Centre Eau Terre Environnement

SEMI-PASSIVE TREATMENT OF NITRATE IN MINE-IMPACTED WATER IN COLD CLIMATES

TRAITEMENT SEMI-PASSIF DES NITRATES ISSUS DE DRAINAGE MINIER EN CLIMATS FROIDS

By

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RÉSUMÉ

Le nitrate est un contaminant retrouvé couramment dans les eaux souterraines et peut avoir des impacts négatifs sur l'environnement, la santé humaine et animale. L'exploitation minière peut contribuer à la libération dans l'environnement de nitrates part l'utilisation d'explosifs et le traitement des minéraux. Les eaux impactées peuvent être traitées à l'aide de diverses technologies actives, passives et semi-passives. Les systèmes de traitement passifs et semi-passifs reposent sur des processus biologiques et ont été identifiés comme des techniques préférentielles, en particulier dans les scénarios de post-fermeture. Cependant, ces processus peuvent être inhibés par les conditions extrêmes associées au climat subarctique du Nord du Canada. L'objectif de ce projet est d'adapter un système de traitement des nitrates aux conditions d'une mine de cuivre subarctique dans le territoire du Yukon, au Canada. L'inoculum prélevé dans la mine a été développé dans une série de bioréacteurs à l'échelle laboratoire et s'est avéré capable d'éliminer efficacement les nitrates par dénitrification. Divers substrats disponibles localement dans le Nord ont été testés en tant que sources de carbone pour soutenir la dénitrification biologique. Les bioréacteurs d'une capacité de 5 litres ont été conçus pour faire face aux concentrations moyennes rencontrées à la dite mine ainsi qu'au pire scénario envisagé par ce partenaire minier. Toutes les sources de carbone testées ont soutenu la dénitrification à des degrés divers. Une plante invasive, *Melilotus albus*, a été sélectionnée comme source de carbone optimale pour d'autres tests de bioréacteur en raison de sa propension à coloniser les sites miniers perturbés. Des tests en colonne ont été réalisés pour évaluer l'impact de la température et du temps de rétention hydraulique sur la capacité d'élimination des nitrates de l'inoculum avec *Melilotus albus* comme source de carbone. Deux bioréacteurs ont été maintenus à 5°C et comparés aux deux fonctionnant à température ambiante. Deux colonnes ont été opérées avec un temps de rétention hydraulique court d'environ 15 heures, tandis que les deux autres ont été maintenues avec un temps de rétention longs d'environ 115 heures. Les résultats suggèrent que l'augmentation du temps de rétention hydraulique peut favoriser l'élimination des nitrates par temps froid. Les résultats des tests à l'échelle du laboratoire orienteront la conception d'un système de traitement à l'échelle pilote à l'avenir. En abordant les défis posés par la contamination par les nitrates dans les environnements miniers, cette recherche contribue non seulement à l'avancement des pratiques de remédiation durable, mais aussi aidera à répondre aux besoins de l'industrie et aux exigences réglementaires

Mots-clés : Bioremédiation ; remédiation minière ; technologies semi-passives ; climat subarctique ; bactéries nitrifiantes ; bactéries dénitrifiantes ; nitrates.

ABSTRACT

Nitrate is a widespread groundwater contaminant and can have negative impacts on human and wildlife health. Mining can contribute to nitrate contamination through blasting, mineral processing, and other activities. Impacted water can be treated using various active, passive, and semi-passive technologies. Passive and semi-passive treatment systems rely on biological processes and have been identified as preferred techniques to treat mine-impacted water, particularly in post-closure scenarios. However, these processes can be inhibited by the extreme conditions associated with the subarctic climate of Northern Canada. The objective of this project was to tailor a nitrate treatment system to the conditions of a subarctic copper mine in Yukon Territory, Canada. Inoculum collected at the mine was developed in a series of batch scale bioreactors and was found to successfully remove nitrate via denitrification. Various substrates that were locally available in the North were tested as carbon sources to support biological denitrification. Bioreactors were designed to emulate average and worst-case scenario nitrate concentrations at the mine. All tested carbon sources supported denitrification to varying degrees. An invasive plant, *Melilotus albus*, was selected as the optimal carbon source for further bioreactor tests due to its propensity for colonizing disturbed mine sites. Five-litre lab scale column tests were conducted to evaluate the impact of temperature and hydraulic retention time on the nitrate removal capacity of the inoculum with *Melilotus albus* as a carbon source. Two bioreactors were maintained at 5°C and compared to the two operated at room temperature. One of each of the cold and room temperature columns were operated with short hydraulic retention time of roughly 15 hours, while the other two had long retention times of about 115 hours. Results suggest increasing hydraulic retention time can support increased nitrate removal in cold temperatures. The results from the lab-scale tests will inform the design of a pilot-scale treatment system in the future. In addressing the challenges posed by nitrate contamination in mine impacted water, this research not only contributes to the advancement of sustainable remediation practices but also aligns with industry needs and regulatory requirements.

Keywords: Bioremediation; mine remediation; semi-passive technologies; subarctic climate; nitrfiying bacteria ; denitrifying bacteria ; nitrates.

SOMMAIRE RÉCAPITULATIF

Introduction et contexte de l'étude

L'exploitation minière joue un rôle important dans l'économie, la culture et le développement du nord du Canada. Le climat extrême du nord impose de nombreux défis pouvant être rencontrés à toutes les étapes du processus minier, y compris en ce qui concerne la fermeture des mines. Les longs hivers, les basses températures, les variations saisonnières des précipitations sont parmi les obstacles auxquelles les mines doivent faire face. Suivant les régulations fédérales, provinciales ou territoriales, les mines peuvent être amenées à continuer de traiter l'eau contaminée après la fin des opérations. Il est préférable de s'appuyer sur des systèmes de traitement passifs ou semi-passifs lors de la remédiation post-fermeture. En effet, les systèmes de traitement semi-passifs et passifs reposent sur des processus biologiques et ont donc des exigences de maintenance et de ressources plus faibles par rapport aux traitements actifs qui dépendent de l'infrastructure, de l'énergie et des intrants chimiques. Cependant, les processus biologiques sont inhibés par les conditions extrêmes du nord canadien. Par conséquent, des adaptations et améliorations doivent être apportées aux systèmes de traitement pour permettre une remédiation efficace de l'eau contaminée par les mines dans les scénarios de post-fermeture. Une mine de cuivre dans le territoire central du Yukon a émis le besoin de développer un système de traitement semi-passif pour éliminer les nitrates de l'eau contaminée sur site. L'objectif de ce projet était de concevoir un système de traitement en utilisant des bactéries natives du site minier et en utilisant des amendements carbonés locaux pour soutenir la dénitrification biologique dans des conditions froides

Matériels et méthodes

Phase 1 : Des sédiments ont été collectés sur le site de la mine et amenés au laboratoire pour développer une biomasse dénitrifiante à partir des échantillons de mine. À cette étape, des populations bactériennes ont été développées en utilisant des milieux de croissance synthétiques et en utilisant de l'acétate de sodium comme source de carbone dans des bioréacteurs (BR) de 1 L qui ont été testés pendant trois semaines. Les bioréacteurs ont été considérés comme fonctionnels si un taux élevé d'élimination des nitrates était observé.

Phase 2 : Les bioréacteurs avec les taux les plus élevés d'élimination des nitrates en phase 1 ont été combinés pour créer un inoculum optimal qui a de nouveau été soutenu par des milieux de croissance synthétiques et de l'acétate de sodium. Cet inoculum a été développé dans des bioréacteurs de 1 L qui ont été surveillés pendant trois semaines.

Phase 3 : Cette phase a utilisé le développement d'inoculum de la phase 2 pour tester dans quelle mesure diverses sources de carbone pouvaient soutenir la dénitrification biologique. Des sources de carbone locales telles que la luzerne blanche (WSC) et l'orge queue-de-renard, le compost municipal local, les résidus de brasserie et d'autres amendements carbonés complexes ont été utilisées. Ils ont été comparés à l'acétate de sodium, une source de carbone simple fréquemment utilisée dans les systèmes de traitement biologique. Un ratio carbone/azote de 20:1 a été utilisé pour garantir que le carbone ne limitait pas la dénitrification. Les bioréacteurs ont été conçus pour simuler des concentrations de nitrates moyennes et des scénarios de pire des cas prédits (25 ppm $N\text{-}NO_3^-$ et 100 ppm $N\text{-}NO_3^-$, respectivement).

La caractérisation de l'ADN de la biomasse développée dans cette phase a été réalisée pour obtenir un aperçu de la communauté microbienne dans les bioréacteurs. Les sources de carbone ont été soumises à des tests de lixiviation pour déterminer leur impact éventuel sur le pH et si elles libéraient des métaux pouvant être préoccupants. De plus, des tests de sorption ont été réalisés pour déterminer dans quelle mesure l'élimination des nitrates était due à la sorption par rapport à la dentification.

Phase 4 : La luzerne blanche a été choisie comme source de carbone utilisée dans le test à l'échelle des colonnes en raison de sa disponibilité sur le site de la mine. Les colonnes ont été inoculées avec du liquide provenant des BR de la phase 3 de la WSC. Deux variables ont été testées, la température et le temps de rétention hydraulique, pour comprendre comment ces paramètres influençaient la dénitrification. Les colonnes ont été exploitées à 5°C et à température ambiante avec des temps de rétention hydraulique théoriques de 10 et 96 heures.

Résultats

Tous les BR de la phase 1 ont eu une élimination des nitrates à des degrés divers, ce qui indique que les bactéries dénitrifiantes ont été développées avec succès à partir d'échantillons de sédiments de mine. Les BR de la phase 2 ont résulté en une élimination des nitrates de 97,8% après trois semaines. Les résultats de la phase 3 étaient plus variés, l'élimination étant la plus faible dans les bioréacteurs utilisant des copeaux de bois comme sources de carbone. La meilleure source de carbone local avec laquelle le meilleur rendement a été obtenu fut le résidu de brasserie, qui a permis d'obtenir un abattement de plus de 99% des nitrates avec des concentrations initiales de 25 et 100 ppm de $N\text{-}NO_3^-$. Les BR de WSC ont, pour leur part, présenter un abattement de de 100% de $N\text{-}NO_3^-$ lorsque la concentration initial était de 25 ppm et ~67% d'abattement lorsque la concentration initiale était de 100 mg/L.

L'analyse taxonomique de l'ADN extrait a révélé que des taxons contenant des dénitrifiants connus ont été identifiés dans tous les BR. Les résultats indiquent que les populations microbiennes ont répondu aux conditions changeantes entre les phases des bioréacteurs en batch. Une analyse métagénomique serait nécessaire pour déterminer la fonctionnalité des microbes trouvés dans les bioréacteurs. Une telle analyse pourrait également indiquer comment les enzymes dénitrifiantes sont impactées par les conditions changeantes.

Les tests en colonne ont montré que le TRH et la température ont effectivement impacté l'élimination des nitrates. La plus forte élimination des nitrates a été observée dans la colonne maintenue à température ambiante et au débit le plus lent. La plus faible élimination a été observée dans la colonne avec le débit le plus rapide et maintenue à 5°C (température froide). Cependant, les résultats révèlent que les impacts inhibiteurs de la température froide sur la dentification peuvent être atténués en augmentant le temps de rétention hydraulique. Sur la période s'étalant des semaine 12 à 19, l'abattement des nitrates est passée de 80-100% à environ 40% sur l'ensemble des colonnes. Le carbone limitait probablement la dénitrification car le COT diminuait rapidement au cours des premières semaines de surveillance. Cependant, l'abattement des nitrates n'a pas diminué au même rythme que les concentrations de COT.

Conclusions

Cette étude a démontré qu'un système de traitement semi-passif peut être adapté pour fonctionner à des températures froides, en utilisant des bactéries indigènes et des sources de carbone disponibles dans le Nord. Cependant, plus de recherches sont nécessaires pour comprendre comment le WSC peut être mieux adapté pour agir en tant que source de carbone pour la dentification. Les futures recherches devraient se concentrer sur la conception de bioréacteurs avec de vrais effluents miniers; sur l'adaptation des amendements de carbone et des exigences de TRH aux conditions sur site.

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ABREVIATIONS LIST

ADN	Acide désoxyribonucléique
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
ASVs	Amplicon Sequence Variants
BR	Bioreactor
C/N	Carbon/nitrogen ratio
CW	Constructed wetland
DIW	Deionized water
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
GPS	Global position system
HRT	Hydraulic retention time
ICP-MS	Inductively coupled plasma mass spectrometry
MIW	Mine-impacted water
NOB	Nitrite-oxidizing bacteria
NPOC	Non-purgeable organic carbon
NSERC	National Sciences and Engineering Research Council of Canada
Obs	Observed
ORP	Oxidation-reduction potential
RCP	Reclamation and Closure Plan
rDNA	Ribosomal deoxyribonucleic acid
SSU	Small subunit
T	Temperature
TOC	Total organic carbon
UV-Vis	Ultra-violet visible
WSC	White sweet clover
YMRC	Yukon Mining Research Consortium

1 INTRODUCTION

1.1 Background

Nitrate is one of the most widespread groundwater contaminants and is often associated with mining, both from nitrogen-based explosives used in the extraction process and chemicals used in mineral processing (e.g. cyanide leaching in gold processing) (Dash et al. 2009; Bailey et al. 2013). Passive and semi-passive treatment systems have been identified as a cost-efficient way to treat mine impacted water. Passive systems require minimal attention after installment whereas semi-passive technologies rely on periodic maintenance and additions of materials such as carbon sources (Trumm 2010; Ness et al. 2014; Eppink et al. 2020). Biological treatment of nitrate contamination relies on naturally occurring microbial populations that transform nitrogen compounds. These biological processes are sensitive to changes in various parameters such as flow rate, pH, and temperature. Therefore, conditions in cold climates, such as Canada's subarctic zone, present various challenges to implementing successful biological technologies to treat nitrates. This emerging field of research has been catalyzed by the need for passive or semi-passive treatment options for closure plans of developing mines as dictated by modern legislation and regulatory bodies. For example, the Yukon Mine Site Reclamation and Closure Policy (2006) dictates that long-term active treatment is not an acceptable option in reclamation and closure plans. Therefore, mines that anticipate the need to remediate water after closure must ensure there are passive treatments options that are tailored to site conditions. Research and advancements in passive technologies will serve to reduce the risks to people and the environment associated with mining.

"Nature's repair mechanisms may be slow but they're thorough. We must find ways to assist and expedite them." – Kalin 2004.

1.2 Mining in Canada's North

Mining has long played a fundamental role in the economies, cultures, and development of Canada's northern regions (Keeling and Sandlos 2015). The importance of mining continues into modern day, as the federal government has indicated that mineral development in the North is a critical part of Canada's development strategy (CanNor 2021). However, the extreme climate and remote nature of these regions pose significant obstacles to mineral development. The arctic and subarctic climates experience seasonal variations in precipitation, insolation, and temperature, which present challenges to mine operations. The region's remoteness affects the infrastructure

needs of the industry, including the road networks, bridges, access to tidewater, and connection to power grids. These needs evolve during the stages of the mine life cycle, from exploration and development to closure and site remediation. addressing the unique challenges of the North is critical to enabling sustainable development in Canada's mining sector.

1.2.1 Yukon Context

Mining has played a central role in the history and economy of the Yukon since the 1880s, when miners, following the California gold rushes, made minor strikes in the Yukon ahead of the Klondike Gold Rush. During the height of the gold rush, approximately $22 million worth of gold was exported from the Klondike region per year (Coates and Morrison 2005). Coates and Morrison (2005) say of gold rushers: "[they] sought only to strike it rich and make enough money to leave the region forever ... for them ... the Yukon was not a home but an opportunity." So, what of those who do call the Yukon home? Yukon communities have seen many mines open and close since the gold rush, and these mines will continue to have social, economic, and ecological impacts for many generations to come.

1.2.2 Mine Closure

Whether due to regional challenges, market fluctuations, or ore depletion, mines inevitably reach closure and some of these mines are abandoned before adequate reclamation has occurred. This can leave unstable sites which may pose a hazard to surrounding areas. The environmental impact of abandoned mine sites in northern Canada is significant with notable examples including the Giant Mine in the Northwest Territories and the Faro Mine in central Yukon. To mitigate further environmental impacts, territorial governments and regulatory bodies have implemented regulations and guidelines regarding the closure and reclamation of mine sites. In Yukon, a Reclamation and Closure Plan (RCP) is required to obtain both mining and water use licenses necessary to operate a mine in the territory (Yukon Government 2013). These plans address reclamation components like site revegetation and recontouring, water treatment, and dust mitigation. This thesis focuses on the water treatment component of reclamation and closure. Under the Yukon Mine Site Reclamation and Closure Policy (2006), passive treatment options are preferred wherever long-term water treatment is necessary in closure, due to lower maintenance and resource requirements. However, long winters and low average temperatures slow biological processes that are frequently relied upon in passive treatment systems. Therefore, research is required to understand how systems can be adapted to function in cold conditions.

1.2.3 Yukon Mining Research Consortium

The Yukon Mining Research Consortium (YMRC) is composed of 6 active hard rock mining companies the Natural Sciences and Engineering Research Council of Canada (NSERC) Industrial Research Chair in Northern Mine Remediation, hosted at Yukon University. This partnership was established to address key remediation challenges faced by the industry through applied research projects. During the inaugural meeting of the YMRC in 2019, water treatment by passive or semi-passive technologies was identified as one of three primary research priorities. The project detailed in this thesis was conducted as part of the applied research program that aims to investigate the feasibility and efficacy of passive and semi-passive technologies in the North, thereby mitigating environmental impacts and enhancing sustainability in Yukon's mining industry.

1.2.4 Minto Mine

Minto Mine is a copper-gold mine located 240 km northwest of Whitehorse, Yukon, on Category A Settlement in the traditional territory of the Selkirk First Nation (Figure 1). The mine operated under several owners from 2007 to its sudden closure in 2023. The most recent owner of the mine, Minto Metals, was a member of the YMRC and acted as the primary industrial partner for this research project. The site had both open pit and underground mining, targeting the high-grade copper sulphide mineralization and native gold hosted in a porphyry deposit (CMC 2018). Froth floatation was used to concentrate minerals on site.

The mine operators identified a need to treat nitrogen compounds at the site by semi-passive technologies, to inform the RCP for the site. Mean annual temperatures at the site are near -5°C, so treatment systems must be adapted to function in the cold. Previous research at the mine includes a pilot-scale constructed wetland (Bouchard et al. 2018) and pilot-scale bioreactors designed to address copper and selenium contamination (Janin et al. 2016). Primary contaminants of concern projected at closure are cadmium, copper, molybdenum, selenium, zinc, and nitrate (Bouchard et al. 2018). Before and after the mine closure, site staff contributed to this project in countless ways, including insight on environmental conditions, data and monitoring, and on-site support and hospitality.

Figure 1 **Location of Minto Mine in Yukon Territory, Canada**

1.3 Objectives

The aim of this study was to design a semi-passive treatment system to address nitrate contamination in mine water in cold climates. The specific objectives were:

Objective 1. To collect sediment from a subarctic mine site, targeting native denitrifying bacteria. Develop these microbial populations in the lab and test their ability to remove nitrate in batch bioreactors.

Objective 2. Identify and characterize carbon sources that can be feasibly sourced in the remote subarctic of Canada. Test these carbon sources with the inoculum sourced from the mine in batch bioreactors at various nitrate concentrations.

Objective 3. Test and optimize treatment technologies based on the on-site conditions of the mine, including temperature and the present and forecasted nitrate concentrations. Investigate nitrate removal capacity in both batch and lab-scale column studies.

Objective 4. Conduct genomic characterization of microbial populations used in biological treatment system.

1.4 Thesis Layout

Chapter 1 introduces the study context and outlines the primary research objectives. Chapter 2 provides a brief overview of the nitrogen cycle, relevant microbial processes, and the impacts of excess nitrogen on environmental and human health. A summary of select studies outline the prevalent technologies used to treat nitrogen compounds, with an emphasis on cold climates. Chapter 3 details the materials and methods used in the study, starting with the collection on sediment at the mine site. The resulting bioreactors were created in four phases, each described in succession. Next, the carbon source characterization is detailed, followed by description of the various analytical methods used throughout the process. The results of each stage of the project are then presented along with discussion about how these relate back to the broader project objectives. This is followed by overarching conclusions and recommendations for future research.

2 LITERATURE REVIEW

2.1 Nitrogen Cycle

Nitrogen (N) is essential for life and is a limiting factor in the growth of plants and microbes (Cabello et al. 2009). The global nitrogen inventory is distributed between the geosphere, biosphere, hydrosphere, and atmosphere. The exchange of nitrogen between these reservoirs is referred to as the nitrogen cycle. The nitrogen stored in geosphere is not believed to play a substantive role in the biological nitrogen cycle (Kuypers et al. 2018; Hall 2019; Mysen 2019). Most nitrogen actively cycled in the nitrogen cycle is found in the atmosphere as N_2 gas; this gas comprises 78% of the atmosphere. However, gaseous nitrogen (N_2) is largely unavailable to organisms due to the strength of triple covalent bonds between nitrogen atoms. The nitrogen in the atmosphere can be integrated into the biosphere via transformation to nitrogen oxides by lightning or microbial nitrogen fixation (Kuypers et al. 2018). In ecosystems, N occurs in various forms, including, but not limited to organic compounds like amino acids and DNA. It also exists in inorganic forms, ranging from ammonia (the most reduced form) to nitrate (the most oxidized form) (Figure 2) (Cabello et al. 2009). The transformations of nitrogen from one compound to another are largely facilitated by bacteria and other microbes (Table 1) (Kuypers et al. 2018).

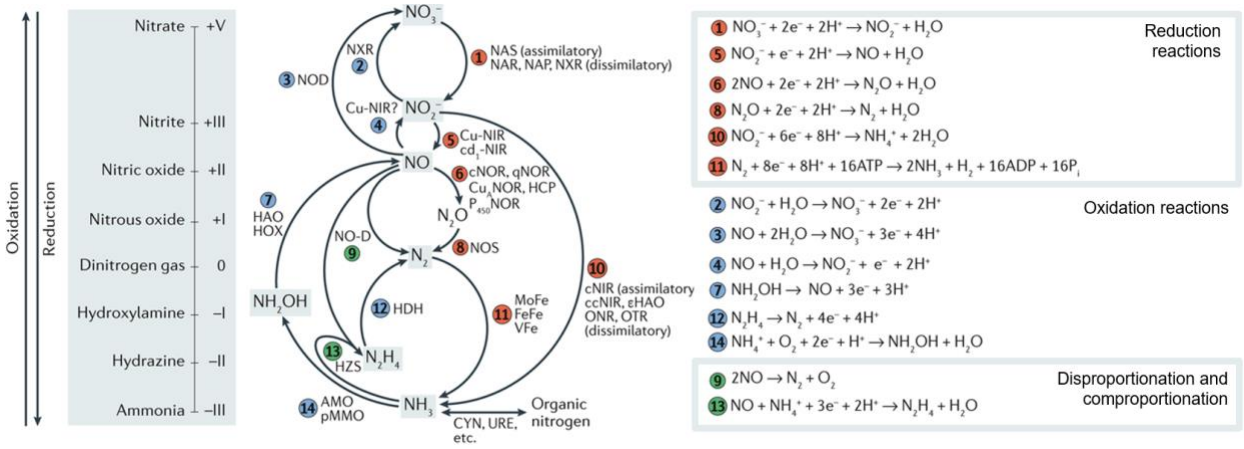


Figure 2 Microbial transformations of nitrogen compounds (Adapted from Kuypers et al. 2018)

2.2 Nitrogen Fixation

Nitrogen fixation is the process by which specific organisms use the enzyme nitrogenase to catalyze the formation of two molecules of NH_3^+ by reduction of N_2 (Equation 1). Both bacteria and archaea can carry these enzymes and are therefore capable of nitrogen fixation, a critical step in the biological nitrogen cycle. Metallic cofactors for nitrogenases include molybdenum, iron, and vanadium. Of these cofactors, molybdenum and iron are considered more likely to be a limiting factor in terrestrial and marine nitrogen fixation (Vitousek and Howarth 1991). Identified nitrogen-fixing bacteria include cyanobacteria (e.g. *Anabaena* and *Nostoc*), species from the genera *Azotobacter*, *Azospirillum, Frankia,* and others (Newton 2007). *Rhizobium* is a genus of nitrogen-fixing bacteria that live symbiotically in specialized root nodules of legume species and may be useful in remediating degraded soil (Franco and De Faria 1997).

Equation 1 $N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$

2.3 Nitrification

Nitrification is a process in which ammonia or ammonium is oxidized to nitrite (Equation 2, Equation 3, Equation 4) which is subsequently oxidized to nitrate (Equation 4) (Sanmugasunderam et al. 1987). This process is catalyzed by enzyme reductases in ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), as well as specific archaea (ammonia oxidizing archaea or AOA), heterotrophic bacteria, and fungi (Prosser 2007; Cabello et al. 2009; Wei et al 2018). The growth rates of nitrifying bacteria are impacted by ammonia and oxygen concentrations, temperature, pH, and light exposure (US EPA 2002). Nitrification is generally an aerobic process but can occur in low oxygen environments by specific bacteria such as *Nitrosomonas eutropha* (Prosser 2007).

Equation 2 $NH_3 + O_2 + \rightarrow NO_2^- + 3H^+ + 2e^-$

Equation 3 $NH_4^+ + O_2 + 2e^- \rightarrow NO_2^- + 2H^+ + H_2O$

Equation 4 $NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$

2.4 Anammox

Anaerobic ammonium oxidation (anammox) occurs when specific bacteria use ammonium as an electron donor and nitrite as an electron receptor to produce nitrogen (Equation 5) (Cabello et al. 2009; Bonassa et al. 2021). Anammox bacteria growth rates are sensitive to temperature, pH, and ammonia and oxygen concentrations (Jermakka 2015). It is suggested that anammox bacteria may be likely to be outcompeted by denitrifying bacteria in some environments (Herbert et al. 2014; Jermakka 2015). A study by Herbert et al. (2014) found that in pilot-scale bioreactors operating at low temperatures, the removal of nitrogen compounds from mine water was due primarily to denitrification rather than anammox processes, as determined by gene expression analysis. Table 1 summarizes the main reactions and microorganisms involved in the transformation of nitrogen.

