search results were refined to “good quality” and a length “< 1200”. At 2 mismatches, there are stray sequences, but specificity was achieved through an optimal annealing temperature of 60°C and was validated with positive and negative controls. Three known *Halomonas* and four non-*Halomonas* (*Psychrobacter*, *Halobacillus*, *Jeotgalibacillus*, and *Bacillus*) isolates were selected to verify the custom primers.

Table S4. *Halomonas* screening primers 406F (5'- ATACCCATTAGGAAAGACATC-3') and 530R (3'-TTATCAAGCCACCTACGC-5') RDP search results with 2 mismatches.

Class	**Family**	**Number of matches**
Gammaproteobacteria	*Alteromonadaceae*	3/14993
Unclassified Gammaproteobacteria Gammproteobacteria	*Halomonadaceae*	1365/9778
	Genus:	
	Halomonas	1326/2895
	Halovibrio	5/29
	Unclassified_Halomonadaceae	34/288

Halomonadaceae *isolate growth rates*

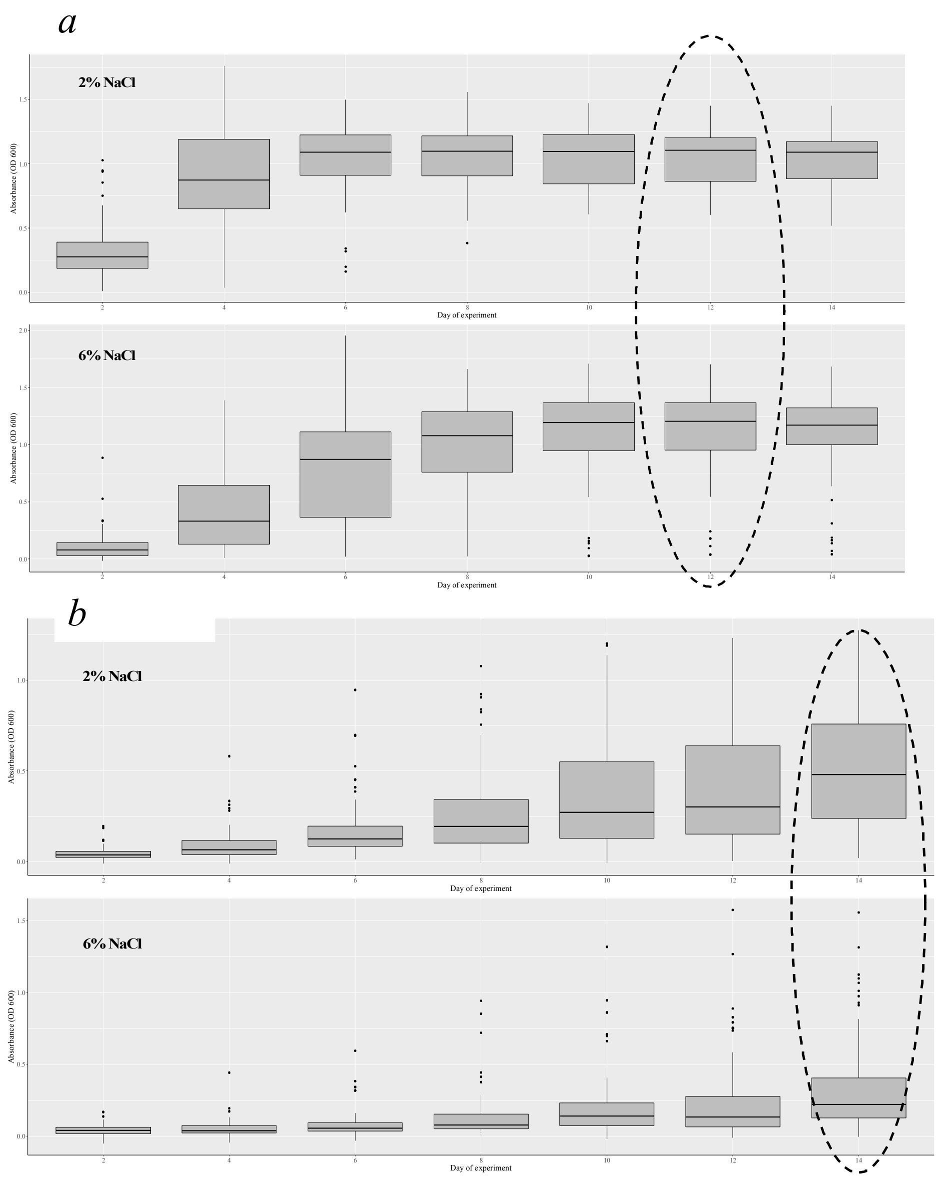

Figure S1. (a) Warm (18 °C) growth rates of each isolate over 14-day period to determine which dataset would be used for the remainder of the paper. Day 12 was selected for both 2 and 6% NaCl and (b) Cold (5 °C) growth rates of each isolate over 14-day period to determine which dataset would be used for the remainder of the paper. Day 14 was selected for both 2 and 6% NaCl.

Deep sequencing results

Table S5. Top 13 ASVs that had 90% prevalence or greater in all samples sequenced (n = 113).

Sequence ID	Order	Family	Genus	Pielou's evenness	Variance	Mean (% relative abundance)	Median (% relative abundance)
ASV1	Burkholderiales	*Comamonadaceae*	Unclassified	0.88	3.16	1.59	0.5
ASV2	Burkholderiales	*Comamonadaceae*	*Hydrogenophaga*	0.85	5.54	1.65	0.43
ASV3	Burkholderiales	*Comamonadaceae*	*Rhodoferax*	0.85	4.56	1.24	0.63
ASV6	Rhodobacterales	*Rhodobacteraceae*	*Tabrizicola*	0.91	1.04	0.97	0.51
ASV8	Flavobacteriales	*Flavobacteriaceae*	*Actibacter*	0.86	0.42	0.48	0.16
ASV17	Burkholderiales	*Comamonadaceae*	*Rhodoferax*	0.91	0.37	0.51	0.28
ASV24	Steroidobacterales	*Steroidobacteraceae*	Unclassified	0.9	0.11	0.31	0.15
ASV33	Flavobacteriales	*Flavobacteriaceae*	*Flavobacterium*	0.88	0.22	0.35	0.18
ASV37	Ignavibacteriales	Unclassified	NA	0.93	0.05	0.22	0.15
ASV51	Pseudomonadales	*Halieaceae*	Unclassified	0.94	0.05	0.25	0.17
ASV53	Micrococcales	*Micrococcaceae*	*Pseudarthrobacter*	0.93	0.05	0.24	0.18
ASV68	Pedosphaerales	*Pedosphaeraceae*	Unclassified	0.97	0.01	0.21	0.2
ASV107	Chitinophagales	*Saprospiraceae*	Unclassified	0.93	0.03	0.18	0.12

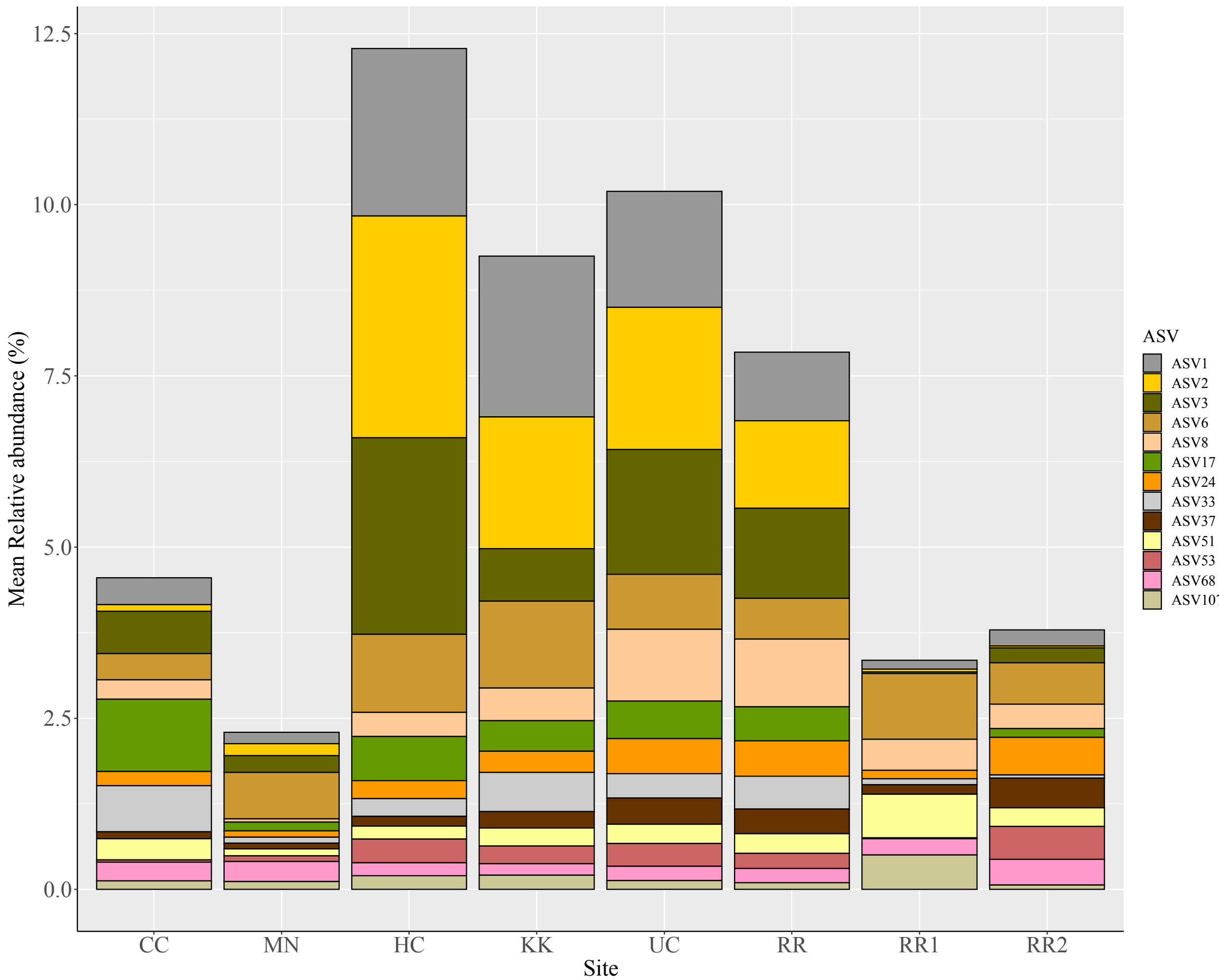

Figure S2. Mean relative percent abundance of the top 13 abundant and prevalent ASVs across study site sediment deep sequenced. CC, MN, and RR2 land use were considered rural whereas HC, KK, UC, RR, and RR1 were considered urban sites.

