USING STABLE ISOTOPES TO TRACE THE FLOW OF NITROGEN AND THE ROLE OF DENITRIFICATION IN A RESTORED PRAIRIE WETLAND COMPLEX RECEIVING WASTEWATER EFFLUENT

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ABSTRACT

Wetlands play a crucial role in reducing nitrogen concentrations in wastewater effluent before it is discharged into public waterways. This study aims to trace the inorganic nitrogen input from treated industrial wastewater effluent into the Frank Lake wetland complex in southern Alberta, Canada, its uptake and subsequent movement through the Little Bow River to the Twin Valley Reservoir by analyzing the stable isotope composition of nitrogen in sediment and leaf tissue samples of *Typha latifolia* and *Schoenoplectus acutus*. In addition, this study analyzes the magnitude of denitrification, which removes nitrogen in the wastewater effluent as water moves through the wetland, using nitrogen isotopic compositions of nitrate molecules in the water. The study identified significant nitrogen uptake and processing within Basins of Frank Lake wetland, primarily due to emergent aquatic vegetation uptake and phytoplankton assimilation leading to sediment deposition and denitrification. Although the wetland effectively processed nitrogen, downstream effects on the Little Bow River system were observed, as evidenced by increased $\delta^{15}N$ levels in *T. latifolia* samples. The Twin Valley reservoir also showed elevated $\delta^{15}N$ values, likely due to wastewater discharge from Nanton through Mosquito Creek. Moreover, there was a substantial reduction in nitrate levels in the wastewater effluent within Basin 1, with denitrification removing 27.6 % to 43.7 % of nitrates in June, 12.6 % to 17.0 % in July, and 8.4 % to 14.0 % in August 2023. The study offers valuable insights into the ecosystem service of nutrient uptake provided by the wetland complex.

DEDICATION

To my wonderful mother, who taught her little girl to dream big and be strong.

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LIST OF ABBREVIATIONS

B1-EFF	Basin 1- Effluent input
B1-BB	Basin 1- Bird Blind
B1-E	Basin 1- Middle West Shore
B1-B	Basin 1- Outlet
B2-SS	Basin 2- South Shore
B2-E	Basin 2- East Shore
B3N-B	Basin 3- North of Island
B3W-N	Basin 3- North Shore
B3-E	Basin 3- East
B3-O	Basin 3- Outlet
FLB1	Frank Lake Basin 1
FLB2	Frank Lake Basin 2
GPS	Global Positioning System
LBR-US	Upstream of Little Bow River
LBR-DS	Downstream of Little Bow River
n	Sample size
P	Probability
PON	Particulate organic nitrogen
R^2	Coefficient of determination
SD	Standard deviation
TON	Total Oxidized Nitrogen
TVR	Twin Valley Reservoir
ε	Fractionation factor
$\delta^{15}N$	Stable nitrogen isotopic composition
$\delta^{18}O$	Stable oxygen isotopic composition

Chapter 1: General Introduction

Industrial and municipal wastewater often contains high concentrations of nitrogen, phosphorus and occasionally chlorides (Mitsch & Gosselink, 2000). The elevated nitrogen and phosphorous concentrations contribute to eutrophication and subsequent depletion of dissolved oxygen concentrations in aquatic ecosystems, posing severe threats to overall ecosystem structure and function (Audet et al., 2020; Fowler et al., 2013). Nitrogen contamination in drinking water also poses a significant hazard to human health, potentially inducing several medical conditions such as methemoglobinemia in infants (Sadeq et al., 2008) and increasing the risk of cancer development (McElroy et al., 2008).

Over the years, strategies in watershed management have focused on controlling nitrogen loss at its origin and thereby alleviating transport of nitrogen downstream of the source. This has been achieved through initiatives aimed at restoring and enhancing landscape hydrology, as well as augmenting landscape nutrient retention (Dunne et al., 2013). Coupled with source control measures, ecologically engineered systems such as natural wetlands (Kadlec, 2009), constructed wetlands (Blankenberg et al., 2006), ponds and reservoirs (Collins et al., 2010; David et al., 2006) and denitrification walls serve as effective measures to prevent downstream transport of nitrogen, thereby enhancing water quality (Dunne et al., 2013).

Wetlands are considered one of the world's most productive and valuable ecosystems (Sieben et al., 2018). They provide a wide range of ecosystem services, such as regulating excess nutrients, especially nitrogen, improving water quality, sequestering carbon, and sustaining unique indigenous biota (Herbert et al., 2015). Because of the wetlands' capacity to remove nutrients and improve water quality, they have been used to treat excess nitrogen

in municipal and industrial wastewater effluent before it is released into natural watersheds (Sanchez et al., 2016; Shao et al., 2013; White & Bayley, 2001).

The biogeochemical cycle of nitrogen in wetlands is characterized by numerous biotic and abiotic transformations involving various organic and inorganic nitrogen forms. These transformations are indispensable for the effective functioning of wetland ecosystems (Vymazal, 2007). Although nitrogen exists abundantly in the atmosphere, it is not readily available for biological processes until it undergoes nitrogen fixation. Nitrogen fixation transforms atmospheric nitrogen into ammonium, a reduced compound that releases energy when combined with oxygen. Nitrifying bacteria utilize this energy to convert ammonium into nitrite and subsequently into nitrate during nitrification. Nitrate, being relatively stable, serves as a primary source of biologically available nitrogen in various ecosystems. However, the reversible process of denitrification enables the conversion of nitrate back into atmospheric nitrogen, facilitated by the oxidation of organic matter, thus releasing energy. Moreover, there are several nitrogen-recycling mechanisms, including the uptake of nitrate and ammonium by photosynthetic organisms such as plants and phytoplankton. Microorganisms play a crucial role in nitrogen recycling by converting nitrogen from dead organic matter into ammonium through a process known as ammonification. This ammonium is then nitrified into nitrate and taken up once again by photosynthetic organisms (Lenton, 2016). The nitrogen transformations are pivotal for both the retention and elimination of inorganic nitrogen compounds within wetlands. These processes include sequestering nitrogen in plant biomass, accumulating nitrogen in wetland sediments, and releasing nitrogen as gases into the atmosphere through denitrification and ammonia volatilization (Lu et al., 2009; Zhang et al., 2016) (Figure 1.1).

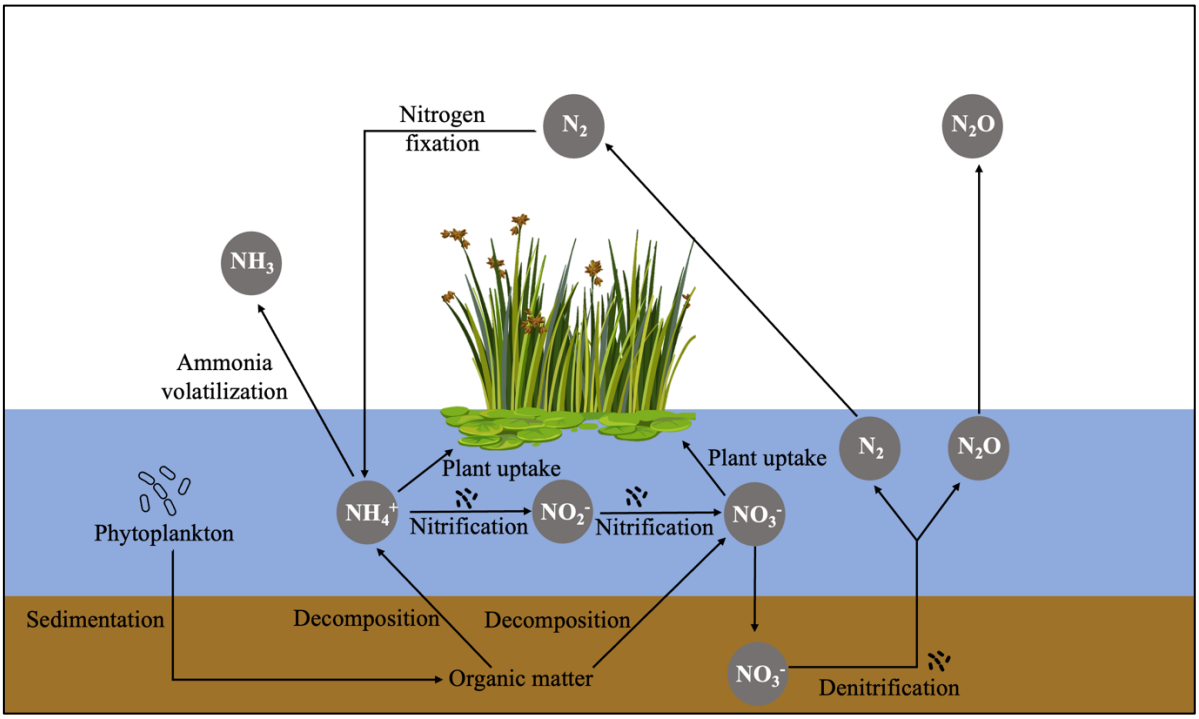


Figure 1.1: A simplified illustration of the nitrogen cycle in a wetland.

Stable nitrogen isotopic composition ($^{15}N/^{14}N$; denoted as $\delta^{15}N$) is increasingly used in research focusing on nitrogen cycling in organisms and ecosystems (Robinson, 2001). The $\delta^{15}N$ values in plants and soil serve as valuable indicators for inferring nitrogen cycling processes that are challenging to measure directly and are difficult to scale accurately. Specifically, the nitrogen isotope ratios in plants are inclined to reflect short-term variations in nitrogen cycling, such as those occurring on annual time scales. Conversely, soil nitrogen isotopes integrate over longer time scales, providing insights into broader trends and processes (Craine et al., 2015). Therefore, these measurements can help to trace nitrogen input from treated wastewater effluent into a wetland and its subsequent transport through the downstream water bodies (Leavitt et al., 2006; Lin et al., 2017). In addition, denitrification is considered the main process that alters the nitrogen isotopic composition of nitrates in water. Therefore, stable nitrogen isotopic composition measurements also

offer insight into denitrification processes that occur within a wetland (Hu et al., 2020; Knoller et al., 2011).

1.1 Objectives

The two main research objectives of this study are:

1. To trace the input of nitrogen into the Frank Lake wetland complex in Alberta, Canada, from industrial wastewater effluent and the subsequent transport of nitrogen through a downstream river (Little Bow River) and on to an associated reservoir (Twin Valley Reservoir) by analyzing the $\delta^{15}N$ value of plant tissue samples of two common wetland plant species; *Typha latifolia* (cattail) and *Schoenoplectus acutus* (hard stem bulrush/ great bulrush)] and sediment samples.
2. To determine whether significant denitrification processes occur as water moves through the Basin 1 of the wetland complex by analyzing the $\delta^{15}N$ of nitrate molecules in the water samples collected from different sampling locations within the Basin 1.

1.2 Study site

1.2.1 Introduction and location description

The Frank Lake wetland complex (Latitude: 50.567º N; Longitude: 113.708º W), a freshwater mineral soil wetland spanning 10.1 km^2, is situated approximately 45 km south of Calgary and 6 km east of High River, Alberta, Canada (White & Bayley, 2001; Zhu et al., 2019). The wetland is composed of three discrete basins (Figure 1.2), each exhibiting distinct ecological features. Basins 1 (5.1 km^2) and 2 (3.6 km^2) feature freshwater marsh vegetation surrounding their open water regions, that are primarily lakes. Conversely, Basin 3 (1.4 km^2) distinguishes itself with a more expansive marsh vegetation and limited open water area (Zhu et al., 2019). The three basins are separated by control weirs positioned at the outlet of each successive basin.

In 1989, Ducks Unlimited Canada restored the wetland complex to promote bird habitat, by supplying treated wastewater from a meat processing facility (Cargill Ltd.) and a sewage treatment facility in High River (Sadler et al., 1995; White & Bayley, 1999, 2001; Zhu et al., 2019). During the period spanning 1989 to 1994, efforts were made to alleviate the effects of the introduced wastewater by also importing water that was diverted from the nearby Highwood River. This strategy was later terminated, resulting in the sole redirection of wastewater to Frank Lake from 1994 onwards (Zhu et al., 2019).

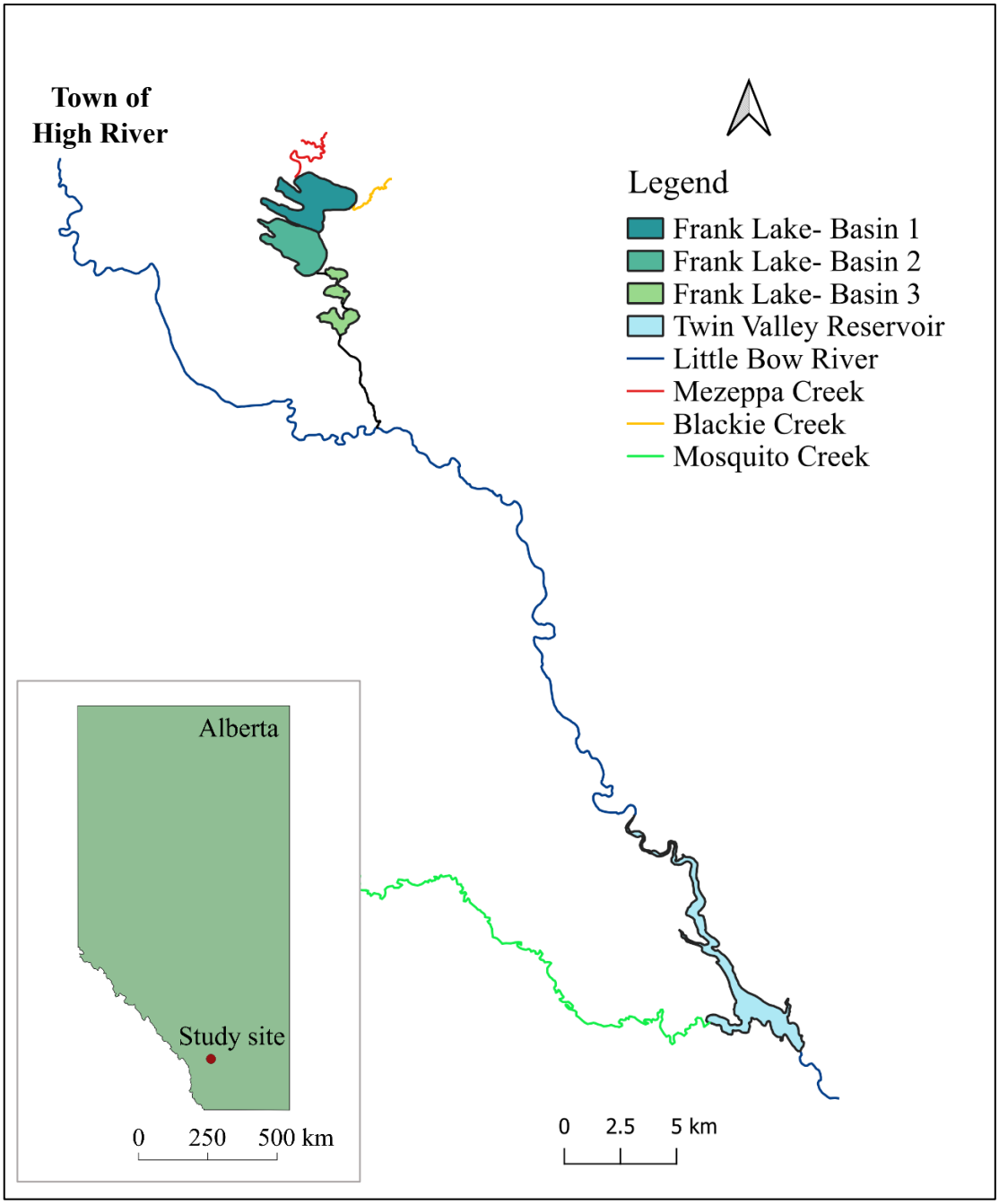


Figure 1.2: Study site map showing the Frank Lake wetland complex, two ephemeral creeks associated with the Frank Lake wetland complex (Blackie and Mazeppa Creeks), Little Bow River and Twin Valley Reservoir and Mosquito Creek. The treated wastewater effluent enters Basin 1 of the Frank Lake wetland complex, flows through Basin 2 and 3, and is released into the Little Bow River, subsequently flowing through the Twin Valley Reservoir.

1.2.2 Hydrology

The treated wastewater effluent sourced from both the municipal sewage treatment plant and meat processing plant enters the wetland through a discharge canal that directly feeds into Basin 1. In 2019, over 3,500,000 m^3 $year^{-1}$ of wastewater effluent was discharged into Basin 1 (Native Plant Solutions, 2021). Additional water sources for the wetland include limited water input from two ephemeral creeks (Blackie and Mazeppa Creek) (Figure 1.2) during the spring and agricultural runoff (White & Bayley, 2001). The two creeks discharge their waters into Frank Lake from the northern and eastern directions of the wetland. There are also other unnamed creeks contributing to the lake, however, the observed discharge from these creeks is notably insignificant relative to the flow from Mazeppa and Blackie (Zhu et al., 2019).

The treated wastewater effluent flows from Basin 1 to Basin 2 and then into Basin 3 before subsequently passing through the Little Bow River, the Twin Valley Reservoir and the Oldman River in southern Alberta (Zhu et al., 2019) (Figure 1.2). The average water depth of Basins 1 and 2 closely aligns, measuring approximately 0.67 m. In contrast, Basin 3 maintains an average water depth of 0.3 m or less. The water level of the wetland follows a seasonal pattern characterized by its lowest values in winter. As spring unfolds, a noticeable increase occurs, often reaching the lake's capacity due to the inflow from ephemeral creeks, although this peak remains unmonitored. Throughout the summer, the water level gradually recedes, returning to its standard operating level (Zhu et al., 2019).

1.2.3 Vegetation

The shoreline vegetation in Basin 1 and Basin 2 of the wetland complex is predominantly characterized by perennial herbaceous plant species, including *T. latifolia*

and *S. acutus* (Hard-stem bulrush). Furthermore, *Myriophyllum exalbescens* (Northern Watermilfoil), a submerged plant, is observed along the shorelines of Basin 1 and Basin 2. Basin 3 is distinguished by its extensive growth of *S. acutus* vegetation while *T. latifolia* is confined to a few small patches within Basin 3.

The shoreline vegetation of the Little Bow River and Twin Valley Reservoir is exclusively comprised of *T. latifolia*, with no observable presence of *S. acutus*. *Typha latifolia* is present as clusters along the river and reservoir.

1.3 Thesis overview

This thesis is structured into three chapters, each presented in manuscript format, addressing key aspects of my research on nitrogen removal in the Frank Lake wetland complex, which receives wastewater effluent and the nitrogen dynamics in the Little Bow River and Twin Valley Reservoir.

Chapter one provides a comprehensive introduction to the nitrogen cycle within wetlands, detailing the mechanisms by which nitrogen is removed in these ecosystems. Additionally, it introduces my study site, the Frank Lake wetland complex- a freshwater mineral soil wetland located in Southern Alberta, Canada.

Chapter two focuses on the use of two common plant species, *T. latifolia* and *S. acutus*, along with sediment as bioindicators to trace nitrogen flow through the Frank Lake wetland complex, Little Bow River and the Twin Valley Reservoir. This chapter also examines the role of these wetland plants in mitigating wastewater nitrogen within a wetland ecosystem.

Chapter three delves into the denitrification processes occurring in Basin 1 of the Frank Lake wetland. It explores the contribution of these processes to nitrogen removal from wastewater. Furthermore, this chapter analyzes the patterns of spatial variation in denitrification and discusses potential reasons for these observed variations.

Chapter 2: Tracing the flow of nitrogen through the Frank Lake wetland complex, Little Bow River and Twin Valley Reservoir

Abstract

Municipal and industrial wastewater effluents often discharge elevated concentrations of nitrogen into natural watersheds. Wetlands have been used to reduce nitrogen concentrations in wastewater effluent before it is discharged into downstream public waterways. This study aims to trace inorganic nitrogen input from treated industrial wastewater effluent into the Frank Lake wetland complex in southern Alberta, Canada, its uptake by the wetland and its subsequent movement through the Little Bow River to the Twin Valley Reservoir using stable isotope composition of nitrogen in sediment and leaf tissue samples of *Typha latifolia* and *Schoenoplectus acutus.* The study revealed significant nitrogen uptake and processing within Basins 1, 2, and 3 of the Frank Lake wetland, attributed to uptake by emergent aquatic vegetation, phytoplankton assimilation leading to sediment deposition and denitrification. Initially, $\delta^{15}N$ values increased in leaf tissue and sediment samples from Basin 1, potentially due to denitrification, followed by a decrease in Basins 2 and 3. Despite effective nitrogen processing within the wetland, downstream impacts on the Little Bow River system were evident, with elevated $\delta^{15}N$ levels in *T. latifolia* samples. Additionally, the Twin Valley reservoir exhibited elevated $\delta^{15}N$ values, likely influenced by wastewater discharge from Nanton via Mosquito Creek. The study offers valuable insights into the ecosystem service of nutrient uptake provided by the wetland complex, the functioning of the network and the transport of nitrogen through the system.