Equation 5 $NH_4^+ + NO_2^- \rightarrow N_2$

Table 1 Summary of relevant nitrogen reaction pathways and associated organisms

Nitrogen reaction pathway	Reactions	Associated microorganisms	References
Nitrification	$NH_3 + O_2 + \rightarrow NO_2^- + 3H^+ + 2e^-$ $NH_4^+ + O_2 + 2e^- \rightarrow NO_2^- + 2H^+ + H_2O$ $NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$	Ammonia-oxidizing bacteria (AOB) (*Nitrosomonas, Nitrosococcus, Nitrospira*), Nitrite-oxidizing bacteria (NOB) (*Nitrobacter, Nitrospina)*	Prosser 2007
Denitrification	$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O$ $NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O$ $2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$ $N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O$	*Paracoccus denitrificans, Pseudomonas*, Archaea, Fungi	Zumft 1997, Saleh-Lakha et al. 2009
Anammox	$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$	Anaerobic ammonium-oxidizing bacteria, Phylum *Planctomycetes*	Kuenen 2008
Nitrogen fixation	$N_2 + 8H^+ + 8e^- + 16$ ATP $\rightarrow 2NH_3 + H_2 +$ 16ADP + $16P_i$	Cyanobacteria, *Rhizobium*, *Azotobacter*	Newton 2007, Franco and De Faria 1997

2.5 Denitrification

Denitrification involves the sequential respiration of nitrate, nitrite, nitric oxide, and nitrous oxide catalyzed by the corresponding reductases by organisms including denitrifying bacteria, some archaea and select fungi (Equation 6, Equation 7, Equation 8, Equation 9) (Cabello et al. 2009). As denitrification occurs as a series of redox reactions, there is potential for the accumulation of intermediate nitrogen compounds (i.e. NO_2^-, NO, and N_2O) if denitrification becomes inhibited (Zumft 1997; Albina et al. 2019). Environmental conditions such as suboptimal pH, toxic concentrations of inhibitory substances (e.g. heavy metals), and imbalance between electron donors and acceptors can impact denitrification pathways (Zumft 1997; Stres et al. 2007; Albina et al. 2019). Nitrite and NO accumulation can inhibit microbial processes, and it is therefore important to understand how environmental factors may impact nitrate removal in remediation applications (Albina et al. 2019).

Equation 6 $NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O$

Equation 7 $NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O$

Equation 8 $2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$

Equation 9 $N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O$

Denitrifying microbes are ubiquitous in anoxic environments where nitrate is present, including soils, marine sediments, and in gut microbiota (Kuypers et al. 2018).

2.5.1 Dissolved Oxygen

Many of the bacteria associated with denitrification are facultative anaerobes, meaning they will preferentially use oxygen as an electron acceptor in respiration when it is available but can use other electron acceptors including nitrate when oxygen is scarce (Lam and Kuypers 2011; Albina et al. 2019). Therefore, denitrification usually occurs in anaerobic conditions, but aerobic denitrification has been observed in some microbes (e.g. *Pseudomonas* sp., *Comamonas* sp., *Paracoccus denitrificans*) (Ji et al. 2015).

Denitrification rate is impacted by dissolved oxygen (DO) concentration, as each enzyme in the successive reduction steps responds differently to the presence of oxygen (Lam and Kuypers

2011). Ji et al (2015) suggest three potential relationships between DO concentration and denitrification (Figure 3). Anaerobic denitrification is inhibited by increases in DO concentration while microbes may exhibit peak denitrification at optimal DO concentrations or be relatively uninhibited by DO concentrations within a certain range (Figure 3) (Ji et al. 2015).

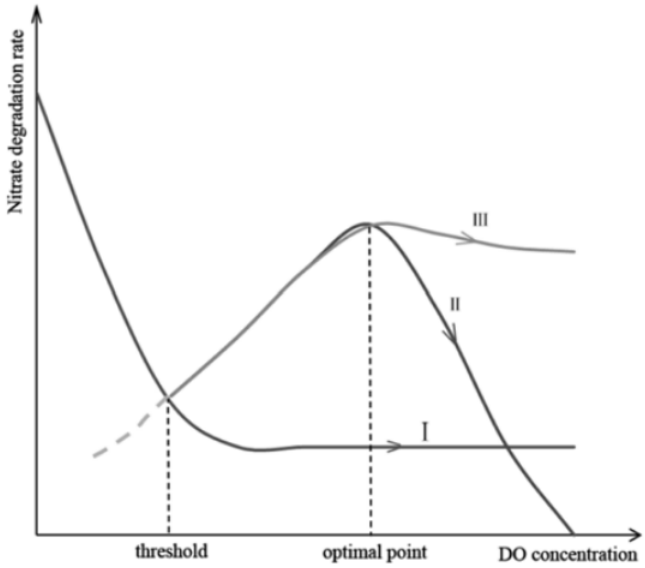


Figure 3 **Three possible relationships between DO concentration and nitrate degradation. (I) represents anoxic denitrification, (II) represents denitrification that decreases after an optimal [DO] is reached, (III) represents denitrification that is not affected by changes in [DO] (Ji et al. 2015)**

2.5.2 Temperature

Temperature (T) can influence the gene expression and subsequent enzyme activity that is responsible for bacterial denitrification (Saleh-Lakha et al. 2009). The optimal temperature for denitrification is 25–37˚C (Saad and Conrad 1993; Ji et al. 2015). However, denitrification can occur in sediment at -2°C if there is liquid water available (Dorland and Beauchamp 1991). Denitrification rates vary according to the availability of liquid water. Therefore, denitrification in frozen soils is impacted by characteristics such as void ratio, particle size, and concentration of water-soluble ions which impact the amount of unfrozen water held in pore spaces (Stres et al.

2007). Additionally, it is thought that low T primarily affects the N_2O reduction step of denitrification, possibly due to the suppression of the N_2O-reductase enzyme (Holtan-Hartwig et al. 2000; Stres et al. 2007). There are numerous ways in which denitrifying bacteria can adapt to function in cold temperatures, including changing membrane composition, and decreasing cell size and water content (Stres et al. 2007).

2.5.3 pH

pH can influence the gene expression and subsequent enzyme activity responsible for bacterial denitrification (Saleh-Lakha et al. 2009). The optimal pH for denitrification is generally regarded as being between pH 7.5 to 9.5 (Zumft 1997; Ji et al. 2015; Albina et al 2019). However, in an extensive review on the impact of soil pH on denitrification, Simek and Cooper (2002) argued that an optimum pH is impossible to determine without understanding how pH impacts factors such as bacterial community composition and acid-tolerance. Denitrification is considerably prohibited when pH is 5 or lower (Saleh-Lakha et al. 2009; Ji et al. 2015). This was determined by Saleh-Lakha et al. (2009) by measuring the gene expression of nitrate reductases and cell density in *Pseudomonas mandelii* in shake flasks tests conducted at pH 5, 6, 7 and 8. The researchers found that low pH interferes with gene expression relating to various enzymes and caused reduced cell density in comparison to the higher pH tests. High pH can also interfere with enzyme activity (Albina et al. 2019). Many bacterial processes, including denitrification, are inhibited by pH >11 (Albina et al. 2019). However, denitrification has been observed in alkaliphilic bacteria at roughly pH 11 (Baumann et al. 1997).

2.5.4 Carbon

Broadly, microbial processes are often described as being governed by the mass balance of carbon, hydrogen, oxygen, and nitrogen (Sobieszuk and Szewczyk 2005). In the case of biological denitrification, efficiency is largely determined by the carbon to nitrogen ratio (C/N), as carbon commonly acts as the electron donor in denitrification (Albina et al. 2019). Sobieszuk and Szewczyk (2005) describe denitrification in the equation below, where v is equal to the balance coefficient and subscripts indicating: S: substrate; N: nitrogen source; X: biomass; P: denitrification product (Equation 10). The stoichiometry of the equation is dependent on coefficient values of a specific biomass yield. An example of this equation in which acetate is the carbon source is presented in **Error! Reference source not found.** (Albina et al. 2019).

Equation 10 $v_sCH_{as}O_{bs}N_{cs} + v_NHNO_3 \rightarrow v_xCH_{ax}O_{bx}N_{cx} + v_pCH_{ap}O_{bp}N_{cp} + v_{H2O}H_2O + v_{CO2}CO_2$

Équation 11 $5CH_3COOH + 8NO_3^- \rightarrow 10CO_2 + 4N_2 + 6H_2O + 8OH^-$

Aerobic and anaerobic denitrification varies in optimal carbon concentration. An extensive review of aerobic denitrification by Ji et al. (2015) found that optimal C/N ratio is between 5–10. However, Her and Huang (1995) used batch bioreactors with various carbon sources to determine that the minimum C/N required for nearly complete anaerobic denitrification (98-100%) was 2. Additionally, the same experiment found denitrification was not impeded with an excess of carbon, up to, and possibly exceeding, 25 C/N (Her and Huang 1995).

2.6 Nitrate and the Environment

Nitrate occurs naturally in freshwater systems at a background concentration of ~ 0–2 mg/L N-NO_3^- (Gomez Isaza et al. 2020). A meta-analysis of the impacts of nitrate on freshwater taxa by Gomez Isaza et al. (2020) found that aquatically respiring organisms such as amphibians and fish are particularly susceptible to elevated nitrate concentrations. These organisms experience adverse effects including reduced growth and survival rates and increased deformity rates under these conditions. Elevated nitrate concentrations are also associated with eutrophication (Erisman et al. 2013). Eutrophication is a process which can lead to algal and cyanobacterial blooms in affected ecosystems, created by elevated nitrate concentrations, which become efficiently assimilated by the phytoplankton (Erisman et al. 2013). Subsequent sedimentation and decay of the algal or cyanobacteria biomass depletes oxygen concentrations throughout the water column (Erisman et al. 2013).

2.7 Nitrate and Human Health

Nitrate contamination is a matter of concern regarding human and wildlife health. In humans, ingesting high levels of nitrate can lead to various health issues. Nitrate can be reduced to nitrite in the gastrointestinal system, which can interfere with red blood cells' ability to carry oxygen, a condition referred to as methemoglobinanemia (Ward et al. 2018). Infants are particularly susceptible to this condition; one study found a 22% increased risk of methemoglobinanemia in infants in an area with drinking water nitrate concentrations over 50 mg/L versus an area with nitrate concentrations under 50 mg/L (Sadeq et al. 2008). Other health risks associated with

nitrate ingestion include adverse pregnancy outcomes in women and infants (Ward et al. 2018). Water nitrate ingestion has also been associated with increased risk of various types of disease, such as colorectal cancer, and thyroid disease, as determined by a review of health studies regarding nitrate ingestion and adverse human health impacts conducted by Ward et al. (2018).

2.8 Nitrate Sources

Nitrate may be present at mining sites as a product of nitrogen-based explosives and as a product of the degradation of cyanide used to leach gold from ore (Akcil and Mudder 2003; Herbert et al. 2014). Holloway et al. (1998) suggest that nitrogen in bedrock of varying lithology can contribute to elevated concentrations of nitrate in surface water through weathering processes. Extractive practices, such as mining in nitrogen rich rock can exacerbate this process by increasing exposure to weathering mechanisms (Bosman 2009). Mining frequently involves the use of various explosives to access and extract target minerals within bedrock. Nitrate based explosives, such as ammonium nitrate fuel oil (ANFO), can generate nitrogen oxides as seen in **Error! Reference source not found.** (Sanmugasunderam et al. 1987; Albina et al. 2019).

Équation 12 $NH_4NO_3 \rightarrow 2H_2O + 2N_2O_{(g)}$

Residues from the blasting can remain at the blasting site if detonation is incomplete, or in waste rock where, following nitrification, nitrate can enter the water system (Bosman 2009). Chemicals used in mineral processing, such as nitric acid (HNO_3), ammonium chloride (NH_4Cl), and ammonium hydroxide (NH_4OH) can also contribute to elevated nitrate levels associated with mining (Bosman 2009). Cyanide (CN) is commonly used to separate gold from host rock in heap leach facilities. Whether by hydrolysis, photodegradation or microbial processes, the degradation of CN generally results in the N transforming into NH_4^+ which can be transformed further to NO_3 by microbial processes (e.g. Equations 13 and 14).

Équation 13 $2CN^- + O_2 \rightarrow 2CNO^-$

Équation 14 $2CNO^- + 2H_2O \rightarrow NH_4^+ + CO_3^{2-}$

2.9 Nitrogen Compound Treatment Technologies

2.9.1 Active Treatment Technologies

Nitrates can be treated by various active technologies. Active treatments are qualified using mechanical and chemical processes to remove contaminants. These processes require regular maintenance and monitoring and rely on infrastructure, and electrical and chemical inputs (US EPA 2014). A selection of these treatments is described in Table 2. Examples include sorption by materials such as zeolite minerals, carbon-based materials (e.g. activated carbon), and chemical denitrification by materials such as zero-valent iron and magnesium (Fe^0 and Mg^0) (Ji et al. 2007; Jermakka et al. 2015). Air stripping and membrane separation are also technologies used to treat nitrogen compounds in mine water (Jermakka et al. 2015). However, these treatment systems are costly, energy intensive and unsuitable for mine closure and reclamation plans.

2.9.2 Passive and Semi-Passive Treatment Technologies

Passive and semi-passive biological treatment technologies employ biological processes to treat nitrates in mine-impacted water (Table 2). Technologies that work passively in temperate and tropical climates may need modifications to function adequately in cold conditions (Jermakka et al. 2015; Ji et al. 2020). Treatment systems are considered “semi-passive” when they require some amount of monitoring, maintenance, or chemical amendments. A review of passive treatment of mine impacted water in cold climates by Ness et al. (2014) found that low temperatures impact these treatment systems in numerous ways including reduced microbial growth and activity, freezing-induced hydraulic failure, and seasonal water quantity fluctuations (e.g. freshet) (Ness et al. 2014). Passive and semi-passive treatments are preferred in mine closure scenarios due to the lower resource and maintenance needs in comparison to active treatments. It is noted however, that passive and semi-passive treatment systems may still require some monitoring and maintenance.

2.9.3 Wetlands

The use of wetlands to treat nitrates has been broadly studied (Bezbaruah and Zhang 2003; Aguirre et al. 2010; Redmond et al. 2014; Austin et al. 2019). Constructed wetlands (CW) are human-made systems within a shallow basin filled with select substrates and macrophytes (aquatic plants). Wastewater is delivered so that it flows either horizontally or vertically through

the substrate (Taylor 2009). Constructed wetland designs vary and can have multiple cells or be combined with other water treatment technologies such as bioreactors. Various studies suggest that success of CWs requires site-specific design (Taylor 2009). The considerations for wetland design include plant selection, substrate, targeted contaminants and associated microbe populations, influent water chemistry, flow rate, and targeted reducing or oxidizing capacity. The mechanisms responsible for nitrogen removal in CWs include plant uptake, volatilization, ammonification, adsorption, nitrification, and denitrification (Wang et al. 2017). Denitrification in wetlands generally occurs in anoxic sediment and anoxic microsites on plant surfaces (Gupta et al. 2016). Vegetation in CWs support microbial activity by providing attachment sites, oxygen and can themselves uptake some contaminants and should therefore be selected carefully (Lin et al. 2002; Taylor 2009). Additionally, the carbon required for denitrification can be provided by the plants and is renewed annually, decreasing the demand for external carbon amendments (Taylor 2009; Haakensen et al. 2015).

This technology is being tested in cold climates. A pilot scale CW installed at Minto Mine in the Yukon successfully removed 97% of nitrates (Bouchard et al. 2018). This CW made use of soil, peat and aquatic plants sourced from the mine site. This pilot project was preceded by site assessment, mine impacted water characterization, and a lab scale study until conditions and design parameters were optimized (Haakensen et al. 2015; Bouchard et al. 2018). Results suggest that microbial communities responsible for denitrification were able to withstand freeze and thaw cycles in subarctic conditions (Haakensen et al. 2013; Haakensen et al. 2015; Bouchard et al. 2018). However, a study conducted by Lin et al. (2002) found that effluent nitrate levels increased when water temperature decreased from 20ºC to 10ºC, supporting the hypothesis that denitrification in CWs is impacted by temperature. Ji et al. (2020) suggest several design modifications to improve CW treatment capacity in cold climates. These include using cold-adapted plants, insulation techniques, and pairing subsurface flow CWs with various functional improvements. This review indicated that recirculation of water can enhance denitrification (Ji et al. 2020). Batch feeding is another strategy suggested to improve nitrification and denitrification in cold climate CWs (Wang et al. 2017; Ji et al 2020). Batch feeding refers to the alternating draining and filling of CWs to promote oxidized conditions when drained, followed by reducing conditions when filled (Wang et al. 2017; Ji et al 2020). This supports sequential nitrification and denitrification in the same CW system (Wang et al. 2017; Ji et al 2020).

2.9.4 Nitrifying and Denitrifying Bioreactors

Biological reactors, or bioreactors (BRs), have been used in a variety of settings to treat nitrate in wastewater. In mine water treatments, BRs are designed to accommodate microbial nitrification and denitrification in the presence of various contaminants, low organic carbon, and non-neutral pH (Jermakka et al. 2015). Bioreactor designs can vary from suspended growth reactors that are actively stirred and aerated and can remediate large quantities of heavily contaminated water, to fixed-film reactors that support the growth of relatively slow establishing microbes and are less sensitive to low temperatures (Jermakka et al. 2015). In cold climates, the addition of carbon is often required to reach treatment objectives (Jermakka et al. 2015).

2.9.4.1 Carbon Sources

As discussed in Section 2.4.4, organic carbon acts as an electron donor in heterotrophic denitrification. Mine impacted water is often low in organic carbon, especially in cold climates (Thurman 1985; Hellman et al. 2019). Therefore, carbon sources are often added to BRs in soluble forms, such as ethanol, methanol, glucose, and sodium acetate. These are referred to as simple carbon sources as they do not require decomposition for the C to be available to microbes (Nielsen et al. 2018). Complex forms of carbon substrate may be used, such as crab chitin, wood chips, and straw (Table 3) (Her and Huang 1995; Greenan et al. 2006; Roser et al. 2018). Complex carbon sources require microbially facilitated decomposition to become bioavailable (Nielsen et al. 2018). Carbon sources can vary in the bioavailability of carbon and in their performance under cold conditions and various HRT (Healy et al. 2012; Nordstrom and Herbert 2017). Substrate choice is dependent on variables such as cost, and type and configuration of the treatment system in use (Ghafari et al. 2008). These carbon sources can be costly and difficult to source in remote areas. Therefore, there is a need to investigate local sources of carbon (Jermakka et al. 2015; Hellman et al. 2019).

Table 2 Summary of nitrogen compound treatment technologies

Technology	Mechanisms for removal	Advantages	Disadvantages	Feasibility in cold climates	Sources
Active chemical treatments					
Ion exchange	Nitrate replaces chloride on surface of resin and is removed from solution	Resin can be regenerated by rinsing standard or nitrate selective resins can be used efficient water recovery (up to 97%)	Requires pre-treating to remove suspended solids. Resins must be rinsed to avoid nitrate in effluent. Waste brine from rinsing	Potential	Rezvani et al. 2017
Zeolite sorption	Sorption of nitrate onto surface of zeolite; ion exchange specifically using zeolite as the resin	Low cost, not temperature dependent	Zeolite must be mined or artificially produced. Pretreatment may be required	Potential	Jermakka et al. 2015
Sorption to carbon-based materials	Sorption of nitrate onto surface of activated carbon	Low cost, not temperature dependent	Pretreatment may be required	Good	Jermakka et al. 2015
Reverse osmosis	Water is forced through a semi-permeable membrane which traps various contaminants	Can remove multiple contaminants	Pre-filtration required, high energy requirements, generates highly concentrated waste. Membrane needs to be monitored and replaced periodically	Potential	Rezvani et al. 2017
Electrodialysis	Potential difference between to electrodes removes salts from wastewater	High water recovery	Generates highly concentrated waste. High energy requirements	Potential	Rezvani et al. 2017
Chemical denitrification	Nitrate is chemically reduced using metals such as aluminum and iron	Potential to remove multiple contaminants. Generates less waste than some other active treatments	Potential for incomplete denitrification and the build-up of nitrite and ammonia. Temperature and pH requirements	Low	Rezvani et al. 2017

Air stripping	pH and temperature of wastewater is raised and aerated or passed through a membrane to transform nitrogen compounds into gaseous forms	Low cost in low volume/high concentration waters	High pH required for optimal efficiency. Temperature dependent. High energy consumption. Less effective for high volumes with low concentrations	Low	Jermakka et al. 2015
Active biological treatments					
Rotating biological contactors	Discs covered in fixed film bacterial growth rotate in basin of wastewater so that film is alternatively exposed to anoxic and aerobic conditions. Partially submerged discs used for nitrification, submerged discs used for denitrification	High biomass concentration, small footprint, nitrification and denitrification abilities	Requires energy to rotate discs, maintenance	Potential	Liu and Liptak 1997
Activated sludge processes	Wastewater is continually mixed with flocculated microbial populations, biological nitrification and denitrification occur	Can be designed to remove nitrogen compounds in sequential aerobic and anoxic zones	Requires energy to mix and aerate, overgrowth of microbes can impact efficiency, may require recirculation of sludge/water	Potential	Liu and Liptak 1997
Moving bed bioreactors	Modified activated sludge process. Constantly stirred tanks with either aerobic or anoxic conditions to support microbial nitrification or denitrification,	Successful at low temperatures, small footprint (compact), Higher biomass concentration than fixed bed reactors, single flow process	High C/N ratio required in low temps. Requires energy to mechanically mix	Successful at 5^{o}C	Dale et al. 2015
Autotrophic denitrification with elemental sulfur (S^0)	Reduction of NO_3^- or NO_2^- to N_2 with energy derived from inorganic oxidation-reduction reactions using reduced	S^0 is readily water-soluble and widely available. No external C source required	Temperature and pH sensitive	Low	Jermakka et al. 2015

	S compounds as electron donor and inorganic C compounds as C source				
Passive and semi-passive biological treatments					
Trickling filter	Microbial nitrification. Water is distributed over a medium with a fixed film of microorganisms, water percolates and organic material is adsorbed onto film	Low-rate trickle filters (1.17 - 3.52 m^3/m^2/day) associated with nitrified effluent and low BOD, natural convection can be used to aerate system. Can be incorporated into a two-stage nitrifying/denitrifying system	Conventionally "active" with mechanized distributer arm, interior of thick film layer can become anaerobic and lead to sloughing of film. Can support growth of 'unwanted' organisms can clog system. Longevity of filter media variable; may need to be changed periodically. Insects and odour can occur in exposed filters	Unknown	Liu and Liptak 1997
Wetlands	Microbial nitrification and denitrification, plant uptake, volatilization, ammonification, adsorption	Can treat large volumes, low costs, low maintenance	Large area required, seasonal, sensitive to influent metal/salt concentrations pre or post treatment may be required	Under investigation, may require design modification (e.g. recirculation, batch feeding) that reduce passivity of the system	Liu and Liptak 1997; Jermakka et al. 2015
Microalgae-based water treatment	Nitrate is consumed by microalgae and cyanobacteria via assimilation or dissimilation pathways. Nitrate/nitrite is reduced to ammonium and then incorporated into amino acids	Potential to remove other contaminants including heavy metals	Light requirements for photosynthesis, long HRT times required, temperature requirements	Unknown	Chai et al. 2021
Fixed bed bioreactors:	Microbial denitrification	low construction and operational costs. Wood chips are	Longevity is unknown, Microbial denitrifcation limited by temperature and carbon availability	Potential: Extending HRT can help manage	Roser et al. 2018; Nordstrom

Wood chip bioreactors		inexpensive and readily available		nitrate under low temperatures, carbon amendments	and Herbert 2019; Hellman et al. 2021
Anammox process	Direct transformation of ammonia to nitrogen gas by anammox bacteria	Can occur in a single reactor. Can treat high ammonia concentrations. no additional C required	Sensitive to nitrite accumulation, slow establishment of anammox bacteria that can be outcompeted by other bacteria, sensitive temperature and pH	Low	Jermakka et al. 2015 Herbert et al. 2014

Table 3 A summary of select studies employing various carbon sources in denitrifying bioreactors

Carbon source	System	C/N*	NO_3^- (mg/L)	HRT (days)	Temperature (°C)	Nitrate removal (%)	References
Crab chitin	Batch	n.s.	1.58	9	approx. 20	58.23	Daubert and Brennan 2007
Cotton	Column	n.s.	19	0.16–0.86	27	85-97	Della Rocca et al. 2005
Cardboard fibres	Batch	280.3	15	180	20	95.8	Greenan et al. 2006
Corn stalks	Batch	42.6	40.5	180	20	91.75	Greenan et al. 2006
Wood chips	Batch	448.9	7.5	180	20	80.13	Greenan et al. 2006
Wood chips and soybean oil	Batch	795.6	12	180	20	85.41	Greenan et al. 2006
Barley straw	Column	65.7	19.5 to 32.5	14	10	99.92	Healy et al. 2012
Cardboard	Column	208	19.5 to 32.5	8.5	10	99.58	Healy et al. 2012
Pine needles	Column	46.54	19.5 to 32.5	9.9	10	99.88	Healy et al. 2012
Woodchips	Column	496	19.5 to 32.5	13	10	99.6	Healy et al. 2012
Ethanol	Field experiment	n.s.	5	0.1	n.s.	100	Jansen et al. 2019
Wood chips	Field experiment	n.s.	8	5.3	n.s.	81.25	Jansen et al. 2019
Wood chips and sodium acetate	Column	n.s.	20	1	5.5	80	Roser et al. 2018
Wood chips and biochar	Column	n.s.	20	1	5.5	3	Roser et al. 2018
Wheat straw	Column	134.8	51.8	140	n.s	96.6	Saliling et al. 2007
Woodchips	Column	393.5	51.9	140	n.s	95.9	Saliling et al. 2007
Biochar	Batch	n.s.	30 to 40	0.5 - 1	27	78.2	Wei et al. 2018
Acetic acid	Batch	1.6	50	0.5	30	100	Her and Huang 1995
Glucose	Batch	3.2	50	0.5	30	99.8	Her and Huang 1995
Methanol	Batch	3.4	50	0.5	30	92	Her and Huang 1995
Sedge	Column	n.s.	22.3 to 32.9	1.5 to 1.8	10	48	Hellman et al. 2021
Straw	Column	n.s.	22.3 to 32.10	1.2 to 2.2	10	42	Hellman et al. 2021
Wood chips	Column	n.s.	22.3 to 32.11	5.2 to 7.2	10	44	Hellman et al. 2021

*n.s = not specified

3 MATERIALS AND METHODS

3.1 Inoculum Collection and Testing

Inoculum sampling was conducted at the mine, targeting native nitrifying, and denitrifying bacterial populations from the site. Sample locations were chosen based on local conditions predicted to be optimal for these targeted bacteria. Denitrifying bacteria were targeted in sediment collected from wetland areas, presumed to be relatively anaerobic and high in organic carbon (Figure 4). Nitrifying bacteria were targeted in surface water samples. The samples were collected close to monitoring wells that were tested regularly by mine staff for a variety of water quality parameters (Figure 5).

Figure 4 **Left: A soil pit dug to 30 cm for sediment collection. Right: A wetland at the mine site, surrounded by waste rock**

Sediment samples were collected by removing the top 30 cm of sediments with a shovel and collecting approximately 5 L of underlying material into clean, unused plastic buckets. One bucket was used per collection site. Shovels were rinsed with deionized water (DIW) between samples.

Water samples were obtained by collecting approximately 5 L of water from the surface of select ponds using clean plastic buckets. All buckets were lidded to prevent contamination. For each location, the GPS coordinates, depth of sample, visual and odour observations were recorded (Appendix I). Both water and sediments samples were stored at 5°C upon return to the laboratory.

Each sample was analyzed for NH_4^+, NO_2^-, NO_3^- and metals (methods detailed in section 3.7). Samples were collected by drawing water from the sediment buckets after they were stored at 5°C for 3 months after initial collection. Water samples were filtered at 0.45 µm and preserved with acid. Samples collected for metal analysis were preserved with 5% v/v trace metal grade 70% nitric acid (HNO_3). Samples collected for nitrogen compound analysis were preserved with concentrated 96% sulphuric acid (H_2SO_4). Filtered and preserved samples were stored in glass bottles at 5°C prior to analysis.