Table S6. All *Halomonadaceae* isolate sequences collected from Milwaukee River Basin and Root River sites maximum percent identities with ASVs categorized by clade and season (Note: does not include isolate H243).

Season	Sequence ID	No clade designation	Clade I	Clade II	Clade III	Clade IV	Clade V
	ATCC positive control	0	0	**1**	0	0	0
Winter							
	ASV144	0	0	**4**	**2**	0	0
	ASV144 & 414	0	0	**1**	0	0	0
	ASV237	0	0	**1**	0	0	0
	ASV414	0	0	0	0	**5**	**15****
	ASV6669	**1**	0	0	0	0	**2**
Spring							
	ASV144	0	0	**15**	0	0	0
	ASV237 & 414		**4**	0	0	0	0
	ASV237, 414, 3199		0	**1**	0	0	0
	ASV414	0	0	0	0	**2**	**9**
	ASV6669	0	0	0	0	**4**	**7**
Summer							
	ASV144	0	0	**20**	0	**2**	**3**
	ASV237	0	0	**4**	0	**1**	0
	ASV237 & 414	**1***	0	0	0	0	0
	ASV414	0	**4**	**6**	0	**2**	**4**

* *Salinicola*

** Contains one *Halovibrio*

Supplemental text II

qPCR assay validation

A quantitative polymerase chain reaction (qPCR) assay was developed to capture the halophilic genera from the family Halomonadaceae. The v4 region did not vary enough from one another that made it challenging to target one genus within the family. Instead, primers and a probe were designed to emphasize the isolates that were cultured out of the study sites: *Halomonas* and *Salinicola,* where *Halomonas* was the dominant genus found consistently across study sites. The total length of the amplicon is on the shorter end of 70 bp (5' - GGAACTGTCAAGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGC GTAGAGATCG – 3').

The forward primer was designed as a degenerate primer as the sequence alignments showed a consistent single base pair change of A to G. The forward primer (sense) GGAACTGTCARGCTAGAG (GGAACTGTCAAGCTAGAG + GGAACTGTCAGGCTAGAG) and probe (anti-sense) CGGGAATTCTACCTTCCTCTCCTGC are specific, whereas the reverse (anti-sense) is less specific (CGATCTCTACGCATTTCA). The probe used was a FAM double-quenched probe containing a 5' fluorophore. The forward primer and probe were checked in the Ribosomal Database Project (RDP--Release 11) Sequence Analysis Tool database, Probe Match function. All search results were refined to "good quality" and a length "< 1200". At 2 mismatches, there are stray sequences, but specificity will be achieved through the optimal annealing temperature determined.

Annealing temperature

A temperature gradient was performed to determine optimal annealing temperature and specificity. A thermocycler setting was created to mimic the qPCR cycles with the first step at 95 °C for four minutes, 40 cycles at 95 °C for 15 seconds and 62 °C for 30 seconds. Each well consisted of 2.5 µL of each primer (forward and reverse) (10 µM), 0.2 µL probe (10 µM), 12.5 µL Master Mix (2x), 2.3 µL of DNase/RNAse-free water, and 5 µL of isolate DNA. DNA templates for each isolate were made by taking a single colony from a streak plate and suspending it in either 30 or 50 µL DNase/RNAse-free water and placed into the –20 degrees C freezer to lyse the cells and release DNA into solution. The DNA templates were centrifuged for 5 minutes at 14,000 g to pellet any cellular material that could interfere with PCR. Each template was read on the nanodrop to obtain DNA concentrations for further dilutions to the desired concentration of ≤ 1.0 ng/uL. The PCR products were ran on 2.5% agarose gel at 125 voltz and the exACTGene 100 bp PCR DNA ladder was ran alongside to verify length.

The optimal annealing temperature was determined to be 62 degrees Celsius where the positive control was amplified and negative controls (e.g., *Pseudomonas*, *Psychrobacter*) were not. Further validation was done with 14 negative control isolates that were closely related to the genera in *Halomonadaceae* and 15 positive controls within the target family on the OneStep qPCR in the GLGC where isolates of interest were amplified, and negative controls were undetected.
In addition, a temperature gradient was set on the OneStep qPCR with temperatures of 61, 61.5, and 62 degrees Celsius to determine efficiency. Efficiency (i.e., slope) was measured by using a positive control (*Halomonas* isolate #111) DNA template and diluting it by 10-fold four times (I.e., 1:100, 1;1000, 1:10000, 1:100000) where the slope could be determined. Although 62 degrees C gave an efficiency of 88.8% resulting in a slight loss in sensitivity, all negative controls were undetected unlike the temperatures of 61 and 61.5 degrees Celsius.

Further validation

All *Halomonadaceae* isolates collected (n = 93) were ran to determine if the qPCR assay created encapsulated all the positive controls against the negative controls. Our assay was able to amplify all *Halomonadaceae* isolates and exclude the negative controls that are closely related to the halophilic family. Note that all DNA templates used in the validation process were read on the nanodrop to obtain DNA concentrations for further dilutions to the desired concentration of ≤ 1.0 ng/uL.

Standard curve and assay efficiency

In preparation for the standard curve, a linearized plasmid with our 70 bp product insert was made. A positive control (*Halomonas* isolate #111) was put through the same end-point PCR explained in the 'Annealing temperature' sub-section. However, a final step was added (72 degrees Celsius for 10 minutes) in order to create 3' adenine overhangs on the product. Once the product was verified, the fresh PCR product was put into a TOPO vector using the Invitrogen TOPO® TA Cloning Kit (pCR 2.1-TOPO Vector) and then introduced to One Shot® TOP10 *E. coli* competent cells to uptake the super-coiled plasmid. Cells were spread on LB solid media containing 100 mg/uL Ampicillin (AMP) and 40 mg/mL X-Gal and incubated at 37 degrees Celsius overnight. White colonies (i.e., transformed cells) were selected and inoculated into LB + AMP broth and shaken at 37 degrees Celsius overnight. Plasmids were then extracted from the cells using the QIAGEN QIAprep Spin Miniprep Kit and then linearized using the restriction enzyme HindIII from BioLabs, Inc.

Aliquots of known copies of the linearized plasmid containing our 70 bp product were made using the known concentration of the stock plasmid at that time of standard curve preparation. Knowing the initial concentration, seven dilutions were created to achieve the certain number of copies: $1.5x10^7$, $1.5x10^6$, $1.5x10^5$, $1.5x10^4$, $1.5x10^3$, $1.5x10^2$, and $1.5x10^1$. A total of nine standard curves were ran from separate standard curve preparations, in triplicates, to establish a Ct threshold as well as the average y-intercepts, slopes, and Ct of each standard curve ran. The nine standard curves produced an average assay efficiency of 99.8%.

Table S7. qPCR v4 assay forward degenerate primer and probe results from RDP with **2 mismatches** primarily targeting the halophilic family Halomonadaceae genera *Halomonas*, *Halovibrio*, and *Salinicola*.

Class	Family	Number of matches
Alphaproteobacteria		2/165068
Gammaproteobacteria	Alteromonadales	3/14993
	Unclassified_Altero monadales	1/178
Unclassfied_Gammaprot eobacteria		
	Halomonadaceae	2530/3873
	Unclassfied_Oceano spirillales	2/606
	Genus:	
	Halomonas	2217/2895
	Halovibrio	11/29
	Carinomonas	15/116
	Chromohalobacter	63/82
	Cobetia	1/224
	Kushneria	57/94
	Salinicola	106/138
	Pistricoccus	1/1
	Unclassfied_Halom onadaceae	59/288

Colony forming units (CFU) across site

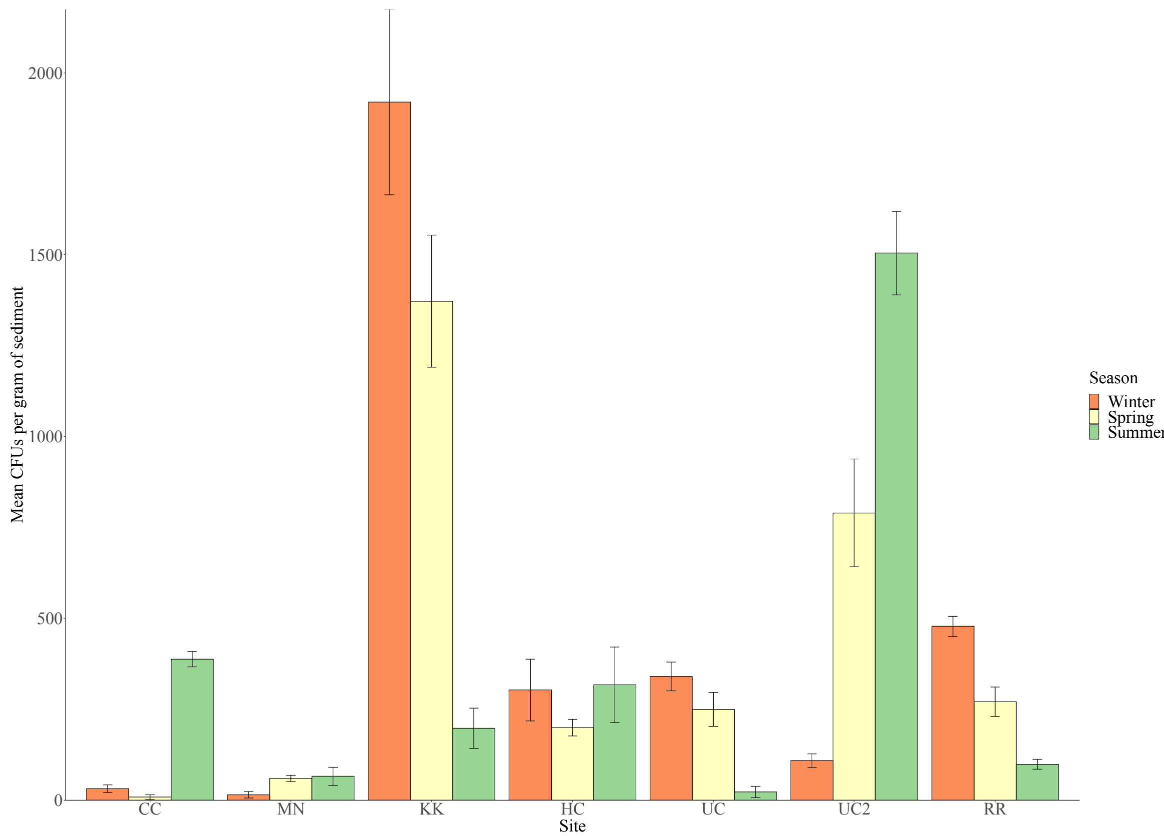

Figure S3. Mean colony forming units (CFUs) per gram of sediment across study site by season with respective standard deviation. Season was decided upon as winter = January, February, and March, spring = April and May, summer = June, July, and August/September sampling events.