2.1 Introduction

Wetland plants rely on nutrients for their growth and reproductive processes. They absorb nutrients from the surrounding water and soil through their root systems, submerged stems and leaves (Brix, 2003, Vymazal, 2011). Given the high productivity of wetland plants, substantial quantities of nutrients can be incorporated into their biomass. This process contributes to the regulation of nutrients in wastewater (Vymazal, 2011).

Wetland plants absorb inorganic forms of nitrogen and convert them into organic nitrogen compounds in plant tissues through a process known as assimilation (Vymazal, 2007; Zhang et al., 2016). Nitrate and ammonium are the two forms of nitrogen that are typically used for assimilation (Vymazal, 2007). The stable nitrogen isotope composition of nitrate and ammonium in wetland plant biomass has proven particularly helpful in tracing the nitrogen input from treated wastewater effluent into aquatic ecosystems (Bruland & MacKenzie, 2010; Leavitt et al., 2006).

In natural wetland complexes receiving wastewater effluent, plants primarily derive nitrogen from two main sources: wastewater and soil (Cecchetti et al., 2020). Consequently, analyzing nitrogen isotopes in plant tissue samples facilitates the identification of nitrogen isotopic ratios within these sources (Cecchetti et al., 2020; Craine et al., 2015). This approach is viable because the isotope ratios in plant tissues generally mirror those of their sources, with no significant fractionation occurring during nitrogen uptake by the plants (Lund et al., 2000).

There are two stable isotopes of nitrogen: ^{14}N and ^{15}N (Robinson, 2001). The nitrogen isotopic compositions are reported in δ notation and expressed in per mil (‰) as follows:

$$\delta\ (‰) = \frac{R_{standard} - R_{sample}}{R_{standard}}$$

Where, the R_{sample} and $R_{standard}$ represent $^{15}N/^{14}N$ for the sample and standard, respectively. The nitrogen gas (N_2) in air serves as the standard for $^{15}N/^{14}N$ (Chen & MacQuarrie, 2005; Hu et al., 2020).

The wastewater-derived nitrogen is frequently enriched in ^{15}N. Therefore, its $\delta^{15}N$ value is distinguishable from other nitrogen sources, such as soils, where $\delta^{15}N$ varies between 0 and 10 ‰ (Cecchetti et al., 2020). However, the precise isotopic composition of wastewater effluent varies depending on the sewage treatment procedures employed. For example, primary wastewater treatment encourages only the large particles and lipids to be removed, resulting in moderately low ^{15}N values in the remaining effluent (DeBruyn et al., 2003; Gaston & Suthers, 2004; Heikoop et al., 2000). In contrast, the secondary and tertiary treatment favors sedimentation, ammonia volatilization, and denitrification with elevated amounts of ^{14}N passed on to the reaction products and associated increases in ^{15}N in the substrate molecules remaining in the wastewater effluent (Bedard-Haughn et al., 2003; Leavitt et al., 2006). Further, biological nutrient removal might slightly increase the ^{15}N concentration in nitrogen molecules remaining in the water (Savage et al., 2004). These higher ^{15}N values in effluent are useful for tracing its movement within a wetland complex and the river system.

Typha latifolia (cattail) is a globally distributed perennial emergent aquatic plant that usually thrives in warm settings with sufficient light (Lin et al., 2017). *Typha* species are frequently encountered in various aquatic habitats, including shallow bays, irrigation

ditches, lakes, ponds, rivers and both brackish and freshwater marshes (Vymazal, 2011). In many studies, *T. latifolia* has been recognized as an efficient treatment species for nutrient removal and water purification in natural and constructed wetlands and lakes (Calheiros et al., 2009; Lin et al., 2017; Liu et al., 2012). *Schoenoplectus acutus* is also a widely distributed sedge species (Sloey & Hester, 2018), that has been studied for its nutrient removing capacity in wetlands (Flanagan et al., 2022; Vymazal, 2011). The isotopic measurements of plant tissues reflect the relatively short-term accumulation of wastewater-derived nitrogen in plant tissue (Craine et al., 2015).

However, macrophyte assimilation is not the sole potential biological assimilation process. Microorganisms and algae also play a role in nitrogen utilization. Upon the death of these organisms and plant macrophytes, a significant portion of their biomass undergoes decomposition, resulting in the release of both carbon and nitrogen (Vymazal, 2007). This decomposition process plays a crucial role in the wetland nitrogen cycle, as it contributes to the release of nitrogen into the sediment (Lin et al., 2017). Therefore, the isotopic measurements of the sediment can be used to study the relatively long-term dynamics of the wastewater nitrogen input to wetlands over several years (Craine et al., 2015).

In this study, *T. latifolia* and *S. acutus* plants were applied as 'bio-monitors', utilizing their stable nitrogen isotope composition to assess the magnitude of effluent-sourced nitrogen across different sites within the Frank Lake wetland complex, Little Bow River and Twin Valley Reservoir during the growing season. Also, $\delta^{15}N$ of the sediment samples were studied to analyze the long-term accumulation of wastewater nitrogen within the Frank Lake wetland complex, Little Bow River, and Twin Valley Reservoir.

2.2 Methods

2.2.1 Vegetation sampling

Plant tissue samples of *T. latifolia* and *S. acutus* were collected as a proxy to estimate the $\delta^{15}N$ in nitrate and ammonium in water and sediment to which the plants were exposed. Plant tissue samples of *T. latifolia* were obtained from Basin 1, 2 and 3 of the Frank Lake wetland complex, from areas within the Little Bow River that were upstream and downstream of the outlet for water input from Frank Lake and in Twin Valley Reservoir. However, *S. acutus* plant tissue samples were collected only from the three basins of the Frank Lake wetland complex as there are no *S. acutus* plants present in the Little Bow River and Twin Valley Reservoir. Sampling sites for *T. latifolia* (Figure 2.1) and *S. acutus* (Figure 2.2) were selected to sample the water flow pattern through each Basin of the wetland, the Little Bow River and Twin Valley Reservoir. The GPS coordinates corresponding to the sampling sites are provided in Appendix 2.1.

Leaf tissue samples of *T. latifolia* from the Little Bow River and Twin Valley Reservoir sampling locations were collected on July 18, 2023. On the same day, I collected leaf tissue samples of both *T. latifolia* and *S. acutus* from Basin 3 outlet of the Frank Lake wetland (B3-O). Leaf tissue samples of *T. latifolia* and *S. acutus* from other Basin 3 sampling locations and Basin 2 sampling locations of the Frank Lake were obtained on July 19, 2023. I collected leaf tissue samples of both plant species from Basin 1 of the Frank Lake on July 20, 2023. To obtain plant tissue samples, plants growing close to the flowing water in the Frank Lake wetland complex, Little Bow River and Twin Valley Reservoir were specifically chosen. This selection was made with the intent of ensuring that these plants serve as the most accurate representatives of the nitrogen content present in the

moving water of the Frank Lake wetland complex, Little Bow River and Twin Valley Reservoir.

Fresh leaf tissue samples of *T. latifolia* and fresh culm samples of *S. acutus* were collected from six replicates (n = 6) at each sampling location, maintaining a ~ 1 m distance between each replicate (Figure 2.3). Plant tissue from the current growing season was collected while disregarding any deceased plant tissue from the previous growing season. The spikelet of the *S. acutus* culms were removed during the collection process.

Upon return to the laboratory, the plant tissue samples were oven-dried in separate paper bags at ~ 60 °C for at least 48 - 72 hours. The oven-dried samples were then cut into small pieces and ground into fine powder using coffee grinders. When necessary, a ball mill was used to obtain a fine powder. The finely ground tissue samples were then transferred into labeled 20 ml scintillation vials. The samples were analyzed for their $\delta^{15}N$ value using gas isotope ratio mass spectrometry at the Stable Isotope Lab at the University of Utah, USA. In addition, carbon content (% C) and nitrogen content (% N) of the plant tissue samples were measured using mass spectrometry. Figure 2.4 summarizes the procedures involved in collecting, preparing and analyzing leaf tissue samples of *T. latifolia* and *S. acutus*.

Apart from that, water samples (n = 3) from wastewater input to Basin 1 were collected on June 7, 2023, July 20, 2023 and August 31, 2023. These samples were analyzed for their nitrate and ammonium concentration and the $\delta^{15}N$ of nitrate and ammonium at the Isotope Science Lab at the University of Calgary (University of Calgary, 2024a; 2024b).

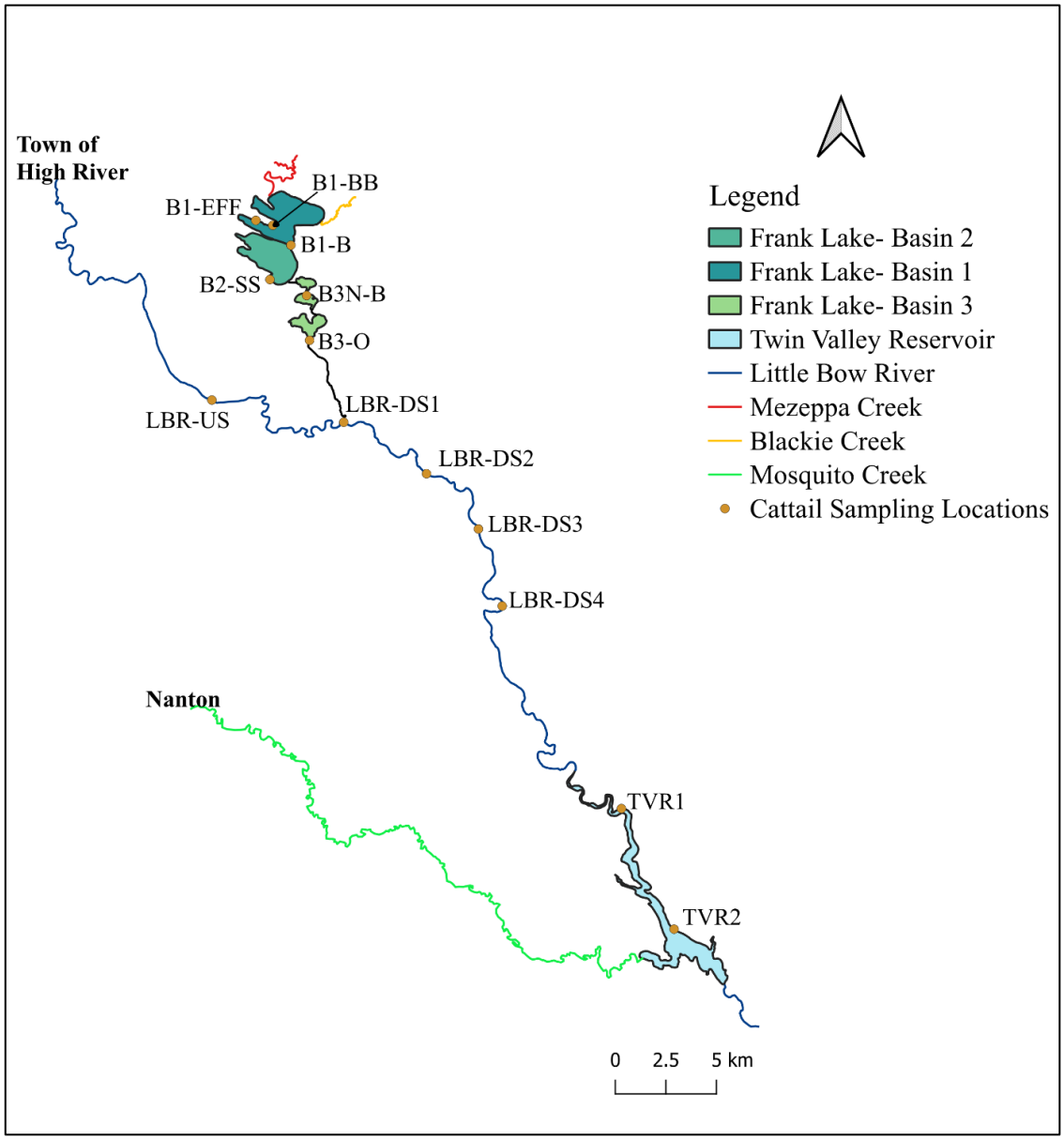


Figure 2.1: *Typha latifolia* tissue sampling locations in the Frank Lake wetland complex, upstream and downstream of the water input from Frank Lake wetland drainage into Little Bow River and in Twin Valley Reservoir. The abbreviations for the specific locations are as follows: B1-EFF, Basin 1- Effluent input; B1-BB, Basin 1- Bird blind; B1-B, Basin 1- Outlet; B2-SS, Basin 2- South shore, B3N-B, Basin 3- North of island; B3-O, Basin 3- Outlet; LBR- US, upstream of the Little Bow River; LBR-DS1-4, downstream of the Little Bow River; TVR1-2, Twin Valley Reservoir. Sediment samples were also acquired from the same locations.

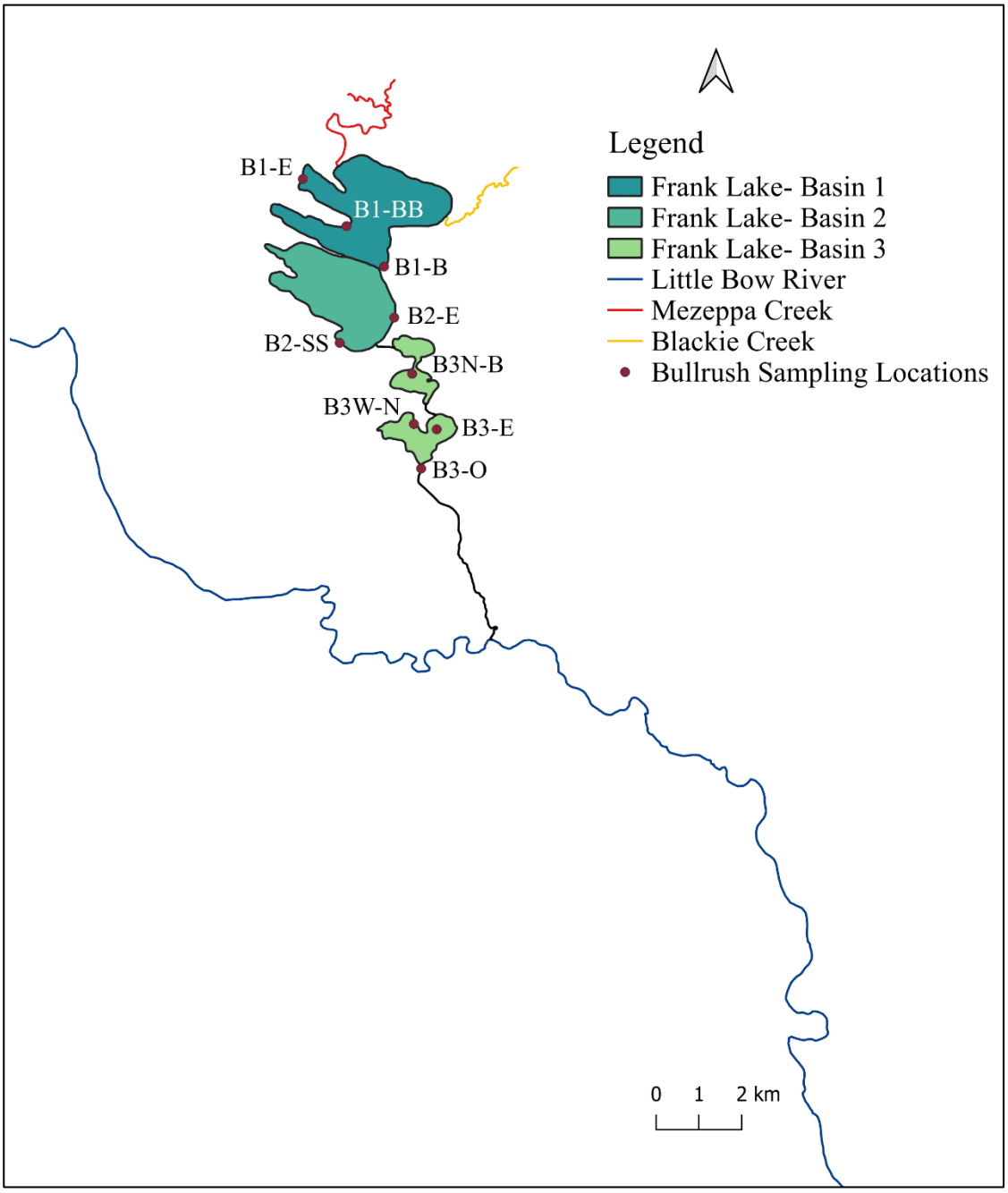


Figure 2.2: *Schoenoplectus acutus* tissue sampling locations in the Frank Lake wetland complex. The abbreviations for the specific locations are as follows: B1-BB, Basin 1- Bird blind; B1-E- Basin 1- Middle West Shore; B1-B, Basin 1- Outlet; B2-SS, Basin 2- South shore, B2-E, Basin 2- East Shore; B3N-B, Basin 3- North of island; B3W-N, Basin 3- North Shore; B3-E, Basin 3- East, B3-O, Basin 3- Outlet. Sediment samples were also acquired from the same locations.

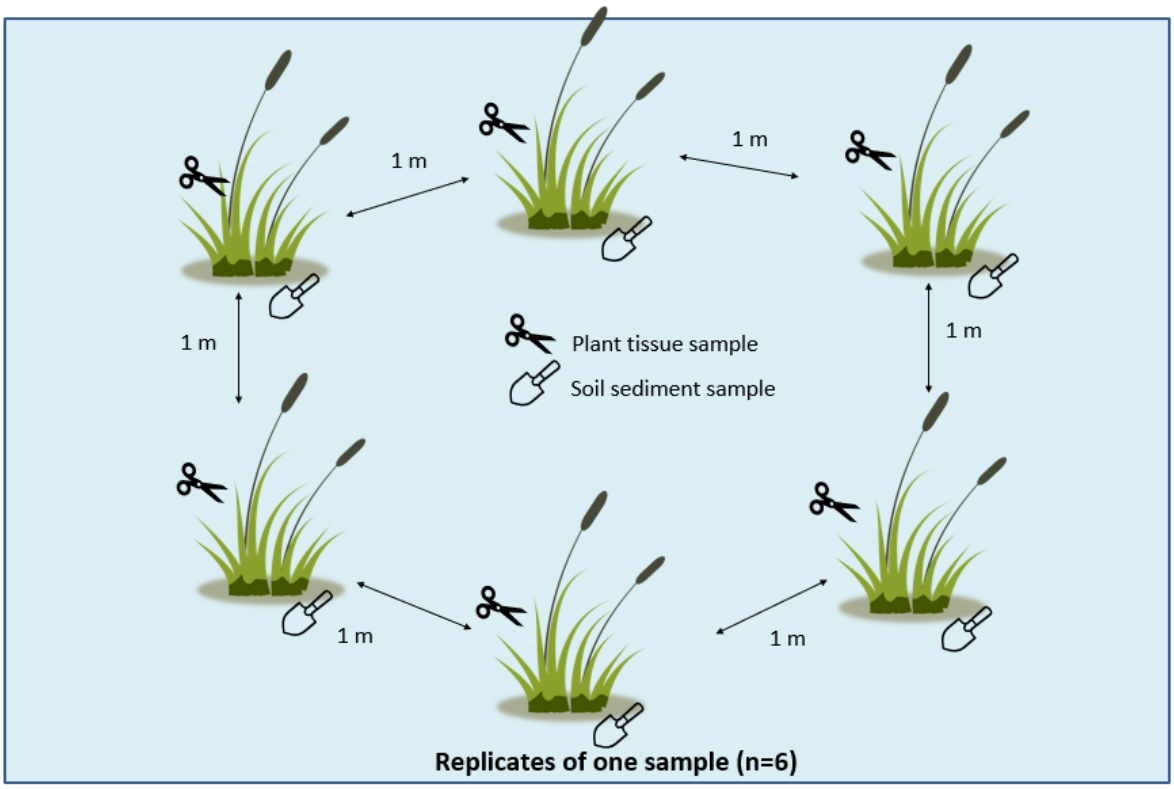


Figure 2.3: Experimental design used to obtain *S. acutus* and *T. latifolia* plant tissue samples and sediment samples from a given sampling location.