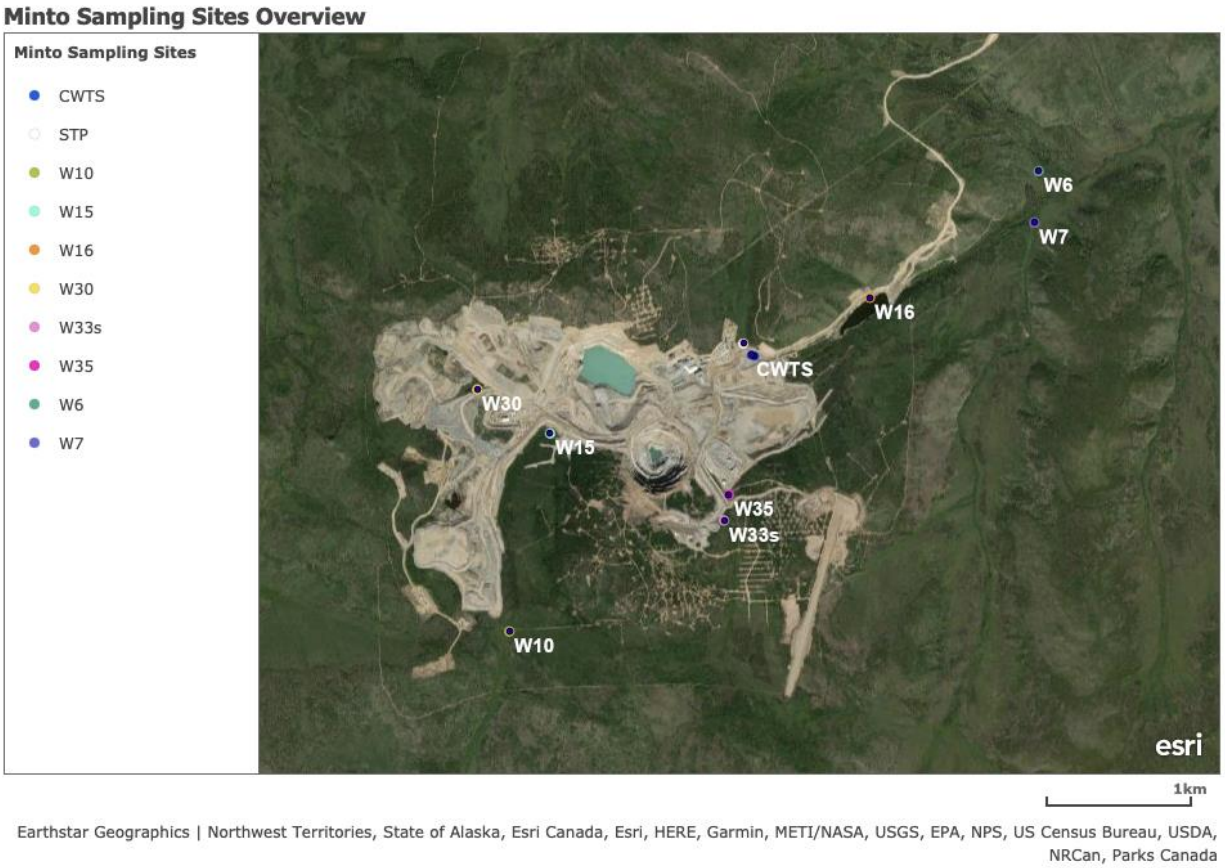


Figure 5 Overview of sampling sites across Minto Mine, located in central Yukon Territory. Note that map is oriented with North at the top of the page

3.2 Bioreactors Phase 1: Developing Inoculum

3.2.1 Growth Media

The medium used to support the growth of heterotrophic denitrifying bacteria consisted of the following nutrients and trace elements: KH_2PO_4, 3.45 g/L (Blaszczyk 1993, Hahnke et al. 2014); $MgSO_4.7H_2O$, 0.49 g/L (Blaszczyk 1993, Hahnke et al. 2014); $FeSO_4.7H_2O$, 0.06 g/L (Blaszczyk 1993); $CaCl_2.2H_2O$, 0.10 g/L (Hahnke et al. 2014); Mn(II), 0.027mg/L; Cu(II), 0.051 mg/L; Mo(VI), 0.096 mg/L; Co(II), 0.059 mg/L; Zn(II), 0.164 mg/L; B(III), 0.005 mg/L; Ni(II), 0.006 mg/L. Soluble carbon was supplied to the bacteria in the form of sodium acetate (CH_3COONa, 7.3027 g/L. The nitrogen source was KNO_3, 1.8 g/L, for an initial $N\text{-}NO_3^-$ concentration of 250 mg/L (Blaszczyk 1993; Du et al. 2003; Hahnke et al. 2014). Vitamins were provided in the form of Difco nutrient broth (0.1 g/L) (Blaszczyk 1993). To ensure that metals remained in solution, a complexing agent was added as well, Na_2-EDTA (5.0 g/L) (Widdel and Bak 1992, Hahnke et al. 2014, Kou et al. 2021).

3.2.2 Bioreactor Design

One bioreactor was made per sediment sample collected at the mine (*n*=16). Each bioreactor was made by combining 900 mL of media with 100 mL of sediment sample in 1 L glass jars (Table 4). The jars and media were autoclaved (Sanyo Labo Autoclave, MLS 3020) at 121°C for 20 minutes and cooled to room temperature prior to the addition of inoculum. Bioreactors were kept at room temperature (20-25°C) (Heylen et al. 2006; Yuan et al. 2021) and covered with an opaque black tarp to prevent the growth of non-targeted microbes (Kou et al. 2021). Each jar was sealed with parafilm that was checked daily and replaced if broken due to gas build-up. Weekly nitrate sampling was conducted by drawing 100 mL of fluid from each bioreactor using sterile plastic syringes. This 100 mL of effluent was replaced with 100 mL of DIW to maintain the volume of the BRs, thereby promoting anaerobic conditions. The BRs were gently inverted about five times before samples were drawn, to ensure a homogenous solution. The 100 mL of sample volume were split into 50 mL duplicate samples. These were filtered using 0.45 µm disc filters, preserved using 50 µL of concentrated 96% sulphuric acid (H_2SO_4) and stored at 5°C prior to analyses. Samples were diluted with a matrix of 0.1% H_2SO_4, up to 100x immediately prior to analysis to reduce interference caused by the coloration of the samples (Figure 6). Dissolved oxygen, oxidation-reduction potential (ORP), conductivity and pH were monitored on a weekly basis

(Section 3.7.1). These parameters were measured from the 100 mL volume collected for nitrogen sampling prior to filtering and preserving. 1 M HCl or 1 M NaOH was used to maintain the pH of each bioreactor between 6.3 to 7.4 (Hahnke et al. 2014; Yuan et al. 2021).

Table 4 Phase 1 bioreactor names and their associated inoculum sources

Bioreactor Name	Inoculum source	Bioreactor Name	Inoculum source
D1	Minto 1	D9	Minto 11
D2	Minto 2	D10	Minto 12
D3	Minto 3	D11	Minto 14
D4	Minto 5	D12	Minto 15
D5	Minto 6	D13	Minto 16
D6	Minto 7	D14	Minto 17
D7	Minto 8	D15	Minto 19
D8	Minto 9	D16	Minto 20

Figure 6 Phase 1 Bioreactors D1 - D4

3.3 Bioreactors Phase 2: Optimizing Inoculum

Fifteen of the sixteen batch BRs that were created to develop the bacterial populations from the mine samples were combined to create an optimized inoculum. Bioreactor D8 that had been inoculated with the Minto 9 sediment was excluded from the optimized inoculum mixture due to low denitrification rates observed in that BR (Section 4.2.2). The optimized inoculum was created by combining 20 mL of each of the 15 select BRs. Two duplicate BRs were created following the same media and method as detailed in section 3.2.2 and inoculated with 100 mL of the optimized inoculum mixture (Figure 7). The weekly sampling protocol was adapted from Phase 1 to account for the lower volume requirements for the analysis due to the dilution. Each week, 30 mL was drawn from each BR using sterile plastic syringes. Four mL of the sample was split into 2 mL duplicate samples and diluted with 18 mL of 1% H_2SO_4 in glass bottles for total organic carbon analysis (TOC). The remaining portion of the sample was then filtered at 0.45 µm. Two 0.5 mL duplicates of the filtered sample were diluted to 50 mL with 0.1% H_2SO_4 to reach a 100x dilution for nitrogen compound analysis. General chemistry parameters were recorded following the methodology detailed in section 3.7.1.

Figure 7 Phase 2 batch bioreactors, D17a and D17b

3.4 Bioreactors Phase 3: Carbon Testing

Various local carbon sources, as well as sodium acetate and molasses, were tested in two batches of BRs. One batch was conducted at 25 mg/L N-NO_3^-, the contemporary average nitrate concentration at the industrial partner's mine site, based on long-term water quality data from the mine (data not shown). The second batch was conducted at 100 mg/L N-NO_3^- the projected worst-case scenario concentration, as based on discussions with the mine staff. The BRs were created following the same media and method as detailed in Section 3.3.1 and 3.2.2, with the modifications. Rather than 900 mL of media and 100 mL of inoculum, 950 mL and 50 mL were used, respectively. Fluid from the Phase 2 BRs were mixed 1:1 and used as the inoculum for these BRs. Additionally, the sodium acetate was omitted from the media and replaced with various soluble and solid carbon sources (details in section 3.6). One carbon source was used per duplicate bioreactor set, following the table below (Table 5). The amount of carbon substrate necessary to achieve a 20 C/N molar ratio was added, to ensure that carbon will not be a limiting factor in the development of the biomass (Appendix II). Carbon calculations were based on the results of the CHN analysis detailed in section 3.4 (Table 6). A duplicate set of BRs were created at 100 mg/L N-NO_3^-, that were not inoculated nor had carbon added, to act as a control. Weekly sampling of the BRs was conducted following the protocol detailed in section 3.3.

Table 5 Summary of design parameters for Phase 3 bioreactors

Bioreactor Name	Carbon source	Initial [N- NO_3^-,]	Inoculum source
Phase 3.1			
D20	Foxtail Barley	25	D17
D21	Compost	25	D17
D22	Brewery Residue	25	D17
D23	Wood shavings	25	D17
D24	White sweet clover	25	D17
D25	Wood chips	25	D17
D26	Sodium acetate	25	D17
D27	Molasses	25	D17
Phase 3.2			
D28	Foxtail Barley	100	D17
D29	Compost	100	D17
D30	Brewery Residue	100	D17
D31	Wood shavings	100	D17
D32	White sweet clover	100	D17
D33	Wood chips	100	D17
D34	Sodium acetate	100	D17
D35	Molasses	100	D17
C100	-	100	D17

Table 6 Mass of each carbon source added to Phase 3 bioreactors

Carbon Source	Mass (mg) in Phase 3.1	Mass (mg) in Phase 3.2
Foxtail barley	953.3	3813.9
White sweet clover	966.8	3868.2
Wood chips	900.5	3602.9
Brewery residue	898.5	3595.0
Wood shavings	884.9	3540.3
Compost	1438.3	5754.5
Molasses	1409.4	5638.9
Sodium acetate	1390.1	5561.5

3.5 Phase 4: Column Tests

Column tests were conducted to investigate the nitrate removal capacity of the denitrifying inoculum developed in Phase 3 BRs. White sweet clover was used as the carbon source due to the promising results of Phase 3 (Section 4.4).

3.5.1 Column Design

Four columns were constructed using acrylic cylinders with the interior dimensions of 15.2 cm diameter and 27.4 cm length with a volume of 5 L (Dimension 3 Plastics Ltd, Burnaby, BC, Canada). Filters made from fiberglass insulation and rigid plexiglass were installed at the outlet end of each column to prevent material from clogging the outlet. The fiberglass was cut to the circumference of the columns and was approximately 5 cm thick, uncompressed. The plexiglass was 3 mm thick, cut to the circumference of the columns and had a series of holes drilled through to allow for influent to pass through (Figure 8).

Figure 8 Empty column with fibreglass and plexiglass filter

White sweet clover (WSC) was used as the carbon source to support denitrification (Table 7). The amount of WSC added was determined by the carbon content determined by CHN analysis (section 3.6.3), multiplied by the amount of nitrate expected to pass through the columns over a 4-month period while maintaining a 20:1 carbon to nitrogen ratio. The WSC added in two size fractions. The larger fraction ranged from 5 – 10 cm and accounted for 14.7% of the mass and about 50% of the volume of WSC added to each column. The smaller fraction was ground using a countertop coffee grinder (Black & Decker Coffee Grinder, Towson, Maryland, USA) and ranged from approximately 0.1 – 3 cm in size and made up 85.3% of the mass and 50% of the volume of WSC. Volumes were rough estimations made by packing dry WSC in glass beakers.

Table 7 Design parameters for the four Phase 4 column bioreactors

Column ID	THRT* (hr)	Temperature (C°)	Mass WSC (g)
BR1	10	5	666
BR2	10	20	666
BR3	96	5	133.2
BR4	96	20	133.2

* Theoretical hydraulic retention time.

Columns were inoculated with 500 mL (10% v/v) of a 1:1 mixture of all four WSC BRsfrom Phases 3.1 and 3.2. The influent was a 25 mg/L N-NO_3^- solution made fresh twice weekly by dissolving 3.61 g of KNO_3 in 20 L of DIW. Influent was stored in 20 L containers and pumped through tubing (C-Flex L/S 16 Tubing 50A) using two peristaltic pumps (Cole-Parmer, Masterflex L/S, Model No: 07528-30). Both inlet and outlets of the columns were connected to the tubing with barbed nylon adapters (¼" NPT - 1/8" ID). Influent was pumped from the bottom to the top of the columns to minimize the formation of preferential pathways and avoid the ingress of oxygen into the system. The effluent from each column was collected in a bucket and weighed before disposal to monitor the flow rate (Appendix III). The weighing and disposal of the volume in the effluent buckets occurred while the effluent from each column was redirected to sample bottles during the twice-weekly sampling events.

3.5.2 Column Operations

Two variables, hydraulic retention time (HRT) and temperature, were tested in the column tests. The targeted HRT for Bioreactors 1 and 2 was 10 hours, based on studies detailed in Table 3. The targeted HRT for Bioreactors 3 and 4 was 96 hours, also based on studies described in Table 3. Flow rates were also informed from pump volume data collected at the mine (data not shown). Hydraulic retention time was controlled by the flow rates of the two peristaltic pumps (Cole-Parmer, Masterflex L/S, Model No: 07528-30). Pump A was set to 7.6 rpm and Pump B was set to 1.3 rpm (Figure 9). Flow rate was calculated by determining the drainable porosity of each column by filling with the allocated WSC, filling each with DI water, soaking for 24 hours, then allowing them to drain for 24 hours and measuring the drained volume (Hellman et al. 2021). Prior to this step, WSC was dried at 60°C for 24 hours.

The operating temperature for BRs 1 and 3 was 5°C, to mimic average groundwater temperatures in the general region of the mine (Gartner Lee Ltd. 2003). A steady temperature of 5°C was maintained by housing BR1 and BR3 and the influent for these columns in a laboratory refrigerator (Fisher Scientific, Isotemp, 11-670-214). The other two columns (BR2 and BR4) were maintained at room temperature (~20°C) for the duration of the experiment.

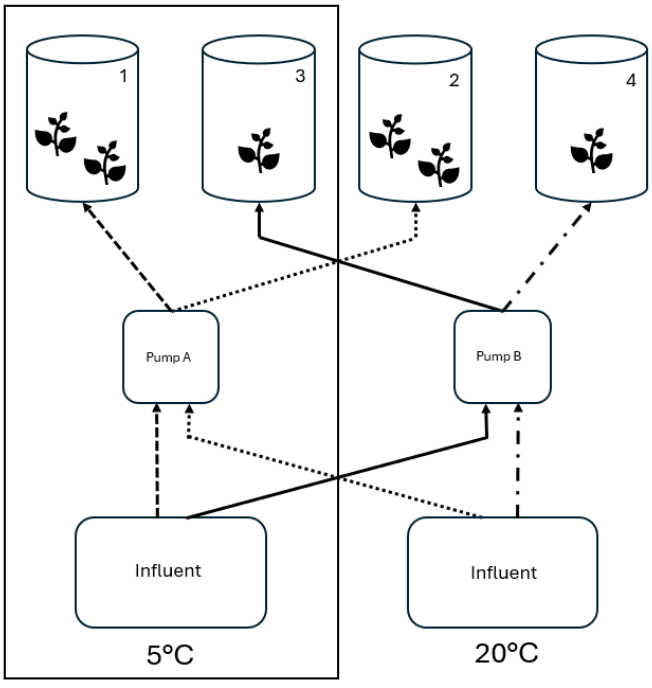


Figure 9 Schematic of the column bioreactor operating conditions

3.5.3 Column Sampling

Effluent from each column was sampled twice weekly for the first 7 weeks of operation and weekly from week 8 to the end of column operation at week 19. The sampling was conducted by collecting effluent directly into sterile sample bottles. Roughly 40 mL of effluent was tested for pH, ORP, DO and conductivity using the methods detailed in Section 3.7.1. Duplicate samples for carbon analyses were collected by aliquoting 20 mL of unfiltered effluent into glass bottles and preserved with 50 µL of concentrated H_2SO_4. Duplicate samples for nitrogen compound were filtered at 0.45 µm and preserved with the same proportion of H_2SO_4 used in the carbon samples. The influent was sampled each time it was made, following the same procedure. All samples were stored at 5°C prior to analysis.

3.6 Carbon Source Characterization

3.6.1 Carbon Source Collection and Preparation

Various simple and complex carbon sources were tested as carbon sources in the denitrifying BRs. Sodium acetate (Fisher Chemical) and molasses (Crosby's 100% Natural Fancy Molasses; Crosby Molasses Company Ltd., St. John, NB, Canada) were the two simple carbon sources tested. Brewery residue was collected from Yukon Brewing, Whitehorse, YT, Canada. Local compost was sourced from a municipal compost facility (Municipal Compost, YukonGrow, Whitehorse, YT, Canada). Wood chips were collected from a cleared block of mixed white spruce and lodgepole pine forest in Whitehorse, YT, Canada. Bark was removed from the wood chips, and excess soil and needles were brushed off the chips. Wood shavings were composed of a mix of local tree species (trembling aspen, *Populus tremuloides*; white spruce, *Picea glauca*; lodgepole pine, *Pinus contorta*) and were donated by Ibex Valley Wood Products (Whitehorse, YT, Canada). White sweet clover *(Melilotus albus*) and foxtail barley (*Hordeum jubatum*) were harvested in various locations around Whitehorse, YT, Canada. These species are considered invasive to the area and were collected in and around disturbed areas including parking lots and roadsides. Solid carbon sources were ground into a powder using a Black & Decker Coffee Grinder (Towson, Maryland, USA) and dried at 60°C for 48 hours prior to analysis and use in BRs. The grinder was rinsed with isopropyl alcohol and rinsed with DIW between carbon sources.

3.6.2 Carbon Source Leach Test

Shake tests were conducted to determine how each carbon source would impact the pH of BRs. Four grams of each carbon source were combined with 400 mL of DIW in baffled flasks and

agitated for 10 days (Eberback Corporation, Model No: E6010.00). pH was measured at the start of the experiment and after 10 days. Each test was duplicated for each carbon source. All resulting solutions were sampled for metal leaching after the 10-day period. Sixty mL of each leachate was collected and preserved with 5% HNO_3 and stored at 5°C prior to analysis at a commercial lab.

3.6.3 CHN Analysis

The percentage of total carbon in each carbon source was determined by CHN analysis (FlashSmart Elemental Analyzer, Thermo Scientific).

3.6.4 Sorption Capacity Test

A sorption capacity test for each carbon source was conducted to determine how much nitrate removal was chemical versus biological. The initial carbon to nitrogen ratio was held constant at 20:1. Sorption capacity tests were run for two hours on an agitation table (Eberback Corporation, Model No: E6010.00), using $N\text{-}NO_3^-$ concentrations of 25, 50 and 100 mg/L in DIW (Table 8). Carbon/N ratios in these tests were made to mimic the BRs, except for one trial in which mass of carbon source was held constant at 1 g per flask. The nitrate solution and carbon sources were combined in baffled flasks that were sealed with parafilm for the duration of the experiments. Samples collected for analysis were vacuum filtered using 0.45 µm filters. Twenty mL of filtered samples were aliquoted into sample bottles and preserved with 50 µL of H_2SO_4 acid and stored at 5°C prior to analysis. These samples were not diluted during initial sampling, but some were diluted with a 0.25% H_2SO_4 matrix if required for analysis.

Table 8 Sorption test design details

Sample ID	Complex carbon source (sorbant)	Mass of sorbant (g)	Initial nitrate concentration (mg/L N-NO_3^-)	Duration of agitation (hr)
AC-1	Foxtail barley	0.48	25	2
AC-2	White sweet clover	0.48	25	2
AC-3	Wood chips	0.45	25	2
AC-4	Brewery waste	0.45	25	2
AC-5	Wood shavings	0.44	25	2
AC-6	Compost	0.72	25	2
AC-7	Foxtail barley	0.48	100	2
AC-8	White sweet clover	0.48	100	2
AC-9	Wood chips	0.45	100	2
AC-10	Brewery waste	0.45	100	2
AC-11	Wood shavings	0.44	100	2
AC-12	Compost	0.72	100	2
AC-13	Foxtail barley	1	50	2
AC-14	White sweet clover	1	50	2
AC-15	Wood chips	1	50	2
AC-16	Brewery waste	1	50	2
AC-17	Wood shavings	1	50	2
AC-18	Compost	1	50	2

3.7 Analyses

3.7.1 General Chemistry

Water quality parameters were measured using a benchtop meter equipped with pH, ORP and conductivity probes (HI5522, Hanna Instruments). Dissolved oxygen was measured using a portable probe (Oakton WD-35643-10 DO 6+). All meters were calibrated daily using certified calibration solutions. The pH probe was calibrated using pH 4.01, 7.00 and 10.01 Oakton pH Buffer Standards (Catalog Number 00654-00, -04, and -08 respectively). The conductivity probe was calibrated to 1413 µS/cm and 12880 µS/cm using Hanna Instruments Conductivity Standards (HI7031 and HI7030). The dissolved oxygen probe was calibrated using Oakton Zero Oxygen Solution (Catalog Number 00653-00). The ORP probe was calibrated using Zobell's Solution 220 mV ORP Standard (LabChem, Catalog Number LC273101). The ORP was measured using a single junction ORP electrode with 3.5M KCl fill solution.

3.7.2 Carbon Concentration Analysis

Carbon concentrations were determined using the Non-purgable organic carbon (NPOC) method on both Shimadzu TOC-VCPH and Skalar FORMACS HT instruments.

3.7.3 Nitrogen Compound Analysis

Ammonia concentration analysis was determined by flow injection analysis on two instruments. The QuikChem® Method 10-107-06-2-B was used on the Quikchem FIA + 8000 Series instrument (Lachat Instruments, Zellweger Analytics Inc). Analysis conducted on the Smartchem 170 instrument followed the Smartchem 170 Method AMM-002-A (Unity Scientific, Brookfield, CT, USA).

Nitrite and nitrate were analyzed in tandem using flow injection analysis on two instruments. The QuikChem® Method 31-107-04-1-A was used on the Quikchem FIA + 8000 Series instrument (Lachat Instruments, Zellweger Analytics Inc). Analysis conducted on the Smartchem 170 instrument followed the Smartchem 170 Method NO3-001-B (Unity Scientific, Brookfield, CT, USA).

It is important to note that the methodology for nitrogen compound analysis used in this study advises that samples be analyzed within 30 days of collection. This holding time was exceeded by about 2-3x for many samples due to capacity limitations. Therefore, samples of known concentrations (e.g. media, influent) taken at the same time as the unknowns were used to determine if samples had degraded. This was not observed to be the case in any set of the samples analyzed at INRS or Yukon University. However, the results of these analysis may not be as accurate as they otherwise would be if protocol was followed regarding holding times.

3.7.4 Metals

Metal concentrations were measured by Inductively Coupled Plasma Mass Spectrometer (ICP-MS) in the INRS laboratory (XSeries 2, Thermo Fisher Scientific) or by a commercial laboratory, ALS, in Burnaby, BC, Canada, using the EPA 200/6020B method.

3.8 DNA Characterization

Both BRs from Phase 2 and the WSC BRs from Phase 3 were sampled for genomic DNA to gain insight to the microbial populations within the bioreactors. Microbial material was collected from the batch BRs by submerging sampling bags made from roughly 2 g of light cotton cheese cloth

in selected BRs.. The sampling bags were affixed to glass stir rods with fishing line to keep the bags submerged at the bottom of the BRs. The bags, fishing line and glass rods were autoclaved at 121°C for 15 minutes prior to being placed in the BRs to avoid contamination. Sampling bags were left in the BRs for 7 days, then collected and stored at -86°C. Multiple samples were collected from each BR. After 7 days, the sample bag in each BR was collected and replaced with a new one. Four to six samples were collected per BR.

Samples were thawed prior to DNA extraction. Pieces approximately 10 x 10 mm were cut from each sample bag using scissors sterilized with ethanol between sample. These pieces were used for DNA extraction using DNeasy PowerSoil Pro Kit (QIAGEN), following the manufacturer's instructions. Positive controls were created by using 50 µL of an *E. coli* culture in the place of the cotton cloth in the extraction kits. Negative controls were included by using 250 µL of nuclease free water in the place of the cotton cloth in the extraction kits. One positive control and one negative control were processed for every ten BR samples. A NanoDrop ND-2000 Ultraviolet-visible (UV-Vis) spectrophotometer (NanoDrop Technologies) was used to measure the concentration of nucleic acid and the purity of the DNA. Nucleic acid concentrations were measured at the wavelength of 260 nm. Absorbance values at 230 nm and 280 nm were measured to detect the presence of organic contaminants and protein contaminants, respectively. Ideal purity levels for DNA samples are indicated by absorbance ratios of A260/A280 between 1.8 and 2.0, and A260/A230 values should be greater than 2.

3.8.1 Microbial Community Analyses

Bacterial and archaeal small subunit (SSU) rRNA gene (rDNA) fragments from the extracted genomic DNA were amplified using primers 515F and 926R. Sample preparation for amplicon sequencing was performed as described previously (Caporaso et al. 2011). The amplicon library was analyzed on an Agilent Bioanalyzer using a high-sensitivity dsDNA assay to determine approximate library fragment size and to verify library integrity. Sequencing was conducted on an Ilumina MiSeq at the Life Sciences Institute of the University of British Columbia.

3.8.2 Bioinformatic Analysis

Sequences were processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) software package (Bolyen et al. 2019). Denoising, chimera checking, and clustering were performed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) plugin tool and denoise-paired instruction (Callahan et al. 2016). For taxonomic annotation, the SILVA database

(release_138) was used as the reference (Quast et al. 2013), together with the naïve-Bayes-algorithm-based trained classifier for a taxonomic assignment at 99%, using feature classifier classify-sklearn instructions (https://docs.qiime2.org/2022.2/data-resources/). All data were visualized in Qiime2 Viewer and Excel. For α- and β -diversity measures, all samples were subsampled to the lowest coverage depth and standard indices were calculated in Qiime2.