qPCR assay results

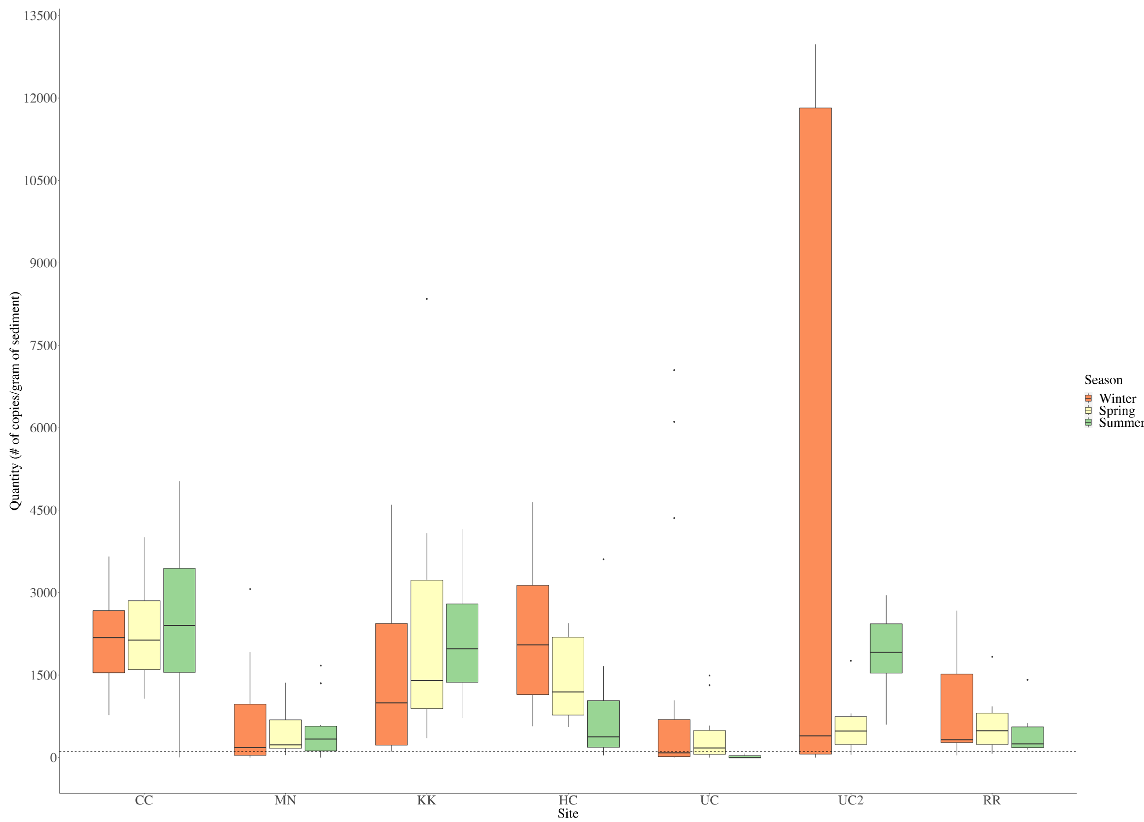

Figure S4. qPCR data for Milwaukee River Basin sites by season. Season was decided upon as winter = January, February, and March, spring = April and May, summer = June, July, and August/September sampling events. The dashed line represents the limit of detection. Note that UC2 January 2022 sampling event had unusually high copies per gram of sediment, which explains the spread of the boxplot.

Halomonadaceae ***phylogenetic tree and isolate record***

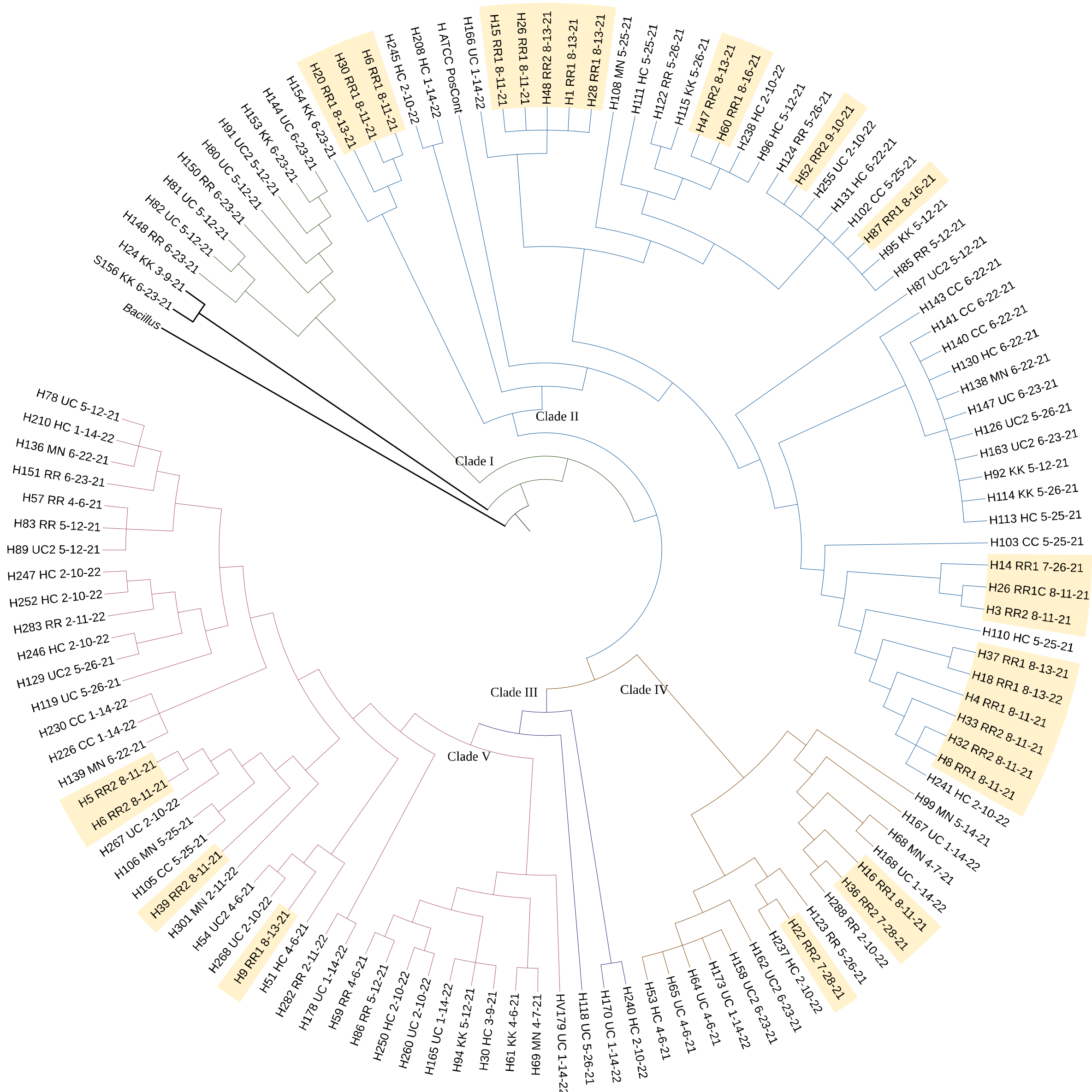

Figure S5. A phylogenetic 16S rRNA gene tree of all Halomonadaceae isolates collected from the duration of the study on 6% NaCl modified M9 solid media (n = 92 plus ATCC *Halomonas* positive control, does not include isolate H243). The isolates highlighted in green were ones cultured out from the Root River upstream and downstream high frequency sampling (n = 29). The rest not highlighted are from the Milwaukee River Basin study sites. Note that once Root River isolates were placed in the tree, there is a shift of clade III now being more closely related to clade V.

Dataset 1. All *Halomonadaceae* isolates collected throughout the whole study with site and isolation method including Milwaukee River Basin study sites (n = 92 plus ATCC *Halomonas* positive control, does not include isolate H243) and Root River high frequency sampling collection of isolates (n = 29 isolate sequences).

Isolate_Classifications.xlsx

Appendix B

3. Increase awareness of chloride impact on our environment

3.1 Abstract

We collaborated with WI Salt Wise, an Oconomowoc, WI high school teacher, and Waukesha County personnel to take our research findings and create educational materials to better increase awareness of the environmental toll chloride has in surface waters. To accomplish this, we took two different modes of action: 1.) created educational video series following the scientific process, and 2.) published a Data Nugget, an interactive classroom lesson, for teachers to use that would also go along with the video series. With receiving funding through the Cooperative Institute of Great Lakes Research (CIGLR), we worked with UWM's Doc | UWM film department to create the video series and used our real data collected to produce the interactive lessons for students to analyze in the classroom. Our final outreach products will be used for social media platforms (e.g., WI Salt Wise), published through the Data Nugget website, and overall help spread awareness of chloride impact on our environment in schools.

3.2 Introduction

There is still much to learn when determining how these aquatic ecosystems are impacted throughout the year after the seasonal chloride pollution subsides (e.g., road salts). There is currently no ecological indicator that can be observed to pinpoint waterways being over-salted. Our research discovered salt-loving bacteria (i.e., halophiles) in freshwater sediment are present throughout the year in southeastern WI (one of many Lake Michigan's drainage basins). True halophiles, like the family *Halomonadaceae*, have been found in all study sites and may serve as a suitable candidate indicator for assessing chloride impaired streams and rivers. To better raise awareness of our findings and chloride pollution in general, we created educational outreach materials.

We partnered up with Wisconsin Salt Wise, a coalition of organizations (e.g., City of Milwaukee, City of Madison, municipal stormwater coalitions, and watershed organizations), a high school teacher from

Oconomowoc WI, and a Waukesha County employee, with the overarching goal to educate communities on the importance of reducing salt pollution through best management practices and the adverse impacts on the environment. To accomplish these educational materials, we received funding through Cooperative Institute of Great Lakes Research (CIGLR) Engagement, Career Training, and Outreach (ECO) program.

Our educational outreach final products created short videos to increase awareness of chloride impact on surface waters and document the rationale, progress, and impact of our research. In addition, we used real scientific datasets from our research on halophilic bacteria to create a Data Nugget lesson plan geared toward junior/senior year high schoolers. Data Nuggets (https://datanuggets.org/about-nuggets-2/) is a free online library of lesson plans where educators can download a variety of science topics to incorporate in their classroom. The main mission of Data Nuggets is to engage students with a scientist's research and follow the scientific method. This process gets students comfortable working with data that may be messy or produce unexpected results. In addition to the lesson plan, teacher resources were incorporated, which included the short video series to go along with the chloride pollution lesson.