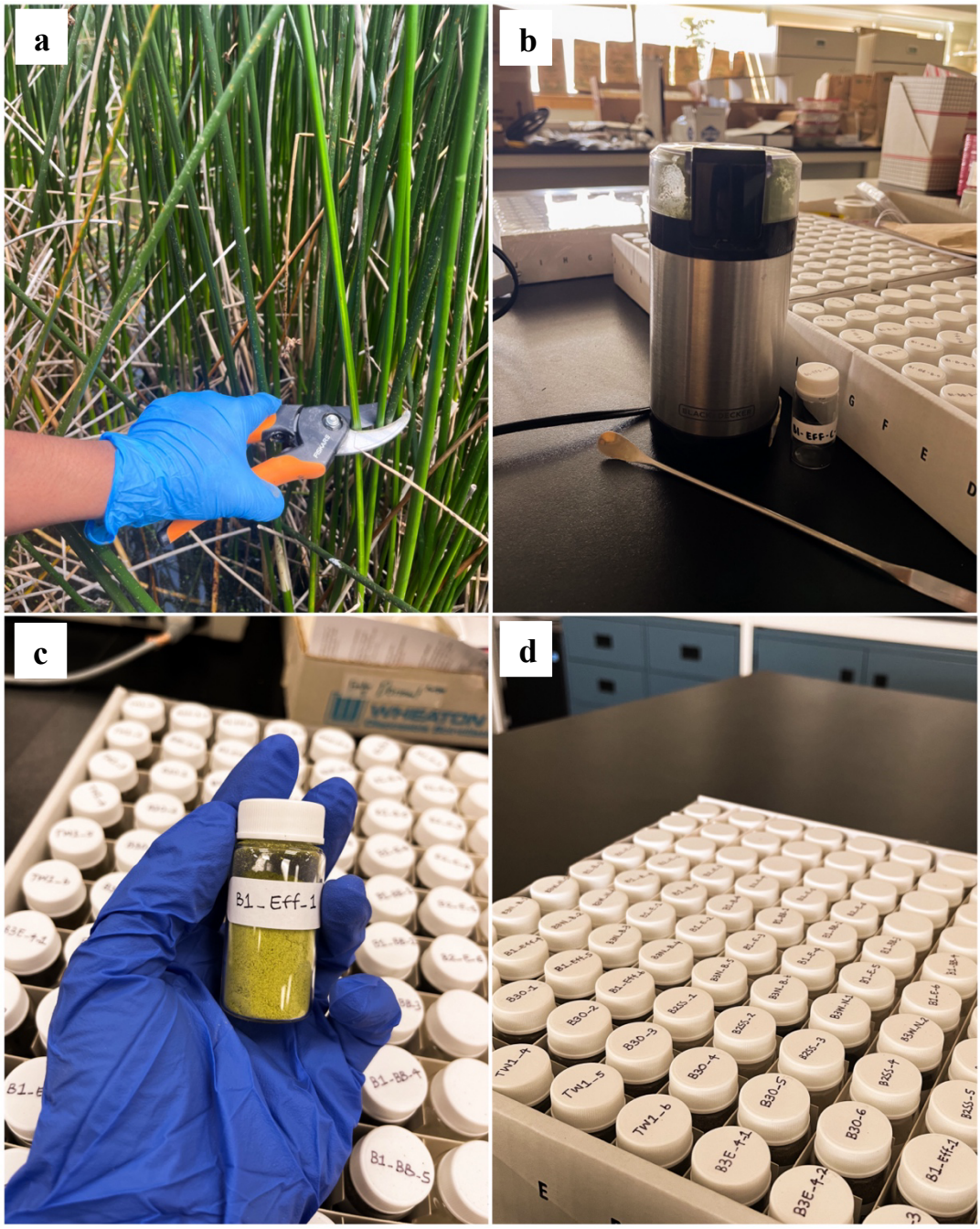


Figure 2.4: a) Fresh leaf tissue samples were collected from six replicates (n = 6) at each sampling location. b) Upon return to the laboratory, the samples underwent oven-drying and were ground into a fine powder using coffee grinders. c) The finely ground tissue samples were subsequently transferred into labeled 20 ml scintillation vials. d) Samples were analyzed for their $\delta^{15}N$ values using gas isotope ratio mass spectrometry at the Stable Isotope Lab at the University of Utah, USA.

2.2.2 Sediment sampling

I collected sediment samples (n = 6) from the Little Bow River and one Twin Valley Reservoir sampling location (TVR2) on May 18, 2023. Sediment samples from the other sampling location in the Twin Valley Reservoir (TVR1) were collected on July 18, 2023. I collected sediment samples from the area of Basin 2 and 3 on July 19, 2023. Basin 1 of the Frank Lake wetland was sampled for sediments on July 20, 2023, simultaneously with the collection of leaf tissue samples (Figure 2.3). A soil core sampler with a diameter of 2 cm was used to collect the top 10 cm of the sediment at each location. Efforts were made to locate areas with more permeable substrates suitable for sediment core sampling. In instances where penetration of the sediment core was impeded by the presence of gravel beneath the sediment, particularly in riverbeds, core samples were collected from two closely adjacent insertions, aiming to attain a cumulative sediment column of 10 cm. Samples were placed in labelled plastic bags.

Upon return to the laboratory, the collected sediment samples were initially air-dried for a period of 48 - 72 hours and subsequently were oven-dried at approximately 60 °C for another 48 - 72 hours. Dried sediment samples were then sieved using a soil sieve with an 850 µm aperture. In cases where samples contained substantial amounts of sand, a soil sieve with a 425 µm aperture was employed to separate and eliminate the sand fraction. The samples were then transferred into 20 ml scintillation vials and sent for analysis of $\delta^{15}N$ values using gas isotope ratio mass spectrometry at the University of Utah, USA. In addition, % carbon and % nitrogen content of the samples were determined using mass spectrometry. Figure 2.5 summarizes the procedures involved in collecting, preparing and analyzing sediment samples.

Figure 2.5: a) Sediment samples were collected from six replicates (n = 6) at each sampling location. b) Upon return to the laboratory, the samples underwent air and oven-drying. c) Dried sediment samples were then sieved using a soil sieve with an 850 µm aperture. d) The samples were then transferred into 20 ml scintillation vials and sent for analysis of $\delta^{15}N$ values using gas isotope ratio mass spectrometry at the University of Utah, USA.

2.2.3 Statistical analysis

Box and whisker plots were employed to represent the plant tissue and sediment data collected in this study. The box plots were formatted as follows: the median was depicted by a horizontal line inside the box, while the lower and upper edges of the box corresponded to the 25th percentile (first quartile) and 75th percentile (third quartile) of the dataset respectively. The whiskers extended to the minimum and maximum values in the dataset, and the outliers were indicated by black closed circles.

The normality of $\delta^{15}N$ values in the *T. latifolia* and *S. acutus* plant tissue and the sediment samples, were examined using the Shapiro-Wilk test. Concurrently, Levene's test was employed to evaluate the equality of variances across the sampling locations.

The $\delta^{15}N$ data of *T. latifolia* leaf tissue samples followed a normal distribution (Shapiro-Wilk test; $P > 0.05$). Also, the variance across groups was not significantly different (Levene's Test for Homogeneity of Variance; $P > 0.05$). Consequently, assumptions of normal distribution and equal variance were met for the $\delta^{15}N$ data of *T. latifolia* leaf tissue, allowing the application of one-way ANOVA to assess the statistical significance of differences among mean $\delta^{15}N$ values across sampling locations. Subsequently, Tukey's Honestly Significant Difference (HSD) test was applied to examine specific pairwise differences among the sample means. The use of ANOVA allowed for a comprehensive examination of group variations, while Tukey's HSD test provided detailed insights into the specific pairs that contributed to significant differences. Due to the considerable variability observed in the dataset, a separate one-way ANOVA was conducted for the plant tissue data collected from both the Little Bow River and Twin Valley Reservoir,

to distinguish them from the observations within the Frank Lake wetland complex and describe the distinct patterns of $\delta^{15}N$ variation in them.

The $\delta^{15}N$ dataset derived from *S. acutus* leaf tissue samples also demonstrated a normal distribution (Shapiro-Wilk test; $P > 0.05$). The variance across groups was not also found to be statistically different (Levene's Test for Homogeneity of Variance; $P > 0.05$). This validation of normal distribution and equal variance conditions allowed the application of one-way ANOVA for *S. acutus* leaf tissue samples. Tukey's HSD test was used to assess the distinct pairwise differences among the sample means.

The $\delta^{15}N$ values of the sediment samples were not normally distributed (Shapiro-Wilk test; $P < 0.05$). Statistically significant differences were observed in the variance across groups as well (Levene's Test for Homogeneity of Variance; $P < 0.05$). Given the non-compliance with the assumptions of normal distribution and equal variance, the Kruskal-Wallis test, a non-parametric alternative, was employed to evaluate the statistical significance of variations among the mean $\delta^{15}N$ values of sediment samples across sampling locations. The Wilcoxon test was used to calculate pairwise comparisons across sampling locations. In addition, the $\delta^{15}N$ values of the sediment samples of the Little Bow River and Twin Valley Reservoir were normally distributed (Shapiro-Wilk test; $P > 0.05$) with an equal variance across groups (Levene's Test for Homogeneity of Variance; $P > 0.05$). Therefore, a separate one-way ANOVA was conducted for the sediment data from the Little Bow River and Twin Valley Reservoir to separate them from the observations within the Frank Lake wetland complex and describe the distinct patterns of $\delta^{15}N$ variation in them.

The wetland sediments are mainly composed of organic carbon and nitrogen derived by decomposition of the plants, animals and phytoplankton (Avramidis et al., 2015). Therefore, a regression analysis was also performed to determine if there was any significant relationship between the $\delta^{15}N$ values and the sediment carbon content (% C) of the soil samples. The objective of this analysis was to investigate whether the fluctuations observed in $\delta^{15}N$ values could be attributed to the organic matter content present within the sediment samples. All the statistical tests and the graphs were generated using R (R version 2022.12.0, ©Posit Software, PBC).

2.3 Results

2.3.1 Variation of $\delta^{15}N$ in *T. latifolia* plant tissue

2.3.1.1 Pattern of $\delta^{15}N$ variation in the Frank Lake wetland complex

The $\delta^{15}N$ values in nitrate and ammonium present in the wastewater entering Basin 1 were anticipated to be enriched in ^{15}N due to isotopic fractionation associated with denitrification and ammonia volatilization during the wastewater treatment process preceding its discharge into Frank Lake wetland complex (Kendal et al., 2007; Leavitt et al., 2006). As expected, the effluent water input in Basin 1 exhibited average (± SD, n = 3) $\delta^{15}N$ values of 15.0 ± 1.0 ‰ for dissolved nitrate (total oxidized nitrogen) and 21.8 ± 4.1 ‰ for dissolved ammonium. The average concentration (± SD, n = 3) of nitrate (309.1 ± 29.4 mg NO_3^- L^{-1}) in the wastewater input significantly exceeded the average concentration of ammonium (5.3 ± 2.2 mg NH_4^+ L^{-1}). The substantially higher concentrations of nitrate in wastewater input led to a weighted average (± SD, n = 3) $\delta^{15}N$ value of 19.8 ± 0.2 ‰ for both nitrate and ammonium combined, as determined by mass balance.

Across all sites, median values of $\delta^{15}N$ in *T. latifolia* plant tissue ranged from approximately 3.5 to 23.0 ‰ (Figure 2.6). Consistent with the higher $\delta^{15}N$ values of inorganic nitrogen input to the Frank Lake wetland complex, the $\delta^{15}N$ values of *T. latifolia* plant tissue in the Basins 1, 2 and 3 were significantly higher than the Little Bow River (Figure 2.6; $F = 164.2$, $P < 0.0001$). The median $\delta^{15}N$ values observed across all Basin 3 sites decreased compared to those in Basin 2, yet they remained higher than the median $\delta^{15}N$ values of those found in the Little Bow River and Twin Valley Reservoir (Figure 2.6). In addition, the $\delta^{15}N$ values of *T. latifolia* leaf tissue samples from Basin 1-bird blind and Basin 1 outlet were significantly higher than the $\delta^{15}N$ value in the wastewater input into Basin 1.

As water exited Basin 3 of the Frank Lake Complex, there was a reduction of 43 % in the $\delta^{15}N$ values compared to the original levels present in the wastewater input to the Basin 1.

2.3.1.2 Pattern of $\delta^{15}N$ variation in the Little Bow River and Twin Valley Reservoir

The lowest $\delta^{15}N$ values were recorded in sampling sites of Little Bow River with the median values approximately ranging from 3.6 to 5.2 ‰ (Figure 2.6). The $\delta^{15}N$ value of the *T. latifolia* leaf tissue samples from the upstream of the water input from Frank Lake wetland drainage into Little Bow River was significantly lower than in sampling locations in the downstream of the Frank Lake water input ($F = 7.0$, $P < 0.05$).

The $\delta^{15}N$ values from the *T. latifolia* leaf tissue samples in both Twin Valley Reservoir sites were significantly higher than any of the Little Bow River sites ($F = 23.1$, $P < 0.0001$).

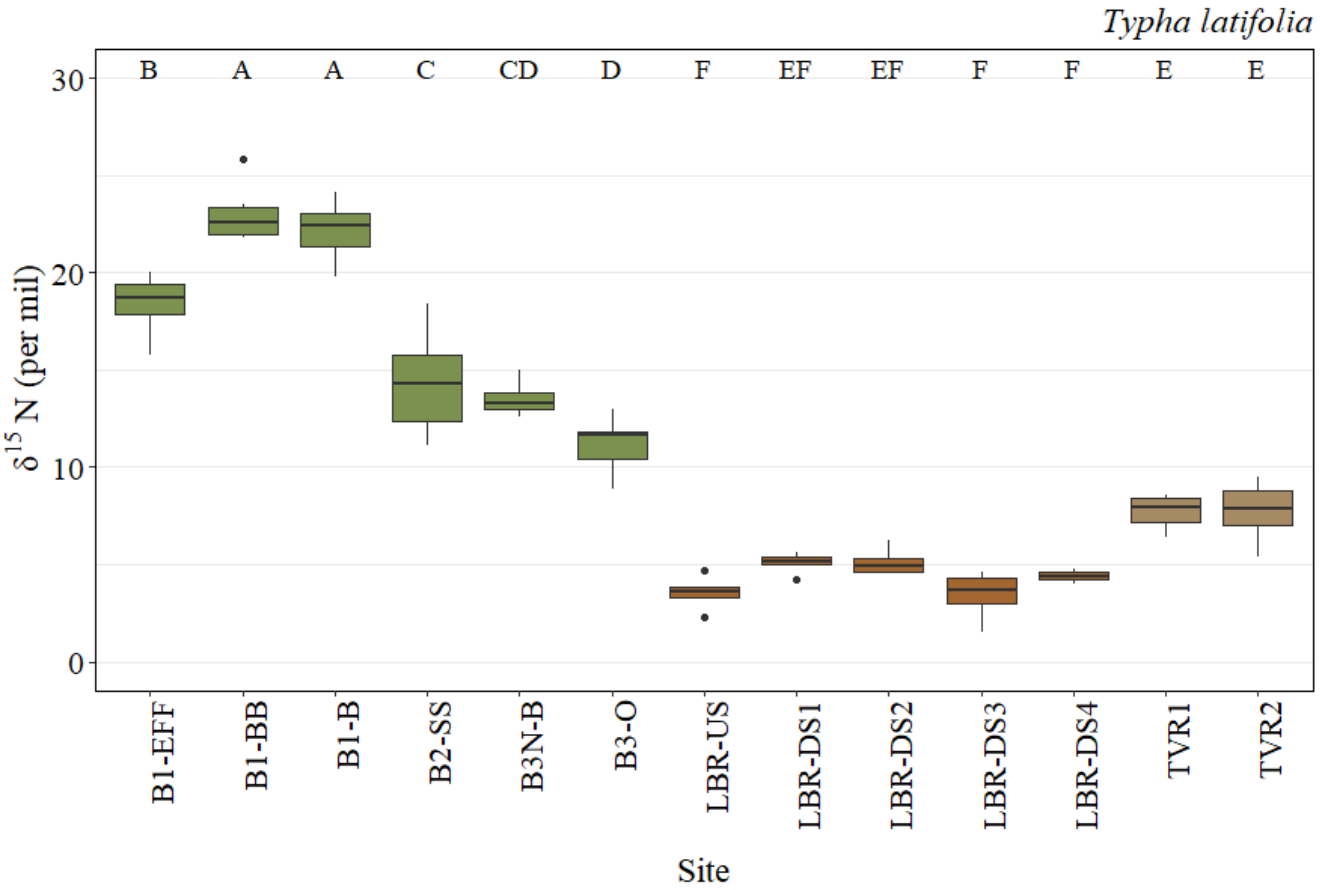


Figure 2.6: Comparison of $\delta^{15}N$ values in *Typha latifolia* plant tissue samples (‰) among sites within Frank Lake wetland complex, upstream and downstream of the water input from Frank Lake wetland drainage into Little Bow River and in the Twin Valley Reservoir. Statistical significance was determined based on Tukey's HSD test after a one-way ANOVA test ($F = 164.2$, $P < 0.0001$) and denoted by different letters located within the upper border of the graph box. The specific locations for the Frank Lake wetland complex, Little Bow River and Twin Valley Reservoir are shown in Figure 2.1.

2.3.2 Variation of $\delta^{15}N$ in *S. acutus* plant tissue

Across all sites in the Frank Lake wetland complex, median values of $\delta^{15}N$ in *S. acutus* plant tissue varied approximately from 7.9 to 22.1 ‰. The $\delta^{15}N$ values of the *S. acutus* plant tissue differed significantly across the sampling sites of Frank Lake wetland complex (Figure 2.7; $F = 57.4$, $P < 0.0001$). Samples of *S. acutus* from Basin 1 bird blind (22.5 ‰) and Basin 1 outlet (19.1 ‰) showed the highest median $\delta^{15}N$ values (Figure 2.7). The bird blind samples exceeded the $\delta^{15}N$ value recorded in the wastewater effluent input to Basin 1 (19.8 ± 0.2 ‰). Although the Basin 1 exhibited higher $\delta^{15}N$ values, Basin 1 - East had a significantly lower $\delta^{15}N$ value in comparison to the other sampling sites within Basin 1 (Figure 2.7; $F = 57.4$, $P < 0.0001$).

Additionally, Basin 3 displayed substantial variation in $\delta^{15}N$, with the highest median $\delta^{15}N$ observed in *S. acutus* plants growing in Basin 3-North of the island at 15.1 ‰ and the lowest in Basin 3 East at 8.1 ‰.