4 RESULTS AND DISCUSSION

4.1 Inoculum Collection and Characterization

Sixteen locations were sampled across the Minto mine following discussions with mine staff to determine where conditions would be suitable to find denitrifying bacteria. GPS coordinates, detailed sampling locations and descriptions can be found in Appendix I. It is important to note that the winter preceding this fall field visit had been an extraordinary year for snowfall and the hydrology of the site was not typical because of increased meteoric water on site. The snow water equivalent of the central Yukon was roughly 135% higher that year than the historical median (Yukon Government 2021).

4.1.1 Nitrogen Compounds

The average NH_4^+ concentration among the 16 inoculum samples was 10.2 mg/L N-NH_4^+ (Table 9) This is much higher than the average concentrations recorded in MIW at the mine (W62 monitoring data not shown), 0.045 ± 0.116 mg/L. This may be due to the time elapsed between collecting samples and testing for nitrogen compound concentrations, approximately 3 months stored at 5°C. Nitrite concentrations were below the limit of detection (0.2 mg/L) for all samples, except for Minto 16 at 0.22 mg/L (Table 9). Nitrate results varied from below the limit of detection (0.2 mg/L) to 16.0 mg/L and 40.10 mg/L in Minto 5 and Minto 6, respectively.

Table 9 Nitrogen compounds recorded in sediment samples collected to target denitrifying bacteria at the Minto Mine site

Inoculum source	N-NH_4^+ (mg/L)	N-NO_2^- (mg/L)	N- NO_3^- (mg/L)
Minto 1	12.9	< LOD*	0.47
Minto 2	12.1	< LOD	0.36
Minto 3	9.52	< LOD	< LOD
Minto 5	10.5	< LOD	40.1
Minto 6	11.2	< LOD	16.0
Minto 7	13.9	< LOD	0.56
Minto 8	12.4	< LOD	< LOD
Minto 9	5.55	< LOD	1.60
Minto 11	9.21	< LOD	0.81
Minto 12	8.17	< LOD	0.31
Minto 14	11.5	< LOD	< LOD
Minto 15	9.41	< LOD	0.80
Minto 16	12.6	0.22	< LOD
Minto 17	0.14	< LOD	0.46
Minto 19	12.9	< LOD	0.66
Minto 20	12.4	< LOD	1.59
Minimum	0.14	0.22	0.31
Maximum	13.87	0.22	40.10
Average	10.25	0.22	5.31
Standard Deviation	3.44	-	11.80

* Limit of detection.

4.1.2 Metal Concentrations

High concentrations of As, Co, Cr, and Ni can inhibit denitrification by interfering with enzymes associated with denitrification or by indirectly impacting soil properties (Li et al. 2018;). Concentrations of these elements were below the limit of detection for all inoculum samples (Table 10). The sample collected from Minto 15 was lost due to a spill was therefore not analyzed for metals.

Copper is an essential micronutrient in the growth of denitrifying bacteria, but it can have inhibitory effects at elevated concentrations (Cabello et al. 2004; Li et al. 2018). Copper concentrations recorded in MIW at the mine (W62) averaged 0.049 ± 0.013 mg/L (data not shown). The Cu concentration in the inoculum samples was lower than that of the MIW for all samples except for Minto 5 and Minto 6, at 0.146 mg/L and 0.068 mg/L, respectively (Table 10). These samples were both collected in a wetland located in a topographic depression adjacent to an ore pad. This

indicates that out of the various locations sampled, this area might be the most influenced by site geochemistry. This is supported by higher-than-average nitrate concentrations when compared to the other samples collected at the mine (Table 9).

Sulphur was similarly elevated above average values in Minto 5 and Minto 6 (Table 10). This might further indicate that this area was impacted by the geochemistry of the copper sulphide ore. Sulphur was also elevated in the samples Minto 16 and Minto 17 at 53.6 mg/L and 84.3 mg/L, respectively (Table 10). These samples were collected in a wetland adjacent to waste rock pile, however, Minto 14, collected within the same wetland, had just 1.9 mg/L S, and none of the samples collected in the area had high Cu concentrations. Additionally, variable Al, Cu and S results are likely due to the heterogeneity of environments from which sediments were collected.

Phosphorus is essential to the growth and metabolism of denitrifying bacteria (Vitousek and Howarth 1991). The concentrations of phosphorus in the samples ranged from 0.03 to 0.16 mg/L (Table 10), suggesting that phosphorus would not be a limiting factor regarding the presence of the targeted bacteria.

Table 10 Select metal results (mg/L) from sediment collected to target denitrifying bacteria

Inoculum sample	Metal concentration (mg/L)													
	Al	As	Ca	Cd	Co	Cr	Cu	Fe	Mo	Ni	P	Pb	S	Zn
Minto 1	0.09	<0.04	83.9	<0.01	<0.01	<0.01	0.013	0.11	0.03	<0.01	0.12	<0.03	31.3	<0.01
Minto 2	0.17	<0.04	85.5	<0.01	<0.01	<0.01	0.007	0.89	0.01	<0.01	0.16	<0.03	1.73	<0.01
Minto 3	0.12	<0.04	107	<0.01	<0.01	<0.01	0.008	0.21	0.01	<0.01	0.04	<0.03	4.49	<0.01
Minto 5	0.46	<0.04	111	<0.01	<0.01	<0.01	0.146	0.56	0.02	<0.01	0.05	<0.03	62.6	<0.01
Minto 6	0.09	<0.04	151	<0.01	<0.01	<0.01	0.068	0.08	0.02	<0.01	0.05	<0.03	105	<0.01
Minto 7	0.10	<0.04	32.9	<0.01	<0.01	<0.01	0.012	0.31	<0.01	<0.01	0.07	<0.03	4.35	<0.01
Minto 8	0.07	<0.04	58.9	<0.01	<0.01	<0.01	0.009	0.74	<0.01	<0.01	0.09	<0.03	1.21	<0.01
Minto 9	0.53	<0.04	44.0	<0.01	<0.01	<0.01	0.014	0.47	<0.01	<0.01	0.09	<0.03	7.84	<0.01
Minto 11	0.54	<0.04	69.4	0.08	<0.01	<0.01	0.016	0.59	<0.01	<0.01	0.05	1.43	1.61	0.05
Minto 12	0.39	<0.04	100	0.08	<0.01	<0.01	0.036	0.53	0.02	<0.01	0.09	1.41	6.26	0.05
Minto 14	0.19	<0.04	106	0.08	<0.01	<0.01	0.011	0.21	0.02	<0.01	0.08	1.40	1.9	0.05
Minto 16	0.21	<0.04	118	0.07	<0.01	<0.01	0.014	0.39	0.02	<0.01	0.07	1.33	53.6	0.05
Minto 17	0.22	<0.04	117	0.08	<0.01	<0.01	0.013	0.19	0.02	<0.01	0.05	1.54	84.3	0.05
Minto 19	0.17	<0.04	87.7	0.08	<0.01	<0.01	0.015	0.10	0.04	<0.01	0.10	1.44	4.81	0.05
Minto 20	0.89	<0.04	31.4	0.08	<0.01	<0.01	0.012	1.38	<0.01	<0.01	0.09	1.49	1.83	0.05
Minimum	0.07	-	31.4	0.07	-	-	0.007	0.08	0.01	-	0.04	1.33	1.21	0.05
Maximum	0.89	-	151	0.08	-	-	0.146	1.38	0.04	-	0.16	1.54	105	0.05
Average	0.28	-	87.0	0.08	-	-	0.026	0.45	0.02	-	0.08	1.43	24.9	0.05
Standard Deviation	0.23	-	34.3	0.00	-	-	0.036	0.35	0.01	-	0.03	0.07	34.7	0.00

4.2 Bioreactors Phase 1: Developing Inoculum

To determine if the sampled sediments contained the targeted denitrifying bacteria, 16 anaerobic batch BRs were inoculated with the sediments and monitored over three weeks.

4.2.1 General Chemistry

Each week, ORP, DO, pH, and conductivity values of each BR were recorded. This data can be found in Appendix IV.

All but one BR had a negative ORP value by Week 3. The outlying BR, D8, was inoculated with sediment that was not normally submerged in typical years. This suggests that this sediment sample was collected from a location that did not have conditions required to support denitrifying bacteria. After three weeks of monitoring, D8 was the only BR that did not have reducing conditions. This is further supported by the results of DO monitoring, with D8 having the highest DO concentration of all BRs, at 27% DO, compared to the next highest at 14.8% DO (D10) (Appendix IV).

4.2.2 Nitrogen Compounds

The initial nitrate concentration in each BR was approximately 250 ppm. All the 16 BRs were successful in removing NO_3^- with little to no increase in NH_4^+ or NO_2^- (Figure 10). The highest removal rates were observed in D1, D3, D4, D6, D7, D13, D14, and D15, with nitrate removal rates between 98-100% in these BRs (Figure 10). This suggests that all BRs may have been inoculated with denitrifying bacteria, and these populations varied in nitrate removal capacity. The lowest removal rate was in D8, with a nitrate removal rate of just 22.8% (Figure 11). The sediment used to inoculate D8 was sandy material that had no odour and though it was submerged when it was collected at the mine, this location was not usually underwater in previous years (personal communication with mine staff). This suggests that this location may not normally have the anaerobic conditions required to support denitrifying bacteria. Therefore, D8 was excluded in the inoculum source in the succeeding phases of the experiment. Additionally, there was little to no accumulation of ammonium or nitrite in the BRs, suggesting complete dentification. However, this cannot be confirmed without the capture and measurement of gaseous N compounds such as N_2O and N_2.

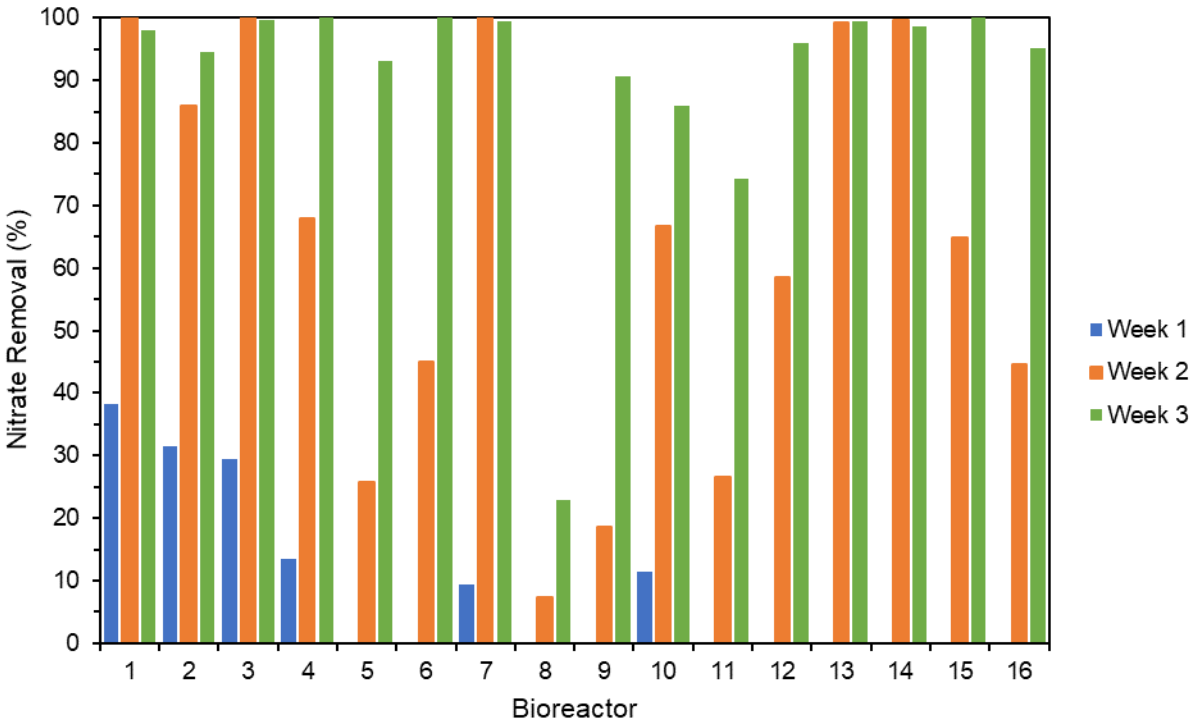


Figure 10 **Nitrate removal percentage in 16 denitrifying bioreactors**

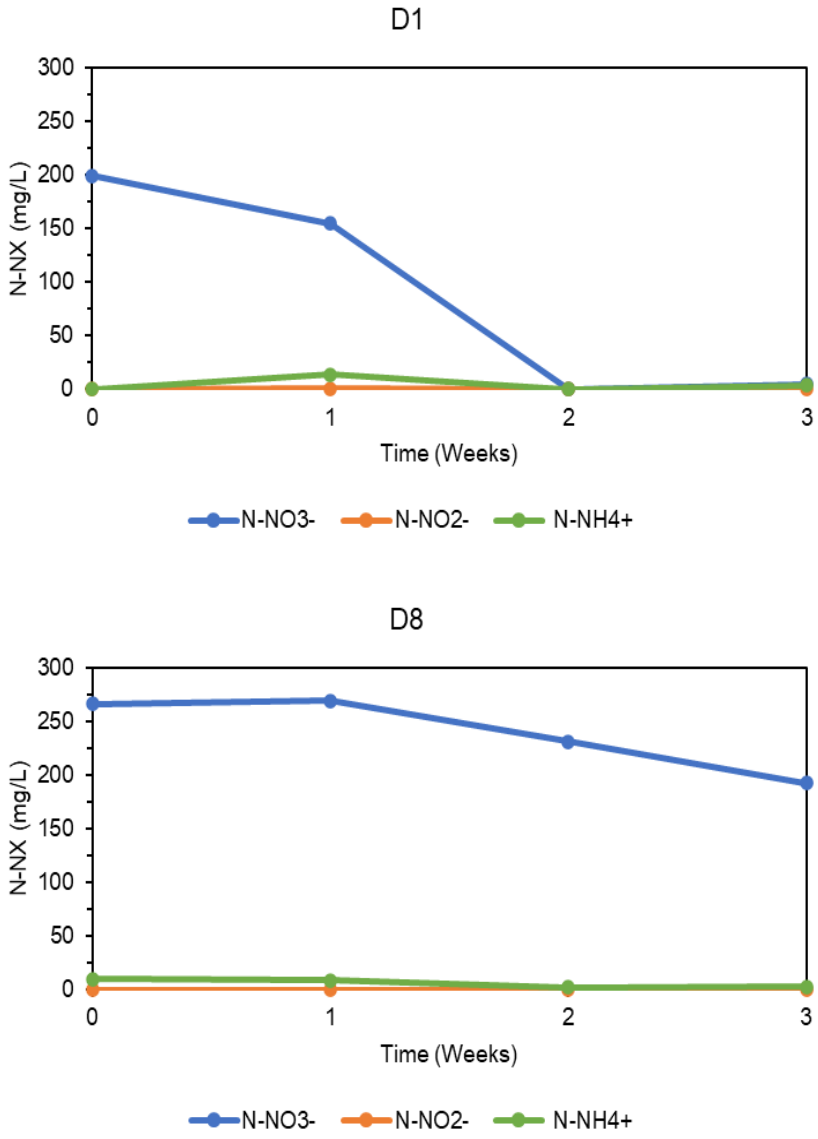


Figure 11 **Concentrations of nitrate, nitrite, and ammonium in 2 of 16 denitrifying bioreactors**

4.3 Bioreactors Phase 2: Optimizing Inoculum

The second phase of the batch BRs were inoculated using the combined inoculum from Phase 1. The media designed to support the bacterial populations was the same as Phase 1, including the initial nitrate concentration. There were two BRs in this phase, duplicates of each other. Therefore, the results presented in this section are the average values from the two BRs.

4.3.1 General Chemistry

The complete results for pH, ORP, DO and conductivity for these BRs can be found in Appendix IV. The pH of the BRs remained near neutral for the duration of the experiments, with an average pH value of 7.67 ± 0.36 across all sampling events. The ORP values were negative throughout the experiment (Figure 12), suggesting reducing conditions. Dissolved oxygen concentration values decreased during the first week of the experiment from 74% to 6.3% and remained relatively low through to week 3 (Figure 12).

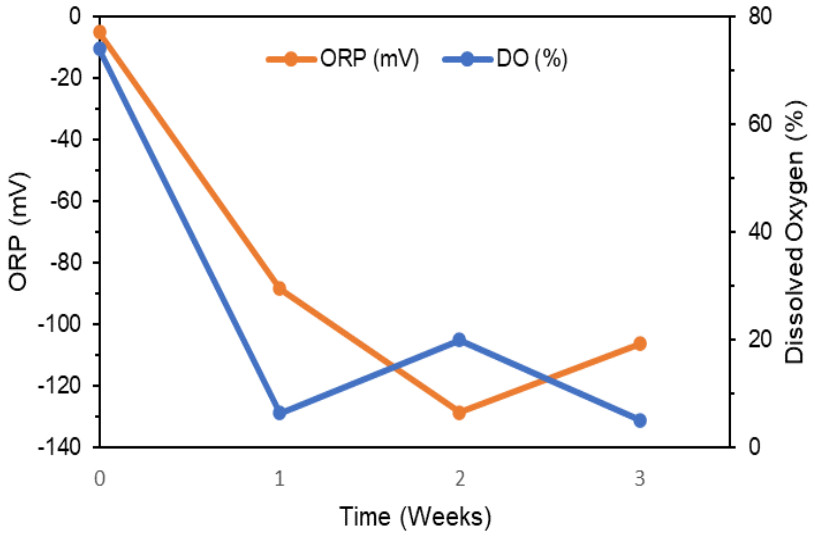


Figure 12 Averaged ORP and DO values for duplicate Phase 2 denitrifying bioreactors

4.3.2 Carbon Concentration

The carbon concentration in both BRs decreased over the three weeks of monitoring (Table 11). This suggests that carbon was being utilized in the dentification process but did not decrease so much as to be a limiting factor.

Table 11 Carbon concentration in Phase 2 bioreactors

Week	Bioreactor	
	D17 TOC (g/L)	D17 duplicate TOC (g/L)
0	5.38	5.41
1	2.43	2.32
2	2.23	2.13
3	2.08	1.94

4.3.3 Nitrogen Compounds

Nitrate concentration decreased 89.8% after one week, 97.5% after two weeks and 97.8% after three weeks (Figure 13). This supports the findings of the Phase 1 BRs, that denitrifying bacteria were present in the inoculum and these populations were successfully developed in Phase 2. Additionally, there was little to no accumulation of ammonium or nitrite, suggesting complete dentification. However, gases including N_2O and N_2 were not captured so the final fate of N within the BRs cannot be confirmed.

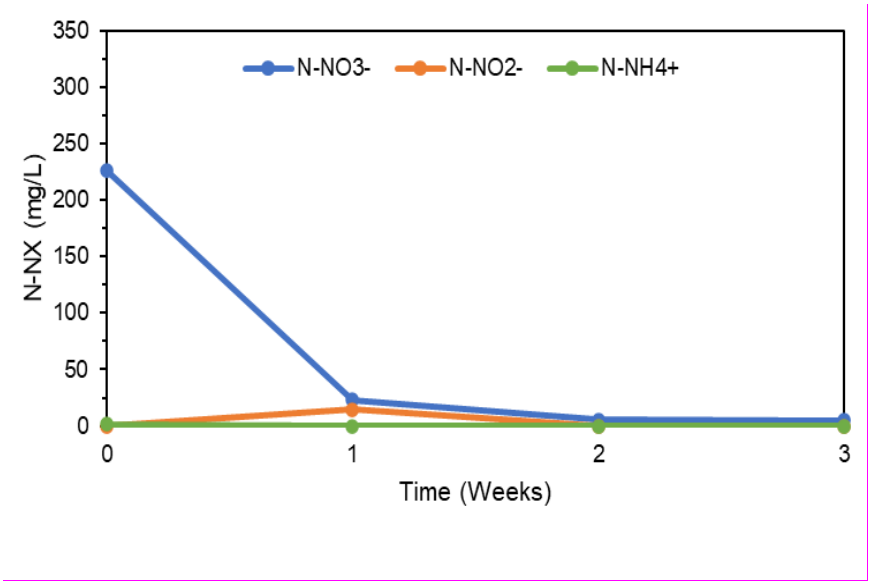


Figure 13 **Averaged nitrogen compound concentration for duplicate Phase 2 denitrifying bioreactors**

4.4 Phase 3: Carbon Testing Bioreactors

The previous two phases of BRs utilized sodium acetate as a carbon source for the denitrification. Local carbon sources (detailed in Section 3.6) were used in this phase to test their ability to support bacterial denitrification.

The initial nitrate concentrations were also changed in this phase. Based on discussions with mine staff, BRs were tested with nitrate concentrations of 25 and 100 mg/L $N\text{-}NO_3^-$**.** All BRs were made in duplicate; the results presented are average values from the duplicate BRs. Carbon amendments were adjusted to maintain the 20:1 carbon to nitrogen ratio.

4.4.1 General Chemistry

Reducing conditions were indicated by negative ORP (-102 ± 29 mV) and relatively low DO concentrations (0.2 to 10% DO) in all BRs, omitting those with compost, wood shavings, and wood chips as their carbon sources in Phase 3.1 (Appendix IV). Bioreactors with compost, wood shavings and wood chips have positive ORP values (36 ± 31 mV) and DO values ranging from 14.9 to 47.4% DO.

Similar results were found in Phase 3.2, except for the compost BRs, which had negative ORP values rather than the positive values observed in Phase 3.1. pH was near neutral for all BRs (data not shown). The results for the BRs that had WSC as the carbon source are displayed below as they are most relevant to subsequent phases of the project (Figure 14). In Phase 3.1, the ORP in the WSC BRs was negative from weeks 1 – 3 and pH remained neutral. Phase 3.2 WSC BRs also had negative ORP values, but pH was lower than in Phase 3.1.

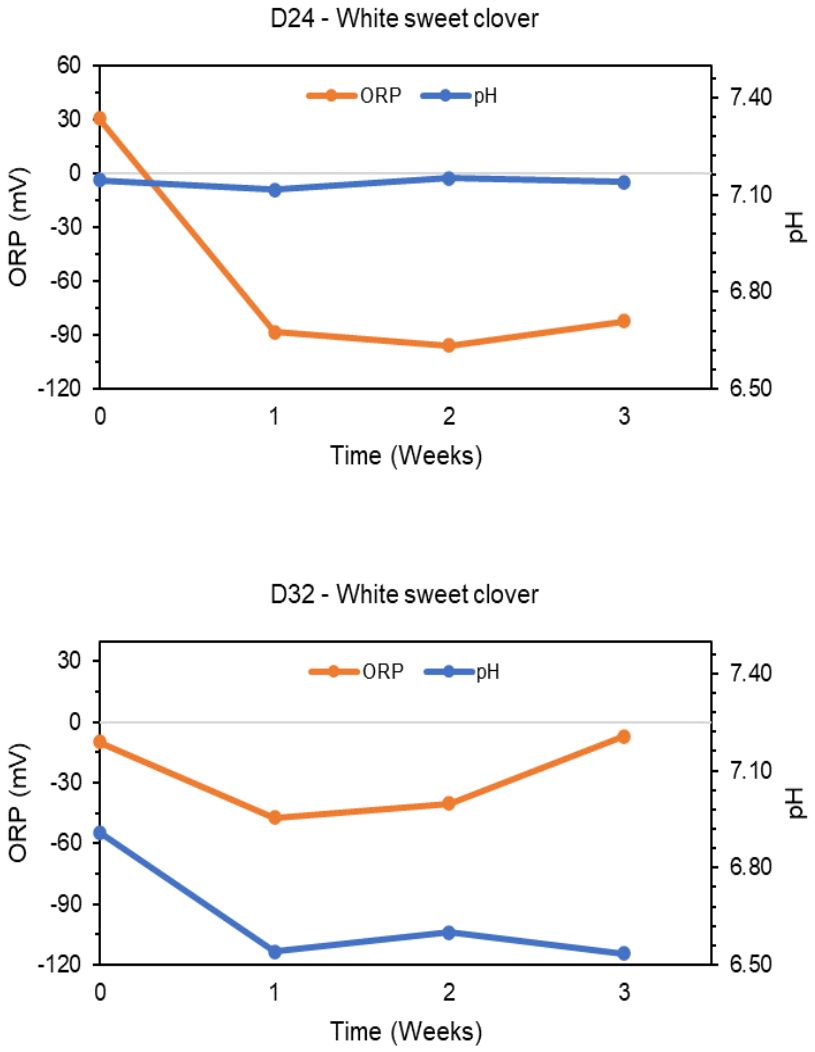


Figure 14 ORP and pH values for Phase 3 bioreactors with WSC

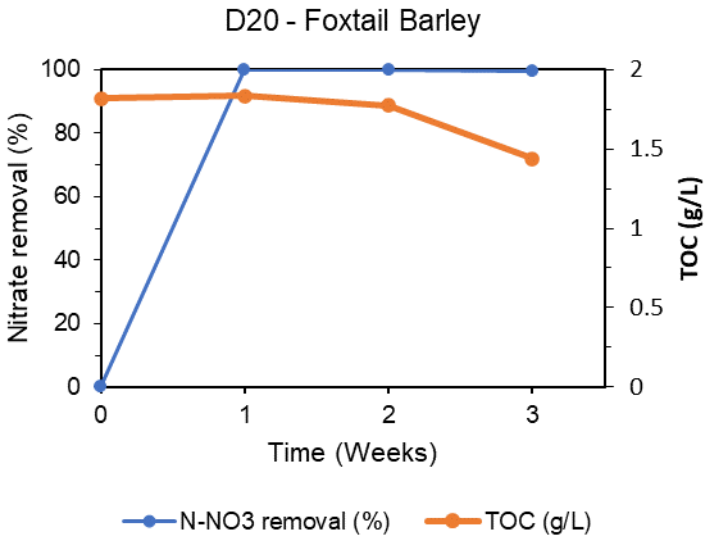
4.4.2 Carbon Concentration

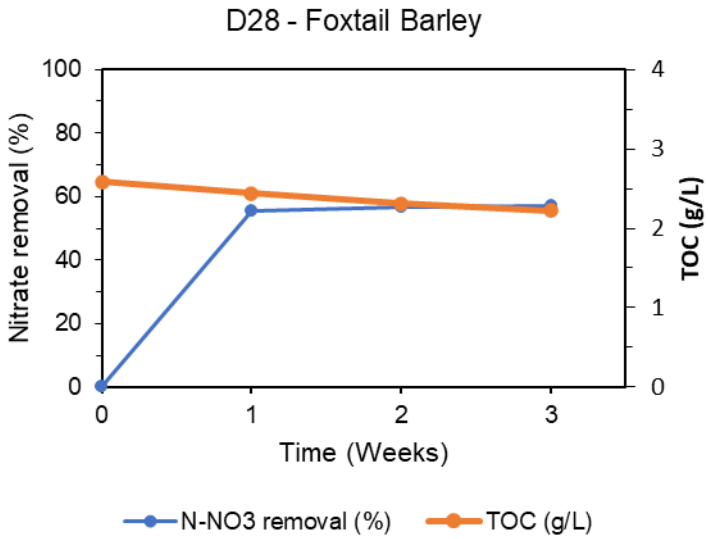
Carbon concentrations decreased between the first and third week in all BRs except in one of the Phase 3.2 BRs with WSC. However, this may be an outlying data point, as all other WSC BRs had a 13-22% decrease in TOC concentration over the three weeks.