3.3 Methods

3.3.1 Educational videos

We collaborated with UWM Peck School of the Arts, Doc | UWM film department. Through this, we were able to involve a group of UWM film students on the project, which offered experience in science communication to those students. The project was split into three short videos to follow the flow of the scientific method. The first video filmed began with field sampling to show the viewer where the samples came from. The second was processing the samples in the laboratory, and the third video ended the video series with how science can inform policy decisions when it comes to winter maintenance and best management practices. Additionally, Doc | UWM created a broad overview video for social media purposes that captured the extent of our research. Future impact measures of these videos would be measured through video views once published on social media platforms (e.g., WI Salt Wise).

3.3.2 Data nugget lesson plan

The data nugget lesson plan used our non-specific colony forming unit (CFU) per gram of sediment data collected from one urban and rural Milwaukee River Basin sites (KK and MN). We decided to put an emphasis on land usage (e.g. impervious surfaces versus green space) in relation to the chloride concentration that was observed at the sampling date, where students had to make connections with the bacterial CFUs. The Data Nugget lesson plan template consists of four major parts. The first component is setting the stage with our narrative as to what we were investigating and why. Following the narrative, the scientific question can be extrapolated and students are provided with data which they can eventually answer the question with at the end. Third, students are prompted to identify the independent and dependent variables. Lastly, they must analyze the data through graphs and answer thought-provoking follow up questions. Our Data Nugget lesson was published onto the Data Nugget website, and future measurements of impact will be tracked by how many times the lesson plan is downloaded and the location in which it was used.

3.4 Final products

3.4.1 Salty sediments? What bacteria have to say about chloride pollution

Videos

Doc | UWM three-part video series and summary project video can be found here:

Password: SFS:

Field sampling:
https://vimeo.com/685634995

Laboratory:
https://vimeo.com/686346782

Policy:
https://vimeo.com/686519215

Project summary video:
https://vimeo.com/686856425

Or on WI Salt Wise Youtube channel:
https://www.youtube.com/channel/UChExOSekqfegfFicF7l6RXg/featured

Lesson plan

The final product of our lesson plan can be found here:

- https://datanuggets.org/2022/03/salty-sediments/

Appendix C

4. A strategy of valuing chloride reductions in Southeastern Wisconsin surface waters

4.1 Abstract

Cost-benefit analysis (CBA) is a systematic assessment method that quantifies in monetary terms all impacts of a policy, including impacts on ecosystem services. The broad purpose of CBA is to help decision-making and to increase social wellbeing. We reviewed available CBAs and related literature on road salt (e.g., chloride compounds) and other winter maintenance applications (e.g., brining technologies), to assess the extent to which CBAs capture the impacts of deicers on aquatic ecosystems and propose a framework for improving the economic valuation of these impacts. To reduce chloride loading, many states like Wisconsin have been experimenting with different winter maintenance routines or implementations of Best Management Practices (BMPs). Our findings suggest there is currently limited CBAs able to get at environmental-related values. More specifically, economic valuation work on road salt pollution is lacking, let alone whether the benefits of changes in winter maintenance actions (e.g., reduction in chloride loading) outweigh costs. We propose a framework using a non-market valuation approach to address CBA knowledge gaps. Here, we describe potential attributes that could be included in a future discrete-choice stated preference survey (DCSP), to determine respondent's willingness to pay for potential policy alternatives in southeastern WI.

<u>Abbreviations:</u>

BMPs = Best Management Practices

WI = Wisconsin

TMDL = Total Maximum Daily Load

CBA = Cost-Benefit Analysis

WTP = Willingness to Pay

TEV = Total Economic Value

RWIS = Road Weather Information Systems

MS4 = Municipal Stormwater permits

CMA = Calcium Magnesium Acetate

MPY = milli-inch penetration per year

WTA = Willingness to Accept

4.2 Introduction

Chloride pollution in surface and groundwaters can come from a variety of anthropogenic sources like fertilizers and water softeners (Kelly et al. 2012). However, road salts continue to rank as the largest input because of the amounts that are applied during the winter season (Kelly et al. 2012). When large pulses of road salt enter freshwater streams and rivers, many organisms cannot handle the change in salinity, which results in a decrease in richness and overall biodiversity (Schuler et al. 2019). A common misconception of road salt pollution is that the consequences associated with chloride entering the body of water are short lived in the water column. Currently, there is not a clear understanding of the ecological consequences from a long-term perspective after the bulk of chloride has passed or stored away in shallow groundwater (e.g., food web dynamics, population recovery of benthic organisms, etc.).

Chloride can be toxic above certain concentrations in freshwater systems, for example one teaspoon of road salt is estimated to permanently contaminate five gallons of freshwater (MPCA, n.d.). These toxicity thresholds are also known as acute (single exposure where adverse effects are observed) and chronic (long-term exposure where adverse effects are observed) (Casarett and Doull, 1975). In 1988, the U.S. EPA established chloride acute and chronic toxicity criteria of 860 and 230 mg/L, respectively. These criteria have not been revised despite marked and ongoing increases in road salt usage (EPA 1988; USGS 2018). However, more recently in 2014, the Wisconsin Department of Natural Resources (DNR) revised chloride acute and chronic toxicity standards to concentrations of 757 and 395 mg/L for freshwater systems, respectively.

To reduce the amount of road salts entering freshwater systems, municipalities can modify winter road maintenance routines or implement various roadside best management practices (BMPs). In Wisconsin (WI), recent technological advancements such as equipment for applying brine solutions (i.e., anti-icing), have made it possible to cut salt material usage while still maintaining road maintenance objectives (WI DOT, 2020). Use of brine, often as a partial substitute for road salt under certain environmental conditions, has proven to reduce chloride runoff entering freshwater systems (Haake and Knouft 2019) and, in some instances, to be more cost-effective than only using dry road salt (Fay et al. 2015). Other alternative strategies like structural BMPs, retention ponds and riparian buffer zones, can also help mitigate chloride loading from snowmelt runoff (Fay and Shi 2012). Both methods facilitate accumulation of road salt compounds while allowing for infiltration, however there is still a looming concern of these chemicals contaminating groundwater supply (Fay and Shi 2012). In issued Wisconsin Discharge Elimination System (WPDES) permits, municipal Stormwater (MS4) permit language does not require monitoring of chloride at stormwater outfalls but in some instances require the submittal of salt reduction strategies and other structural BMPs. In addition, there are no established Total Maximum Daily Loads (TMDLs) freshwater streams, rivers and lakes should be receiving for chloride at this time (WI DNR, n.d.).

There are estimates for the expenditures needed to update winter road maintenance equipment, however the benefits associated with the improved road conditions and ecosystem health are more difficult to quantify. Economists have developed several methods, collectively known as nonmarket valuation, that can be used to estimate these benefits. Estimate benefits can then be used to evaluate the relative costs and benefits of various policy options. This analysis reviews the nonmarket valuation and cost-benefit analysis (CBAs)-related literature on road-salt deicers, and brine applications to identify the extent to which they capture the impacts of road salt and brine on aquatic ecosystems. Findings suggest 1) nearly all studies recognize the ecological costs of road salt application, 2) and few studies attempt to estimate or proxy for these costs. We, thus, propose a framework in valuing reduction in chloride loading, which could, if implemented, help inform winter road maintenance and stormwater management decisions.

4.3 Methods

4.3.1 Evaluate the extent to which cost benefit analyses (CBAs) and related literature capture the impacts of road salt and brine on freshwater ecosystems

4.3.1a Nonmarket valuation

Nonmarket valuation methods are used by economists to place monetary values on goods and services not traded in markets, like improvements in environmental quality. The value a person places on a change in a nonmarket good or service can be measured by their willingness to pay (WTP) for the change; these values can, in turn, be used in CBAs.

CBAs are a crucial process in policymaking, which is why it is important we understand where people place their values.-However, if there is no monetary value associated with a change in an environmental good or service then the benefits (or costs) associated with that change will not be reflected in the CBAs (US EPA, 2010). Nonmarket valuation methods can be split into two main categories: Revealed and stated preference methods (Baker and Ruting, 2014).

Revealed preference methods are techniques that use information on people's actual behavior to estimate nonmarket values (Baker and Ruting, 2014). The most common revealed preference methods are the travel-cost and hedonic property methods. The travel-cost method analyzes peoples' travel behavior and expenditures to estimate average WTP to visit a particular place, such as visiting a park to walk the trails or enjoy the scenery (Baker and Rutting 2014). The hedonic property method is different in that it estimates WTP by analyzing how home values vary with structural, neighborhood, and environmental amenities (e.g., green space around the house) (US EPA 2010). Stated preference methods differ from revealed preference methods in that they estimate WTP based on peoples' responses to hypothetical choices presented in surveys. These surveys ask participants to make trade-offs between environmental conditions and income, which allows the researcher to estimate WTP (Baker and Ruting, 2014).

Economists use the total economic value (TEV) framework to classify different types of values. The TEV divides values into use and non-use values. Use values pertain to the benefits a person receives from utilizing an environmental resource whereas non-use values pertain to benefits that are independent of use, such as the benefits associated with knowing that an environmental resource exists (Price et al. 2020). Revealed preference methods can be used to estimated use values, while stated preference methods can estimate use and non-use values.

Stated preference methods are a fitting technique to estimate WTP for programs that mitigate ecological damage from chloride loading (e.g., substituting brine solution for rock salts) so it can be incorporated into CBAs. Revealed preference methods are not appropriate for this purpose as travel behavior and housing decisions are not likely to be affected by chloride pollution at prevailing concentrations. Overall, there are many improvements needed in water quality regulations, specifically in non-point source pollution (e.g., road salt runoff in stormwater) because it is difficult to address in terms of measuring clean-up costs (Kieser et al. 2019). By using nonmarket valuation methods, this will help produce accurate CBAs that can inform policymakers considering chloride-reduction programs or general stormwater water regulations.

However, the use of stated preference methods does have many uncertainties. One of the most common biases that stated preference surveys face is hypothetical bias. Since a stated preference survey is essentially creating a hypothetical market, survey participants will likely select a larger WTP option than what they would likely pay in reality (Murphy et al. 2005). In addition, many stated preference surveys that have been conducted on environmental-related issues struggle to find representative samples (Zhang and Sohngen 2017). This was demonstrated in a stated preference survey by Zhang and Sohngen (2017) sent to recreational anglers on Lake Erie to determine their WTP to reduce harmful algal blooms (HABs). Out of all the surveys sent, more than half of the participants that responded were over the age of 45 years old and were households with higher education and income (Zhang and Sohngen 2017). Although still useful information, this highlights the potential concerns with statistical inference. For example, a stated preference survey was sent to 17 eastern states near Chesapeake Bay and surrounding lakes to determine a households WTP for a pollution reduction program to improve water quality (Moore et al. 2015). Due to a larger study area, this was able to reach a widespread demographic to better understand the socioeconomic variability. Although this study was more far-reaching, there were many biases that needed to be taken into account. The participant responses needed to be considered and filtered for four types of biases that were classified as: Protest, warm-glow, hypothetical bias, and scenario rejection (Moore et al. 2015). With these bias assumptions established, Moore et al. (2015) were able to remove survey responses that fell into these categories in order for the results to be the most representative.