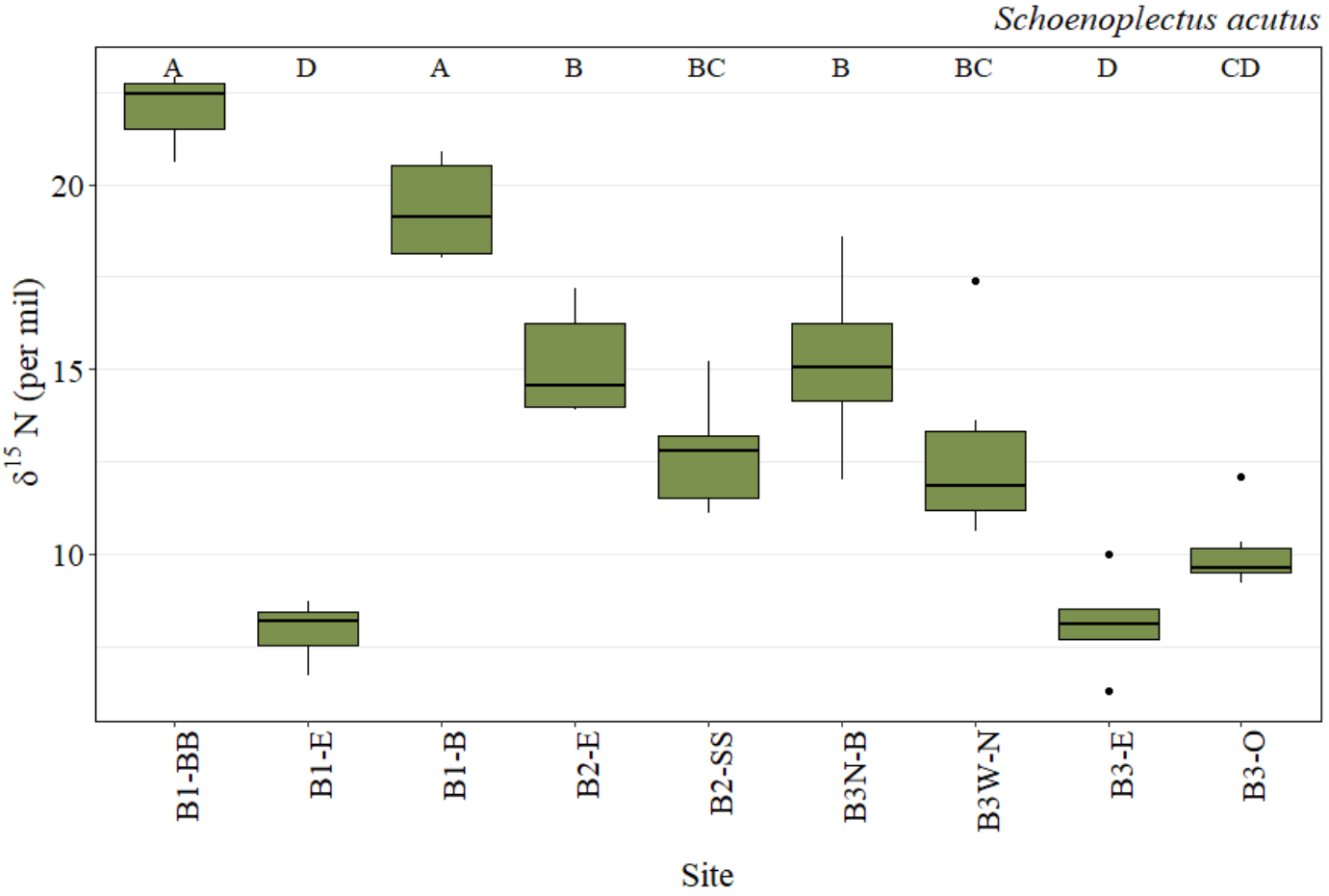


Figure 2.7: Comparison of $\delta^{15}N$ value of *Schoenoplectus acutus* plant tissue samples (‰) among sites within Basin 1, Basin 2, and Basin 3 Frank Lake wetland complex. Statistical significance was determined based on Tukey's HSD test after a one-way ANOVA test ($F = 57.4$, $P < 0.0001$) and denoted by different letters located within the upper border of the graph box. The specific locations for the Frank Lake wetland complex are shown in Figure 2.2.

2.3.3 Variation of $\delta^{15}N$ in sediment

The median $\delta^{15}N$ values in sediment samples ranged approximately from 4.1 to 19.2 ‰ across all locations within the Frank Lake wetland complex, Little Bow River and Twin Valley Reservoir. There was statistically significant variation among the different sites for $\delta^{15}N$ in sediment (Figure 2.8; Chi-square $_{(16,102)} = 97.79$, $P < 0.0001$).

The mean $\delta^{15}N$ values of sediment in Basin 1 bird blind and Basin 1 outlet were significantly higher than the Basin 1 effluent input (Figure 2.8). Moreover, sediment samples obtained from Basin 1 East demonstrated a significantly lower $\delta^{15}N$ values, aligning with the observations made in the plant tissue samples of *T. latifolia* and *S. acutus*. Basin 3 outlet demonstrated a significant decrease of $\delta^{15}N$ in sediment when compared to Basin 1 effluent input (Figure 2.8).

The median $\delta^{15}N$ in sediment from downstream of the water input from the Frank Lake wetland drainage into the Little Bow River was observed to be higher compared to that from upstream of the Frank Lake water drainage (Figure 2.8). The $\delta^{15}N$ at both sites within the Twin Valley Reservoir was significantly higher compared to all sampling locations along the Little Bow River ($F = 58.08$, $P < 0.0001$).

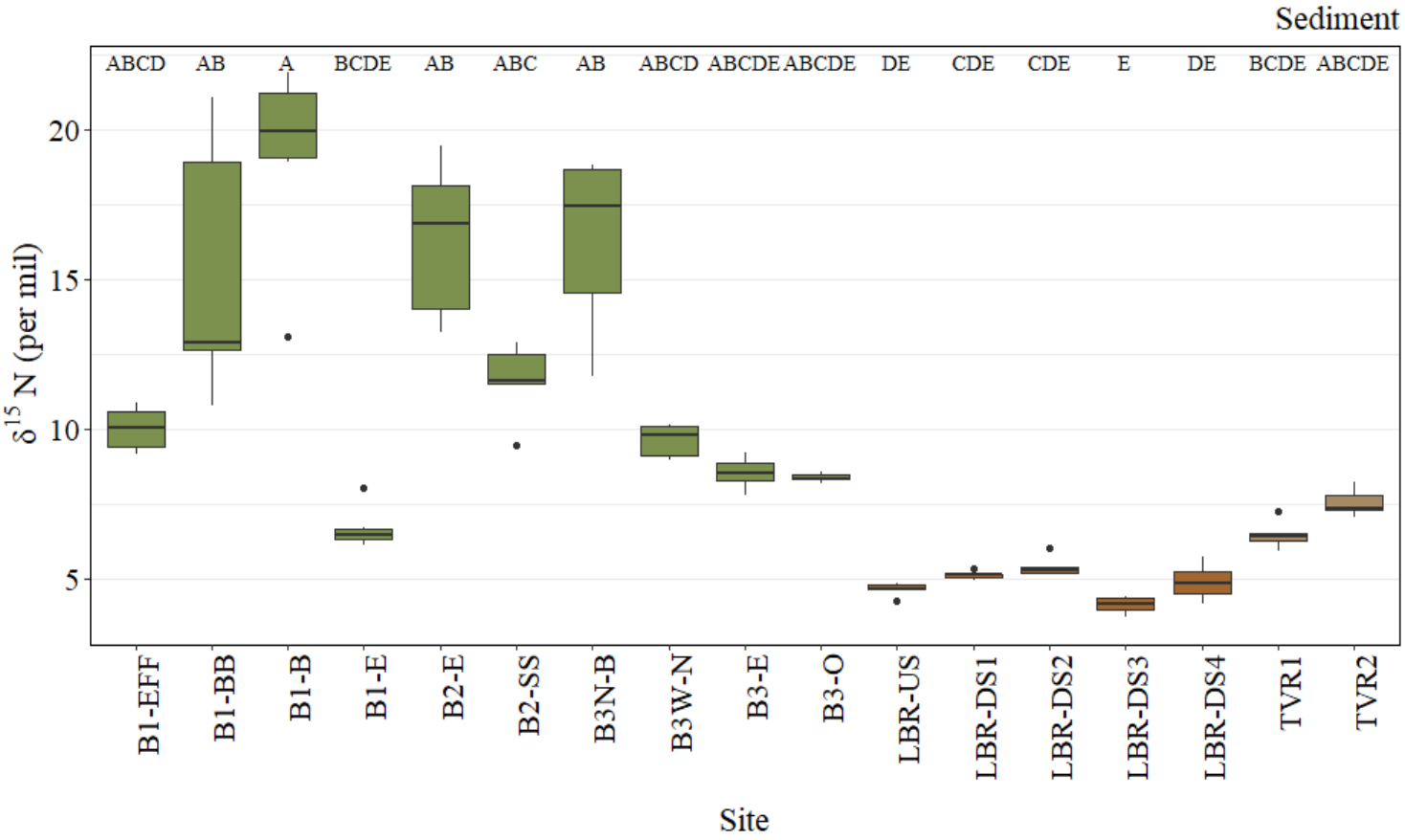


Figure 2.8: Comparison of $\delta^{15}N$ values in sediment samples (‰) among sites within Frank Lake wetland complex, upstream and downstream of the water input from Frank Lake wetland drainage into Little Bow River and Twin Valley Reservoir. Statistical significance was determined based on multiple comparison tests after a Kruskal-Wallis test (Chi-square $_{(16,102)} = 97.79$, $P < 0.0001$) and denoted by different letters located within the upper border of the graph box. The specific locations for the Frank Lake wetland complex, Little Bow River and Twin Valley Reservoir are shown in Figure 2.1 and Figure 2.2.

A regression analysis was conducted to test for a significant linear relationship between the $\delta^{15}N$ and the sediment carbon content. The results showed a significant linear relationship between the $\delta^{15}N$ value of soil sediment samples and the weight percent carbon content in the sampling locations (Figure 2.9, Linear regression: $R^2 = 0.3634$, $P < 0.05$).

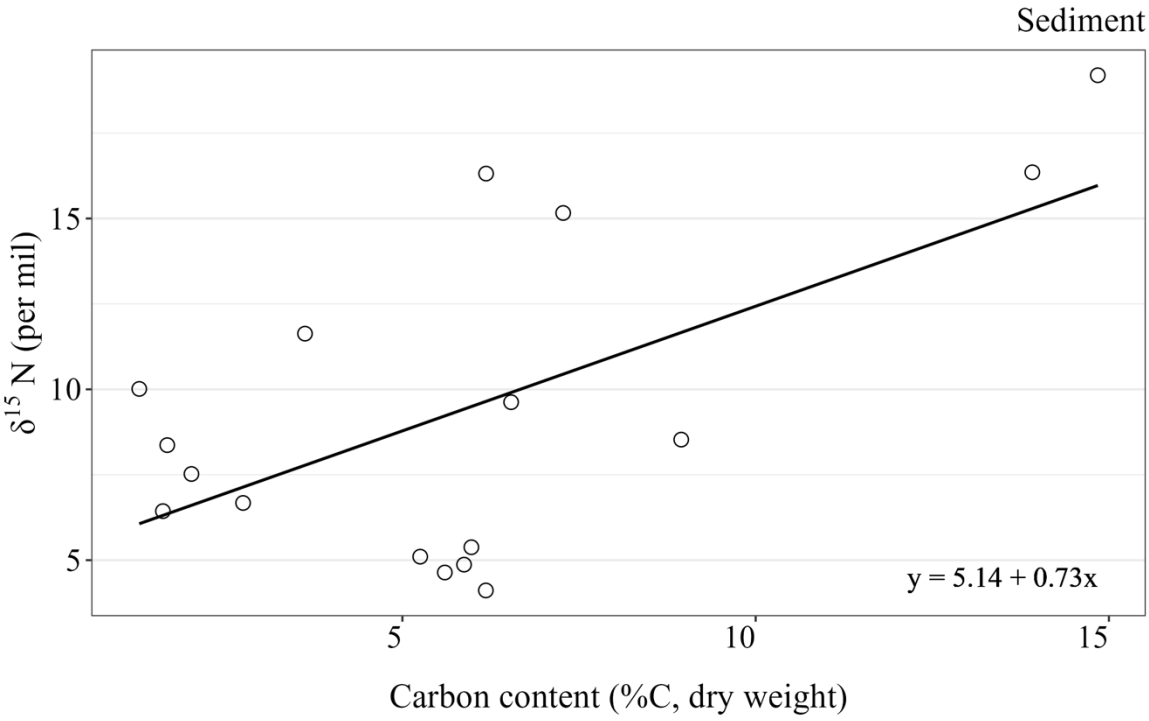


Figure 2.9: The relationship between the carbon content (% C) and the $\delta^{15}N$ value of sediment samples (‰). The line represents a linear regression fitted to the data points: $y = 0.73x + 5.14$ ($F = 8.5644$, $P < 0.05$).

2.4 Discussion

2.4.1 Factors influencing plant $\delta^{15}N$ variability

The $\delta^{15}N$ of *T. latifolia* and *S. acutus* leaf tissue were expected to be enriched in $\delta^{15}N$ due to the isotopic fractionation occurring during denitrification and ammonia volatilization processes in the wastewater treatment process in the Town of High River before discharge into the Frank Lake wetland (Leavitt et al., 2006). This was evident in the *T. latifolia* leaf tissue collected within a few meters of wastewater input into Basin 1 of the Frank Lake wetland, showing a high median $\delta^{15}N$ value of 18.7 ‰.

However, further enrichment in median $\delta^{15}N$ was observed in *T. latifolia* leaf tissue near the Basin 1- Bird blind (B1-BB) and Basin 1- Outlet (B1-B) (22.6 ‰ and 22.4 ‰ respectively), as well as in *S. acutus* leaf tissue near the Basin 1- Bird blind (B1-BB) (22.5 ‰). Previous studies highlighted the role of denitrification as a crucial process for removing nitrogen from wastewater input into the Frank Lake wetland (White and Bayley, 2001; Zhu et al., 2019). Additionally, Flanagan et al. (2022) estimated that denitrification could eliminate a substantial portion, ranging from 40.8 % to 65.7 % of nitrogen input to Frank Lake from industrial wastewater effluent. Based on these findings, I suggest that denitrification processes within the Frank Lake Basin 1 significantly impact the $\delta^{15}N$ levels in plant tissue by increasing $\delta^{15}N$ in the nitrogen accessible for plant uptake. The denitrification process within a wetland is shaped by various factors, such as oxygen levels in sediment, the availability of carbon sources, temperature, concentrations of nitrate, and the composition of plant species (Lu et al., 2009). In Basin 1- Bird blind (B1-BB) and Basin 1- Outlet (B1-B), the water was characterized by stagnant conditions with abundant dead plant material. These environmental conditions facilitate anaerobic processes, thereby

promoting denitrification in the sediment. Consequently, the higher $\delta^{15}N$ values observed in these two locations are likely linked to the greater denitrification rates taking place in those areas.

However, the median $\delta^{15}N$ value of *S. acutus* leaf tissue samples from Basin 1- Middle West Shore (B1-E) was notably low (8.2 ‰). Denitrification rates can vary across a transverse gradient within a wetland. Typically, denitrification rates are higher in open water areas compared to shallow marshes or edge areas near the ground. Areas close to the edges of lakes are not regularly submerged, resulting in aerobic conditions that are not favorable for denitrifiers. Conversely, along the edges of emergent vegetation, sediment becomes anaerobic, fostering conditions favorable for denitrification (Song et al., 2013). This is accompanied by a higher organic carbon content of the sediment, derived from the increased sedimentation of organic matter following the death and decomposition of phytoplankton, further enhancing denitrification processes. Hence, the low $\delta^{15}N$ of *S. acutus* leaf tissue samples observed in Basin 1- Middle West Shore (B1-E) may be attributed to sampling near the outer edge of the wetland as it transitions into grassland, due to the inaccessibility of the edges of emergent vegetation during the vegetation sampling at that location. Similarly, the reduced $\delta^{15}N$ value recorded in the *S. acutus* leaf tissue samples collected from Basin 3- East (B3-E) must be attributed to Basin 3- East experiencing a period of drying during the sampling period. Flanagan et al. (2022) also observed higher $\delta^{13}C$ in Basin 3 *S. acutus* biomass, suggesting that biomass production was water-limited in Basin 3 compared to Basins 1 and 2.

While denitrification typically leads to an enrichment of the stable nitrogen isotopic composition of water in Basin 1, it has been noted that the $\delta^{15}N$ values of *T. latifolia* and *S.*

acutus leaf tissue samples decline after an initial rise as water passes through Basin 1, 2, and 3 of the wetland. This is attributed to the significant uptake and processing of nitrogen from wastewater effluent as it traverses the three basins of the wetland. This trend is further supported by the significant reduction of the primary inorganic nitrogen compound found in wastewater effluent into the Frank Lake wetland complex, namely nitrate, even within Basin 1 of the wetland (This is discussed in detail in Chapter 3 of this study). Flanagan et al. (2022) identified three potential mechanisms influencing the retention and removal of nitrogen influx into Frank Lake from municipal and industrial wastewater effluent. These mechanisms include the absorption of nitrogen by emergent aquatic vegetation, assimilation of nitrogen by plankton, which subsequently becomes part of organic sediment deposition in the lake and denitrification processes.

Zhu et al. (2019) reported that approximately 244 tons of nitrogen were annually introduced into the Frank Lake wetland through wastewater effluent. However, merely 5% of this amount was found to be discharged into the Little Bow River, indicating the high nitrogen treatment efficiency within the Frank Lake wetland. Nonetheless, there was still a significant impact on the Little Bow River system, as evidenced by an increase in $\delta^{15}N$ in *T. latifolia* leaf tissue samples from 3.6 ‰ in the sampling locations upstream of the water input from Frank Lake wetland drainage into Little Bow River to 5.2 ‰ in the area downstream of the Frank Lake water input.

In general, the $\delta^{15}N$ values of the *T. latifolia* leaf tissue samples collected from the sampling sites along Little Bow River were relatively low, ranging approximately from 3.6 to 5.2 ‰. In river ecosystems, denitrification is influenced by multiple factors, including the presence of nitrate and organic carbon, sediment oxygen level dynamics, particle size

and the dynamics of the sediment-water interface and hyporheic zone (Kreiling et al., 2019). Moreover, land cover and land use play a pivotal role in shaping the function of riverine ecosystems (Kreiling et al., 2019). Increased agricultural and urban development within watersheds can lead to higher potential denitrification rates compared to streams with native vegetation, primarily due to elevated surface water nitrate concentrations (Findlay et al., 2011). In addition, streams draining agricultural and urban areas often undergo changes in the particle size, shape and composition of riverbed sediments, typically resulting in a prevalence of finer sediments (Kreiling et al., 2019). The presence of fine particles can enhance denitrification by creating low oxygen conditions and providing greater amounts of organic carbon that are available for microbial processes (Kaushal et al., 2014). However, in an earlier investigation, Little et al. (2003) observed a non-significant correlation between land use and nitrate concentrations in Little Bow River. These findings suggest a potentially low denitrification rate in the Little Bow River, which may contribute to maintaining $\delta^{15}N$ levels at relatively low and relatively constant levels.

The Little Bow River serves as the primary water source for the Twin Valley Reservoir. However, Mosquito Creek is also known for carrying treated wastewater from the town of Nanton into the Twin Valley Reservoir (Town of Nanton, 2024). The relatively high $\delta^{15}N$ values of the *T. latifolia* leaf tissue samples collected from Twin Valley Reservoir could be attributed to the nitrogen content originating from Mosquito Creek. Furthermore, the Twin Valley Reservoir exhibits a prolonged water residence time, estimated at 2.3 years (Brinkmann & Rasmussen, 2010). According to Kreiling et al. (2019), regions with extended hydraulic retention times can foster low redox conditions, potentially enhancing

denitrification processes. The high $\delta^{15}N$ value of Twin Valley Reservoir may also have resulted from past events, such as the mobilization of nitrogen during the 2013 flood.

While denitrification serves as the primary mechanism altering $\delta^{15}N$ values in plant tissue in the Frank Lake wetland complex, Little Bow River and Twin Valley Reservoir, the variability in foliar $\delta^{15}N$ observed can be influenced by numerous other factors (Craine et al., 2015). These factors include nitrogen deposition, groundwater nitrogen supply and ecosystem nitrogen losses.

Nitrogen deposition

Nitrogen deposition can induce alterations in plant $\delta^{15}N$ when plants directly uptake nitrogen compounds from leaf surfaces through precipitation (wet deposition) and atmospheric particle deposition (dry deposition). Ecosystems subject to significant precipitation originating from marine sources tend to exhibit elevated $\delta^{15}N$ values, reflecting the influence of marine-derived nitrogen (Craine et al., 2015). Conversely, regions where precipitation is primarily derived from inland sources tend to exhibit lower $\delta^{15}N$ values, typically falling within the range of -3 to +1 ‰ (Houlton & Bai 2009). The nitrogen isotopic composition of atmospheric ammonium is also likely influenced by marine sources (Craine et al., 2015). The ammonium in bulk precipitation sourced from non-marine origins typically demonstrates lower $\delta^{15}N$ values than nitrates (Xiao & Liu 2004; Zhang et al. 2008). In a recent study, Zhu et al. (2019) indicated that nitrogen deposition has a minimal impact on the Frank Lake wetland complex, Little Bow River and Twin Valley Reservoir. This assertion is supported by observations indicating that solute concentrations in inland precipitation are generally low (Freeze & Cherry, 1979), and the

nitrogen dry deposition in Alberta is negligible (total N dry deposit < 0.1 % of total mass) (Government of Alberta, 2006).