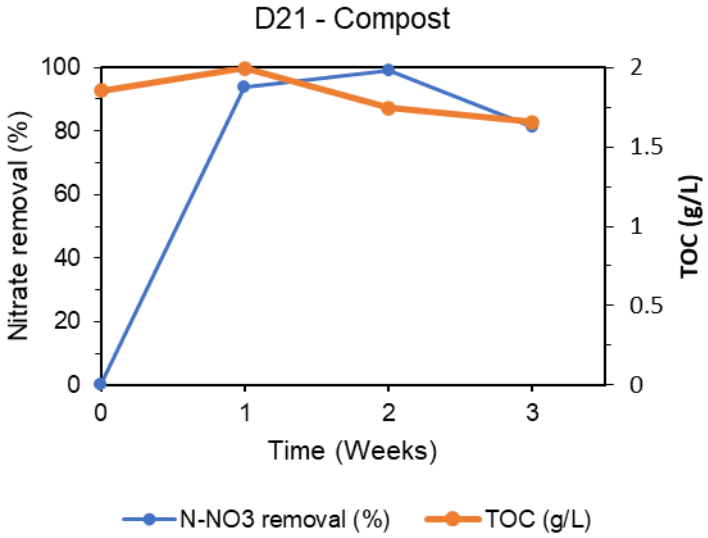
Carbon concentrations ranged from roughly 1.5 to 2 g/L in Phase 3.1 across all carbon sources (Figure 15). Phase 3.2 BRs ranged from 1.7 to 3.5 g/L TOC, despite starting with masses of carbon sources four times greater than in Phase 3.1 (Table 5). These results were not limited to the complex carbon sources, as the sodium acetate behaved similarly. This suggests that there may be a difference in carbon utilization between the denitrification occurring at 25 mg/L N-NO_3^- and 100 mg/L N-NO_3^-.

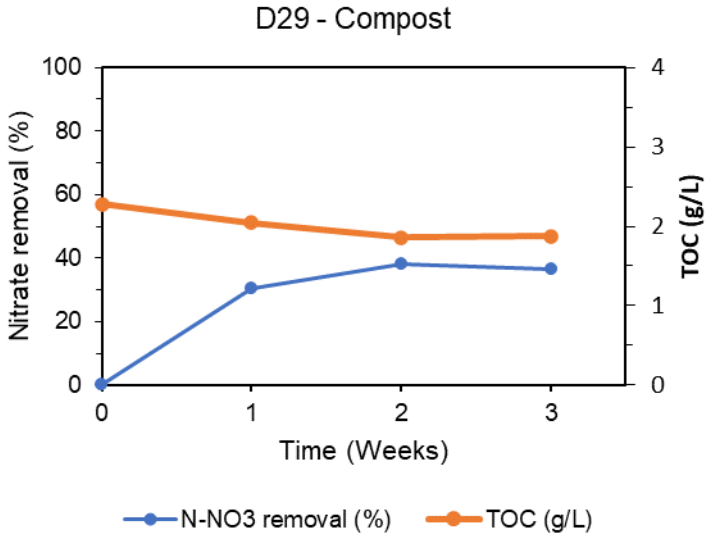
All BRs in Phase 3 were inoculated with 50 mL of fluid from the Phase 2 BRs. As the Phase 2 BRs did not see a complete reduction of carbon, the inoculum from these BRs would have introduced additional carbon into the Phase 3 BRs and may partially account for the discrepancy in expected versus observed TOC concentration.

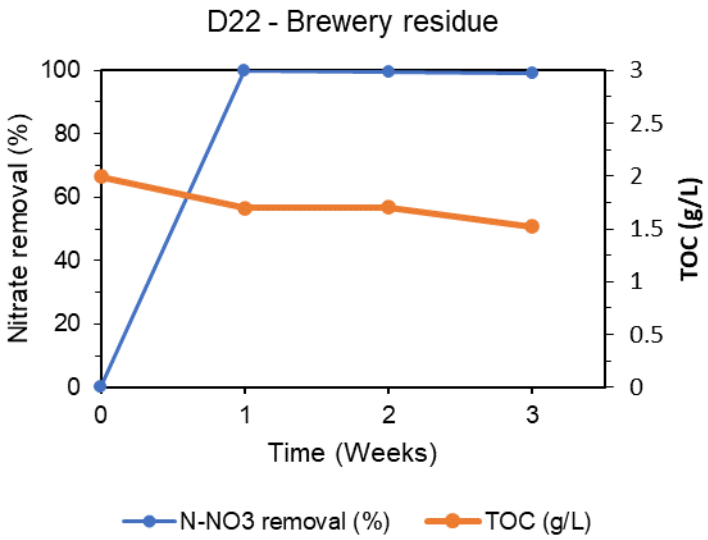
There was carbon present in the control BRs, despite the lack of intentional carbon addition to these BRs. This indicates that there were other sources of carbon in the media, likely including the Difco nutrient broth. However, the carbon concentration in the control BRs did not change more than 0.05 g/L over the course of the experiment (Figure 15).

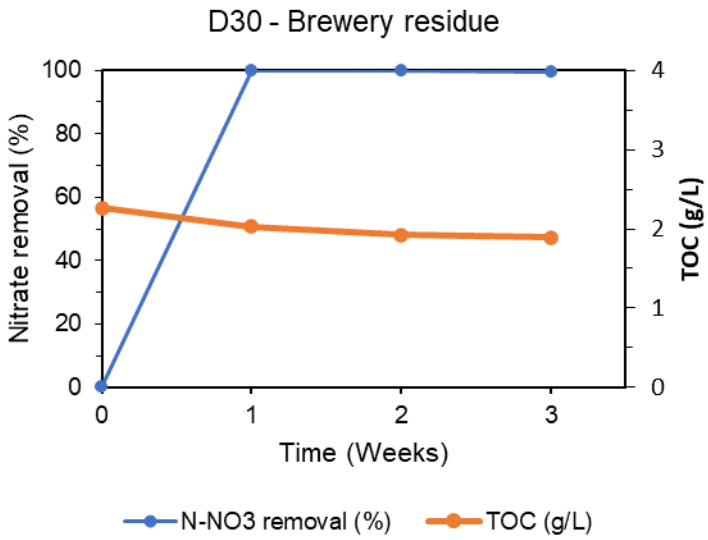

D20 - Foxtail Barley
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)

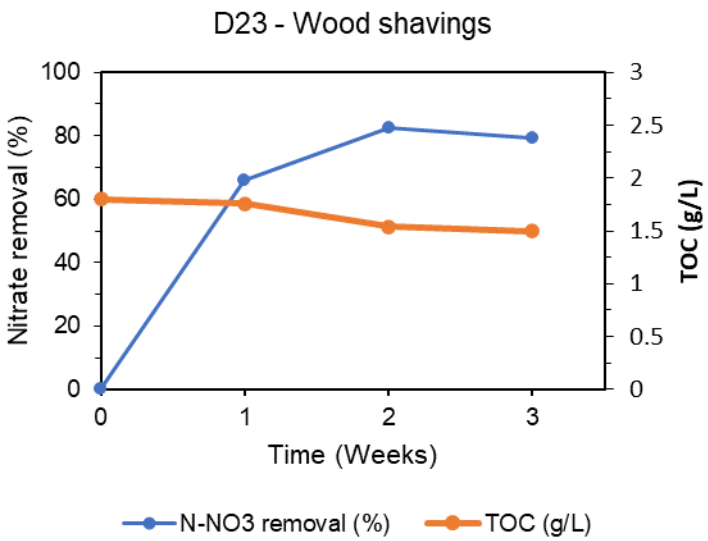

D28 - Foxtail Barley
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)

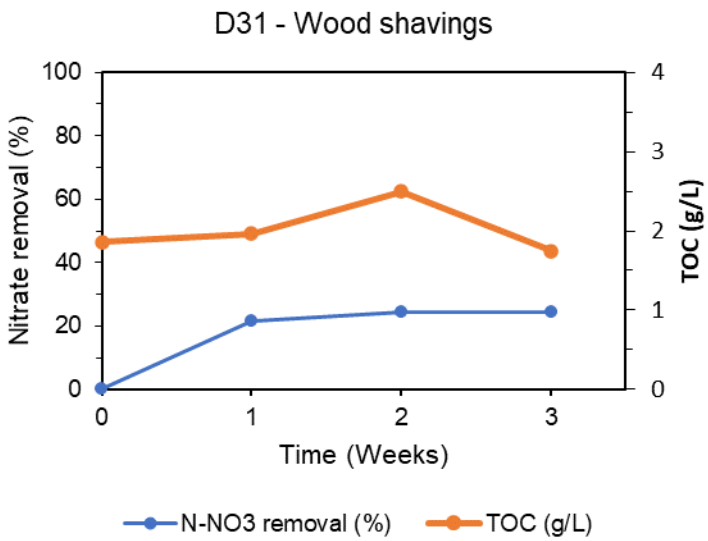

D21 - Compost
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)

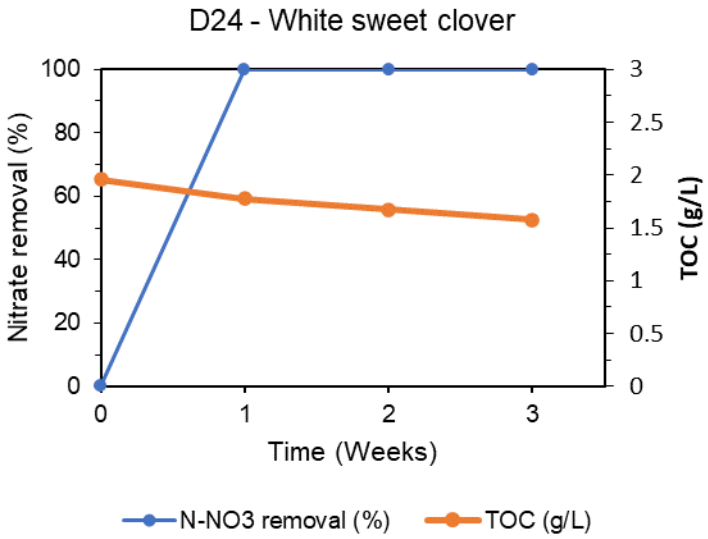

D29 - Compost
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)

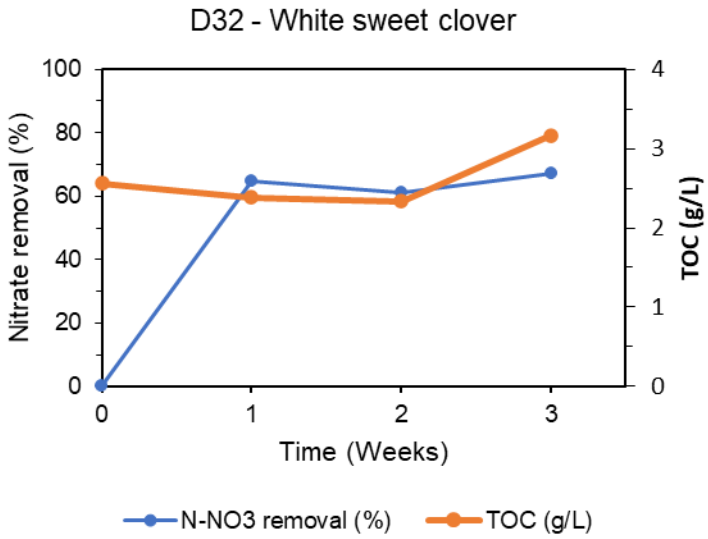

D22 - Brewery residue
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)

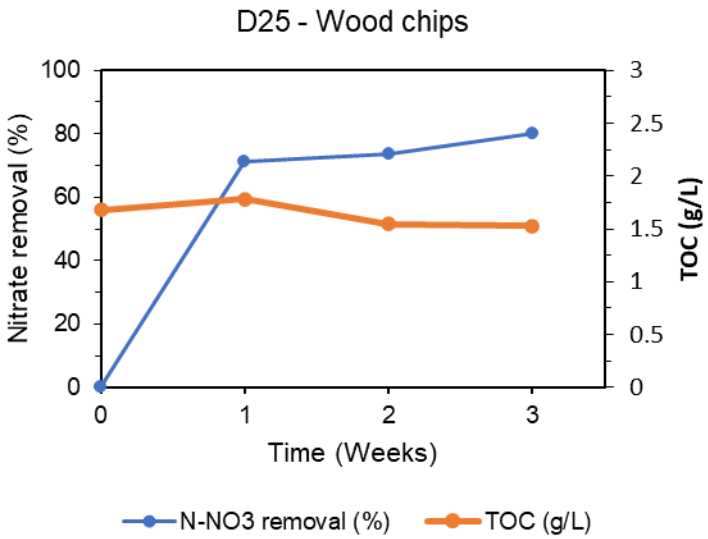

D30 - Brewery residue
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)

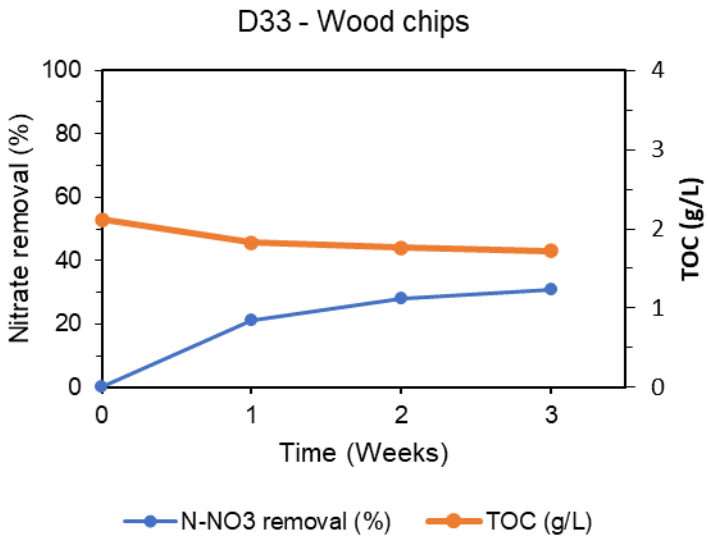

D23 - Wood shavings
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)

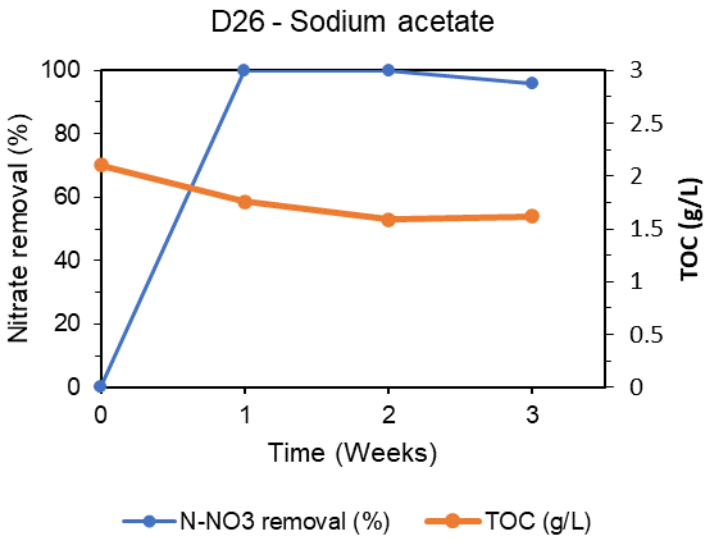

D31 - Wood shavings
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)

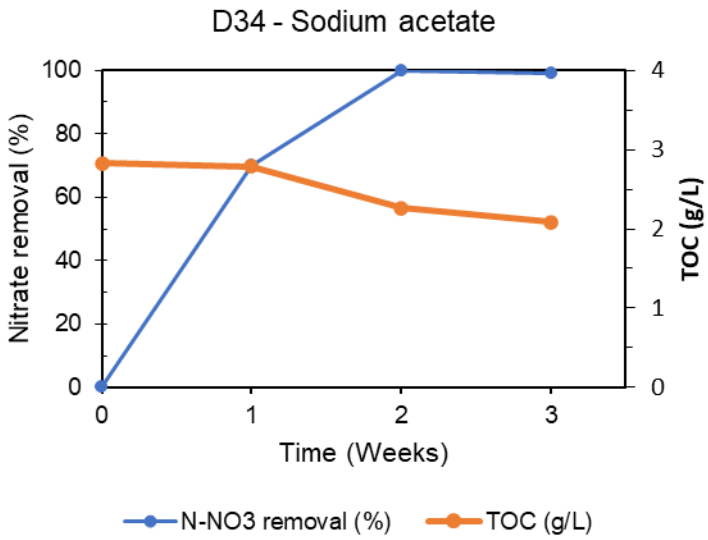

D24 - White sweet clover
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)

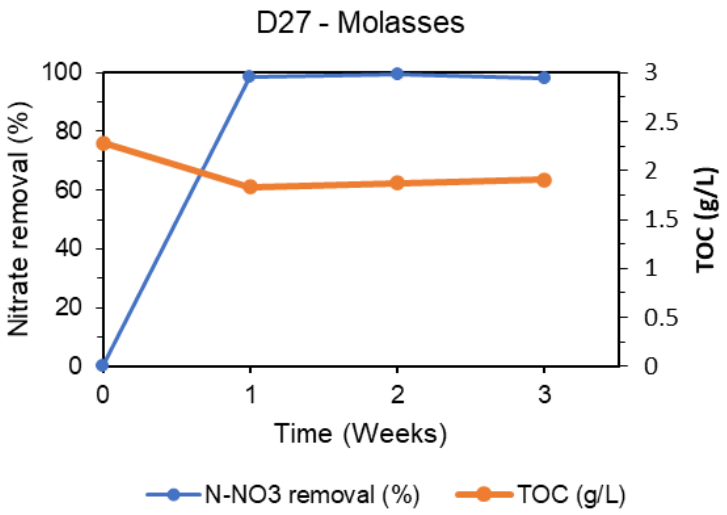

D32 - White sweet clover
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)

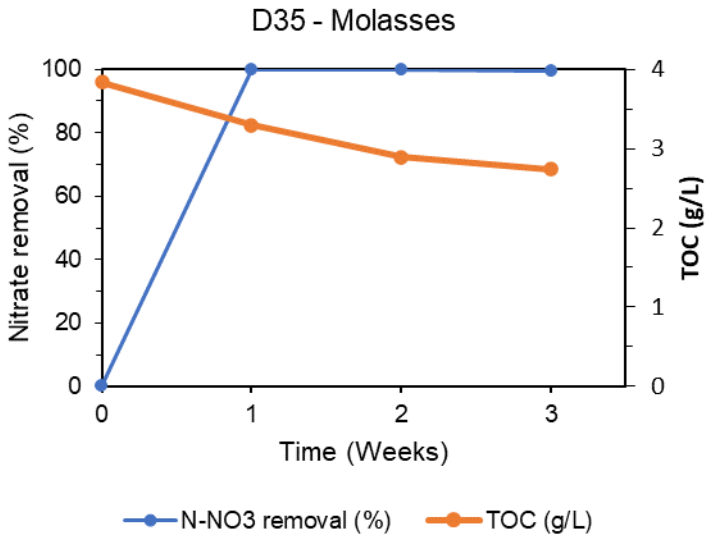

D25 - Wood chips
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)


D33 - Wood chips
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)


D26 - Sodium acetate
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)


D34 - Sodium acetate
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)


D27 - Molasses
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)


D35 - Molasses
Nitrate removal (%)
TOC (g/L)
Time (Weeks)
N-NO3 removal (%)
TOC (g/L)

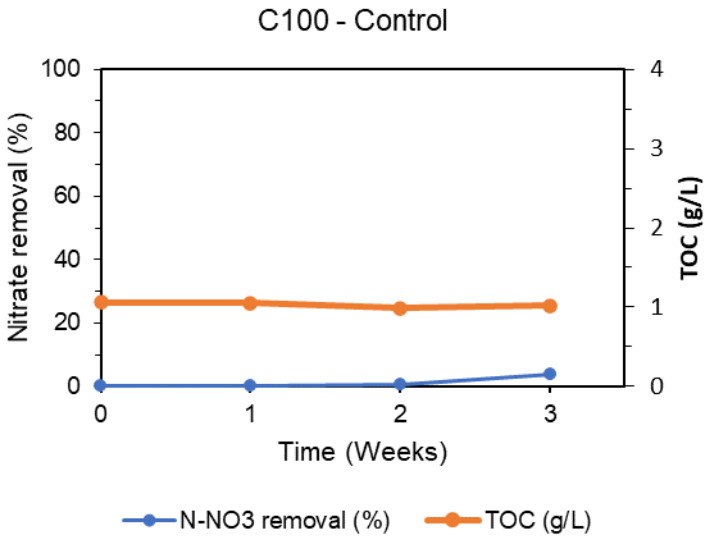


Figure 15 **Carbon concentrations plotted with nitrate removal percentage in Phase 3 bioreactors**

4.4.3 Nitrogen Compounds

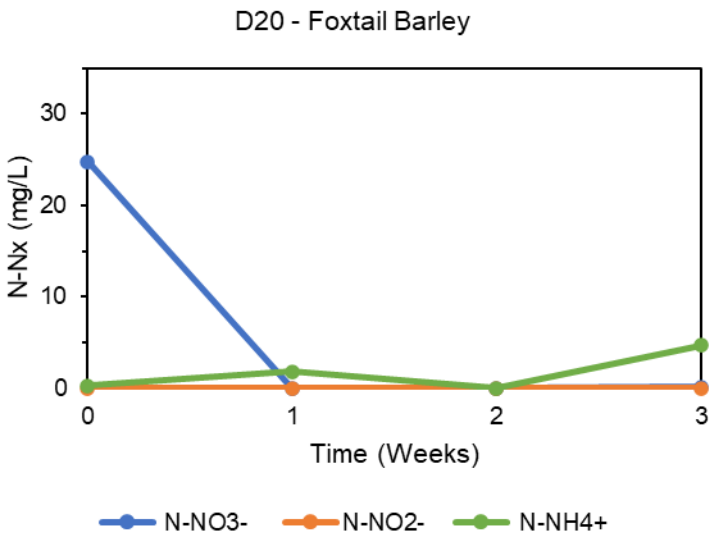
The nitrate removal in the Phase 3.1 BRs ranged from 100 – 80% removal of nitrate. The highest nitrate removal was seen in the BRs that used white sweet clover, foxtail barley, and brewery residue (Table 12). Conversely, the nitrate removal in the Phase 3.2 BRs was highest in the BRs using brewery residue, molasses, and sodium acetate as carbon sources. Generally, nitrate removal results were more variable in Phase 3.2 ranging from 24.4% removal in wood shavings BR to 99.5% in molasses BRs. Based on the two batches, brewery residue performed the best out of the locally available carbon sources (99.1 – 99.5% nitrate removal) (Table 12). White sweet clover BRs had 100% removal of nitrate after three weeks in Phase 3.1, but this decreased to 67.1% removal after three weeks in the Phase 3.2 BRs. Despite having less nitrate removal than the brewery residue when initial $N\text{-}NO_3^-$ was 100 mg/L, the WSC was chosen as the carbon source to be used in the column scale BRs. This decision was informed by the availability of WSC at the Minto Mine site, and its propensity to grow readily in disturbed areas around the region.

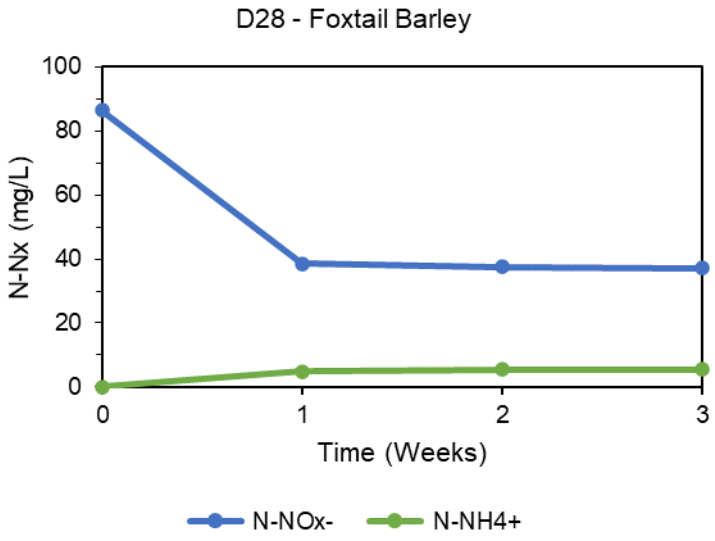
All results for the Phase 3.2 BRs are expressed in values of NO_x as analysis was only conducted for nitrite + nitrate for these samples. It is assumed that NO_2^- concentrations are negligible based on the results from Phase 3.1, which did not indicate any substantial accumulation of NO_2^- (Figure 16). Discrepancies can be observed between target media $N\text{-}NO_3^-$ concentration and actual $N\text{-}NO_x^-$. In the case of Phase 3.1, the higher than targeted $N\text{-}NO_3^-$ concentrations may be due to residual $N\text{-}NO_3^-$ in the inoculum. For Phase 3.2, $N\text{-}NO_3^-$ was lower than the targeted 100 mg/L concentration. This could be due to the inoculum having a dilution effect on nitrate concentration.

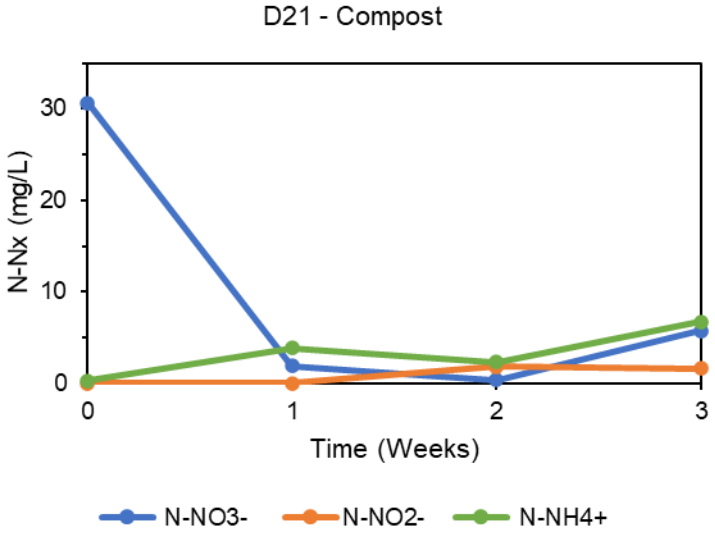
Little change was seen in N-NOx concentrations in the control BRs. This suggests that the decrease in N-NOx concentrations does not occur in the absence of either inoculum or a carbon source.