4.3.1b Road salt and other alternatives

There are many factors that go into determining the best methodology to control snow and ice to keep the roads safe while also considering impacts on infrastructure and the environment. Moreover, winter road maintenance can pose challenges because the most effective course of action differs based on the circumstances. In Wisconsin's Department of Transportation (WI DOT) winter report for 2019-2020, it was estimated a statewide total of 425,558 tons of salt were applied with 12.2 tons of salt used per lane mile (WI DOT, 2020). In addition to traditional road salt, the WI DOT's statewide material use of brine solutions was estimated to be 11,398,968 gallons in the forms of anti-icing, pre-wetting and deicing application. Anti-icing is the application of brine solution before the winter storm, whereas deicing applications of brine solutions occur during and after the storm (Dane County Winter Maintenance Application Rate Guidelines, Fortin Consulting 2017). Pre-wetting is a technique where dry salt application can be wetted in order to speed up the melting process and adhere more effectively to the road surface (Minnesota Local Road Research Board, 2012). Brine solution is water typically mixed with sodium chloride—but select counties in WI have used other chloride salts such as calcium and magnesium chloride or even a combination as these chemicals work effectively at different temperatures (WI DOT, 2020; Shi et al. 2015). A survey participant from Fay et al. 2015 stated the use of brine solutions cut the agency's use of salt materials in by 30%. Similarly, a report done on Westchester County, NY, and other NE communities found that 25% less salt material was needed when brining practices were implemented (Kelly et al. 2010). In addition to potential cuts in chloride loads, brining technologies have been determined to be cost-effective in multiple case studies due to reductions in overtime pay for staff and road salt expenditures (Hakake and Knouft 2019; City of Ballwin, Missouri, 2017).

4.3.1c Winter maintenance benefits

There are many benefits associated with road maintenance decisions that are made during the winter season. The information that various technologies provide, such as the common use of road weather information systems (RWIS) can assist winter maintenance staff reduce the overall risk of car accidents by acting in a timely manner (Fay and Shi 2012; Midema and Wright 1995). A review done by Boselly (2001) analyzed the cost savings associated with RWIS and found that many states like Minnesota, North Dakota, and West Virginia were saving thousands of dollars in winter maintenance activities with the help of RWIS technology. Furthermore, a study determined that road surface conditions prove to be the greatest relative risk of winter accidents when compared to the other major weather factors such as gusty winds (Usman and Fu 2012, report prepared for the Salt Institute) making RWIS an appealing technology to keep roads safe. By keeping the roads clear during the winter, other benefits like fuel savings (i.e., less travel time) have been shown. An analysis done by Midema and Wright (1995) calculated the indirect benefits associated with RWIS and broke it down by two and four lane roads of three different cities in the state of Indiana. Their results with the implementation of RWIS found that per vehicle kilometer on a two and four lane road of reduced fuel consumption came out to $0.0007 and $0.0003 in benefits, respectively (Midema and Wright 1995). Overall, Midema and Wright's (1995) total indirect benefits from the usage of RWIS for a two and four lane roads considering accidents, travel times and fuel consumption gave a total savings of $192,320. If we break this monetary value of indirect benefits down to the individual level for reduced fuel consumption, a two-lane highway gives $662 whereas a four-lane resulted in $506 (Midema and Wright 1995). From winter road maintenance practices in general, the WI DOT reported from the 2019-2020 winter season, there was a decrease in accidents by 31% from the previous year demonstrating the importance of clearing roads properly using many different winter maintenance techniques. Although technologies like RWIS are useful, Ye et al. (2013) determined that brining and dry salts help prevent accidents from occurring regardless when compared to a sand and salt combination. A sensitivity analysis done of their findings was able to demonstrate that the use of liquid versus dry deicer types may be more effective in reducing crashes, with their overall recommendation suggesting that the use of brine is an ideal winter maintenance BMP at this point in time (Ye et al. 2013).

4.3.1d Winter maintenance costs

Although there are benefits of safer roads during the winter, winter road maintenance is costly. In the state of Wisconsin, the WI DOT reported $84,639,241 of total costs from the 2019-2020 winter season. The number one contributor of this total cost was the salt materials, with equipment costs ranking second, and labor costs as the third major source of cost (WI DOT, 2020). In terms of brining systems, the costs up front are expensive, but many municipalities have been able to demonstrate the investments were well worth it. Through the Minnesota Pollution Control Agency (MPCA) Smart Salting Training sessions, they were able to obtain information on select municipalities who utilize brining technologies to determine the associated costs and returns. For example, an MS4 stormwater permittee, the University of Minnesota, located in the metropolitan area of the Twin Cities had upfront costs of $10,000 to implement brining in their winter maintenance activities—they immediately saw returns in the first year of a total of $55,000 while also almost reducing their dry salt usage in half (MPCA Minnesota Stormwater Manual, last edited 2017; Fortin Consulting, 2014). Another Midwest city, Webster Groves outside of St. Louis, Missouri, budgeted to retrofit dump trucks the city already owned that was used for carrying aggregate materials to also be used in the winter months equipped for brining (City of Webster Groves, 2013). The city of Webster Groves estimated that they would be able to cut salt usage by 20-30% with the use of brining techniques (City of Webster Groves, 2013).

WI salt expenditures

Rock salt prices increased in the last decade when looking at the state of Wisconsin expenditures. It was calculated that Milwaukee County cities from the 2007-2008 winter season compared to the 2018-2019 season were paying an extra $30.55 per ton for rock salt (5.7% increase) (WI DNR, n.d.; City of Cudahy Public Works Department, n.d.). With the higher prices per ton of rock salt, it has pushed many municipalities to re-evaluate their implemented BMPs to reduce expenditures on rock salt while also keeping roads safe. Much research has supported that brining is an excellent alternative to cut salt usage while not compromising road safety (Ye et al. 2013; WI DNR, 2020). More specifically, the City of Cudahy, WI, in Milwaukee County Public Works Department, found prior to the 2016-2017 winter season they were using approximately 17.8 tons of salt for each winter event. However, the city began implementing brining techniques and were able to decrease salt usage by 13.8 tons for each winter event with a total savings of $40,602 according to 2018 data (WI DNR, n.d.; City of Cudahy Public Works Department, n.d.).

Deicer corrosion

In terms of corrosion to infrastructure and vehicles, different deicer compounds will do more damage than others. Winter maintenance staff must consider the different options available that are best suited for the winter storm event while also considering the chemicals corrosive properties. A study done by Fay et al.

(2007) looked at different types of deicer compounds and their corrosive properties using electrochemical analysis by exposing the deicer type to steel material. The results found that acetate-based deicers were the least corrosive with chloride and ag-based compounds causing the greatest damage to the material (Fay et al. 2007). The same study done by Fay et al. (2007) also determined that chloride-based deicers were highly corrosive to the pavement with acetate-related deicers ranking the least. To lessen the corrosive properties of chloride-based deicers, many municipalities have explored corrosive inhibitor compounds that are added to the winter treatment. For example, the WI DOT stated that the addition of an organic compound, such as glycols, to brining solutions reduces the corrosion of winter maintenance equipment (WI DOT 2020; Fay and Shi 2012). Another report stated that in general, any addition of an ag-related product to the brine treatment can help mitigate corrosion (Frisman, n.d.) whereas most common inhibitor ingredients in products on the market are not very clear what their inhibitor mixture contains. A different study by Xie et al. (2017) exposed concrete cores taken from bridge decks to either sodium chloride or potassium acetate deicers. What was found was that potassium acetate caused more structural damage than the sodium chloride exposed cores, but regardless it is important to note that both deicer compounds caused some level of damage to the concrete (Xie et al. 2017).

Deicer toxicity

Studies of deicer alternatives such as the compound calcium magnesium acetate (CMA), and other acetate compounds, have shown contradicting findings about whether their environmental impact is less than chloride salts. Vitaliano (1992) suggests that CMA is a better option from an environmental and cost-effective perspective. However, a study on different deicer compounds found that sodium chloride compounds had less of an environmental impact than CMA when comparing their entire life cycle (i.e. mining the compound(s) to its final stage of usage on the roads) (Fitch et al. 2013). In terms of toxicity, a study done on several types of deicers exposed to fathead minnows was able to demonstrate that the compound potassium acetate was the most toxic with sodium chloride ranking the least toxic compared to calcium and magnesium chloride compounds (Pilgrim 2013). However, it should be noted that toxicity by mass plus deicing effectiveness should be taken into account for toxicity evaluations. Additionally, another toxicity study found that beet juice deicer as an alternative was more toxic than salt brine at the half maximum effective concentration (i.e., EC50) when exposed to mussel larvae (Gillis et al. 2021). This raises another complicating factor in the fact that different organisms will exhibit different sensitivities to different deicer types. Organic-related deicers are a concern as they can deplete oxygen in freshwater systems and promote prolific biofilm growth on streambeds (Nott et al., 2020), however, chloride compounds may not rank the highest in terms of its toxicological impacts but its free-flowing capabilities make it a tricky compound that allows it to linger in the environment for unknown periods of time (Fay and Shi 2012).

Aquatic ecosystem impacts

One of the many challenges is trying to convey to the general public that chloride loading into waterways is negatively impacting the ecosystem as the ramifications are typically not visual. However, some research has been able to point to some indirect impacts. A study found that waterbodies that receive substantial amounts of stormwater containing a combination of nutrients and high salinity may result in more salt-tolerant algae that can then lead to harmful algal blooms (Tonk et al. 2007). In addition, chemical stratification can occur in small lakes that experience high loads of road salt runoff creating oxygen depleted lakes (Novotny and Stefan 2012). With little dissolved oxygen, this could lead to fish kills and other downstream ecosystem issues that are still not fully understood with road salt pollution (Hintz and Relyea 2019). More locally, the Milwaukee River Basin's rivers and streams are essential for Lake Michigan's aquatic life, including the fish population that encompasses a large fishing industry. The Milwaukee Riverkeeper estimates about 54 Lake Michigan fish species use the Milwaukee River Basin as important habitat, as most freshwater fish like Chinook Salmon or Walleye rely on streams and rivers to spawn (Milwaukee Riverkeeper, n.d.). This is important to consider as in total, the Great Lakes fishing industry is estimated to be worth $7 billion on a yearly basis (Great Lakes Fishery Commission, n. d.). Other visual impairments from chloride pollution may be dying vegetation along urban streams and rivers and roadside plants, with the highest recorded plant tissue chloride concentration at 11,000 mg/L about 60 meters from the highway in a road salt review done in Canada (Cain et al. 2001).