Groundwater nitrogen supply

In addition, groundwater can also influence the plant $\delta^{15}N$. Approximately 99.9% of the Earth's fixed nitrogen is contained within rocks (Craine et al., 2015). This geological reservoir of nitrogen undergoes turnover over millions of years, driven by high pressures and temperatures that release nitrogen from rock formations. The significant contribution of rocks to ground and surface waters and soil systems are widely acknowledged (Craine et al., 2015). In addition, recent research revealed an increase in nitrate concentrations in shallow aquifers in regions with intensive agriculture. This rise is attributed to nitrogen fertilizers used in crop production and nitrogen from animal waste. However, Zhu et al. (2019) effectively argued that groundwater flows had no significant net contribution to the Frank Lake wetland complex. This argument was supported by the observation of low hydraulic conductivity in the soil, resulting in low hydraulic gradients, as well as the presence of 10-350 m of glacial till in the sediment beneath the Frank Lake Basins. Therefore, I suggest that the $\delta^{15}N$ value of nitrate and ammonium in Frank Lake is unlikely to be influenced by groundwater, and consequently, the $\delta^{15}N$ of the wetland plants.

Ecosystem nitrogen losses

Ecosystem nitrogen losses through gas emissions and hydrological pathways have the potential to influence the $\delta^{15}N$ values of plant tissue. The process of ammonia volatilization, where ammonium is converted into ammonia and released into the atmosphere, often leads to a significant isotopic fractionation effect (Craine et al., 2015). This phenomenon can cause the remaining ammonium in the water to become enriched

with ^{15}N, thereby affecting the $\delta^{15}N$ values of plant tissue that absorbs nitrogen from the water and sediment (Bruland & MacKenzie, 2010). However, in my study, I observed that the average concentration (± SD, n = 3) of ammonium (5.3 ± 2.2 mg $NH_4^+ L^{-1}$) in the wastewater effluent into Basin 1 was minimal compared to the concentration of nitrate (309.1 ± 29.4 mg $NO_3^- L^{-1}$). Therefore, I suggest that ammonia volatilization had no significant effect on the $\delta^{15}N$ value of the wetland plant tissue. Additionally, in alkaline conditions, ammonium produced from the mineralization of organic matter in the wetland can undergo volatilization into the atmosphere as ammonia resulting in an isotopic fractionation effect (Craine et al., 2015). According to Fernández et al. (2009), the annual conversion of organic nitrogen in the topsoil into ammonium through mineralization amounts to only 1-3 %. This proportion is deemed insignificant compared to the nitrogen input from wastewater effluent into Basin 1. Therefore, I also propose that this minimal impact had no significant influence on the $\delta^{15}N$ value of the wetland plant tissue.

Nitrification is another process that can influence the $\delta^{15}N$ value of plants (Bruland & MacKenzie, 2010). However, the relatively low concentration of ammonium in the wastewater effluent entering Basin 1 of the Frank Lake wetland complex, along with the minimal ammonium concentration produced during organic matter mineralization, typically limits nitrification in the Frank Lake wetland. Consequently, this leads to minimal impact on the $\delta^{15}N$ value of the wetland plant tissue.

Additionally, hydrological losses such as erosion and leaching do not appear to be accompanied by fractionation and thus do not impact the $\delta^{15}N$ value of the wetland plants.

Other water input pathways

Surface water runoff may also impact the $\delta^{15}N$ levels in a wetland, potentially influencing the $\delta^{15}N$ values of plant tissue. However, in 2023, surface water runoff into Basin 1 of Frank Lake was minimal due to the dry conditions experienced that year. This decrease in runoff can be attributed to the limited flow from Mazeppa and Blackie creeks, which typically supply water to Frank Lake during the spring but cease during mid-summer in dry years (Zhu et al., 2019). Consequently, these water input pathways are expected to have minimal significance on the $\delta^{15}N$ values of plant tissue in 2023.

2.4.2 Factors influencing sediment $\delta^{15}N$ variability

In this study, the observed pattern of $\delta^{15}N$ variation in sediment within the Frank Lake wetland, Little Bow River and Twin Valley Reservoir closely aligned with the pattern of $\delta^{15}N$ variation in leaf tissue samples of *T. latifolia* and *S. acutus.* This was because the mechanisms governing the changes in sediment isotopic ratios largely overlap with those affecting plants (Craine et al., 2015). Depletion of ^{14}N from available pools usually enriches the remaining nitrogen pool with ^{15}N leading to $\delta^{15}N$ enrichment in plants, microbes, phytoplankton and the organic matter they produce upon their decomposition. However, as soil organic matter turnover occurs on slower timescales, the $\delta^{15}N$ of soil organic matter, and thus the sediment, likely reflects longer-term processes compared to plant $\delta^{15}N$ (Craine et al., 2015).

Even though the sediment $\delta^{15}N$ followed the same pattern of variation in $\delta^{15}N$ of *T. latifolia* leaf tissue, the median foliar $\delta^{15}N$ values of the *T. latifolia* were higher than the median sediment $\delta^{15}N$ values in all sampling locations of the Frank Lake wetland complex except for the Basin 3- North of Island (B3N-B). Nonetheless, the $\delta^{15}N$ values of *T. latifolia*

leaf tissue samples collected from the Little Bow River and Twin Valley Reservoir locations closely resembled the $\delta^{15}N$ values of the sediment. The $\delta^{15}N$ of sediment is not a good indicator of the signature of the pool that serves as a source of nitrogen to plants annually because of the lengthy turnover rates of sediment (Craine et al., 2015). The isotopic signature of the plant-available nitrogen pool relies upon the rate at which nitrogen is supplied to the wetland, with wastewater effluent serving as a prominent nitrogen source in this scenario.

In this study, a significant relationship was observed between the $\delta^{15}N$ value of the sediment and its carbon content, also indicating a significant relationship between $\delta^{15}N$ value and organic matter content. The variability in the $\delta^{15}N$ value of the sediment across the Frank Lake wetland, Little Bow River, and Twin Valley Reservoir is likely associated with changes in organic matter content in the study area.

2.4.3 Limitations of the study

This study reveals that denitrification processes occurring in the Frank Lake wetland influence the availability of $\delta^{15}N$ within the wetland, thereby reflecting in the $\delta^{15}N$ values observed in the leaf tissue samples of *T. latifolia* and *S. acutus*. Consequently, utilizing plants growing in a wetland impacted by denitrification offers only a preliminary indication of the movement of wastewater nitrogen. However, it provides a more comprehensive understanding of the overall nitrogen flow within the system.

In this study, I presented evidence suggesting that the rates of mineralization and nitrification in the Frank Lake wetland complex are insignificant, resulting in low concentrations of ammonium and nitrate in the soil. It is observed that significant fractionation is more likely to occur only when the available nitrogen concentration in the

soil is high (Bruland & MacKenzie, 2010). Therefore, plants in the Frank Lake wetland are less likely to undergo isotope fractionation during uptake. However, in wetlands with high available nitrogen concentration in their root zones, consideration should be given to the isotope fractionation during plant nitrogen uptake as well.

2.4.4 Future research

Denitrification played a critical role in removing nitrogen from wastewater, particularly noticeable in Basin 1 of the Frank Lake wetland complex. This was evident from the increased enrichment in plant $\delta^{15}N$ compared to the wastewater effluent. However, there is concern about the extent to which denitrified nitrogen transitioned entirely into dinitrogen gas and how much was transformed into nitrous oxide, a potent greenhouse gas (Flanagan et al., 2022; Harrison et al., 2012). Further research is required to study nitrous oxide emissions from Basin 1 of the Frank Lake wetland, to evaluate whether there are any negative climate consequences.

2.5 Conclusion

The nitrogen influx from wastewater effluent into the Frank Lake wetland complex undergoes substantial uptake and processing as water passes through Basins 1, 2, and 3. Three potential mechanisms have been identified to influence this retention and removal process: (i) absorption by emergent aquatic vegetation, (ii) assimilation by plankton leading to organic sediment deposition and (iii) denitrification. Initially, the $\delta^{15}N$ values of T. *latifolia* and *S. acutus* leaf tissue and sediment samples from Basin 1 of the Frank Lake wetland complex increased, possibly due to denitrification and then decreased as the water passed through Basins 2 and 3 of the wetland. Despite the efficient nitrogen processing and removal within the Frank Lake wetland, there remains a significant impact on the downstream Little Bow River system, evidenced by elevated $\delta^{15}N$ levels in *T. latifolia* leaf tissue and sediment samples in the downstream of the Frank Lake water drainage. Moreover, the Twin Valley reservoir displays higher $\delta^{15}N$ values in both *T. latifolia* leaf tissue and sediment samples, potentially influenced by wastewater discharged from Nanton via Mosquito Creek.

Chapter 3: Determining the magnitude of denitrification in the Frank Lake wetland complex

Abstract

Municipal and industrial wastewater effluents often discharge elevated concentrations of nitrogen into natural watersheds. These high nitrogen concentrations in aquatic ecosystems are associated with severe environmental issues, including eutrophication, human health problems and alterations in overall ecosystem structure and function. Wetlands have been used to reduce nitrogen levels input from wastewater effluent before water is discharged into downstream public waterways. Wetland processes, such as nitrogen sequestration in plant biomass, accumulation in sediments, and the production of nitrogen gases via denitrification and ammonia volatilization play a vital role in retaining and removing inorganic nitrogen compounds. This study analyzes the magnitude of denitrification, that removes nitrogen as water moves through the wetland, by assessing the nitrogen isotopic composition of nitrate molecules in the water. The study revealed a substantial reduction in nitrate levels in the wastewater effluent following its discharge into Basin 1 of the wetland complex. Denitrification accounted for the removal of 27.6 % to 43.7 % of nitrate in June, 12.6 % to 17.0 % of nitrate in July, and 8.4 % to 14.0 % of nitrate in August 2023. The study provided valuable insights into the magnitude and seasonal variation in denitrification, an important ecosystem service that removes nitrogen input to the wetland from wastewater effluent.

3.1 Introduction

Even after the treatment process, wastewater discharge can retain a significant amount of nutrients (Kim et al., 2011). Among the main nitrogen forms found in treated wastewater are organically bound nitrogen, nitrate, nitrite and ammonium (Kim et al., 2011; Yousefi & Mohseni-Bandpei, 2010). The main processes contributing to the removal of inorganic nitrogen in wastewater include assimilation in plants and microorganisms, denitrification, sedimentation and volatilization (Kim et al., 2011; Lund et al., 2000; Søvik & Mørkved, 2008). However, denitrification is considered the primary mechanism for nitrogen removal in most wetlands (Hu et al., 2020; Søvik & Mørkved, 2008; Yousefi & Mohseni-Bandpei, 2010). Denitrification offers a significant advantage as it acts as a "permanent" sink, leading to the irreversible elimination of nitrogen from the wetland in gaseous forms (Lund et al., 2000). Nitrogen assimilated in wetland vegetation is regarded as a "temporary" sink, as it is expected to undergo re-mineralization sooner or later unless the vegetation is harvested (Søvik & Mørkved, 2008).

Stable nitrogen isotope measurements have proven highly effective in delineating nitrate sources, identifying nitrate transport pathways, and discerning nitrogen transformation processes such as nitrification or denitrification (Chen & MacQuarrie, 2005; Fukada et al., 2003; Knoller et al., 2011; Søvik & Mørkved, 2008; Xue et al., 2009). Denitrification is the microbial reduction of nitrate, where nitrate serves as an electron acceptor under anaerobic conditions. Nitrite produced during this reaction is subsequently reduced to nitric oxide, nitrous oxide, and ultimately to dinitrogen gas (Buyanjargal et al., 2023; Reinhardt et al., 2006; Zhang et al., 2016). Denitrification is known to induce isotopic changes in the residual nitrate pool, as bacteria selectively reduce nitrate molecules

containing lighter isotopes of nitrogen (^{14}N) (Buyanjargal et al., 2023; Hu et al., 2020; Søvik & Mørkved, 2008). This occurs because breaking or forming chemical bonds involving ^{15}N requires more energy compared to processes using ^{14}N (Robinson, 2001). Consequently, the residual nitrate pool becomes enriched with heavier nitrogen isotopes (^{15}N) due to kinetic isotope effects (Granger et al., 2008). The difference in mass-dependent characteristics leads to a phenomenon termed isotopic fractionation (Buyanjargal et al., 2023).

A closed-reaction system undergoing isotopic fractionation can be studied using the Rayleigh fractionation model. According to the model, if a substrate and its product retain an identical $^{15}N/^{14}N$ ratio, no fractionation occurs. In this scenario, the ratio of $\delta^{15}N$ of the product to $\delta^{15}N$ of the substrate, also known as the enrichment factor (α), equals unity (Figure 3.1). Any deviations in α from unity signify isotopic fractionation (Robinson, 2001; Søvik & Mørkved, 2008). To express fractionation on the δ scale, α values can be converted as follows:

$$\varepsilon = 1000 (1- \alpha)$$

Where, ε is the fractionation factor representing the difference in $\delta^{15}N$ between a substrate and its immediate product.

For a given reaction, ε varies depending on external factors, such as temperature (Robinson, 2001). The fractionation factor becomes negative when the substrate is isotopically heavier than the product (Søvik & Mørkved, 2008). The magnitude of fractionation plays a crucial role in determining the isotopic enrichment during nitrate reduction. A precise understanding of ε is essential for accurately characterizing and

quantitatively assessing nitrate reduction during denitrification (Knoller et al., 2011). Several studies offer comprehensive overviews of isotope fractionation factors derived from numerous culture experiments and field studies in natural settings. Lehmann et al. (2003) demonstrated a wide range of variation in ε values for nitrogen isotopes during denitrification, from -5 ‰ to -40 ‰, indicating a significant dependence of ε on diverse experimental and environmental conditions, as well as reaction pathways and the enzymes involved. Granger et al. (2008) conducted a comprehensive investigation into the nitrogen isotopic fractionation of nitrate during denitrification by studying a diverse array of laboratory cultures of denitrifying bacteria. The Granger et al. (2008) study documented a range of 5.46 ± 0.3 ‰ to 26.66 ± 0.5 ‰ in ε for nitrogen within nitrate molecules during denitrification.

During denitrification, the $\delta^{15}N$ of residual substrate changes according to the Rayleigh fractionation model as follows:

$$\delta_S = \delta_0 + \varepsilon \ln (1-f) \qquad \textit{Equation 1}$$

Where, δ_S is the $\delta^{15}N$ value of the residual nitrate molecules at time t, δ_0 is the initial $\delta^{15}N$ value of nitrate molecules and f is the fraction of the substrate that has been converted into product (Figure 3.1) (Robinson, 2001). Simultaneously, $\delta^{15}N$ of the product (δ_P) changes as follows:

$$\delta_P = \frac{\varepsilon (1-f) [\ln (1-f)]}{f} \qquad \textit{Equation 2}$$

Previous research conducted at the Frank Lake wetland complex has suggested that denitrification is a potentially significant mechanism for nitrogen removal from wastewater

input (White & Bayley, 2001; Zhu et al., 2019). Furthermore, it has been calculated that denitrification may account for the removal of approximately 40.8% to 65.7% of nitrogen input into the Frank Lake wetland complex from industrial wastewater inputs (Flanagan et al., 2022). In this chapter, the focus was on examining the contribution of the Frank Lake wetland complex to the removal of wastewater nitrogen via denitrification. The Rayleigh fractionation model was employed as a robust method to enhance the accuracy and refinement of the findings from earlier research.

While denitrification plays a significant role in nitrogen removal from wastewater effluent, the uptake of nitrogen by plants and phytoplankton also holds significance in wetland ecosystems (Lund et al., 2000). Chlorophyll 'a' concentration in phytoplankton serves as a widely recognized indicator of phytoplankton biomass in aquatic environments. Given that phytoplankton biomass substantially contributes to the Particulate Organic Nitrogen (PON) pool, there exists a direct correlation between chlorophyll 'a' concentration and PON concentration in water (Desortová, 1981). Hence, previous studies have utilized chlorophyll 'a' concentration to gauge the extent of nitrate-nitrogen consumption by phytoplankton (Desortová, 1981). This approach allows the quantification and comparison of nitrogen removal by various processes within the ecosystem.

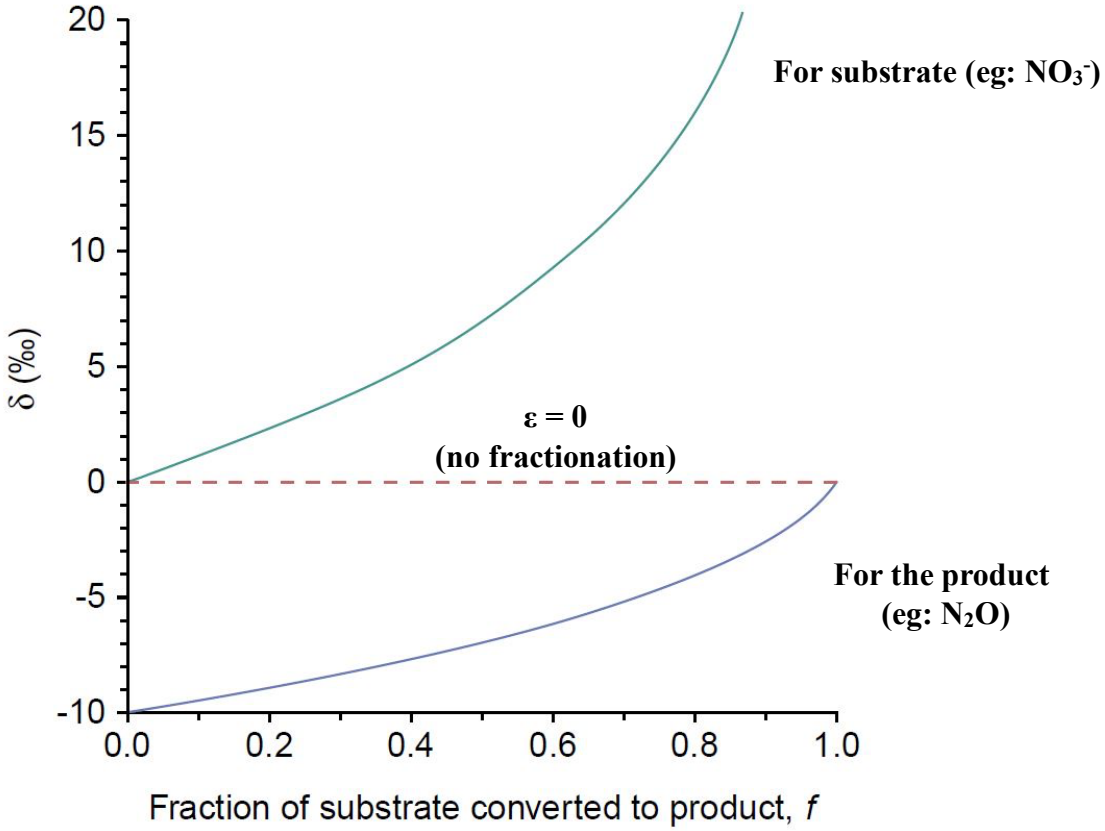


Figure 3.1: The relationship between the δ value (‰) and the fraction of sustrate converted into product (*f*) with a ^{15}N fractionation factor (ε) of 10 ‰, according to the Rayleigh fractionation model (Robinson, 2001). In this case, the $\delta^{15}N$ of residual substrate (NO_3^-) is described by the *equation 1* (upper curve) and the $\delta^{15}N$ of of the accumulated product (N_2O) is decribed by the *equation 2* (lower curve).