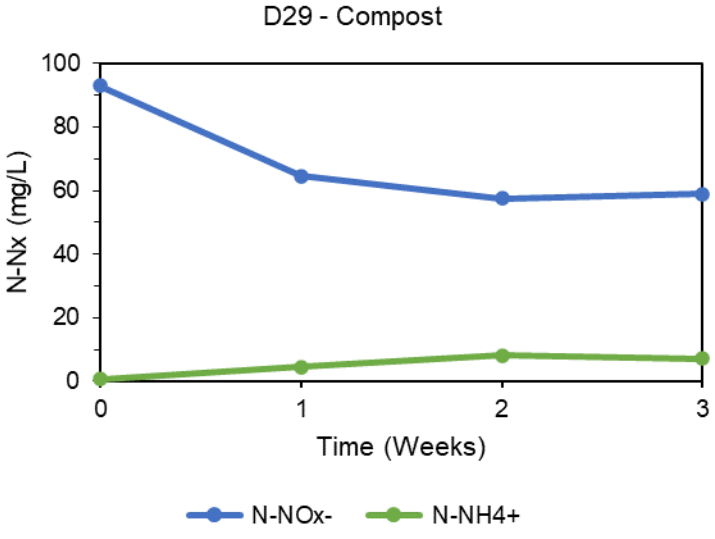
Table 12 Nitrate removal in Phase 3 bioreactors after 3 weeks

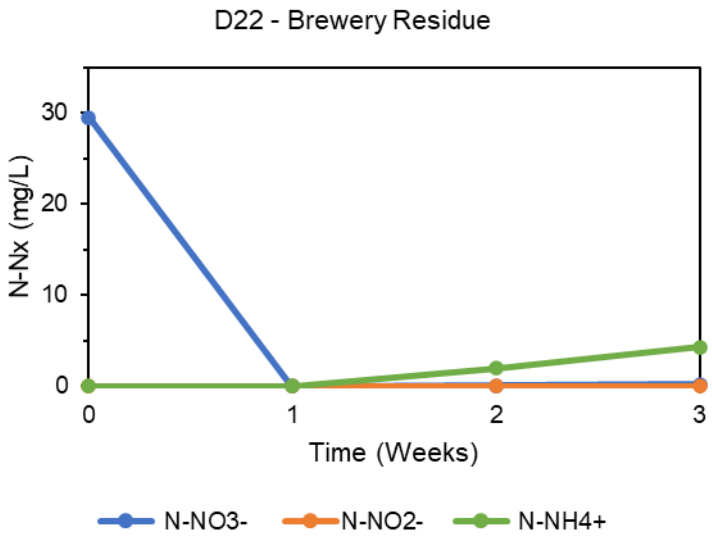
Carbon source	$N\text{-}NO_3^-$ Removal (%) Phase 3.1	N-NOx Removal (%) Phase 3.2
White Sweet Clover	100.0	67.1
Foxtail Barley	99.3	57.1
Brewery Residue	99.1	99.5
Molasses	98.3	99.5
Sodium Acetate	95.7	99.2
Compost	81.4	36.6
Wood Chips	80.0	30.7
Wood Shavings	79.3	24.4
Control	-	3.8

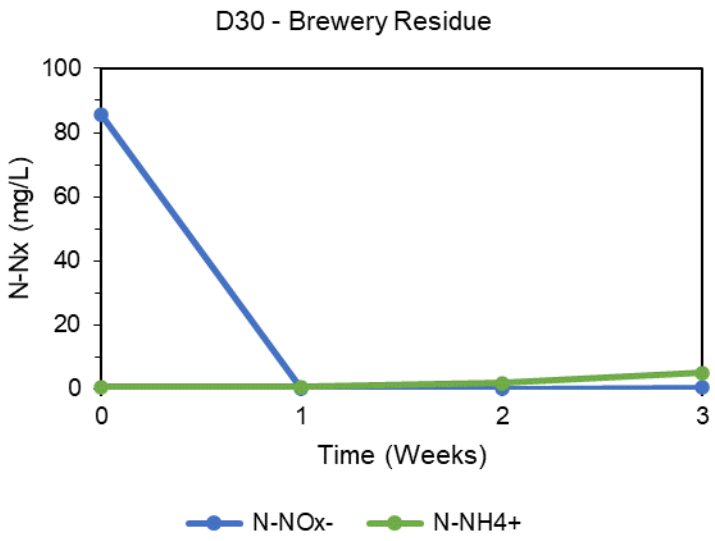

D20 - Foxtail Barley
N-Nx (mg/L)
30
20
10
0
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+

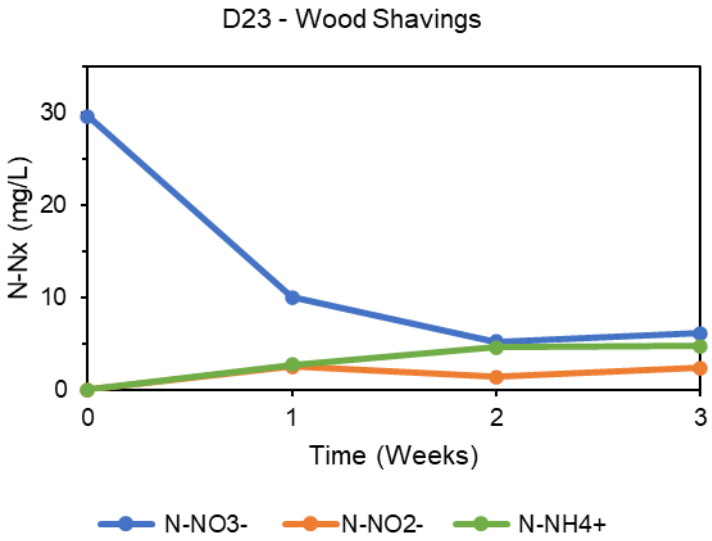

D28 - Foxtail Barley
N-Nx (mg/L)
100
80
60
40
20
0
0
1
2
3
Time (Weeks)
N-NOx-
N-NH4+

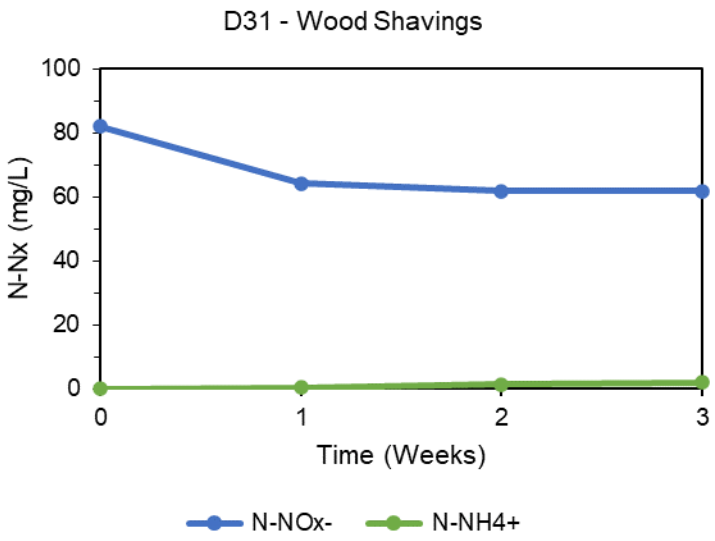

D21 - Compost
N-Nx (mg/L)
30
20
10
0
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+

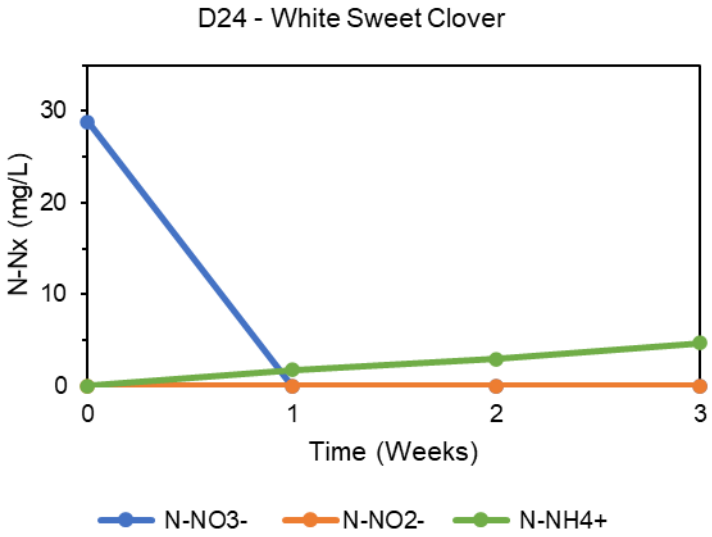

D29 - Compost
N-Nx (mg/L)
100
80
60
40
20
0
0
1
2
3
Time (Weeks)
N-NOx-
N-NH4+

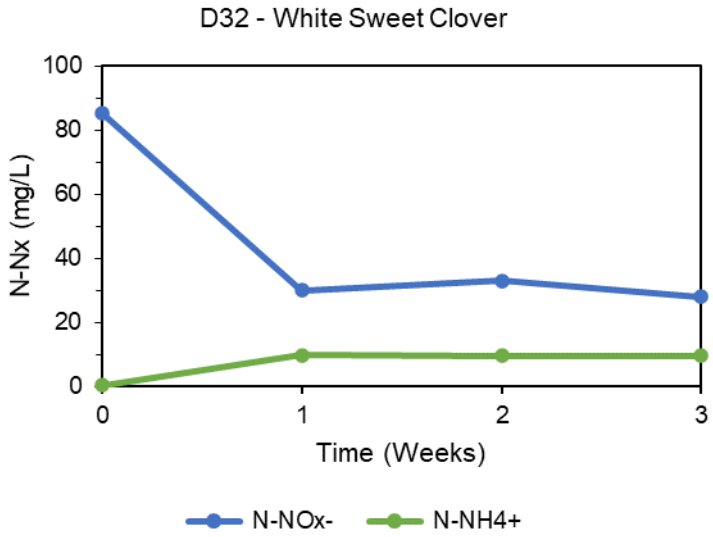

D22 - Brewery Residue
N-Nx (mg/L)
30
20
10
0
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+

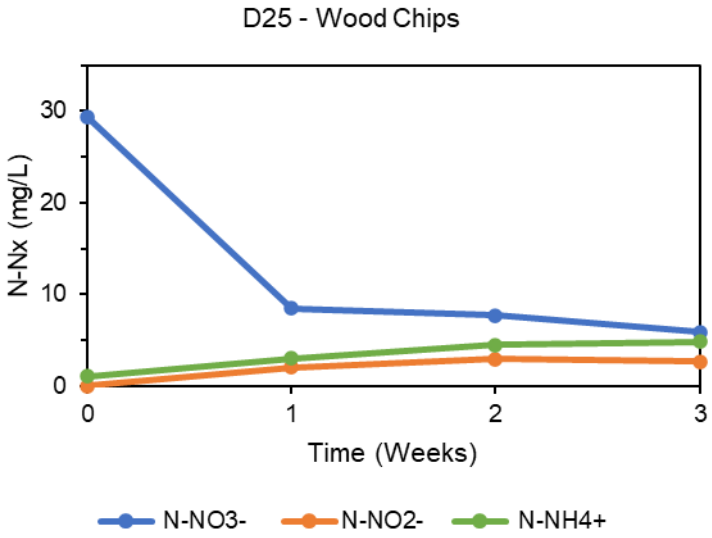

D30 - Brewery Residue
N-Nx (mg/L)
100
80
60
40
20
0
0
1
2
3
Time (Weeks)
N-NOx-
N-NH4+

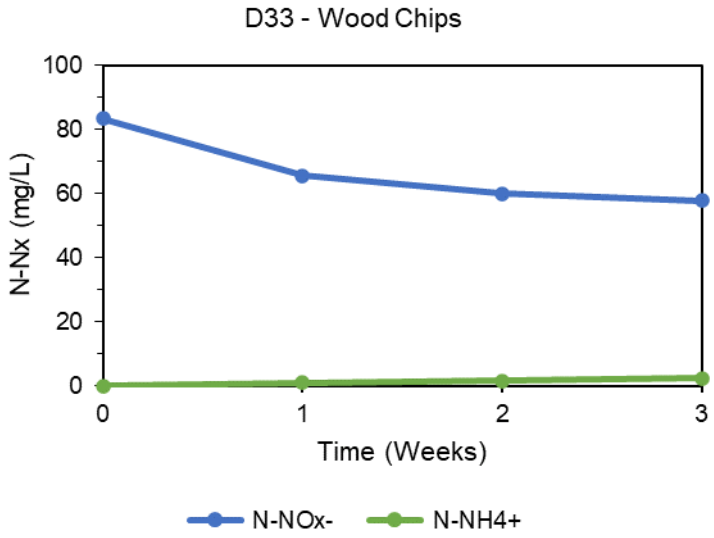

D23 - Wood Shavings
N-Nx (mg/L)
30
20
10
0
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+

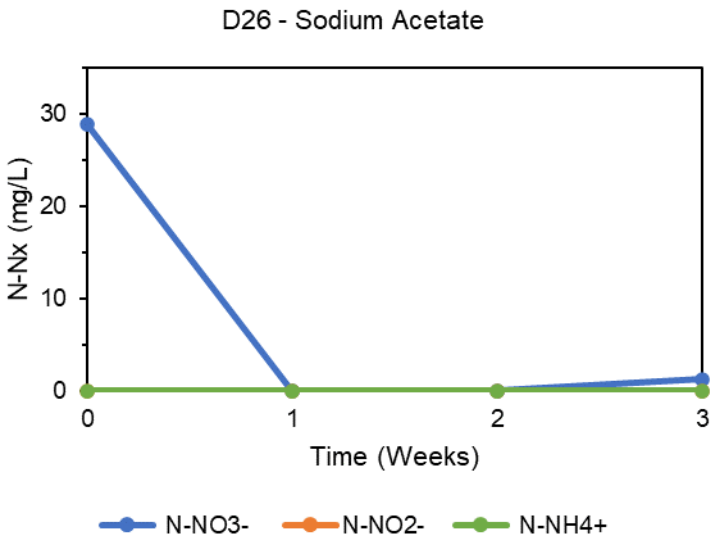

D31 - Wood Shavings
N-Nx (mg/L)
100
80
60
40
20
0
0
1
2
3
Time (Weeks)
N-NOx-
N-NH4+

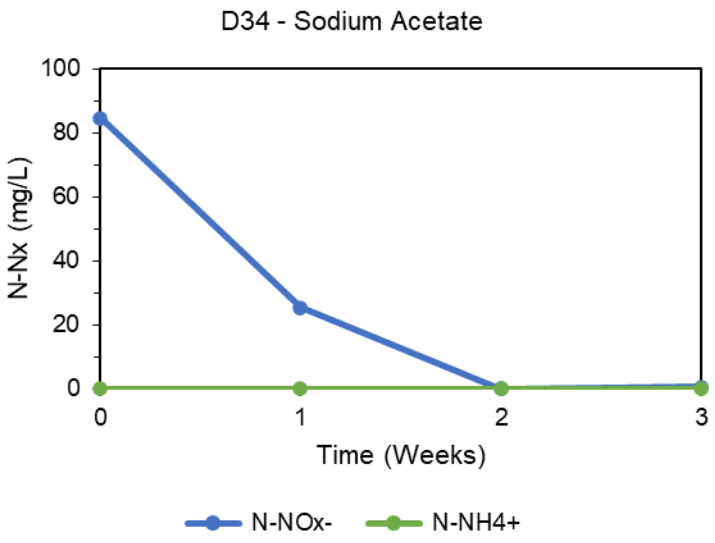

D24 - White Sweet Clover
N-Nx (mg/L)
30
20
10
0
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+

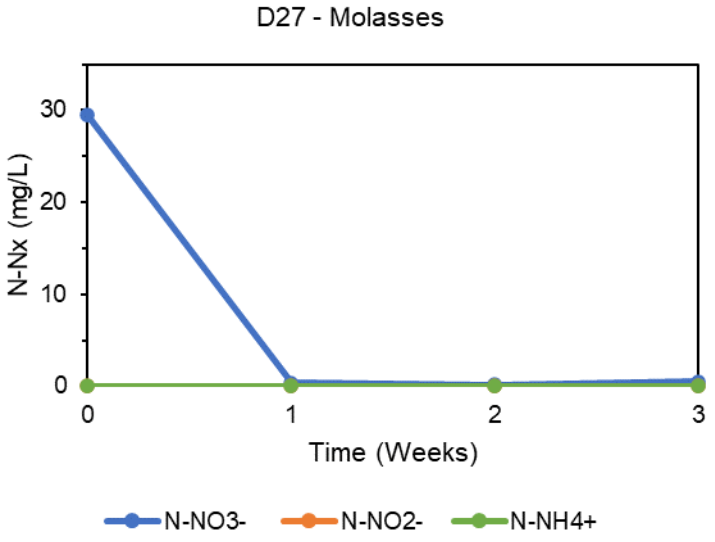

D32 - White Sweet Clover
N-Nx (mg/L)
100
80
60
40
20
0
0
1
2
3
Time (Weeks)
N-NOx-
N-NH4+

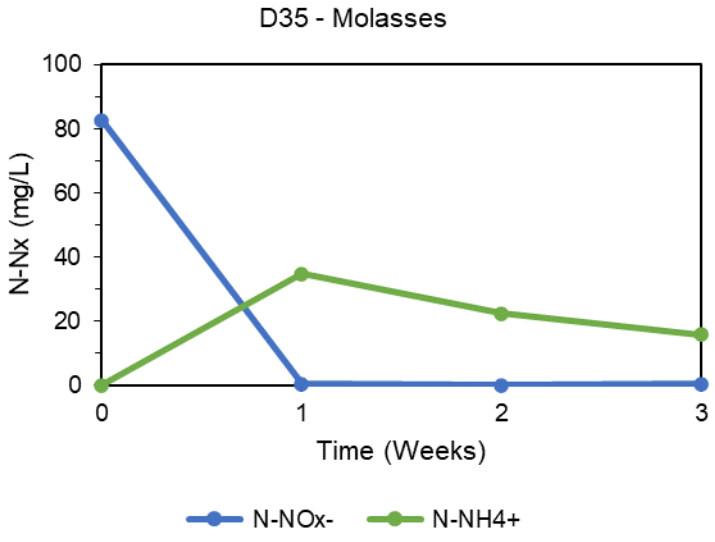

D25 - Wood Chips
N-Nx (mg/L)
30
20
10
0
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+


D33 - Wood Chips
N-Nx (mg/L)
100
80
60
40
20
0
0
1
2
3
Time (Weeks)
N-NOx-
N-NH4+


D26 - Sodium Acetate
N-Nx (mg/L)
30
20
10
0
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+


D34 - Sodium Acetate
N-Nx (mg/L)
100
80
60
40
20
0
0
1
2
3
Time (Weeks)
N-NOx-
N-NH4+


D27 - Molasses
N-Nx (mg/L)
30
20
10
0
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+


D35 - Molasses
N-Nx (mg/L)
100
80
60
40
20
0
0
1
2
3
Time (Weeks)
N-NOx-
N-NH4+

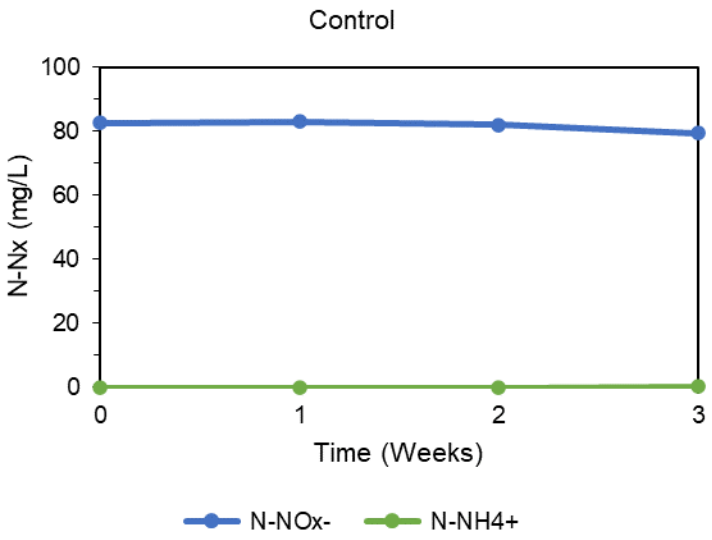


Figure 16 Concentrations of nitrogen compounds in Phase 3 bioreactors

4.5 Phase 4 Column Tests

4.5.1 Column Operations

The results of the volumes tests at the beginning of column operations were 2.295 L, 2.366, 4.115 L, and 4.095 L, for BRs 1 - 4, respectively. These values were used to calculate HRT. These volumes may have changed over the course of experiment as the WSC may have degraded over the 19 weeks of operation.

The targeted HRT for the fast flow rate columns, BR 1 and 2, was 10 hours. However, the average HRT for these columns was 14 ± 1 and 17 ± 6 hours for BR 1 and 2, respectively (Figure 17). The targeted HRT for the slow flow rate columns, BR 3 and 4, was 96 hours. The average HRT for these columns was 130 ± 13 hours for BR 3 and 116 ± 18 hours for BR 4 (Figure 17). For all columns, HRT was higher than targeted. Hydraulic retention time may have been impacted by minor leaks that occurred in all columns over the first few weeks of operation.

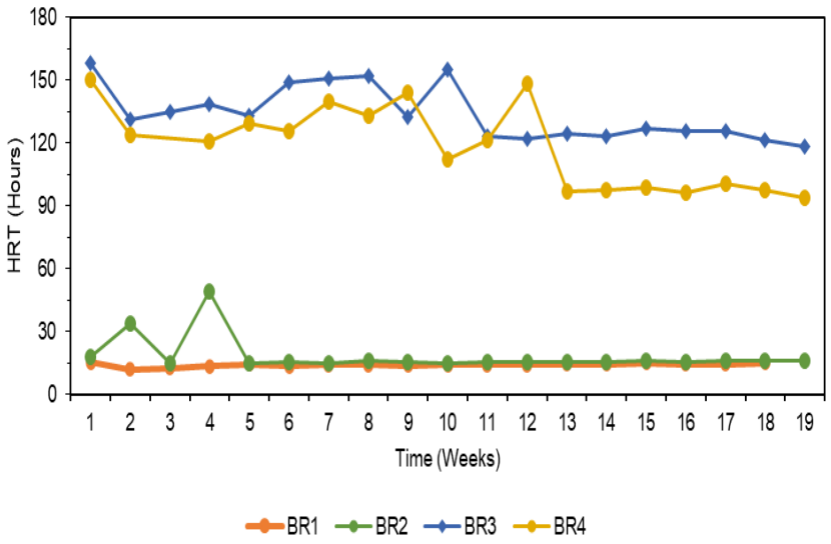


Figure 17 Hydraulic retention time over 19 weeks of Phase 4 column bioreactor operation

4.5.2 General Chemistry

The general chemistry measurements were recorded from effluent from the columns. As stated in Section 3.5.3, there were no probes installed inside the columns, therefore, results might not be representative of conditions within the columns for some parameters as a result of being exposed to the air. For example, dissolved oxygen values varied only slightly over the course of column operations, perhaps indicative of the sampling method not capturing conditions within the columns (data not shown). Effluent pH also remained unchanged over the 19 weeks, remaining near neutral in all columns (data not shown).

Notable changes in general chemistry parameters relate to the change in ORP over time. Both columns operating in cold temperatures (BR1 and 3) have negative ORP values from weeks 2 to 6, with ORP becoming positive by week 7 (Figure 18). Conversely, despite starting with negative ORP values in week 1, the two room temperature BRs (BR2 and 4) have higher ORP values than the cold BRs in weeks 2 to 7. Effluent from all columns have ORP values over 129 mV from week 9 onwards (Figure 18).

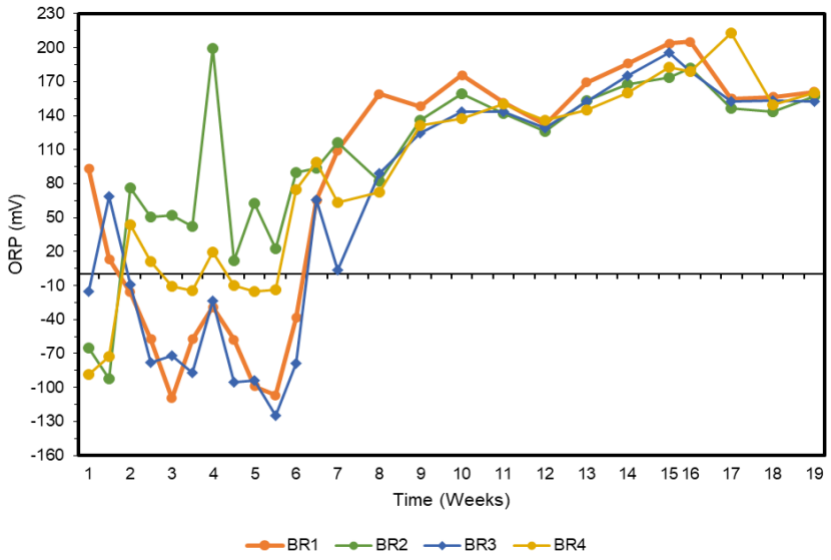


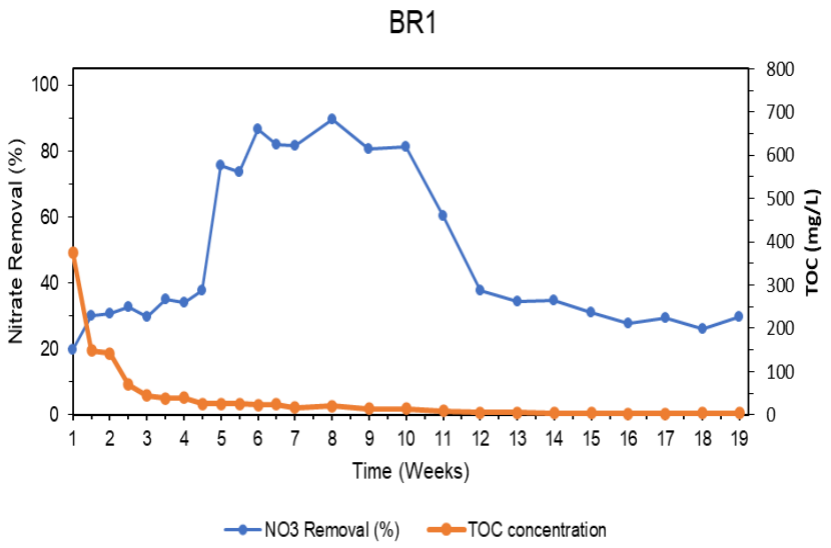
Figure 18 ORP values in Phase 4 column bioreactors

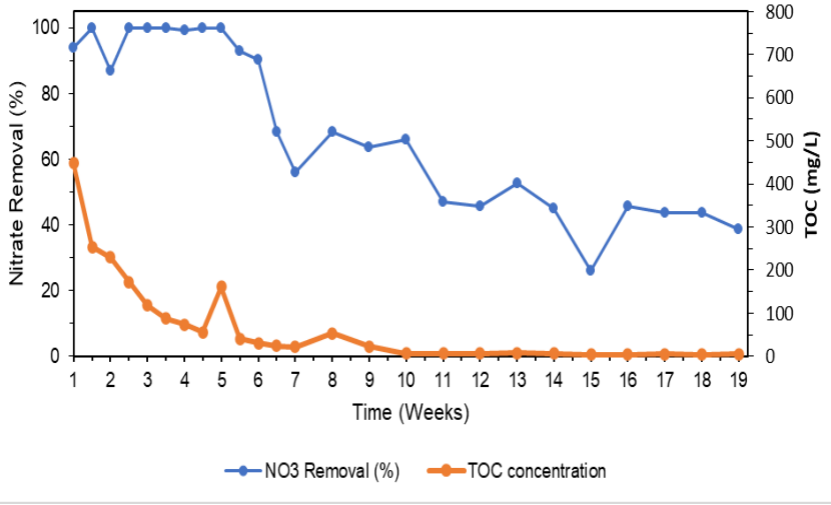
4.5.3 Carbon Concentration

Carbon concentrations in all four columns started high in week 1 in the range of 689 and 376 mg/L TOC in BR4 and BR1, respectively (Figure 19). The effluent from the columns was slightly odorous and a pale orange in colour for the first 1-3 weeks of operation, before odour dissipated, and effluent became fairly colourless for the remainder of column operations. By week 4, TOC concentrations had dropped below 100 mg/L in all columns (Figure 19). Carbon concentrations remained low, close to or below 10 mg/L from week 11. This suggests that initially, large quantities of carbon were leaching from the WSC in the columns. This decrease in carbon concentration in the effluent may be due to a variety of factors. The bio-accessible carbon in the WSC may have been exhausted, resulting in C limiting denitrification. If this were the case, it would be expected to see a rapid decrease in nitrate removal. While we do see a decrease in nitrate removal, the decrease does not occur at the same rate as decrease in carbon concentration. The C/N ratio may be a factor in this relationship.