Public health implications

From a human health standpoint, anthropogenic inputs of chloride are being observed in groundwater due to its chemical properties of staying in solution (McNaboe 2017). Because of chlorides free flowing (and lingering) ability, research done in Toronto, Canada, calculated that within their study area only 45% of the chloride was flushed out annually with the rest suspected to be stored in the groundwater (Howard and Haynes 1993). Furthermore, the presence of road salts in waterways has been shown to displace heavy metals that are attached to sediment releasing them back into the water column or to seep into groundwater (Schuler and Relyea 2018). This raises great concern for wells used for drinking water or agricultural purposes located in areas where these interactions may be occurring. Chloride in humans is primarily absorbed by the small intestine, but little is known on the impacts of long-term exposure to those exposed to road salt (e.g., NaCl) contaminated drinking water (WHO 2003). In Madison, WI, Well #14 was indefinitely shut down because of its increasing chloride and sodium concentrations that are suspected to be contaminated from road salts where health officials were mainly concerned of hypertension (City of Madison, WI , n.d.).

4.3.1e Review of literature

Attempts to capture environmental benefits from reduction of salt usage in the form of another alternative, such as brining, is lacking—especially when focusing on the quality of our freshwater resources. One of the very few papers, Vitaliano (1992) does attempt to calculate Adirondack Park campers' WTP for road salt damaged vegetation. Vitaliano (1992) used a travel cost model which only encompasses those who use Adirondack Park and fails to consider non-users of the park. Although Vitaliano's (1992) travel cost study is solely focused on dying vegetation from road salt, it can provide insight into methods that could be applied to addressing waterways. More specific to chloride, Groothius et al. (2021) used a stated preference survey method and found that a respondent's WTP to reduce chloride pollution in streams by 10% was $15.16, by 25% to be $37.90, and reduce it by 50% to be $78.80. Another survey-based study conducted through the Colorado Department of Transportation (CDOT) focused on the users of deicer applications worldwide (Fay et al. 2007). Fay et al. (2007) surveyed 24 total winter maintenance staff in the United States, including two participants from different countries. Interestingly, the participants were asked to rank several types of deicers based on their adverse impacts on the environment (Fay et al. 2007). The results from this survey question in Fay et al. (2007) found that participants ranked chloride-based deicers as the most harmful to the environment.

Most road salt related CBAs take into account road maintenance expenditures, corrosion costs, and benefits associated with improved road conditions, but not the environmental impacts of chloride loading. A CBA review paper done on anti-icing and deicing studies by Fay et al. (2010) was not able to report cost-benefit ratios as many of the benefits were unknown. For example, a study researching anti-icing and deicing practices highlighted the need to weigh in the costs associated with environmental impact, but no estimates of these costs were available (Shi et al. 2009). It should be noted that any chemical applied on roads is going to face an associated cost, but what should be looked at more closely is if the benefits of practicing anti-icing and other alternatives may outweigh the costs in terms of its environmental impact. More importantly, how do the costs and benefits of programs that would reduce chloride usage or loading compare to existing practices. Overall, there is a lack of CBAs evaluating these issues, where most literature (whether a review, cost-savings or formal CBA) did not provide Net Present Values (NPV)-- and if they were formulated, it was not clear how they arrived at that number.

Our literature review found that there are many different tradeoffs when determining the best deicing method from costs or cost-savings, corrosion properties, and toxicity on the environment (Table 4). Much of the available research is able to acknowledge the gap in understanding the environmental impact and the need to quantify the unknown values to create stronger CBAs down the road.

Table 4. Literature review findings done on deicer compounds with emphasis on anti-icing technologies.

Paper	Purpose	Environmental values? *	Costs?	Benefits?	Recommendation
Wisconsin Department of Transportation (WIDOT) (2020)	An annual winter maintenance report comparing cost savings with emphasis on brine technologies from 2019-2020 winter season.	Mentioned	90% of Wisconsin's counties have equipment for anti-icing (i.e. brining) where total winter costs (labor, salt materials, equipment, administration, and furnished material costs) were estimated at $84,639,241. Of this amount, only 2% was anti-icing related costs. In other words, statewide costs associated with applying anti-icing chemicals was totaled out to be $1,750,115.	Saw a 19% decrease in salt material costs from the previous winter season statewide. Other savings comparing it to the previous winter season were a 33% reduction in both equipment and labor costs. In addition, it was estimated the state of WI saved $8.1 million due to brine applications.	The WIDOT is recommending to counties to use liquid forms of salt rather than dry application.
	Revealed preference method approach to determine WTP.	Yes. Values estimated for roadside vegetation, but not aquatic ecosystems.	Used a travel cost model to estimate WTP of campers in Adirondack Park WTP to avoid damaged	None quantified.	Switching from rock salt to CMA would be socially efficient for state highway agencies and improve roadside

Vitaliano (1992)			vegetation from the usage of road salt. The cost calculated was determined on statistics from the New York Department of transportation (NYDOT) of the number of trees and the damage from a certain amount of salt applied. The cost was determined to be $75 per ton of aesthetic damage.		vegetation aesthetics.
Fortin Consulting, Inc. (2014)	A winter maintenance report comparing cost savings with emphasis on brine technologies.	Mentioned	**University of Minnesota Twin Cities:** Implementation of new smart salting BMPs cost about $10,000 (new equipment costs). **Waconia, MN**: None listed. **Prior Lake, MN**: Invested heavily on the educational side of salting and the equipment for anti-icing (estimated cost of $250,000).	**University of Minnesota Twin Cities:** Salt reductions of 41%, and saved $55,000 in their first year. **Waconia, MN**: A 70% reduction in salt usage per pound. Saving a total of $8,600 a year. Brining practices cut per lane mile costs to $1.80 (from updated equipment associated with brine applications and calibrations).	Education and implementation of new BMPs (e.g., brining) is crucial to reduce chloride loads entering our freshwater resources. These methods have been also proven to cut winter maintenance costs for municipalities.

				Prior Lake, MN: Overall 42% salt use reduction. Applied 500 pounds of salt per lane mile but with the switch to pre-wet salting practices this number was cut down to about 200 pounds per lane mile. It was estimated around $2000 is saved per winter storm event.	
Kelting and Laxson (2010)	Comparison of deicer compound cost savings in Adirondack Park and attempt to quantify environmental costs using values derived from Costanza et al. (1997) that summarized ecosystem service benefits with their land use and value expressed in acre/year.	Yes, derived from Costanaza et al. (1997) values, but these values were also not specific to chloride.	The average direct costs for two lane highway was $30 per lane kilometer per event and $21 for freeways. These costs take into account the equipment, labor time, and material aspects associated with winter maintenance. Note these calculated costs exclude weather anomalies like an extreme storm event.	**Salt only treatment**: Paid for itself only the first 25 and 35 minutes on two lane highways and freeways respectively (CBA ratio of 12:1 for two lane highway and 3:1 for freeways). **Salt/abrasives treatment**: Did not pay for itself and proven not to be cost effective with a CBA ratio of 0.8:1 for the two lane highway.	Overall, the two different treatments did not differ in terms of vehicle collisions on a two lane highway, but the treatments were effective in the freeway setting. Overall, the salt only treatment pays for itself quicker than the salt/abrasive mixture but the authors stated a larger dataset would need to be analyzed to make further conclusions.

				However, the freeway treatment produced a CBA ratio of 2.8:1, but took a 6-hour period to pay for itself during the winter maintenance treatment.	
Kuemmel and Bari (1996)	Formal cost benefit analysis performed to determine if the usage of salt only or salt/abrasive mixtures were cost effective	Mentioned	Costs associated with winter activities like the labor time, equipment, and materials for the specific highways a part of this study in Idaho resulted in a total of $1,545,108.	Benefits determined that crashes were reduced resulting in a savings of $8,559,200 and a CBA ratio of 7.76 was determined.	Overall, road safety on these select highways produced a favorable CBA ratio suggesting the costs associated with winter maintenance are outweighed by the benefits.
Ye et al. (2013)	Formal cost benefit analysis performed to determine if winter maintenance activity benefits outweighed the costs	Mentioned	Costs associated with winter activities like the labor time, equipment, and materials for the specific highways a part of this study in Idaho resulted in a total of $1,545,108.	Benefits determined that crashes were reduced resulting in a savings of $8,559,200 and a CBA ratio of 7.76 was determined.	Overall, road safety on these select highways produced a favorable CBA ratio suggesting the costs associated with winter maintenance are outweighed by the benefits.

Groothuis et al. (2021)	Stated preference survey to determine WTP for a stormwater management program (emphasis on increased temperature and road salt pollution). Experimented with four different types of survey formats.	Yes	None mentioned.	Found in their survey that a respondent's WTP to reduce chloride pollution in streams by 10% was $15.16, by 25% to be $37.90, and reduce it by 50% to be $78.80. Using the more conservative WTP from one of the more successful survey types, it was estimated to be $20.99 for the implementation of a stormwater management plan to reduce road salt and thermal pollution.	Found that surveys given to respondents without figures gave them a better understanding of the stormwater pollution problem, but rather explained with some short science of the issue. Their survey design could help serve others to be used in future CBAs.

* = **Yes**: meaning they attempted to quantify environmental-related values, **Mentioned**: mentioned the need to incorporate environmental -related values but did not quantify, and **No**: does not include or mention environmental-related values.

4.3.2 Propose a strategy to address knowledge gaps in the literature

4.3.2a Stated preference survey method

The main goal of a stated preference survey is to evaluate people's preferences for policy alternatives, and in doing so estimate WTP for changes in environmental resource conditions (Holmes et al. 2017). Survey attributes should be carefully selected, as each attribute is defined as a characteristic of the topic being addressed in the survey. Much work goes into developing these surveys, with a crucial step using focus groups. These groups are beneficial when refining the survey attributes, especially participants that are a mix of policymakers and the general public (Holmes et al. 2017). Once attributes of the survey are decided upon, the levels of each would then be addressed so that participants are given choices to choose from in the final survey product (Holmes et al. 2017).

Our literature review on available CBAs and cost-saving comparisons of different road salt alternatives has been able to identify the lack of nonmarket values on this topic, with only one study that was able to estimate these values (Groothuis et al. 2021). It is clear that the majority of related literature will acknowledge the ecological impacts but will rarely attempt to quantify them (Table 1). Stated preference surveys can be used to estimate WTP for changes in environmental goods and services resulting from programs changes, where programs could be presented and established to reduce chloride levels in impaired Wisconsin waterways. Associated benefits of reductions in chloride result in greater variety and abundance of different freshwater organisms, create more aesthetic environments (e.g., vegetation and soils), and a healthier environment for wildlife in general. This could be especially relevant to WI since warmer temperatures in winter may result in increased runoff events. The amounts of road salt being applied from private businesses', that are not monitored through permits like municipalities, also raises concern in contaminating groundwater supplies as chloride's chemical properties allow it to permanently linger in solution. Subsequently, there are currently no technologies that can remove chloride compounds from our waterways once exposed (MPCA, n.d.).