3.2 Methods

3.2.1 Water sample collection

To determine the magnitude of denitrification in the Frank Lake wetland complex, water samples were collected along two transects within both Basin 1 and Basin 2 of the wetland complex. The first transect extended from the wastewater effluent input in Basin 1 to the Basin 1 outlet, while the second transect stretched from the Basin 1 outlet to the Basin 2 outlet. Both transects were 1740 m in length. I collected water samples along the transects with three replicates from each of the sampling locations. Sampling locations were placed at equal distances (435 m) along the transects and at least 20 m away from the shore. The three replicates of each sampling location were spaced 20 m apart from one another (Figure 3.2). Additional water samples were collected from the Basin 1 wastewater effluent input. Sample collections were carried out on June 7, 2023, July 20, 2023 and August 31, 2023.

Prior to water sample collection, new collection bottles underwent an acid wash using a diluted hydrochloric acid solution. The solution was swirled to ensure it coated all internal surfaces of the bottles, allowed to soak for 48 hours, followed by a thorough rinse with deionized water. The bottles were then air-dried in a controlled, clean environment. The collected water samples were stored in ice until they reached the laboratory.

Upon arrival at the laboratory, collected samples were filtered through a 0.45 μm Whatman capsule filter (Hu et al., 2020) and transferred into appropriately labeled clean bottles. These bottles were then stored in a refrigerator until they were transported for analysis. The $\delta^{15}N$ values of the nitrate molecules in all the water samples were analyzed at the Stable Isotope Lab at the University of Calgary, Alberta, Canada using bacteria denitrifier method (University of Calgary, 2024a) (Figure 3.3).

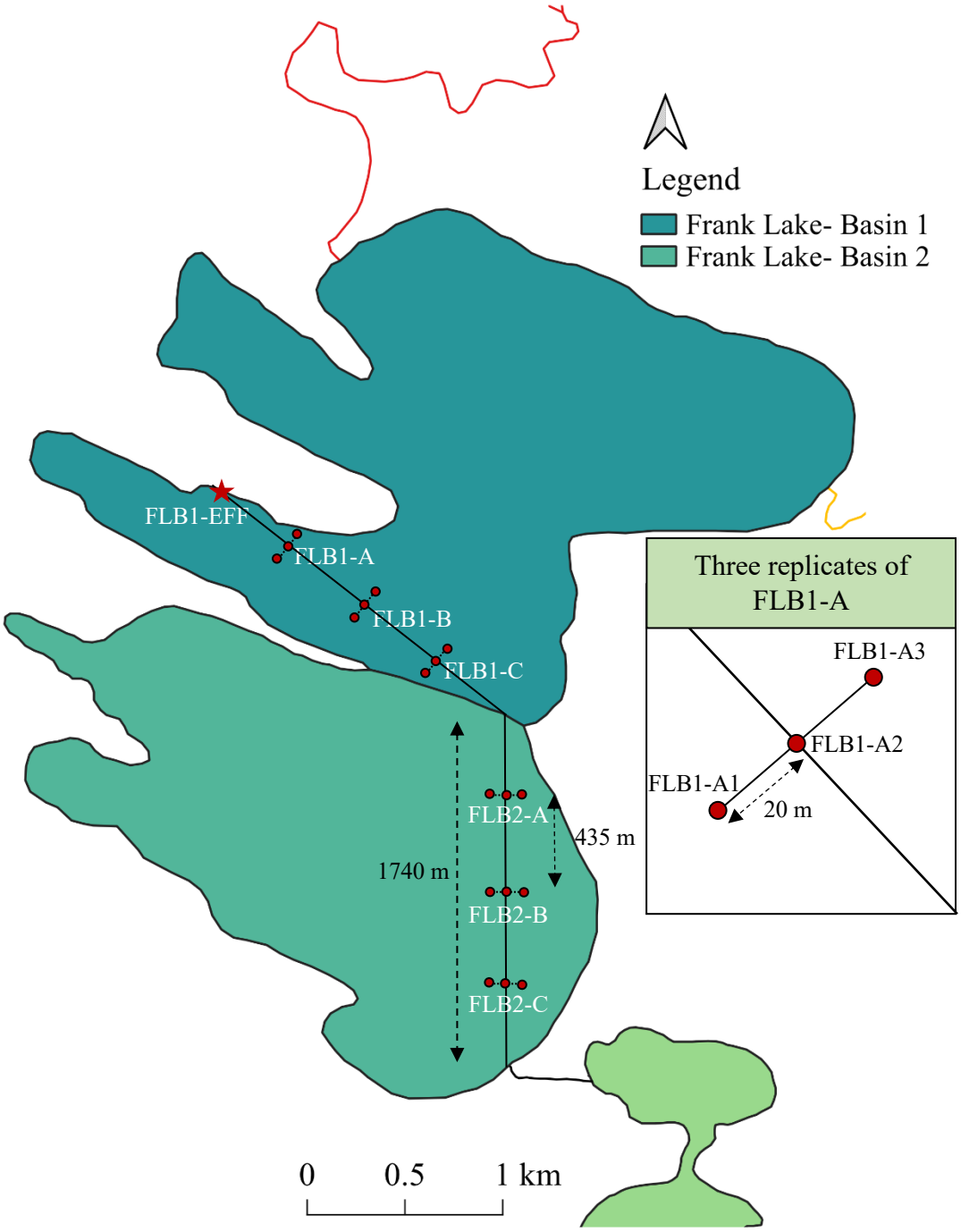


Figure 3.2: Water sampling locations within Basin 1 (FLB1) and Basin 2 (FLB2) of the Frank Lake wetland complex. Two transects, each spanning 1740 m, stretched from the Basin 1 wastewater effluent to the Basin 1 outlet and from the Basin 1 outlet to the Basin 2 outlet. Sampling locations were placed at equal distances (435 m) along the transects, with replicates positioned 20 m apart from each other.

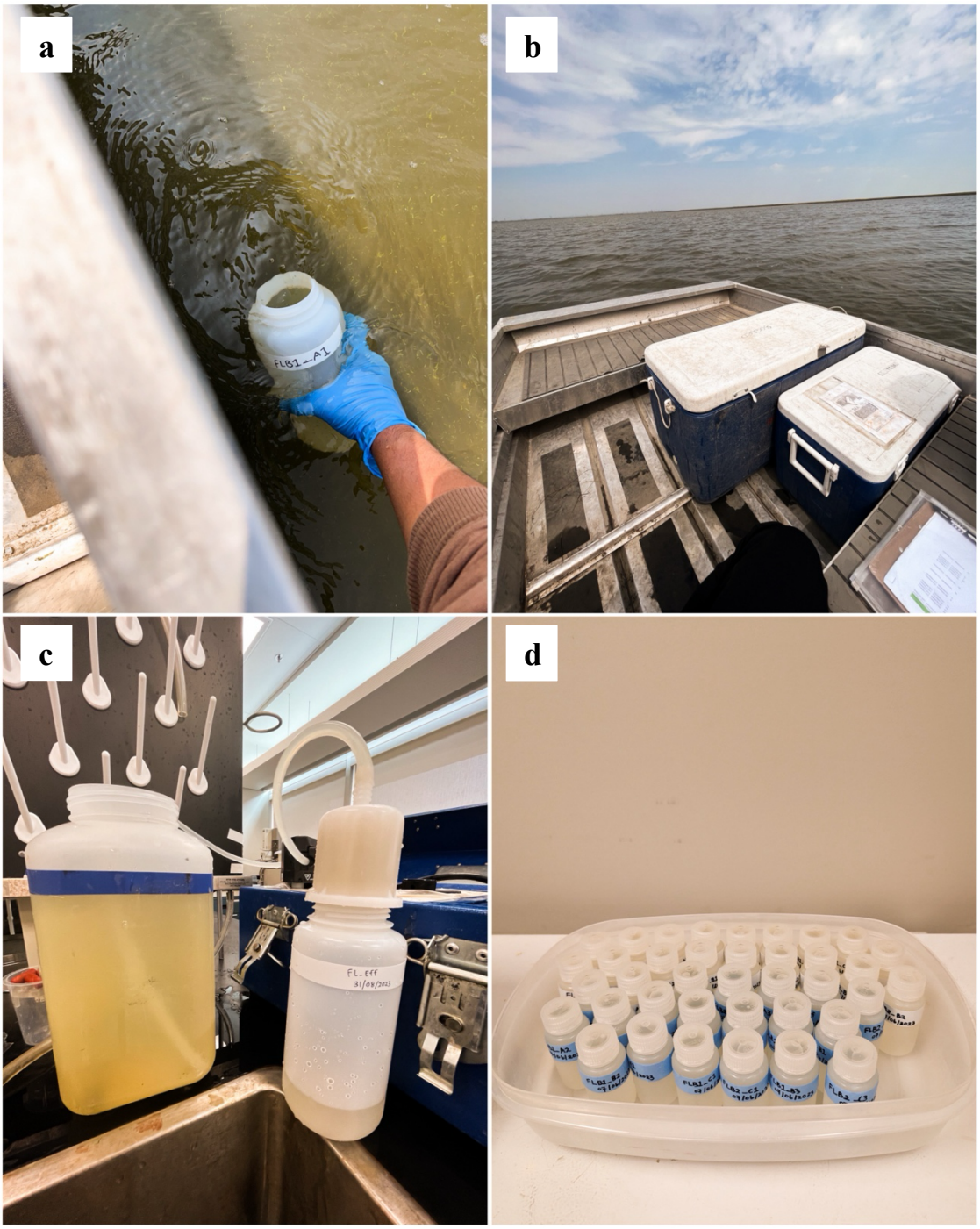


Figure 3.3: a) Water samples were collected along two transects from three positions each in Basin 1 and 2. b) The collected water samples were stored on ice until they reached the laboratory. c) The water samples were then filtered through a 0.45 µm membrane filter and transferred into appropriately labeled clean bottles. d) The $\delta^{15}N$ values of nitrate in all water samples were analyzed at the Stable Isotope Lab at the University of Calgary, Alberta, Canada.

In addition, water temperature, specific conductivity, pH and dissolved oxygen concentration were measured at all sampling locations using a multiparameter water quality sonde (YSI EXO1), which was calibrated on site each time.

3.2.2 Statistical Analysis

The total nitrate concentrations along the three transect positions of Basin 2 of the Frank Lake wetland complex were notably low, with most measurements falling below the detection limit. Consequently, the statistical analysis was exclusively directed toward samples from Basin 1. Bar charts were used to visualize the variation in total nitrate concentration along the three transect positions of Basin 1 during June, July and August. Additionally, a regression analysis was performed to test for a significant relationship between the total nitrate concentration of Basin 1 and the distance from the wastewater effluent input in each month. All the statistical tests and the graphs were generated using R (R version 2022.12.0, ©Posit Software, PBC).

As the wastewater entered Basin 1, nitrate concentrations were drastically reduced in each month. In addition, minimal fluctuations in both total nitrate concentrations and nitrogen isotopic measurements were observed across all the transect points of Basin 1. As a result, the total nitrate concentration and $\delta^{15}N$ values from all transect points of Basin 1 were averaged to derive single representative values for Basin 1, which were then utilized for subsequent analysis.

The Rayleigh fractionation model was applied to nitrogen isotopic measurements to estimate the percentage of total nitrate consumed during denitrification in each month.

Granger et al. (2008) provided the groundwork for this research by determining the fractionation factor (ε) of nitrogen in nitrate molecules during denitrification.

The chlorophyll content data in the water from Basin 1 of the Frank Lake wetland was used to assess the potential role of phytoplankton nitrogen uptake in nitrate removal. Chlorophyll 'a' is often used as an indicator of phytoplankton biomass (Desortová, 1981). Therefore, chlorophyll 'a' data from Basin 1 were used to estimate the amount of nitrogen in organic matter produced by phytoplankton. This approach allowed a more quantitative comparison between uptake of nitrogen (nitrate-nitrogen) by phytoplankton compared to the amount of nitrogen (nitrate-nitrogen) converted to nitrous oxide or dinitrogen gas via denitrification. The chlorophyll data utilized in this study were acquired from the Bogard lab through their routine water sampling procedures conducted in Basin 1 of the Frank Lake wetland in June, July and August 2023.

3.2.3 The nitrate ^{15}N fractionation factor for denitrification

Granger et al. (2008) conducted a detailed investigation involving individual experiments with five denitrifying bacterial species measured under different nitrate concentrations in both fresh and sea water conditions to analyze the fractionation of nitrate ^{15}N during denitrification. The fractionation factor (ε) employed in the current study was derived from the findings of Granger et al. (2008), yielding a mean (± SD) value of 17.8 ± 5 ‰ (Figure 3.4). This value was subsequently utilized to estimate the magnitude of denitrification in Basin 1 of the Frank Lake wetland complex using the Rayleigh fractionation model.

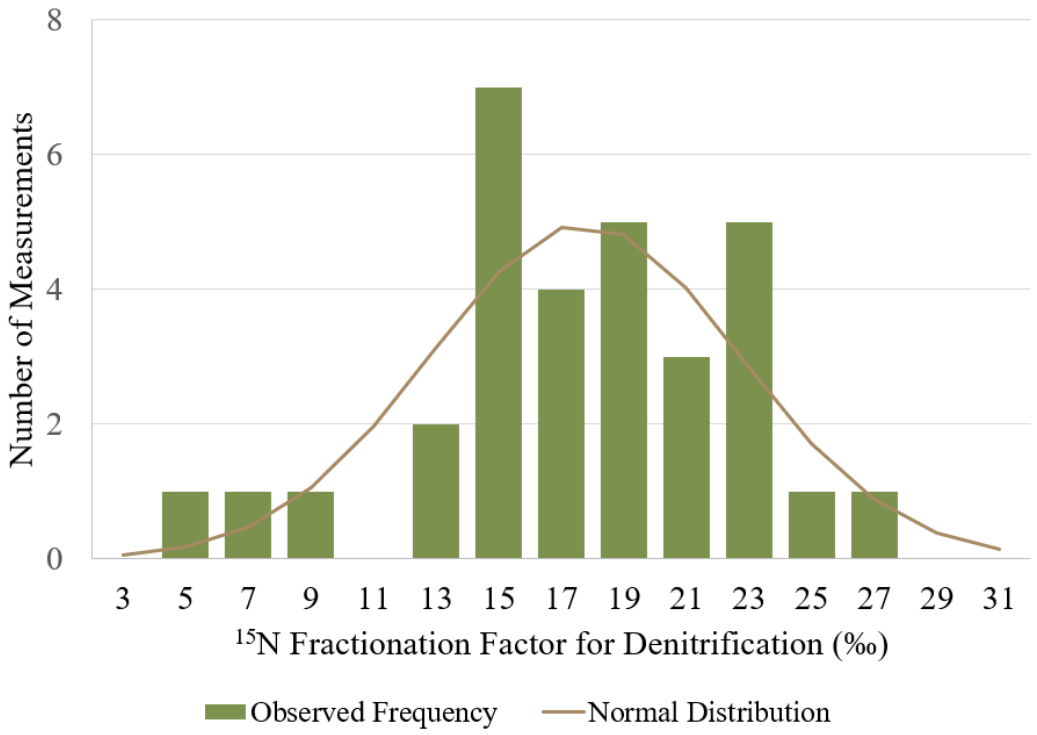


Figure 3.4: Frequency distribution of fractionation factors (ε) for denitrification obtained from individual experiments with denitrifying bacterial species (n = 5) under different nitrate concentrations in both fresh and sea water conditions by Granger et al. (2008) with an observed mean (± SD) of 17.8 ± 5 ‰.

3.2.4 Calculations

Determining the magnitude of denitrification

The reduction of total nitrate concentration (*f*) within the Basin 1 was calculated as follows:

$$f = \frac{TON_{FLB1}}{TON_{FL\text{-}EFF}}$$

Where, $TON_{FL\text{-}EFF}$ is the total nitrate concentration in the wastewater effluent input into Basin 1 of the Frank Lake wetland complex and TON_{FLB1} is the average total nitrate concentration along the transect points of Basin 1.

Considering the potential contribution of Basin 1 vegetation to nitrate removal, the fraction of nitrate removed by emergent plants was subtracted from the original total nitrate reduction fraction. This yielded the fraction of nitrates removed during denitrification and other nitrate removing processes such as phytoplankton uptake (f') (Figure 3.5).

$$f' = f - f_P$$

Where, f_P is the fraction of nitrate removed by the Basin 1 plants. Flanagan et al. (2022) calculated this value to be 8.7%.

The Rayleigh fractionation model was employed to derive the corresponding fraction of nitrate removed based on the average $\delta^{15}N$ values across the transect points within the Basin 1 (f'') (Figure 3.5). Then, the percentage of nitrate removed during denitrification was calculated as follows,

$$\text{Denitrification (\%)} = \frac{f''}{f'} \times 100$$

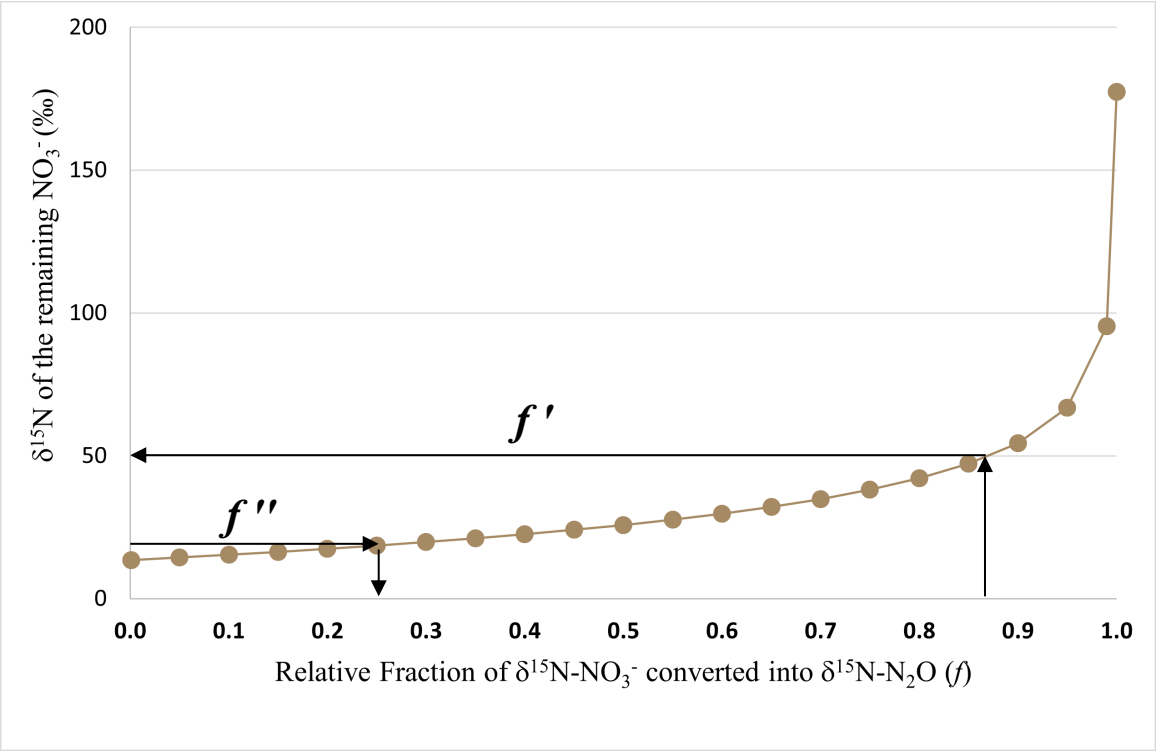


Figure 3.5: Rayleigh fractionation model showing the exponential relationship (ε = 17.8) between the $\delta^{15}N$ of the remaining nitrate molecules in water (‰) and the fraction of nitrate converted into product (*f*).

Estimating Particulate Organic Nitrogen (PON) concentration

I estimated Particulate Organic Nitrogen (PON) concentration in the water from the measurements of phytoplankton chlorophyll 'a' as follows.