The WSC in the columns appeared, by visual inspection, relatively unchanged over the 19-week period. Therefore, it may be assumed that substrate remained in the columns, though the less bioavailable carbon may have been available to the denitrifying microbes. Although an herbaceous annual or biennial plant, WSC is a woody species (Chen et al. 2021; Sowa-Borowiec et al. 2022). Hu et al. (2019) suggest that the high lignin content in woody biomass may inhibit

the release of carbon available to microbes in denitrifying BRs. Alkali and acidic pretreatments may be applied to enhance the breakdown of lignin in woody species, thereby increasing the carbon required for denitrification (Hu et al. 2019). However, if such pretreatments were to be applied to WSC, the treatment system would become less passive and therefore less ideal for post-closure MIW remediation. Another option to decrease the lignin content in the WSC would be to harvest the plants earlier in the growing season before the stems become woody (Sowa-Borowiec et al. 2022). Ideally, the WSC would also be harvested before going to seed, to avoid the unintentional spread of this invasive species.


BR1
Nitrate Removal (%)
TOC (mg/L)
Time (Weeks)
NO3 Removal (%)
TOC concentration


BR2
Nitrate Removal (%)
TOC (mg/L)
Time (Weeks)
NO3 Removal (%)
TOC concentration

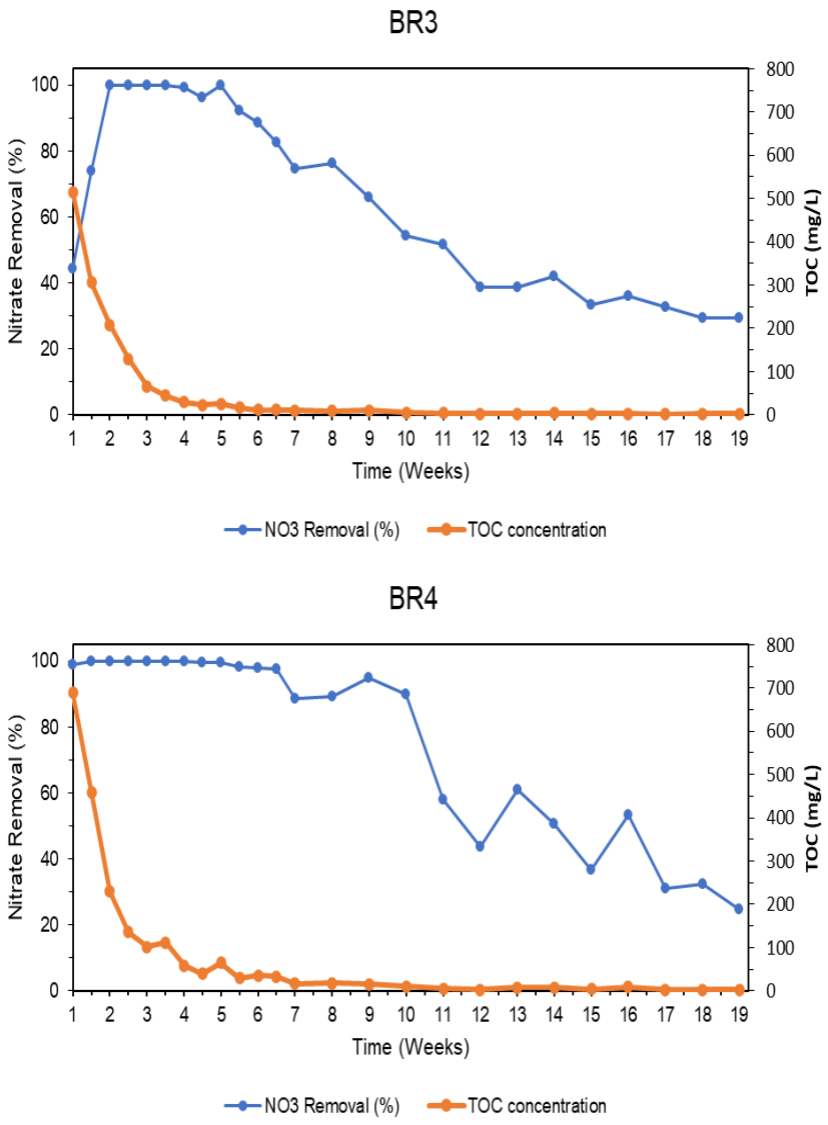


Figure 19 TOC plotted against nitrate removal percentage in Phase 4 column bioreactors

4.5.4 Nitrogen Compounds

Bioreactor 1 was kept at 5°C and had the lowest HRT of all the columns at 14 hours. Nitrate removal in BR1 noticeably peaked from weeks 5 to 10, ranging from 73.8 to 89.8% N-NO_3^- removal during that period (Figure 20). Before and after that period, N-NO_3^- removal averaged 33 ± 8% in BR1. This delayed peak was not observed in the other 3 columns. The lag in nitrate removal in BR1 may be due to the microbial populations needing to acclimate to the cold temperatures before being able perform denitrification at low HRT, or vice versa. Ammonium and nitrite concentrations were very low for the duration of BR1 column operation, with maximum values of 0.66 and 0.05 mg/L, respectively (Figure 20).

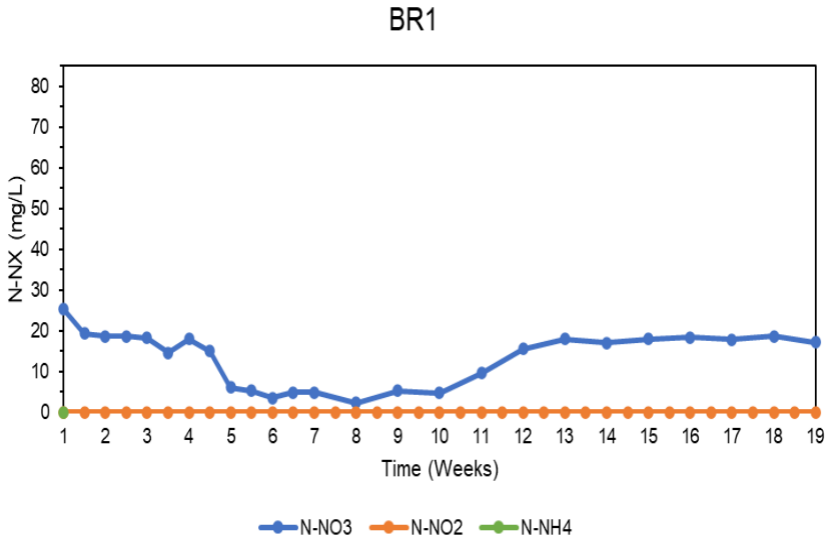


Figure 20 Concentrations of nitrate, nitrite, and ammonium in BR1

Bioreactor 2 was kept at room temperature with an HRT averaging 17 hours. This BR had relatively high rates of nitrate removal, ranging from 87.1 to 100% removal over the first 6 weeks of operation (Figure 21). From week 7 onwards, nitrate removal slowly decreased from 68.4% to a minimum of 26% in week 15, averaging 50.8 ± 11.7% removal during that period. Ammonium was relatively high in BR2 over the first few weeks of operation, before falling below 1 mg/L from week 9 onwards (Figure 21). The high N-NH_4^+ concentrations released in the BRs may be due to the nitrogen fixation ability of WSC (Chen et al. 2021). As a legume species associated with

nitrogen-fixing rhizobacterium, the relatively high N content of WSC (section 4.6.2) may contribute to the release of ammonium into the effluent. Nitrite concentrations were low throughout column operations, with concentrations <1 mg/L in all but the first two weeks, in which N-NO_2^- ranged from 3.9 to 1 mg/L (Figure 21).

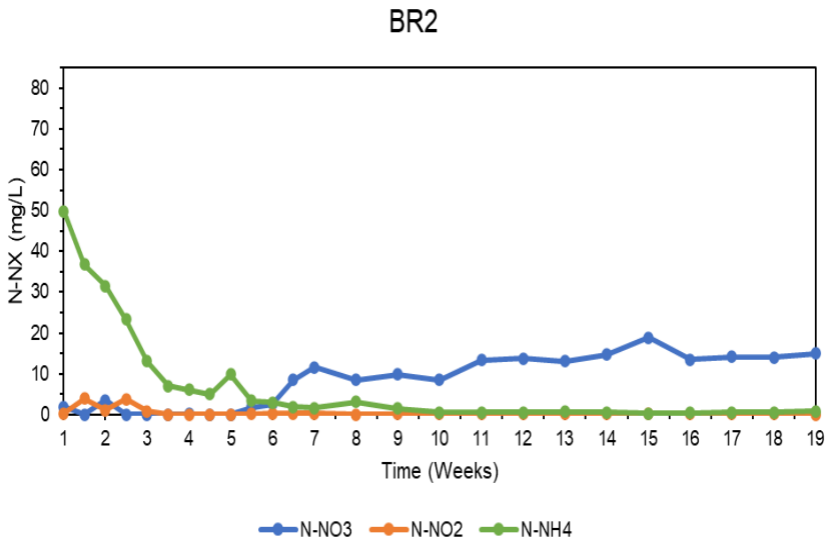


Figure 21 Concentrations of nitrate, nitrite, and ammonium in BR2

Bioreactor 3 was maintained at 5°C with BR1, and similarly had a brief delay before reaching peak nitrate removal (Figure 22). However, BR2 reached peak nitrate removal after just two weeks, rather than the five-week delay seen in BR1. This suggests that in cold temperatures, a longer HRT can support nitrate removal, as HRT in BR3 averaged 130 hours in comparison to the BR1 HRT of 14 hours. However, nitrate removal began to decrease by week 7, dropping from a high of 100% in week 5 to 74.8% in week 7. From then, nitrate removal steadily decreased until the end of column operations (Figure 22). Interestingly, overall nitrate removal in BR3 is comparable to BR2. The average nitrate removal over the entire 19 weeks is 67.2 ± 2.4% in BR3 and 71.0 ± 24.7% in BR2.

Additionally, BR3 had relatively high ammonium concentrations compared to BR1. Ammonium concentration was highest in week 1 at 63.3 mg/L, then steadily decreased until week 8, when concentrations decreased below 1 mg/L and remained low for the duration of column operations (Figure 22). Nitrite concentrations were <1 mg/L for the duration of the experiment.

Bioreactor 4 was kept at room temperature with had the longest HRT of all the columns, averaging 130 hours. This BR had the longest period of high nitrate removal, ranging from 88.6 to 100% removal over the first 10 weeks of operation (Figure 23). This period of high nitrate removal was 4 weeks longer than the in BR2. From week 11 onwards, nitrate removal slowly decreased from 60.9% to a minimum of 24.6% in week 19, with an average removal of 43.5 ± 12.2% removal during the final 9 weeks of operation (Figure 23). This BR had the highest ammonium concentrations of all the BRs, with an initial $N\text{-}NH_4^+$ concentration of 81.9 mg/L in week 1. This high initial concentration dropped rapidly, decreasing by almost half in week 2 at 43.7 mg/L $N\text{-}NH_4^+$. By week 7 and onwards, $N\text{-}NH_4^+$ concentrations were <1 mg/L. Nitrite concentration was 3.8 mg/L in week 3 but was otherwise <1 mg/L $N\text{-}NO_2^-$, like the other three columns.

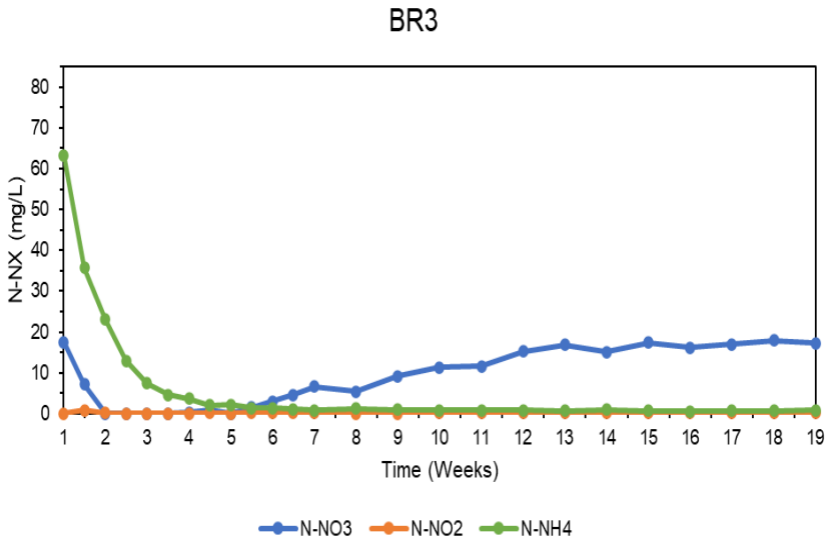


Figure 22 Concentrations of nitrate, nitrite, and ammonium in BR3

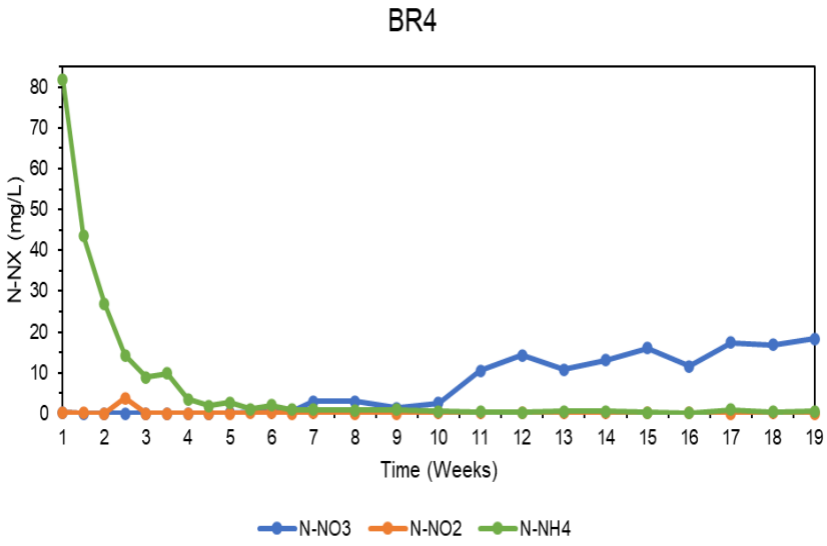


Figure 23 Concentrations of nitrate, nitrite, and ammonium in BR4

4.6 Carbon Source Characterization

4.6.1 Carbon Source Leach Test

The impact of each complex carbon source on BR pH was determined through shake tests. Nearly all substrates lead to a decrease in pH after 10 days, varying from 5.83 in the wood chips to 6.87 in white sweet clover (Table 13). Compost was the only substrate that increased from neutral to 8.25 after 10 days.

Table 13 Change in pH after 10-day shake flask test of carbon sources

Carbon source	Initial pH (Day 0)	Final pH (Day 10)
Foxtail Barley	6.48	5.54
Foxtail Barley duplicate	6.39	4.19
White Sweet Clover	6.87	7.70
White Sweet Clover duplicate	6.17	4.93
Wood chips	6.13	5.64
Wood chips duplicate	6.07	5.54
Brewery residue	7.42	5.98
Brewery residue duplicate	6.69	4.77
Wood shavings	6.50	5.25
Wood shavings duplicate	5.83	5.39
Compost	8.04	7.30
Compost duplicate	8.25	7.66

Notable results from the leaching tests include relatively high calcium results in the WSC leachate duplicates, in comparison to the other carbon sources, at 81.1 and 117 mg/L (Table 14). This may be related to the growing conditions of the WSC. The WSC was harvested from various areas within the City of Whitehorse, YT. Studies have suggested that urban soils often have higher Ca concentrations than non-urban soils, due to weathering of concrete surfaces and run-off from various human activities (Grella et al. 2018).

As discussed in Section 4.1.2, As, Co, Cr, Cu, and Ni can impact the enzymes associated with denitrification. Concentrations of all these elements are negligible in all carbon source leachates (Table 14).

Table 14 Select metal results from carbon source leaching test

Carbon Source	Metal concentration (mg/L)													
	Al	As	Ca	Cd	Co	Cr	Cu	Fe	Mo	Ni	P	Pb	S	Zn
Foxtail Barley	0.114	0.00046	18.1	0.0000342	0.00077	0.00144	0.0378	0.104	0.00634	0.0016	22.3	0.000421	6.38	0.134
Foxtail Barley duplicate	0.111	0.00069	24.2	0.0000248	0.00076	0.0009	0.0334	0.050	0.00256	0.00144	29.0	0.000357	6.50	0.160
White Sweet Clover	0.615	0.00177	117	0.00045	0.00376	0.00207	0.0771	0.887	0.0149	0.0212	11.5	0.00181	14.3	0.184
White Sweet Clover duplicate	0.344	0.00176	81.1	0.00037	0.00326	0.00107	0.0573	0.249	0.00155	0.0186	16.2	0.000703	11.3	0.121
Wood chips	0.57	0.00096	4.85	0.000126	0.0006	0.00304	0.0106	0.442	0.00131	0.00251	0.25	0.000983	0.78	0.032
Wood chips duplicate	0.39	0.00098	5.00	0.00016	0.00062	0.00204	0.00781	0.392	0.000155	0.00232	0.22	0.000491	0.78	0.0314
Brewery residue	0.196	0.00098	12.9	0.00031	0.00071	0.00059	0.0512	0.402	0.00346	0.00054	15.9	0.000408	13.9	0.275
Brewery residue duplicate	0.282	0.00061	15.5	0.000284	0.0016	0.00051	0.0464	0.530	0.00143	<LOD	32.1	0.000552	14.6	0.401
Wood shavings	0.145	0.00034	1.77	0.0000154	0.00011	<LOD	0.00335	0.057	0.000064	<LOD	0.085	0.000112	0.78	0.0066
Wood shavings duplicate	0.132	0.00135	1.82	0.0000109	0.00011	<LOD	0.00444	0.085	<LOD	<LOD	0.053	0.000166	0.64	0.0073
Compost	2.46	0.0157	71.8	0.000959	0.00311	0.00453	0.0306	1.650	0.00297	0.00679	7.86	0.0207	3.99	0.379
Compost duplicate	2.36	0.0154	51.3	0.000784	0.00258	0.0044	0.0379	1.890	0.00397	0.00735	6.00	0.0185	4.50	0.256

LOD = Limit of detection.

4.6.2 CHN Analysis

The carbon, hydrogen, and nitrogen content of each carbon source was analyzed to inform how much of a carbon source would need to be added to BRs in Phase 3 and 4 to maintain the targeted carbon to nitrogen ratios. Most carbon sources averaged around 45% carbon, except for the compost (Table 15). Molasses and sodium acetate were not analyzed as the carbon content of these substances has been determined to be 28.9% for molasses (Nielsen 2019), and 29.3% for sodium acetate. White sweet clover had the second highest nitrogen content after brewery waste at 3.4 and 4.1 - 4.9% N, respectively.

Table 15 Sorption capacity results of various carbon sources averaged among different nitrate concentrations

Sample	Mass (mg)	% N	% C	% H
Foxtail Barley	1.032	1.8	43.2	6.3
Foxtail Barley duplicate	1.173	1.9	42.1	6.0
White Sweet Clover	1.088	3.4	42.3	6.3
White Sweet Clover duplicate	0.955	3.4	41.9	6.2
Wood chips	1.007	0.8	45.7	6.2
Wood chips duplicate	0.978	0.5	44.7	5.9
Brewery residue	1.182	4.1	45.5	6.9
Brewery residue duplicate	1.098	4.9	45.1	6.9
Wood shavings	0.988	0.5	46.1	6.3
Wood shavings duplicate	1.068	0.6	45.9	6.1
Compost	0.962	2.8	29.5	3.6
Compost duplicate	1.068	2.8	27.1	3.2

4.6.3 Sorption Capacity Test

Sorption tests were conducted on the solid carbon sources to determine if any of the nitrate removal observed in the BRs was due to sorption, rather than biological denitrification. See Appendix VI for full results. As in Section 4.4.3, nitrogen compounds concentrations are expressed in values of NO_x as analysis was only conducted for nitrite + nitrate for these samples. It is assumed that NO_2 concentrations are low to negligible based on the results from the initial nitrate solutions being close to the targeted values.

Average removal of nitrate via sorption was low for all carbon sources, ranging from averages of 2.1 – 5.1%. The mg of ions adsorbed per g of carbon source after two hours (represented by Q_t) varied only slightly among carbon sources. The results for white sweet clover are skewed by results in 1/3 tests indicating an increase in $N\text{-}NO_x$. This could be an outlier value or may have been due to an error in the sampling protocol or during analysis. The low removal rates of nitrate in all tests suggest that sorption of nitrate by the carbon sources would not be a large contributing factor in the overall nitrate removal occurring in the bioreactors.

4.7 DNA Characterization

DNA characterization of inoculum from Phase 1 BRs and the WSC BRs of Phase 3.1 and 3.2 was conducted to gain insight to the microbial communities within these batch BRs. Positive and negative controls were included in the methodology (Section 3.8). All negative control amplified, indicating contamination (data not shown). However, the sequencing revealed that there was negligible overlap in species composition between the negative controls and the BRs, so the negative controls were not included in the results displayed in the following sections. For results of the UV-Vis analysis of extracted DNA, see Appendix VII. The replicates for BR samples were determined to be like one another at the species level, and therefore average values from these replicates are presented below (Appendix VII).

4.7.1 Microbial Diversity

The total observed numbers (Obs) of Amplicon Sequence Variants (ASVs) was 37 ± 16 with an alpha diversity (Chao1) of 36 ± 15 (Figure 24), which is typical for BRs, but far less than the number of dominant ASVs in soils or vegetated wetlands (Hellman et al. 2021; Gillespie et al. 2023). This suggests that the microbial composition of the original sediments collected at the mine became less diverse during biomass development in Phases 1 to 3. Microbial diversity was highest in BRs D17a and D17b and was lowest in BRs D32a and D32b. This further indicates that microbial community composition was impacted by conditions in the BRs and that species diversity.

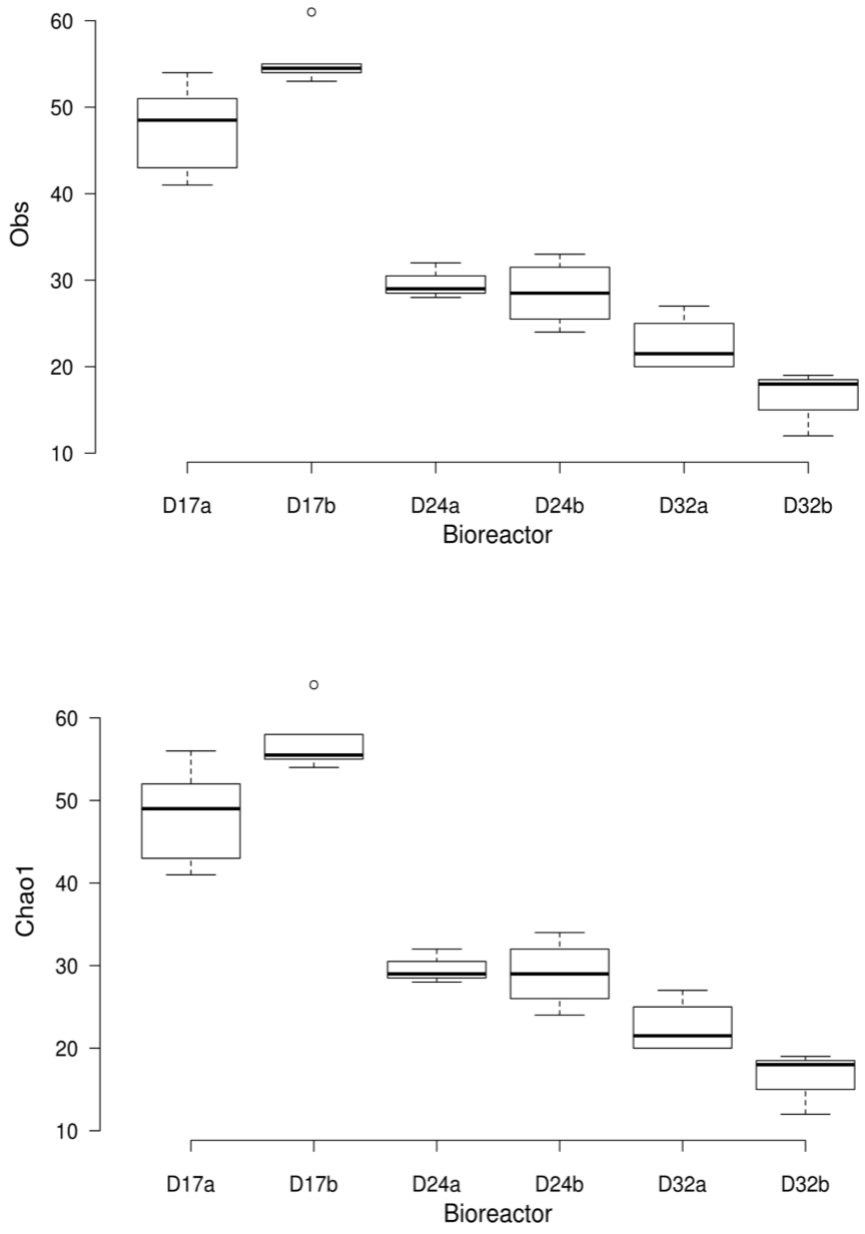


Figure 24 Observed ASVs and alpha diversity in select bioreactors

4.7.2 Microbial Community Analysis

For all BRs the microbial population was constrained to 3 major phyla (Figure 25). The most abundant being the Proteobacteria with an average relative abundance of 98.1 ± 2.3% across all samples. The other two phyla, Actinobacteria and Firmicutes, averaged 1.7 ± 2.2% and 0.02 ± 0.3% across all samples, respectively. Bioreactors D17a and D17b showed increased amounts of Actinobacteria and Firmicutes in comparison to the other BRs. Within the phylum Proteobacteria, the Gammaproteobacteria were most dominant, followed by Alphaprotoebacteria at an average of 91.2 ± 11.4% and 6.9 ± 9.1% relative abundance across all BRs, respectively. At the class level, BRs D17a and D17b have increased abundance of Alphaproteobacteria and Actinobacteria (Figure 26).