Stated preference surveys are useful in understanding peoples' preferences for road salt management alternatives. Related literature reviewed was able to demonstrate that there is no silver bullet solution as it is a case-by-case basis (e.g., type of winter storm, pavement temperature). Many of the different deicer compounds also propose tradeoffs where one may cause a greater toxicity to freshwater organisms but perhaps its corrosive properties on infrastructure are less.

Furthermore, there are uncertainties from the end results of stated preference survey studies done because they are restricted to their specified study area(s). Benefit transfer is a useful tool under circumstances where a policy alternative's calculated benefits was done a broader scale and not in a specific geographical area (US EPA 2000). This is where there are challenges in being able to transfer the benefits calculated to another area where the study was not performed in terms of informing policy (Bateman et al. 2011; US EPA 2000). This is especially applicable because if there are limited nonmarket valuation estimates related to chloride, benefit transfer is not an option for researchers or policy makers interested in understanding the environmental costs of chloride use. Some researchers like Bateman et al. (2011), attempted to understand this benefit transfer when it comes to non-market valuation methods and how future stated preference methods can design their studies to configure to this. The only stated preference study related to chloride, Groothius et al. (2021), was able to calculate survey respondent's WTP for chloride reductions for that particular geographical area. Since the stated preference survey was specific to a location and program, it makes benefit transfer difficult to apply to for Southeastern WI.

Here, we propose a stated preference survey highlighting the southeastern region of WI that includes the counties: Milwaukee, Ozaukee, Walworth, Racine, Kenosha, Washington and Waukesha. The survey would elicit residents' preferences for reducing chloride entering waterways, while considering the switch to the use of a different deicer compound to reduce corrosion on steel commonly used on vehicles.

4.4 Results

4.4.1 Proposed framework for southeastern (SE) region of Wisconsin

Our literature review revealed three potential attributes that could be used in a stated preferences survey. The first attribute pertains to chloride loading. Because long-term effects of chloride remain unknown, it is difficult to link chloride reductions to specific ecosystem services. As a result, we propose, similar to Groothius et al. (2021), using a person's WTP could be determined for a certain percent reduction in chloride entering freshwater systems. To do this, we need to understand the baseline of how much salt the Southeastern region of WI is using to determine the chloride reductions proposed in a survey question. Publicly available salt data of each Southeastern county with a MS4 permit through the WI DNR was summarized from their 2019 Annual Report, as well as WI DOT county serviced highways in the Southeastern region from the 2019-2020 winter report. The WI DNR MS4 annual report did not specify each deicing compound by chemical type, but rather by the product categories: beet juice, brine, chemelt (products unspecified in WI MS4 annual reports that fall in the category), and pre-wet as liquid deicers. Dry materials are labeled as salt, salt mixture, and sand with salt being the primary winter treatment across all Southeastern counties (Figure 8). For the purpose of this survey, only deicer categories associated with chloride salts, dry and liquid applications, were considered for each Southeastern county. With the collected data, we calculated a total estimate of pounds of chloride applied from the 2019 winter season under conditions of the most commonly used salt, NaCl (Table 5). Therefore, our estimate is likely to be more conservative as our chloride calculation did not account for $MgCl_2$ and $CaCl_2$ compounds but rather grouped them all in one chloride salt category. When we convert this amount to pounds of chloride we can assume 1,105,252 lbs applied from 2019 Southeastern WI data. However, a report done in the Twin Cities Metropolitan Area (TMCA) Minnesota by Stefan et al. (2008) was able to determine about 30% of road salt applied within the TMCA watershed enters the Mississippi River (i.e. surface water), with 70% most likely residing in terrestrial environments like soils, vegetation, or even groundwaters. Other studies represented in different geographical areas have found similar numbers in that even up to 50% of road salts applied can reach our freshwater ways (Meriano et al. 2009; Howard and Haynes 1993; Ruth 2003). More specifically with brining applications, Haake and Knouft (2019) were able to estimate that liquid brine applications reduced chloride loads into waterways by about 45%. Under this assumption, we took 45% of the liquid application chloride amounts. We then took 30% of the total chloride load per Stefan et al. (2008) estimates, and were able to produce and estimate around 325,179 lbs of chloride entering our surface waters seasonally from road salt applications in Southeastern WI. The chloride reduction percentages proposed in a stated preference survey should be considered around these values when looking at a Southeastern WI chloride reduction, with emphasis around the chloride amount specifically entering aquatic systems.

A second attribute that should be considered could be road safety, however, in this column we propose it is left as a constant variable in the survey as safety of the roads should not be compromised. In fact, Ye et al. (2013) findings show the main reason leading up to accidents in the winter was due to driving too fast under poor road conditions.

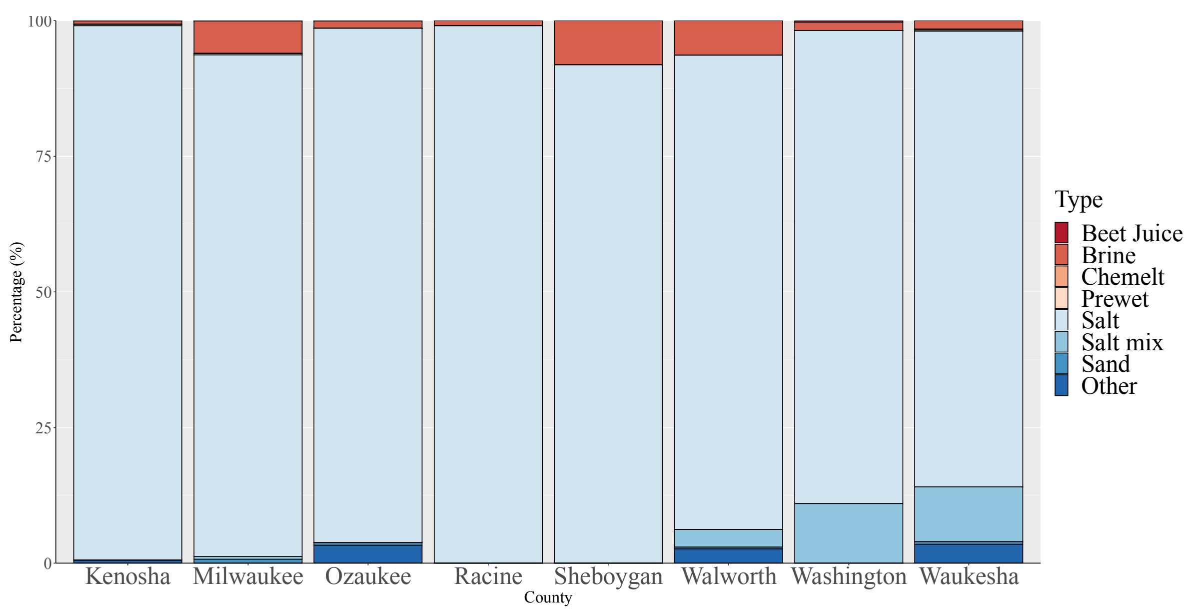

Figure 8. Summarized product type data from the WI DNR 2019 MS4 Annual Reports submitted by each Southeastern county. The MS4 Annual Report only includes months from October through March into the following year. Data is expressed in percentage to demonstrate how much of each product is used by the county.

Table 5. Summarized data from the WI DNR 2019 MS4 Annual Reports submitted by each Southeastern county. The MS4 Annual Report only includes months from October through March into the following year. WI DOT data was collected from their annual winter report of 2019-2020. This data includes only dry and liquid chloride-based salts and does not take into account each individual chloride-based deicer used (e.g., $MgCl_2$, $CaCl_2$, and NaCl) but rather groups them all together.

County	Municipal total liquid use (lbs)	Municipal total dry salt use (lbs)	WI DOT total liquid use (lbs)	WI DOT total dry salt use (lbs)
Kenosha	873,081	103,504,000	136,643	15,890,000
Milwaukee	9,536,940	144,742,000	2,007,855	64,996,000
Ozaukee	373,774	27,606,000	422,504	6,374,000
Racine	473,462	48,686,000	489,375	15,962,000
Sheboygan	3,482,609	39,236,000	-	-
Walworth	606,935	8,606,000	633,973	20,276,000
Washington	437,458	27,420,000	812,358	12,908,000
Waukesha	2,033,984	107,810,000	2,172,487	29,806,000
Total	17,818,243	507,610,000	6,675,194	166,212,000
Add 45% of liquids:	8,018,209.44		3,003,837.40	

Estimate SE WI total salt (lbs)	684,844,047
Total salt (Liters)	310,643,222
Total salt converted to grams of chloride	491,662,239
Estimate Southeastern WI total chloride (lbs)	**1,083,931**
30% of chloride estimate that enters surface waters (lbs)	**325,179**

The third attribute we propose to take into account is corrosion properties of the chloride-based deicer compounds commonly used in SE Wisconsin (NaCl, $CaCl_2$, $MgCl_2$, 23% NaCl brine). We focused on galvanized steel, as many vehicles are made with this common type and can be a relatable attribute to the survey participant. The corrosion attribute would give alternative scenarios where one end of the spectrum is minimal corrosion programs such as the substitution of brine or acetate compounds, or, business-as-usual with dry chloride-based salts. From our literature review, we developed a corrosion ranking, adopted by the findings of Shi et al. (2009, CDOT Report) electrochemical analysis of two common types of steel (i.e. galvanized steel for our purposes) submerged in the deicer. We also used Petkuviene and Paliulis (2010) findings where they used a NaCl brine spray application (23% weight per volume) on galvanized steel. In addition, we gathered Shi et al. (2005) corrosion rates for $CaCl_2$ and NaCl, however, it is important to note the rates were for carbon steel not galvanized due to limited research on this topic. Furthermore, we converted the Shi et al. (2009) and Shi et al. (2005) units of milli-inch penetration per year (MPY) to centimeters per year. Whereas Petkuviene and Paliulis (2010) reported units in grams lost from the steel material. From this, we were able to calculate the volume of the steel strip and determine the height to ultimately find the centimeters lost after the experiment and expressed in comparable units of centimeters per year (Table 6). Lastly, all numbers were reported at a conservative-level by subtracting the uncertainty calculated from Shi et al. (2009), Shi et al. (2005), and Petkuviene and Paliulis (2010). Shi et al. (2009) was able to demonstrate that overall, $MgCl_2$ deicers caused more corrosive damage when compared to the other deicers tested on galvanized steel. When we compare the corrosiveness properties of $MgCl_2$ to Petkuviene and Paliulis (2010) NaCl brine spray, we find that $MgCl_2$ is about 14x more corrosive to galvanized steel. $MgCl_2$-treated galvanized steel was only about 1x more corrosive compared to $CaCl_2$ and potassium acetate, whereas it was 2x corrosive than sodium acetate from the Shi et al. (2009) findings. As for NaCl and $CaCl_2$ treatments from Shi et al. (2005) on carbon steel, its corrosion properties fall very close in line with $MgCl_2$ on galvanized steel.