First, I estimated the mass of phytoplankton chlorophyll 'a' in Basin 1 of the Frank Lake wetland (M_{chla}) (µg) as follows,

$$M_{chla} = C_{chla} \times V_{B1}$$

Where, C_{chla} is the chlorophyll 'a' concentration in Basin 1 (µg L^{-1}) and V_{B1} is the estimated volume of Basin 1 of the Frank Lake wetland (L). I calculated V_{B1} as follows,

$$V_{B1} = A_{B1} \times D_{B1}$$

Where, A_{B1} is the area of the Basin 1 (m^2) and D_{B1} is the average depth of Basin 1 (m). According to Zhu et al. (2019), Basin 1 of the Frank Lake wetland has an A_{B1} value of 5,100,000 m^2 and a D_{B1} value of 0.67 m. This resulted in a V_{B1} for Basin 1 of the Frank Lake wetland of 3,417,000 m^3 or 3,417,000,000 L (1 m^3 = 1000 L). Then, the biomass of the phytoplankton chlorophyll 'a' (B_{chla}) (µg) was calculated assuming chlorophyll 'a' is 1 % of the biomass of Basin 1 of the Frank Lake wetland (Desortová, 1981).

$$B_{chla} = M_{chla} \times 100$$

Then, I estimated carbon content of the phytoplankton chlorophyll 'a' biomass ($Carbon_{chla}$) (µg), assuming the carbon content is ~ 45 % of the biomass (Desortová, 1981).

$$Carbon_{chla} = B_{chla} \times \frac{45}{100}$$

The mass of PON (M_{PON}) (µg) was then estimated using the Redfield ratio of carbon to nitrogen to phosphorus (C:N:P). The Redfield Ratio is the consistent ratio of 106:16:1 of C:N:P observed in phytoplankton (Redfield, 1958).

$$M_{PON} = Carbon_{chla} \times 0.151$$

Then, the concentration of PON (C_{PON}) (µg L^{-1}) was calculated as follows,

$$C_{PON} = \frac{M_{PON}}{V_{B1}}$$

3.3 Results

3.3.1 Variation of water quality parameters in Basin 1

The water temperature in Basin 1 of the Frank Lake wetland complex fluctuated across the water sampling dates of June, July and August, ranging from 17.1 ± 0.2 °C on August 31, 2023 to 20.6 ± 0.8 °C on July 20, 2023. However, the pH of the water in Basin 1 tended to increase slightly over time, with the lowest value of 8.4 ± 0.0 recorded on June 7, 2023 and the highest value of 9.1 ± 0.1 observed on August 31, 2023. The dissolved oxygen concentration of water in Basin 1 also increased over time, starting at 5.4 ± 0.2 mg L^{-1} on June 7, 2023 and plateauing at 7.2 ± 0.9 mg L^{-1} on July 20, 2023.

Table 3.1: The water temperature, pH, conductivity and dissolved oxygen concentration of Basin 1 of the Frank Lake wetland complex in June, July and August 2023 (Values are means ± SD, n = 9).

Date	Temperature (°C)	pH	Dissolved oxygen concentration (mg L^{-1})	Conductivity (µS m^{-1})
June 07, 2023	19.4 ± 0.5	8.4 ± 0.0	5.4 ± 0.2	2654.8 ± 3.4
July 20, 2023	20.6 ± 0.8	8.7 ± 0.0	7.2 ± 0.9	3145.9 ± 12.2
August 31, 2023	17.1 ± 0.2	9.1 ± 0.1	7.2 ± 0.5	3293.9 ± 6.3

3.3.2 Variation of nitrate concentration in Basin 1

The highest total nitrate concentration was observed at the wastewater effluent input, reaching 364.3 mg NO_3^- L^{-1} in June, 299.2 mg NO_3^- L^{-1} in July and 263.9 mg NO_3^- L^{-1} in August 2023 (Figure 3.6). The water exiting Basin 1 exhibited a significant decrease in total nitrate concentration. This reduction was 95.3 % in June and July. Similarly, a drastic reduction of 92.2 % in total nitrate concentration was observed in August.

A regression analysis was conducted to test for a significant relationship between the total nitrate concentration and the distance from the wastewater effluent input along the transect of Basin 1. The results showed a significant relationship between the total nitrate concentration and the distance from the wastewater effluent input in June (Figure 3.7, Exponential regression: $R^2 = 0.7467$, $P < 0.05$) and July (Exponential regression: $R^2 = 0.7757$, $P < 0.05$) and August 2023 (Exponential regression: $R^2 = 0.6488$, $P < 0.05$).

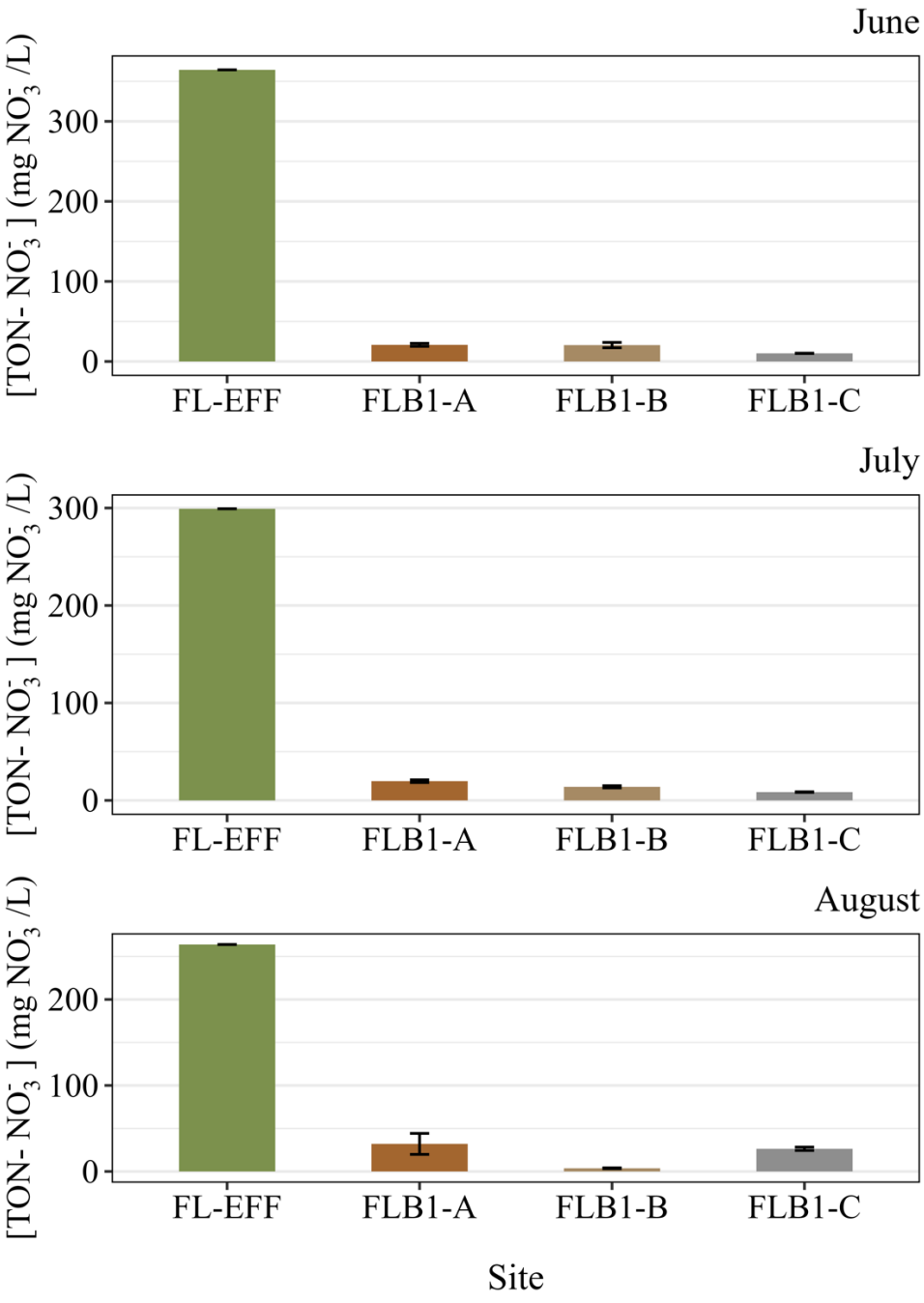


Figure 3.6: The variation of total oxidized nitrate concentration [TON-NO_3^-] (mg NO_3^- / L) along the transect of the Frank Lake Basin 1, which extended from the wastewater effluent input (FL-EFF; distance = 0 m) to the Basin 1 outlet (distance = 1740 m) in June, July and August 2023. Sampling locations (FL- EFF, FLB1-A, FLB1-B and FLB1-C) are shown in Figure 3.2.

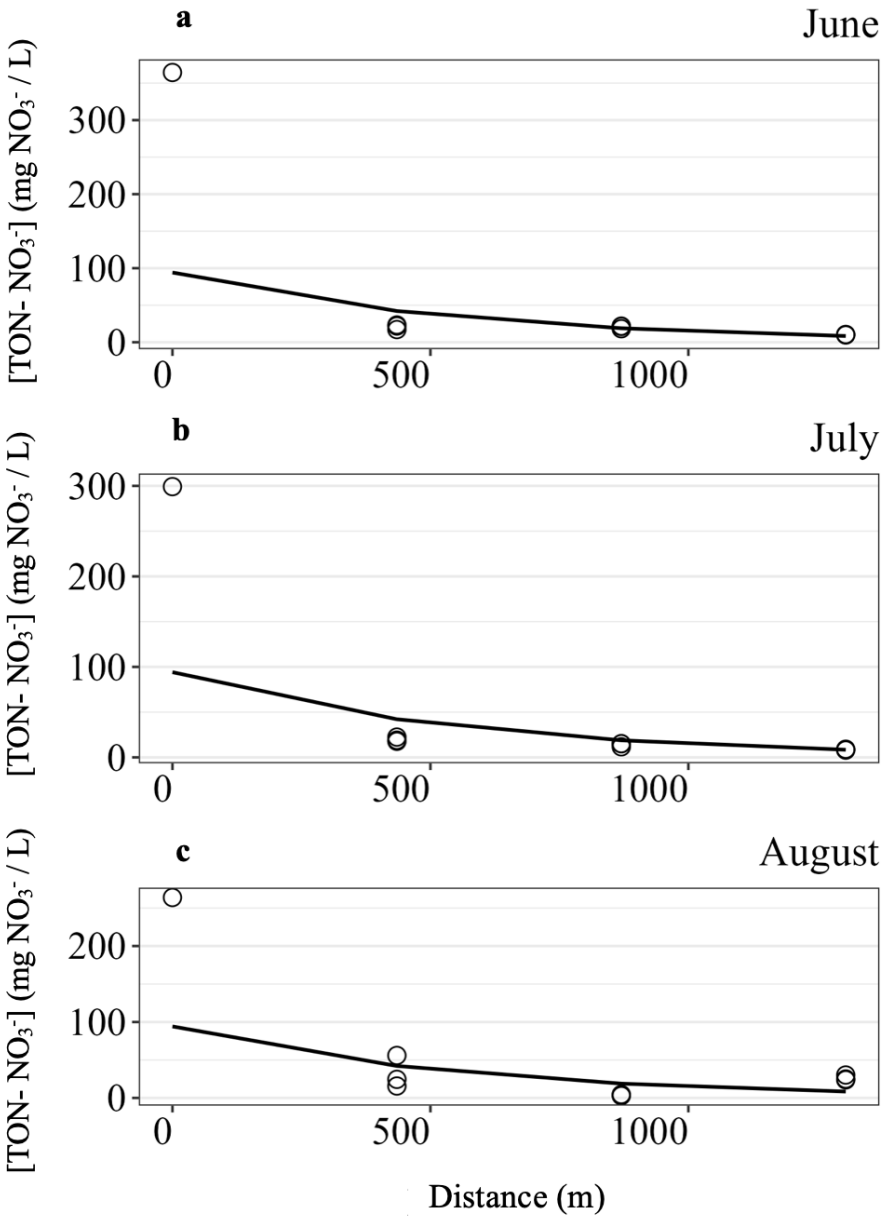


Figure 3.7: The relationship between the total nitrate concentration [TON-NO_3^-] (mg NO_3^- /L) and the distance along the transect of the Frank Lake Basin 1, which extended from the wastewater effluent input (FL-EFF; distance = 0 m) to the Basin 1 outlet (distance = 1740 m) in June, July and August 2023. Sampling locations (FL- EFF, FLB1-A, FLB1-B and FLB1-C) are shown in Figure 3.2. (a) the curve represents an exponential regression fitted to the data from June 2023: $y = 94.134e^{-0.002x}$, $R^2 = 0.7467$, $P < 0.05$ (b) the curve represents an exponential regression fitted to the data from July 2023: $y = 82.899e^{-0.002x}$, $R^2 = 0.7757$, $P < 0.05$ (c) the curve represents an exponential regression fitted to the data from August 2023: $y = 52.007e^{-0.001x}$, $R^2 = 0.6488$, $P < 0.05$.

3.3.3 Variation of $\delta^{15}N$ in Basin 1

The $\delta^{15}N$ of the nitrate molecules of the wastewater input into the Basin 1 of the Frank Lake wetland complex were recorded as 13.5 ‰, 17.0 ‰ and 14.6 ‰ in June, July and August 2023 respectively. The mean $\delta^{15}N$ values of Basin 1 highlighted an enrichment of $\delta^{15}N$ in the remaining nitrate molecules in the water. These increments amounted to 45.3 % in June, 15.2 % in July, and 15.7 % in August 2023 (Figure 3.8).

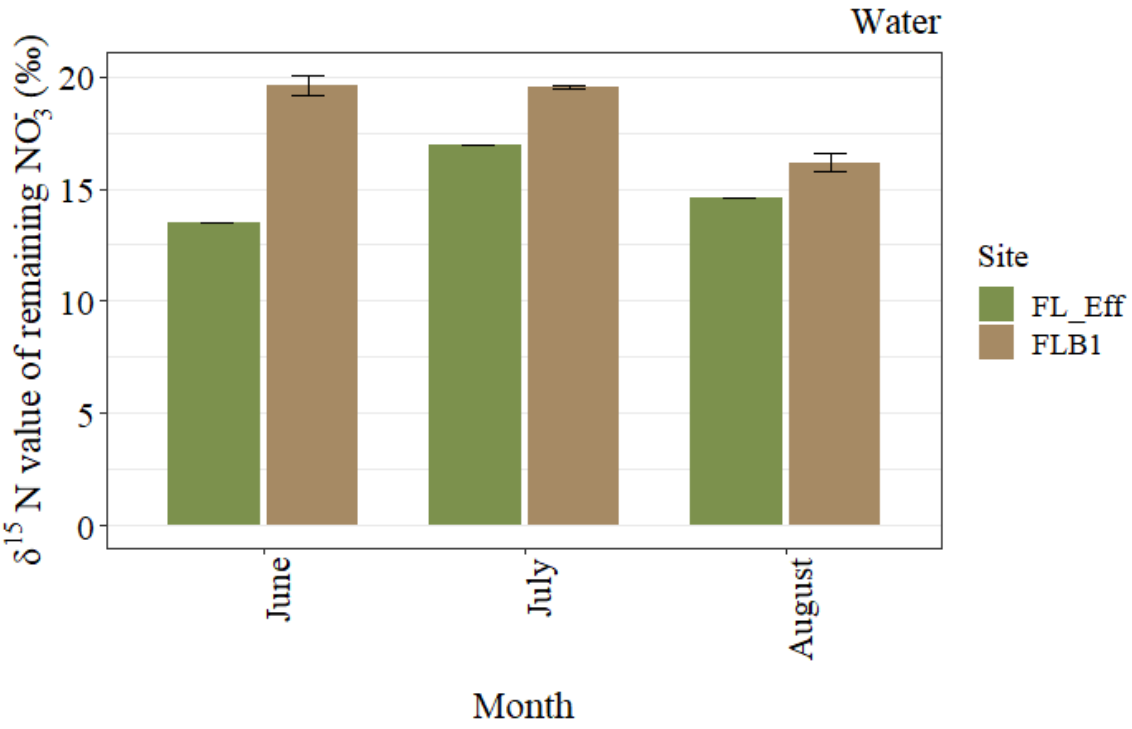


Figure 3.8: The $\delta^{15}N$ of remaining nitrate (‰) at the wastewater input to Basin 1 and the water flowing out of Basin 1. The abbreviations are as follows: FL- EFF: Wastewater effluent input into the Basin 1 (n = 1), FLB1: Frank Lake Basin 1 transect mean (Values are means ± S.E, n = 9).

3.3.4 Magnitude of denitrification in Basin 1

As previously stated, the total nitrate concentration in the wastewater effluent decreased drastically within Basin 1 in June, July, and August 2023. These reductions amounted to 95.3 % in both June and July and 92.2 % in August. Within Basin 1 of the Frank Lake wetland complex, the known rate of nitrogen removal through emergent plant uptake stands at 8.7 % (Flanagan et al., 2022). Therefore, denitrification and other nitrate removal processes contributed to 86.6 % of nitrate removal in June and July, and 83.5 % in August.

According to the Rayleigh fractionation model, if all the nitrate was removed by denitrification, the $\delta^{15}N$ of the remaining nitrate molecules in the water leaving the Basin 1 should be 49.8 ‰ in June, 53.3 ‰ in July and 46.1 ‰ in August (Figure 3.8). However, the observed $\delta^{15}N$ of the remaining nitrate molecules of the water was 19.6 ‰ in June, 19.5 ‰ in July and 16.2 ‰ in August. These findings revealed that denitrification was not the only process removing nitrate as measured $\delta^{15}N$ values were much lower than the values noted above if denitrification was the only process removing nitrates. It was calculated as 27.6 - 43.7 % in June, 12.6 - 17.0 % in July and 8.4 - 14.0 % in August 2023 (Table 1). The rest of the nitrates must have been removed by other nitrate removing processes, particularly phytoplankton uptake.

Table 3.2: Percentage denitrification (%) within the Basin 1 of the Frank Lake wetland complex in June, July and August 2023 ($\varepsilon = 17.8 \pm 5$ ‰).

Month	Percentage denitrification at $\varepsilon = 17.8 \pm 5$ ‰ (%)
June	27.6 - 43.7
July	12.6 - 17.0
August	8.4 - 14.0

3.3.5 Nitrate uptake by phytoplankton in Basin 1

Both the chlorophyll 'a' concentration of the phytoplankton and the PON concentration in the water increased from June to August 2023. In June 2023, the chlorophyll 'a' concentration of the phytoplankton was 24.1 µg L^{-1} and the PON concentration in the water was 163.7 µg N L^{-1}. By August 2023, the chlorophyll 'a' concentration had increased to 144.6 µg L^{-1}, and the PON concentration had risen to 982.7 µg N L^{-1} (Figure 3.9).