In order to include corrosion as an attribute in a stated preference survey, we created hypothetical scenarios of switching to a different deicer to demonstrate the reduction in corrosion per year on galvanized steel using our derived numbers from Shi et al. (2009), Shi et al. (2005), and Petkuviene and Paliulis (2010). Interestingly, we find that the use of NaCl and switching over to the spraying of NaCl liquid brine could reduce yearly corrosion in galvanized steel by 88.5%, with the overall trend showing that brine may likely lead to less steel corrosion (Figure 9).

Table 6. Deicer type and corrosion rates expressed in centimeters per year on galvanized steel.

Deicer type	Paper	Corrosion rate (cm/year)	(+/-) uncertainty (cm)	Conservative estimate (cm/year)
MgCl2	Shi et al. (2009)	0.0432	0.0051	0.0381
CaCl2*	Shi et al. (2005)	0.0250	0.0006	0.0244
NaCl*	Shi et al. (2005)	0.0252	0.0011	0.0241
23% NaCl brine spray	Petkuviene and Paliulis (2010)	0.0037	0.0010	0.0028
Potassium Acetate**	Shi et al. (2009)	0.0432	0.0152	0.0279
Sodium Acetate**	Shi et al. (2009)	0.0229	0.0051	0.0178

* Means the corrosion rate estimates were determined on carbon steel not galvanized steel.
** Note: Potassium and sodium acetates are the most commonly used deicers at airports due to their low corrosion properties.

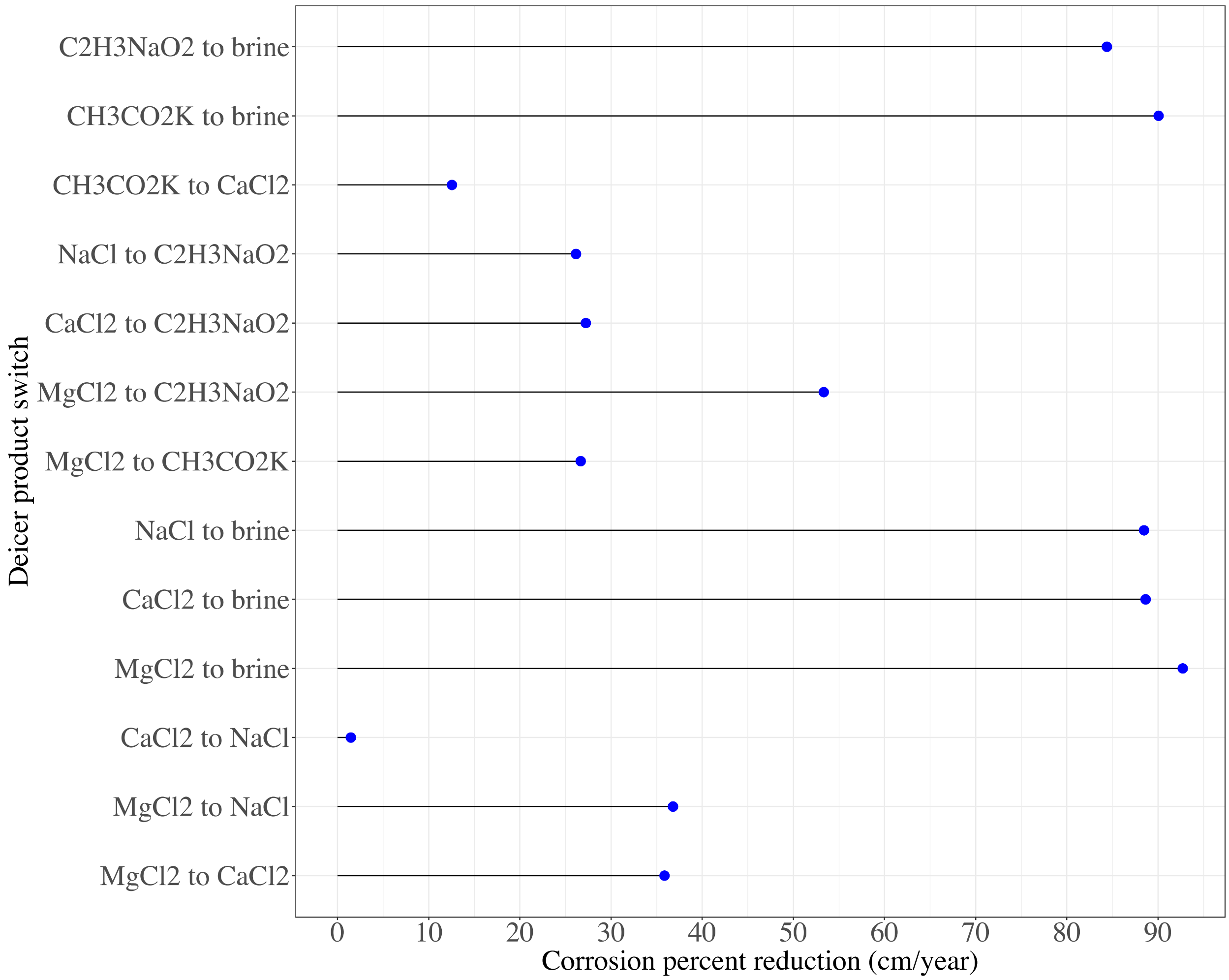

Figure 9. Corrosion percent reduction estimates if one were to switch to a different deicer alternative. Corrosion is based on galvanized steel centimeter per year from Table 3 data. $C_2H_3NaO_2$ = Sodium Acetate, CH_3CO_2K = Potassium Acetate, NaCl = Sodium Chloride, $MgCl_2$ = Magnesium Chloride, $CaCl_2$ = Calcium Chloride.

A final attribute that one should consider is the toxicity of common deicer compounds. We were not able to produce a metric associated with toxicity at this time. Literature on this topic varied greatly with different findings and concentrations reported making it difficult to find a common scale across each deicer. Instead of focusing on toxicity of each deicer product, it may be a beneficial approach to use the stated toxicity standards for chloride as an attribute instead (EPA 1988).

4.4.2 Future tradeoffs to consider

To understand a households WTP for chloride reductions in WI's waterways, an ideal stated preference survey would develop household preferences for winter road maintenance or stormwater management options. The program could reduce loading through some combination of change in application methods (e.g., brining), deicer compound type, and stormwater management (e.g., green infrastructure). The most difficult major tradeoff that should be considered is road salt toxicity to the environment (terrestrial and aquatic) and the quantities used. The common road salt types such as NaCl, $MgCl_2$, $CaCl_2$, and acetate-related deicers like CMA rank at different toxicities depending on how much is used and the mechanisms in which they interact in the environment. Literature reviewed was not able to give a conclusive ranking as it is dependent on the organism and situation, but a laboratory-controlled study by Pilgrim et al. (2013) was able to determine potassium acetate had the greatest toxicity on the test organisms. It is important, however, to note that waterways are receiving a variety of deicer compounds at different rates and concentrations— there is not just one universal deicer compound being used. Unknown synergistic interactions may occur of these compounds that could create a more toxic environment than what is seen in the laboratory when analyzing one compound at a time under controlled conditions. It is also apparent that these compounds differ in the amount that needs to be applied on the roads because of variation in their effectiveness. This should especially be considered with these compounds in the form of brine solutions (e.g. 23% NaCl brine).

The second major tradeoff that should be considered would be accounting for road safety in the form of health risks. A comparison of NaCl, $MgCl_2$, and $CaCl_2$ mixtures were able to demonstrate similar ice melting capacity as dry salts included in the study (Ye et al. 2013). More research should determine if there is any associated risk with using one or the other even though their effectiveness has been shown to be similar. WI DOT annual report from 2019-2020 explained that the mix of dry salt and abrasives, like sand, did not make a difference in accidents where more material was needed rather than the alternative of brine solution. In addition, corrosion properties to infrastructure and corrosion-inhibitor benefits should be further investigated. Similar to the toxicity rankings of each deicer compound frequently used, there is not a conclusive answer in which compound is less corrosive than the other as infrastructure materials and conditions are variable (Casey et al. 2014). However, laboratory-controlled studies, like Shi et al. (2010) have been able to show the importance of adding a corrosion inhibitor on the steel structures in concrete (e.g., rebars) were able to slow the weakening of the concrete structure. In terms of corrosion-inhibitors used, a review done by Casey et al. (2014) report prepared for Idaho DOT found that products being used in Wisconsin to help mitigate corrosion were listed as: ArctiClear Gold, Bio Melt 64, FreezGard, Geomelt, Ice Ban M80, and Ice Bite 55. More specifically in SE Wisconsin the corrosion-inhibitor chemicals associated with anti-icing according to WI DOT (2020) were only the addition of the product Beet Heet in Walworth County. Other than the sole usage of NaCl brine, Kenosha County reported the mix of a $CaCl_2$ and $MgCl_2$ brine whereas Milwaukee, Ozaukee, and Racine used NaCl mixed with $CaCl_2$ (WI DOT 2020).

With our findings, we propose two different discrete-choice stated preference (DCSP) surveys of households within Southeastern WI, with the main objective of understanding respondent knowledge and attitudes about chloride pollution. The first DSCP survey we propose is estimating the respondent's WTP for municipal actions that reduce chloride loading, a similar set up to Goothuis et al. (2021). Our second DSCP survey option we propose is looking at the chloride pollution problem from a different approach by understanding the respondent's willingness-to-accept (WTA) reductions in winter road deicing activities (less deicer used means less chloride loading into aquatic environments). With this second option, we acknowledge the fact that the road safety attribute would need to be re-assessed as this would no longer be a constant variable. Results from these proposed alternatives would be useful to policymakers from a municipal and regional standpoint when making winter maintenance decisions.

4.5 Conclusions

- Our literature review findings on CBAs and related-literature on deicer compounds were limited. Of the papers reviewed. the majority would mention the need to understand environmental-related values, but most did not attempt to quantify these values and/or include in their framework. There is a need to understanding these unknown values to create better water quality CBAs for future policies.
- It is clear that there are winter maintenance alternatives and technologies currently available that could be implemented to reduce chloride loading into waterways—however, what is unknown is whether the economic benefits of these changes outweigh the costs for municipalities—which may explain why most SE WI municipalities are still using dry salt instead of brining technologies.
- We developed a framework by identifying four different attributes for SE WI to assist in the creation of DCSP surveys. DCSP surveys could help estimate the economic value of reducing chloride loading into surface waters and understand participant's knowledge and attitudes of chloride pollution.
- Two DCSP surveys proposed could assess chloride pollution from two different perspectives: 1.) determine participant's willingness to pay for municipal actions that would reduce chloride loading, or 2.) a willingness to accept approach for a reduction in side roads maintained to cut down on materials, therefore reducing the amounts of chloride used.