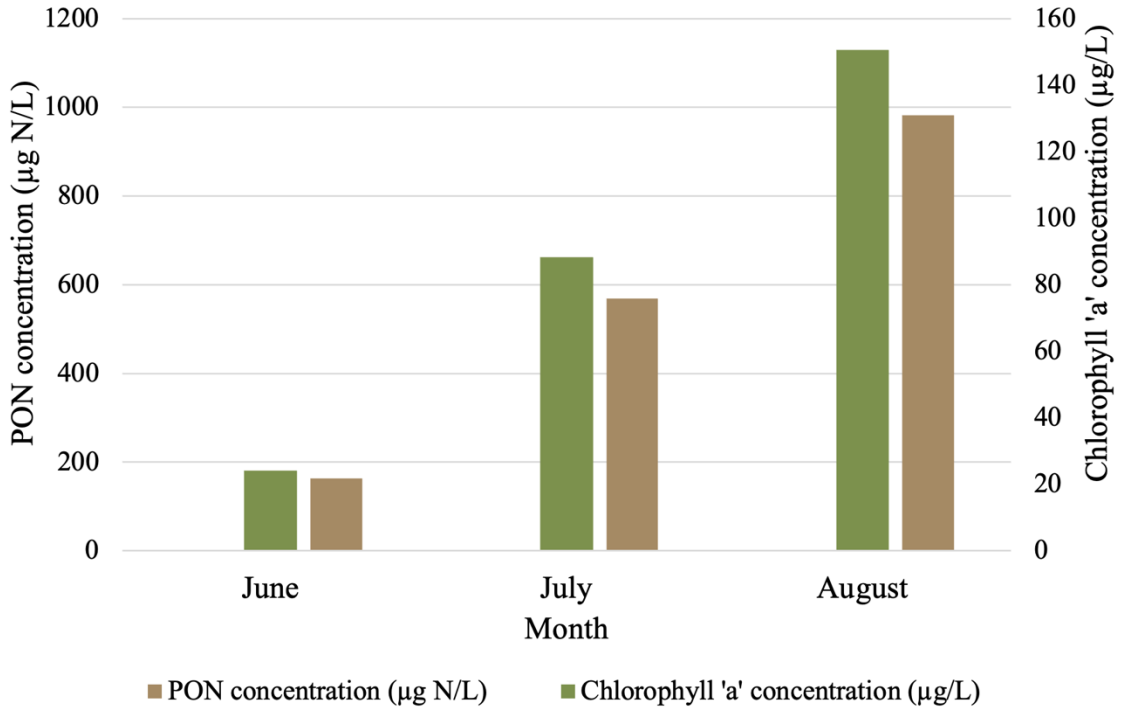
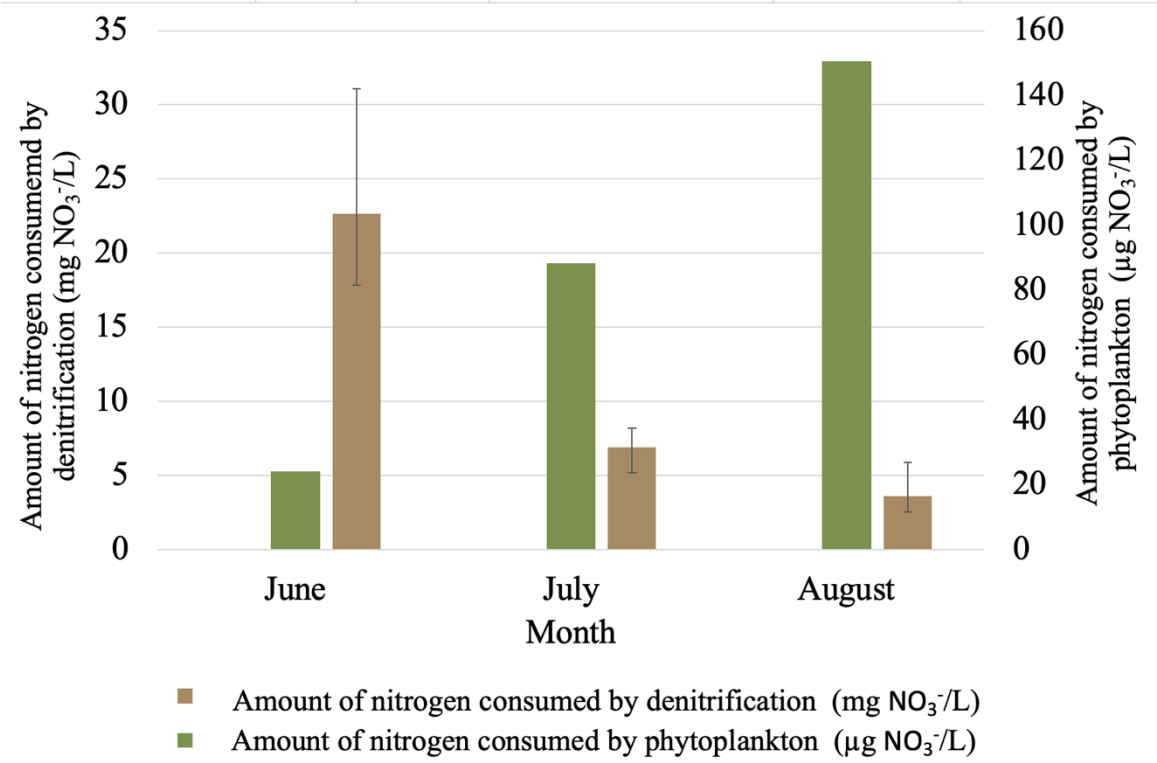


Figure 3.9: The variation of chlorophyll ‘a’ content of the phytoplankton (µg L^{-1}) (green bars) and the PON concentration in the water (µg N L^{-1}) (brown bars) within the Basin 1 of the Frank Lake wetland complex in June, July and August 2023. Both chlorophyll ‘a’ content of the phytoplankton and the PON concentration in the water increased from June to August 2023.

The nitrate-nitrogen uptake by phytoplankton, as indicated by PON concentration, was highest in August 2023 (982.7 µg NO_3^- L^{-1}) and lowest in June 2023 (163.7 µg NO_3^- L^{-1}). In contrast, nitrate-nitrogen consumption during denitrification was highest in June 2023 (22.7 mg NO_3^- L^{-1}) and lowest in August 2023 (3.6 mg NO_3^- L^{-1}). When nitrate-nitrogen consumption by phytoplankton was at its lowest, denitrification reached its highest nitrate-nitrogen consumption. Conversely, during periods of peak nitrate-nitrogen consumption by phytoplankton, denitrification consumed the least nitrate-nitrogen (Figure 3.10).



Fugure 3.10: The variation of nitrate – nitrogen consumption by phytoplankton (µg NO_3^- /L) (green bars) and denitrification (mg NO_3^-/L) (brown bars) within the Basin 1 of the Frank Lake wetland complex in June, July and August 2023. Nitrate-nitrogen consumption by denitrification peaked during periods of low nitrate-nitrogen consumption by phytoplankton and decreased during periods of high nitrate-nitrogen consumption by phytoplankton.

3.4 Discussion

3.4.1 Nitrate removal

The nitrate concentration in the wastewater effluent experienced a significant reduction upon reaching the first transect point of Basin 1 of the Frank Lake wetland complex during June, July, and August of 2023. The decline in nitrate concentration is attributed to processes such as denitrification and assimilation by plants and phytoplankton, which actively consume nitrates in the water. In June 2023, the removal of nitrates from the wastewater effluent into Basin 1 of the Frank Lake wetland complex peaked at 95.3 %. Assuming 8.7 % of these nitrates were taken up by plants (Flanagan et al., 2022), it was determined that denitrification processes removed approximately 27.6 - 43.7 % of the remaining nitrates, as per the Rayleigh Fractionation model. By August 2023, the removal efficiency decreased slightly to 92.2%. It was discovered that only 8.4 - 14.0 % of the nitrates were removed through denitrification processes during the same period.

The phytoplankton abundance, measured by chlorophyll 'a' concentration in Basin 1 of Frank Lake, was at its lowest (24.1 $\mu g\ L^{-1}$) in June 2023 and highest (144.6 $\mu g\ L^{-1}$) in August 2023. Similarly, PON concentration increased from 163.7 $\mu g\ N\ L^{-1}$ in June 2023 to 982.7 $\mu g\ N\ L^{-1}$ in August 2023. The concentration of PON in the wetland's water represents the nitrogen contained within phytoplankton cells and other particulate organic matter. As phytoplankton grow and multiply, they consume nitrogen (nitrate, ammonium and organic nitrogen forms such as urea and amino acids) from the water and convert them into organic nitrogen compounds, thereby increasing the PON concentration (Glibert et al., 2016). This process also leads to an increase in their biomass, which is reflected in their chlorophyll 'a' concentration. Therefore, there is a correlation between PON concentration and chlorophyll

'a' concentration. Higher chlorophyll 'a' levels indicate greater phytoplankton biomass, which corresponds to higher PON levels due to the enhanced incorporation of inorganic nitrogen into biomass (Desortová, 1981).

Since wastewater input into Frank Lake wetland has significantly lower ammonium concentrations compared to nitrate, it is reasonable to assume that phytoplankton primarily rely on nitrate as their nitrogen source in Frank Lake. Consequently, PON concentration and chlorophyll 'a' concentration in the water may indirectly reflect nitrate nitrogen uptake by phytoplankton (Desortová, 1981). When using chlorophyll ‘a’ as an indirect indicator for nitrate nitrogen uptake by phytoplankton and comparing it with the consumption of nitrate nitrogen during denitrification, it becomes apparent that between June and August 2023, the consumption of nitrate nitrogen by denitrification gradually decreased. This decline aligns with a progressive increase in nitrate nitrogen uptake by phytoplankton. The absorption of nitrate by phytoplankton decreased the available nitrate for denitrification, resulting in lower denitrification rates in the water.

However, the calculated amount of nitrate consumed by denitrification (mg $NO_3^- L^{-1}$) was found to be several orders of magnitude higher than the estimated nitrate used in the formation of PON (μg $NO_3^- L^{-1}$). This discrepancy may be attributed to the high turnover rates of PON. Essentially, PON may be rapidly deposited into the sediment, resulting in significant nitrate consumption even if the water consistently has a relatively low concentration of PON. In this case, the concentration of PON would not accurately reflect the flux of nitrate into PON. However, this speculation warrants further investigation.

3.4.2 Factors influencing $\delta^{15}N$ of the nitrate molecules in water

Denitrification is the main process influencing $\delta^{15}N$ of the residual nitrate pool in water (Buyanjargal et al., 2023; Hu et al., 2020; Søvik & Mørkved, 2008). The process of denitrification is strictly an anoxic process and is sensitive to oxygen levels (Lu et al., 2009). Nevertheless, denitrification processes have been observed in wetland ecosystems with detectable dissolved oxygen levels in their surface waters (Phipps & Crumpton, 1994). In this study, the average dissolved oxygen concentrations (± SD, n = 9) in the water column of Basin 1 within the Frank Lake wetland were measured at 5.4 ± 0.2 mg L^{-1} in June, 7.2 ± 0.9 mg L^{-1} in July and 7.2 ± 0.5 mg L^{-1} in August 2023. In similar scenarios, prior research has suggested that denitrification takes place within the microscopic anoxic zone of bacterial biofilms. Using labelled ^{15}N, Reddy et al. (1989) observed denitrification activities in anaerobic sediment located just below the aerobic rhizosphere. Christensen et al. (1989) found that oxygen penetrated approximately 1 mm into the sediment, confining denitrification to the thin, anoxic layer directly beneath the aerobic zone. Hence, it can be reasonably inferred that denitrification took place within the anaerobic sediment of Basin 1 in the Frank Lake wetland, rather than in the oxygenated water within the wetland itself during the period. Considering the rising trend in dissolved oxygen concentration of the water in Basin 1 from June to August 2023 and the findings noted by Christensen et al. (1989) regarding oxygen penetration into a shallow layer of sediment, it is also plausible to assume that declining denitrification in Basin 1 during August was influenced by the increasing dissolved oxygen concentration in the water at that time.

Temperatures ranging from 15 °C to 35 °C are considered favorable for denitrification (Hu et al., 2020). Also, denitrification rates increase with rising temperatures

up to a certain optimum, as higher temperatures enhance the enzymatic activity of denitrifiers (Shan et al., 2018). In this study, the average temperature (± SD, n = 9) of the water column in Basin 1 was recorded as 19.4 ± 0.5 °C in June, 20.6 ± 0.8 °C in July and 17.1 ± 0.2 °C in August 2023. These temperatures fell within the favorable range for denitrification. Considering the lower water temperature observed during the August water sampling compared to June, it is reasonable to assume that the drop in water temperature may have also affected denitrification rates in Basin 1.

Some studies suggest that the influence of soil pH on denitrification is complex, with no specific optimal pH identified (Šimek et al., 2002). However, in a previous study by Foglar & Gašparac (2013), it was observed that denitrification remains optimal within the pH range of 5.9–8.0, with the highest denitrification rate occurring at a pH value of 7.1 (Foglar & Gašparac, 2013). Moreover, denitrification rates declined beyond this optimal pH value. In the current study, the pH of water in Frank Lake Basin 1 increased from 8.4 ± 0.0 to 9.1 ± 0.1 between the sampling dates in June and August 2023. The higher pH value of the water in August may have contributed to the observed decrease in denitrification during the same period.

In general, denitrification increases with increasing nitrate concentration (Lu et al., 2009). The Frank Lake Basin 1 received 364.3 mg NO_3^- L^{-1}, 299.2 mg NO_3^- L^{-1} and 263.9 mg NO_3^- L^{-1} of nitrates through the wastewater effluent in June, July and August 2023, respectively. However, as mentioned above, the nitrate uptake by plants and phytoplankton can influence nitrate availability for denitrification (Lund et al., 2000). Additionally, denitrification increases with soil carbon content as denitrifying bacteria consume organic carbon as their energy source (Li et al., 2021). The denitrification processes in a wetland

are influenced by numerous factors that are challenging to control or detect. Denitrification is primarily shaped by the combined effects of these factors rather than by any single factor alone (Li et al., 2021).

Apart from denitrification, nitrification can alter the $\delta^{15}N$ value of the residual nitrate pool (Bruland & MacKenzie, 2010). Nitrification generally results in a decrease in $\delta^{15}N$ values of nitrate molecules with increasing nitrate concentrations, thereby reducing the isotopic effect (Søvik & Mørkved, 2008). Decomposition processes within wetlands are thought to convert organic nitrogen into ammonium. Subsequently, a portion of this ammonia undergoes conversion into nitrate through nitrification (Lee et al., 2009). However, the annual conversion of organic nitrogen in the topsoil into ammonium through mineralization only amounts to approximately 1-3% of the organic nitrogen pool (Fernández et al., 2009). This percentage is considered insignificant when compared to the nitrate input from wastewater effluent into Basin 1. Moreover, I observed relatively low concentrations of ammonium in the wastewater effluent entering Basin 1 of the Frank Lake wetland complex compared to nitrates, amounting to 9.7 mg $NH_4^+ L^{-1}$ in June, 2.8 mg $NH_4^+ L^{-1}$ in July and 3.4 mg $NH_4^+ L^{-1}$ in August 2023. The comparatively low ammonium concentration in wastewater effluent entering Basin 1, alongside the minimal ammonium concentration produced during organic matter mineralization, typically limits nitrification in the Frank Lake wetland. Phytoplankton typically favor absorbing ammonium over nitrates due to the lower energetic requirements for assimilating ammonium. Consequently, they initially consume available ammonium, reducing the amount of ammonium accessible for nitrification (Glibert et al., 2016).

In addition, nitrates in groundwater can undergo denitrification under anaerobic conditions, leading to higher $\delta^{15}N$ values in wetland water (Chen & MacQuarrie, 2005). However, Zhu et al. (2019) argued that groundwater flow had no significant effect on the Frank Lake water. This was supported by the observed low hydraulic conductivity of the soil, which results in low hydraulic gradients, as well as the presence of 10-350 meters of glacial till in the sediment beneath the Frank Lake Basins (Zhu et al., 2019).

The $\delta^{15}N$ levels in nitrate molecules within Basin 1 of the Frank Lake wetland can also be influenced by surface water runoff. However, in 2023, there was zero surface water runoff into Basin 1 due to the prevailing dry weather conditions. This decline in runoff was linked to the zero flow from Mazeppa and Blackie creeks, which typically supply water to Frank Lake during the spring but cease during mid-summer dry years (Zhu et al., 2019).

3.4.3 The ^{15}N fractionation factor (ε) for denitrification

The Rayleigh model assumes a constant ε throughout the denitrification process. However, this ε can vary significantly due to a multitude of factors such as fluctuations in environmental conditions (e.g., temperature, pH, oxygen levels), shifts in microbial community composition and activity and variations in substrate availability. These dynamic influences can lead to unpredictable isotopic patterns during denitrification, challenging the assumption of a constant ε (Robinson, 2001). Consequently, to better capture the complexities of denitrification, researchers often employ a range of fractionation factors derived from experimental studies or field observations, allowing for a more nuanced interpretation of isotopic data. This approach yields a range of potential values for percent denitrification rather than a single deterministic estimate, reflecting the inherent variability

and uncertainty associated with denitrification processes in natural environments (Granger et al., 2008).

3.4.4 Limitations of the study

In this study, the Rayleigh fractionation model was used to determine the magnitude of denitrification in Basin 1 of the Frank Lake wetland complex. The Rayleigh model applies to unidirectional reactions in closed systems. However, natural systems are neither closed nor strictly unidirectional. In natural systems, the consumed nitrogen source can be replenished through processes such as the decomposition of organic matter (Robinson, 2001).

Furthermore, the approach used to estimate PON concentration in the water based on the chlorophyll 'a' concentration in the water entailed several assumptions, thereby introducing uncertainty to the accuracy of the derived PON concentration values. Hence, it would be beneficial to conduct further research to confirm the correlation between chlorophyll 'a' concentration and PON levels in the water, ensuring that the assumptions made during estimation of PON concentration remain valid across different environmental conditions.

3.4.5 Future Research

Since nitrate molecules consist of both nitrogen and oxygen, several researchers have employed the dual-isotope ($\delta^{15}N$ and $\delta^{18}O$) approach to study denitrification (Fukada et al., 2003; Hu et al., 2020). These studies have revealed a significant correlation between the $\delta^{15}N$ and $\delta^{18}O$. An advantage of analyzing both $\delta^{15}N$ and $\delta^{18}O$ of nitrate is the systematic variation observed in oxygen isotopic compositions with nitrogen isotopic compositions during denitrification (Kendall, 1998). Using the dual-isotopic approach can thus offer

highly complementary and more compelling evidence for the occurrence of denitrification (Chen & MacQuarrie, 2005).

Adequate carbon sources and suitable temperatures (15 - 35 °C) promote the completion of denitrification reactions, resulting in the production of dinitrogen gas (Hu et al., 2020). However, under certain conditions, the reaction may produce and release intermediate nitrous oxide into the atmosphere, which is a potent greenhouse gas (Flanagan et al., 2022; Harrison et al., 2012). Nitrous oxide has a greenhouse effect potential that is 265 times greater than that of carbon dioxide over a 100-year timescale, making it a significant contributor to global climate change (Wang et al., 2024). Further research is required to study nitrous oxide emissions from Basin 1 of the Frank Lake wetland, to evaluate whether there are any negative climate consequences.

3.5 Conclusion

The observed drastic decrease in total nitrate concentration within Basin 1 of the Frank Lake wetland complex during June, July, and August 2023 underscores the significant role of the wetland in mitigating nitrate levels in wastewater effluent. Denitrification emerged as a key mechanism responsible for this reduction, contributing substantially to the removal of nitrate during these months. The observed seasonal variation in denitrification rates, peaking during periods of low phytoplankton activity and diminishing during periods of high phytoplankton activity, highlights the dynamic interplay between biological processes and denitrification in the Frank Lake wetland ecosystem. However, denitrification processes in wetlands are influenced by numerous other factors that are challenging to control or detect. Therefore, denitrification processes are shaped by the combined effects of environmental and biological factors rather than by any single factor alone. These insights not only contribute to our understanding of denitrification dynamics but also emphasize the importance of wetlands as natural systems for nitrogen removal, thus providing valuable information for ecosystem management and conservation efforts.

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Appendix1- Supplementary information for Chapter 2

Appendix 2.1: Geographic coordinates (GPS) of the vegetation sampling locations.

	Site	Sample ID	Plant species collected	GPS coordinates	
				Latitude	Longitude
Frank Lake wetland complex	Basin 1- Effluent input	B1-EFF	*T. latifolia*	50.5661111	113.7255556
	Basin 1- Bird Blind	B1-BB	*T. latifolia, S. acutus*	50.5638889	113.7158333
	Basin 1- Middle West Shore	B1-E	*S. acutus*	50.5741667	113.7286111
	Basin 1- Outlet	B1-B	*T. latifolia, S. acutus*	50.5561111	113.7044444
	Basin 2- South Shore	B2-SS	*T. latifolia, S. acutus*	50.5337170	113.7186000
	Basin 2- East Shore	B2-E	*S. acutus*	50.5458333	113.7000000
	Basin 3- North of Island	B3N-B	*T. latifolia, S. acutus*	50.5337160	113.6929830
	Basin 3- North Shore	B3W-N	*S. acutus*	50.5233330	113.6933330
	Basin 3- East	B3-E	*S. acutus*	50.5222222	113.6858333
	Basin 3-Outlet	B3-O	*T. latifolia, S. acutus*	50.5138889	113.6908333

Appendix 2.1 (Contd.): Geographic coordinates (GPS) of the vegetation sampling locations.

	Site	Sample ID	Plant species collected	GPS coordinates	
				Latitude	Longitude
Little Bow River	Upstream	LBR-US	*T. latifolia*	50.4901500	113.7586670
	Downstream sampling site 1	LBR-DS1	*T. latifolia*	50.4776000	113.6661670
	Downstream sampling site 2	LBR-DS2	*T. latifolia*	50.4553170	113.6104000
	Downstream sampling site 3	LBR-DS3	*T. latifolia*	50.4315670	113.5742170
	Downstream sampling site 4	LBR-DS4	*T. latifolia*	50.3974830	113.5580500
Twin Valley Reservoir	Sampling site 1	TVR-1	*T. latifolia*	50.3078744	113.4753655
	Sampling site 2	TVR-2	*T. latifolia*	50.2548330	113.4389170