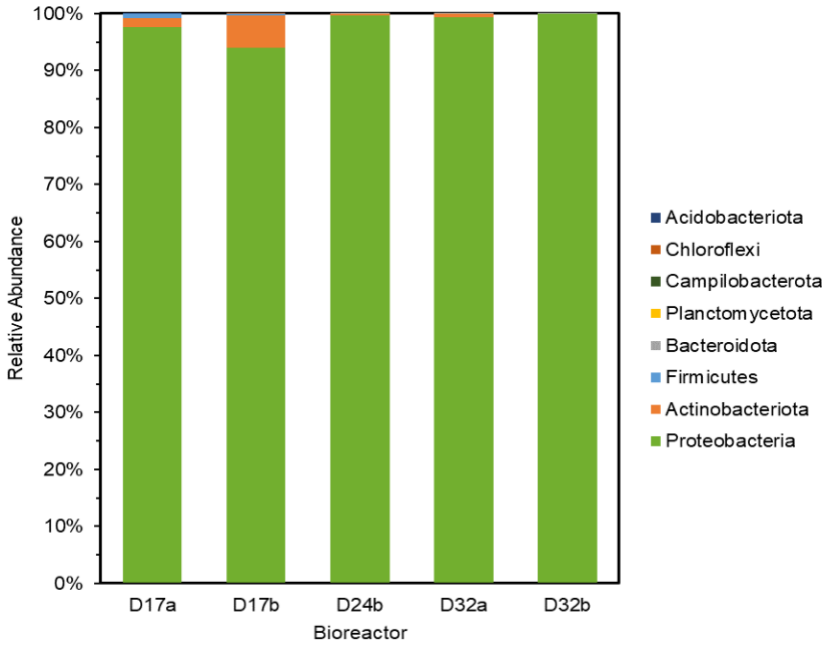


Figure 25 Relative abundance of phyla in select bioreactors

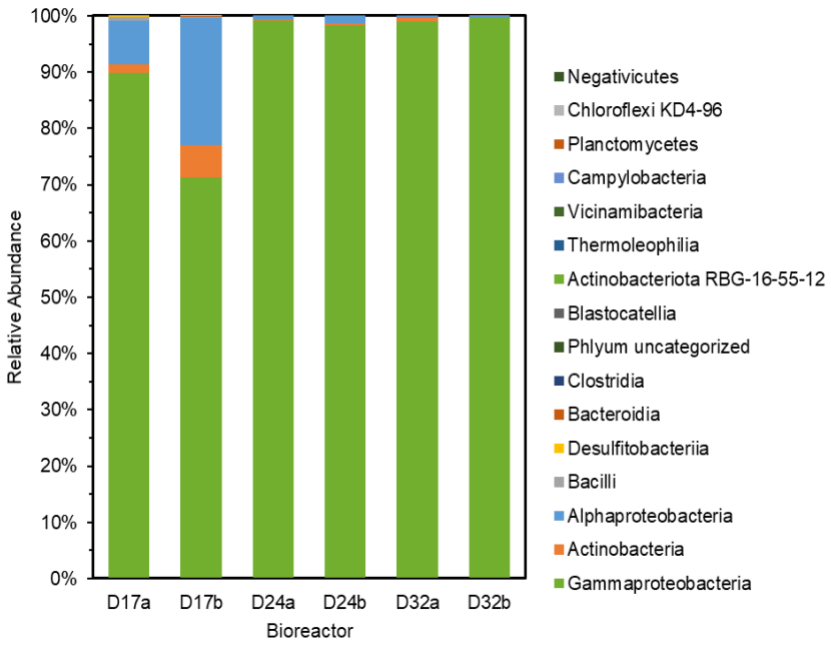


Figure 26 Relative abundance of classes in select bioreactors

At the genus level all BRs feature high relative abundance of *Ochrobactrum, Aeromonas, Janthinobacterium, Alcaligenes*, *Pseudomonas* and *Yersinia*, which may include the known denitrifying species in those groups (Figure 27, Table 16). There is a notable difference between D24 and D32 BRs. Both were inoculated with a 1:1 combination of D17a and D17b. The D24a and D24b BRs were those that tested WSC as a carbon source at 25 mg/L N-NO_3^-. The D32a and D32b BRs were those that tested WSC at 100 mg/L N-NO_3^-. The difference in bacterial communities between these BR treatments suggests that nitrate concentration may impact microbial ecology. However, metagenomics would need to be conducted to determine which genes are being expressed and in what relative abundances are in the BRs. Gene expression analysis can reveal which enzymes in the nitrogen cycle are being produced and could potentially identify enzymes that can break down the complex carbon source.

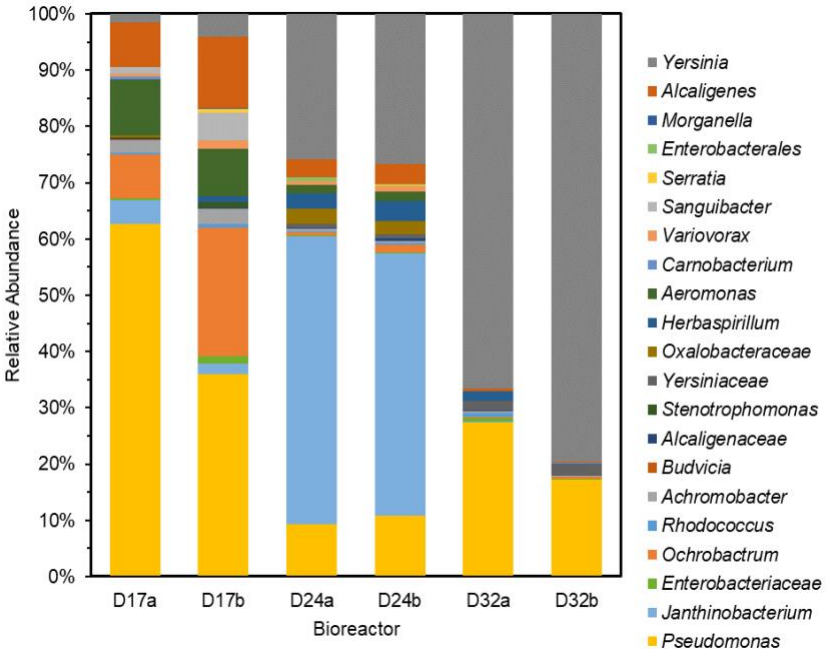


Figure 27 Relative abundance of genera in select bioreactors

Table 16 Percentage abundance of the most abundant genus across all bioreactors

Phylum	Class	Order	Family	Genus	D17a	D17b	D24a	D24b	D32a	D32b
Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae		0.32	1.36	0.16	0.15	0.66	0.05
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Ochrobactrum*	7.67	22.66	0.58	1.32	0.33	0.35
Actinobacteriota	Actinobacteria	Corynebacteriales	Nocardiaceae	*Rhodococcus*	0.30	0.69	0.19	0.33	0.61	0.01
Proteobacteria	Gammaproteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*	2.14	2.63	0.40	0.42	0.34	0.26
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	62.46	35.89	9.33	10.76	27.35	17.26
Proteobacteria	Gammaproteobacteria	Enterobacterales	Budviciaceae	*Budvicia*	0.24	0.00	0.05	0.00	0.00	0.00
Proteobacteria	Gammaproteobacteria	Burkholderiales	Alcaligenaceae		0.10	0.19	0.21	0.42	0.21	0.00
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	0.30	0.96	0.02	0.07	0.03	0.01
Proteobacteria	Gammaproteobacteria	Enterobacterales	Yersiniaceae		0.01	0.01	0.63	0.64	1.70	2.09
Proteobacteria	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae		0.22	0.04	2.72	2.44	0.00	0.00
Proteobacteria	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	*Herbaspirillum*	0.00	0.98	2.71	3.48	1.56	0.10
Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	9.91	8.52	1.44	1.68	0.03	0.03
Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	*Carnobacterium*	0.50	0.00	0.00	0.03	0.02	0.06
Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	*Variovorax*	0.50	1.39	0.65	0.95	0.00	0.00
Actinobacteriota	Actinobacteria	Micrococcales	Sanguibacteraceae	*Sanguibacter*	1.19	4.91	0.00	0.00	0.00	0.00
Proteobacteria	Gammaproteobacteria	Enterobacterales	Yersiniaceae	*Serratia*	0.04	0.67	0.09	0.17	0.00	0.01
Proteobacteria	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	*Janthinobacterium*	4.22	1.82	51.08	46.65	0.10	0.01
Proteobacteria	Gammaproteobacteria	Enterobacterales			0.01	0.08	0.57	0.17	0.00	0.00
Proteobacteria	Gammaproteobacteria	Enterobacterales	Morganellaceae	*Morganella*	0.00	0.22	0.11	0.11	0.00	0.00
Proteobacteria	Gammaproteobacteria	Burkholderiales	Alcaligenaceae	*Alcaligenes*	7.91	12.45	3.09	3.55	0.54	0.25
Proteobacteria	Gammaproteobacteria	Enterobacterales	Yersiniaceae	*Yersinia*	1.52	4.13	25.91	26.65	66.50	79.50

5 CONCLUSIONS

The overarching objective of this study was to design a semi-passive treatment system to address nitrate contamination in mine water in cold climates. The key guiding principle of the project was to harness naturally occurring processes, thereby minimizing the reliance on energy-intensive or chemically driven treatment methods to ultimately support sustainable remediation of mine-impacted water in cold climates. This guiding principle influenced the materials used and the methods followed throughout this project.

First, sediment samples were collected at the mine site, in hopes that these would contain native denitrifying bacteria that would be adapted to the cold. The removal of nitrate observed in all Phase 1 batch BRs indicates the sediments collected at the Minto Mine did contain the targeted denitrifying bacteria. However, these populations were not compared to commercially available denitrifying cultures, so it is unknown if native bacteria are more effective at removing nitrate from cold MIW. Though this does indicate that inoculum can be sourced directly from site in future trials.

The denitrifying biomass was further developed in Phase 2, with high nitrate removal observed. However, it could not be determined how complete the denitrification process was in the first phase, and all succeeding phases of the BRs. The analysis of N compounds was limited to ammonium, nitrite, and nitrate. The final product of denitrification is N_2, which is a gas that would have been able to leave the system undetected. Experimental design changes could permit gases to be captured, therefore allowing for a better understanding of the microbial process occurring within the BRs. However, as ammonium, nitrite and nitrate are the nitrogen compounds monitored in the MIW at the Minto Mine, these were arguably the most important factors to measure.

The objective of Phase 3 was to test local carbon sources against a control (sodium acetate), to determine if denitrification could be adequately supported with substrates available in the North. Nitrate removal occurred in all Phase 3 BRs, to varying degrees. However, the invasive white sweet clover was chosen as the carbon source to be used in the Phase 4 column BRs due to its availability in harvestable quantities on the Minto Mine site and the high rates of nitrate removal seen in Phase 3. Additionally, mines are mandated by various regulations to manage the invasive species on mine sites. Therefore, it can be an added benefit if mines are able to harvest WSC and apply it as a substrate in a water treatment system.

Phase 4 involved further testing of WSC as a carbon source in column BRs. Two variables were tested, temperature and hydraulic retention time. Cold temperature is regarded as an obstacle to MIW treatment by biological denitrification (Jermakka et al. 2015). Therefore, nitrate removal was compared between 5°C and 20°C. It has been identified in several studies that increasing HRT can support biological denitrification in cold temperature (Roser et al. 2018; Nordstrom and Herbert 2019; Hellman et al. 2021). As such, the column with the highest nitrate removal was maintained at 20°C and had the longest HRT. The columns that were most similar in nitrate removal were BR2 and 3. Bioreactor 2 was maintained at 20°C and had a short HRT compared to BR3, which was operated at 5°C and had a longer HRT. This supports the notion that increasing HRT can improve nitrate removal in low T settings. However, BRs were not duplicated in the phase of the experiment. Increasing the number of replicates would enhance the reliability of these findings.

Total organic carbon decreased over time in all columns, suggesting that carbon may have become a limiting factor in denitrification. This may have contributed to the decrease in nitrate removal over the course of column operations. Avenues for future research include how pretreatment and harvest timing may impact the bioavailability of the carbon in WSC.

DNA samples were collected from selected BRs to analyze microbial community composition. The diversity of ASVs in the BRs compared to general soil samples suggests that efforts to develop the targeted biomass were successful. Results indicate that microbial populations responded to the changing conditions between the phases of batch BRs. Taxonomic groups containing known denitrifiers were identified in all BRs. However, metagenomics investigations would be required to understand if the bacteria in the BRs are in fact functionally carrying out denitrification.

This research project has shown that native bacteria from a northern mine site can be developed in the lab and be applied in lab-scale column BRs to successfully remove nitrate in cold temperatures. These findings contribute to the growing body of evidence that passive and semi-passive treatment systems can be adapted to function in Canada’s northern mining sector.

It is also interesting to note that the industrial partner for this project, the owners of the Minto Mine, abandoned the site while this research was being conducted. This highlights the need for research to keep pace with industry so that mine operators and regulators can make informed decisions regarding the use of passive and semi-passive treatment systems in this industry.

5.1 Recommendations

Future research should involve replicate BRs at the column scale, focusing on optimizing substrate amendments and hydraulic retention time parameters to better understand how these BRs can be effectively scaled-up to the pilot scale and beyond. Additional avenues of investigation include subjecting BRs to freeze and thaw conditions to gain insight as to how microbial populations and nitrate removal might be impacted by the temperature variations that would occur at a northern mine site.

Additionally, the influent used in the column tests was made to mimic nitrate concentrations at the Minto Mine but did not emulate the other water chemistry parameters at the site. Future research should investigate how real MIW interacts with the denitrification treatment system. Denitrification can be impacted by pH, nitrate concentrations, heavy metals, HRT, etc., so it is important to understand the characteristics and quantity of the MIW to design an effective treatment system.

A techno-economic assessment should be conducted to understand the costs and benefits of adapting the aforementioned treatment system to the operational scale. It is critical to understand the feasibility of a semi-passive treatment system, particularly when compared with conventional treatment options.

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APPENDIX I

Full description of inoculum sampling locations

Location 1: W30

Date: September 13, 2021

The first sampling location was Well 30 (W30). This location was a wetland with waste rock piles bordering it on two sides with the opposite side being dominated by willows and graminoids.

Table 17 Description of inoculum sampling locations for samples Minto 1 - 3

Sample ID	Coordinates	Depth	Description
Minto 1	62.61771 -137.26627	30 cm	Sample taken below approx. 90 cm of standing open water Cold water temp
Minto 2	62.61777 -137.26735	36 cm	Sample collected in muddy area on western shore of the pond First 15 cm of sediments contained many roots Soil was black in colour Some odour present
Minto 3	62.61757 -137.26868	35 cm	Sample collected at SW end of the pond with no standing water Greyish soil with silty/clayey texture No smell Roots reaching at least 35 cm below the surface of soil

Location 2: W35

Date: September 13, 2021

The second sampling location was at W35. This location was a wetland upstream of W30. This wetland features a small pond that does not dry out during the driest periods of the year. The pond is located below an ore pad and may therefore have a high dissolved copper content. This pond was likely once a part of Minto Creek before disturbance occurred.

Table 18 Description of inoculum sampling locations for samples Minto 5 and 6

Sample ID	Coordinates	Depth	Description
Minto 5	62.61029 -137.23340	30 – 60 cm	Sample taken below approx. 30 cm of standing water Upstream of W35 Cold water temp Rocky, sandy sediment with no smell
Minto 6	62.61015 -137.23383	40 cm	Sample collected from shallow water about 15 cm deep with vegetation including graminoids and willows Top layer of soil features organic material Lower soil layer has sandy texture No odour

Location 3: W16

Date: September 13 & 14, 2021

The third sampling location was at W16, a large water storage pond. All samples were collected on September 13, except Minto 11 which was collected on September 14. This water storage pond in bordered on the north side by the mine access road. The pond is dammed at the northeast end and was experiencing higher than average water levels when sampling occurred.

Table 19 Description of inoculum sampling locations for samples Minto 7, 8, 9, and 11

Sample ID	Coordinates	Depth	Description
Minto 7	62.62096 -137.21887	20 cm	Sample taken below approx. 40 cm of standing open water about 2 m from shore Cold water temp This location was not usually under water during normal years Faint odour Sediment high is fibrous organic content with peaty texture
Minto 8	62.62095 -137.21887	30 cm	Sample collected in muddy area on shore, approx. 3 m from Minto 7 Sediment was dark with fibrous, peaty material Some odour present
Minto 9	62.62307 -137.21518	35 cm	Sample collected approx.. 1 m from shore near the main water pump for the storage pond Sediment was sandy No smell
Minto 11	62.62255 -137.212389	30 cm	Sample was collected about 0.5 m from shore, not usually underwater as indicated by submerged willows Natural edge of the pond Some odour

Location 4: CWTS

Date: September 13, 2021

The fourth sampling location is a constructed wetland on site. The wetland is constructed on the top of a waterproof liner and contains emergent graminoid vegetation.

Table 20 Description of inoculum sampling locations for sample Minto 12

Sample ID	Coordinates	Depth	Description
Minto 12	62.61960 -137.22983	30 cm	Sample taken below approx. 30 cm of standing water No odour detected but has been quite odorous in the past according to mine staff observation Sediment black in colour

Location 5: W15

Date: September 14, 2021

This sampling location was at W15, a large natural wetland. Here, water quality is monitored at the NE end at W15 and at the SW end at W10. This wetland is approx. 1 km in length and is dominated by willows and some spruce. A small creek meanders through the wetland.

Table 21 Description of inoculum sampling locations for samples Minto 14 – 17

Sample ID	Coordinates	Depth	Description
Minto 14	62.61487 -137.25717	30-50 cm	Sample taken on natural edge of pond that had a liner on the opposite shore approx. 50 m away to the NE Shoreline dominated by graminoids and willows Cold water temp Faint odour
Minto 15	62.61465 -137.25839	30 cm	Sample collected in soil above the water table 15 cm layer of moss removed from above the soil At 30 cm depth, a large rock was encountered and could not be removed No odour detected
Minto 16	62.61348 -137.26060	35 cm	Sample collected from bottom of a small creek meandering through wetland Lots of organic material including leaves at surface of sediment No smell Very cold water temperature
Minto 17	62.61146 -137.26309	30 – 54 cm	Sample collected in mossy depression in wetland, under some standing water Very cold water temp Organic content (leaves, roots, branches) present in soil in up to 54 cm of depth Located about halfway between W15 and W10

Location 6: Minto Creek / W6

Date: September 14, 2021

This sampling location included Minto Creek, downstream of the dam at W16. This location is not at a well but is expected to have water quality like W3. The other sample at this location was taken directly at W6 in a Minto Creek tributary.

Table 22 Description of inoculum sampling locations for samples Minto 19 and 20

Sample ID	Coordinates	Depth	Description
Minto 19	62.62815 -137.19377	35 cm	Sample taken from bottom of creek under moving water No odour Sandy sediment
Minto 20	62.63099 -137.19206	40-45 cm	Sediment more clayey/silty in texture in the first 30 cm than at Minto 19 After 30 cm, sediment is sandy in texture, similar to Minto 19 Location considered to be undisturbed but is near to the road No odour

Elemental composition of the inoculum samples

Table 23 Full results from ICP-MS analysis of inoculum samples

Inoculum Sample	Metal Concentration (mg/L)																		
	Al	As	B	Ca	Cd	Co	Cr	Cu	Fe	K	Mg	Mn	Mo	Na	Ni	P	Pb	S	Zn
Minto 1	0.09	<LOD	<LOD	83.93	<LOD	<LOD	<LOD	0.013	0.11	7.00	7.59	0.09	0.0	22.38	<LOD	0.12	<LOD	31.29	<LOD
Minto 2	0.17	<LOD	<LOD	85.50	<LOD	<LOD	<LOD	0.007	0.89	2.78	34.17	2.35	0.01	26.24	<LOD	0.16	<LOD	1.73	<LOD
Minto 3	0.12	<LOD	<LOD	107.09	<LOD	<LOD	<LOD	0.008	0.21	2.82	30.60	1.17	0.01	23.42	<LOD	0.04	<LOD	4.49	<LOD
Minto 5	0.46	<LOD	0	111.37	<LOD	<LOD	<LOD	0.146	0.56	4.40	38.47	0.02	0.0	30.33	<LOD	0.05	<LOD	62.60	<LOD
Minto 6	0.09	<LOD	0	151.30	<LOD	<LOD	<LOD	0.068	0.08	3.71	36.06	0.01	0	47.82	<LOD	0.05	<LOD	105.20	<LOD
Minto 7	0.10	<LOD	<LOD	32.94	<LOD	<LOD	<LOD	0.012	0.31	1.31	35.38	0.03	<LOD	6.39	<LOD	0.07	<LOD	4.35	<LOD
Minto 8	0.07	<LOD	<LOD	58.88	<LOD	<LOD	<LOD	0.009	0.74	1.12	15.12	0.45	<LOD	9.11	<LOD	0.09	<LOD	1.21	<LOD
Minto 9	0.53	<LOD	<LOD	44.00	<LOD	<LOD	<LOD	0.014	0.47	4.14	8.89	0.03	<LOD	7.14	<LOD	0.09	<LOD	7.84	<LOD
Minto 11	0.54	<LOD	0.1	69.35	0.08	<LOD	<LOD	0.016	0.59	1.82	17.74	0.30	<LOD	6.81	<LOD	0.05	1.43	1.61	0.05
Minto 12	0.39	<LOD	0	99.83	0.08	<LOD	< 0,01	0.036	0.53	4.75	31.44	0.22	0.02	49.38	<LOD	0.09	1.41	6.26	0.05
Minto 14	0.19	<LOD	0.1	106.12	0.08	<LOD	< 0,01	0.011	0.21	3.96	33.58	0.19	0.0	17.98	<LOD	0.08	1.40	1.88	0.05
Minto 16	0.21	<LOD	0.1	118.30	0.07	<LOD	< 0,01	0.014	0.39	3.02	33.96	0.55	0.0	14.75	<LOD	0.07	1.33	53.63	0.05
Minto 17	0.22	<LOD	0.1	116.77	0.08	<LOD	< 0,01	0.013	0.19	3.21	30.65	0.23	0.0	17.90	<LOD	0.05	1.54	84.26	0.05
Minto 19	0.17	<LOD	0.1	87.72	0.08	<LOD	< 0,01	0.015	0.10	3.94	38.04	0.33	0.0	19.05	<LOD	0.10	1.44	4.81	0.05
Minto 20	0.89	<LOD	0.1	31.43	0.08	<LOD	< 0,01	0.012	1.38	0.64	7.59	0.03	<LOD	4.55	<LOD	0.09	1.49	1.83	0.05
Minimum	0.07	0.00	0.1	31.43	0.07	0.00	0.000	0.007	0.08	0.64	7.59	0.01	0.01	4.55	0.00	0.04	1.33	1.21	0.05
Maximum	0.89	0.00	0.1	151.30	0.08	0.00	0.000	0.146	1.38	7.00	38.47	2.35	0.04	49.38	0.00	0.16	1.54	105.20	0.05
Average	0.28		0.1	86.97	0.08			0.026	0.45	3.24	26.62	0.40	0.02	20.22		0.08	1.43	24.87	0.05
Standard Deviation	0.23		0.0	34.33	0.00			0.036	0.35	1.63	11.65	0.62	0.01	13.94		0.03	0.07	34.68	0.00

APPENDIX II

Table 24 Carbon calculations for Phase 3 bioreactors

UWS	there is 46.0 % of carbon, or: 460.0 g/kg n=m/M therefore n= 38.3 moles of C per 1 kg for 32.14 mmoles of carbon m= 838.4 mg of carbon source in the 25 N-NO_3 bioreactor for 128.51 mmoles of carbon m= 3352.4 mg of carbon source in the 100 N-NO_3 bioreactor
COWC	there is 28.3 % of carbon, or: 283.0 g/kg n=m/M therefore n= 23.6 moles of C per 1 kg for 32.14 mmoles of carbon m= 1362.8 mg of carbon source in the 25 N-NO_3 bioreactor for 128.51 mmoles of carbon m= 5449.2 mg of carbon source in the 100 N-NO_3 bioreactor
Molas	there is 28.9 % of carbon, or: 288.8 g/kg n=m/M therefore n= 24.1 moles of C per 1 kg for 32.14 mmoles of carbon m= 1335.5 mg of carbon source in the 25 N-NO_3 bioreactor for 128.51 mmoles of carbon m= 5339.8 mg of carbon source in the 100 N-NO_3 bioreactor
Sodium acetate	there is 29.3 % of carbon, or: 292.8 g/kg n=m/M therefore n= 24.4 moles of C per 1 kg for 32.14 mmoles of carbon m= 1317.1 mg of carbon source in the 25 N-NO_3 bioreactor for 128.51 mmoles of carbon m= 5266.4 mg of carbon source in the 100 N-NO_3 bioreactor

FTB	there is 42.7 % of carbon, or: 427.0 g/kg n=m/M therefore n= 35.6 moles of C per 1 kg for 32.14 mmoles of carbon m= 903.2 mg of carbon source in the 25 N-NO_3 bioreactor for 128.51 mmoles of carbon m= 3611.5 mg of carbon source in the 100 N-NO_3 bioreactor
WSC	there is 42.1 % of carbon, or: 421.0 g/kg n=m/M therefore n= 35.1 moles of C per 1 kg for 32.14 mmoles of carbon m= 916.1 mg of carbon source in the 25 N-NO_3 bioreactor On veut 128.51 mmoles de carbone, m= 3663.0 mg of carbon source in the 100 N-NO_3 bioreactor
WWC	there is 45.2 % of carbon, or: 452.0 g/kg n=m/M therefore n= 37.7 moles of C per 1 kg for 32.14 mmoles of carbon m= 853.3 mg of carbon source in the 25 N-NO_3 bioreactor for 128.51 mmoles of carbon m= 3411.8 mg of carbon source in the 100 N-NO_3 bioreactor
YBR	there is 45.3 % of carbon, or: 453.0 g/kg n=m/M therefore n= 37.8 moles of C per 1 kg for 32.14 mmoles of carbon m= 851.4 mg of carbon source in the 25 N-NO_3 bioreactor for 128.51 mmoles of carbon m= 3404.2 mg of carbon source in the 100 N-NO_3 bioreactor

Column Carbon Calculations

25 ppm N-NO3 (10 hr HRT)

WSC there is 42.1 % of carbon, or: 421.0 g/kg

n=m/M therefore n= 35.1 moles of C per 1 kg of WSC

For 23.52857 moles of carbon

m= 670.6 g of WSC in short HRT columns

Parameter	Value	Unit
N-NO3	25	mg/L
C:N	20:01	ratio
Volume	658.8	L
mg/L N-NO3 *Vol	16470	mg
Mg/1000	16.47	g
g/ g/mol N	1.176429	mol
mol N * 20	23.52857	mol C

Target: 20 mol C per 1 mol N
Target [N-NO3] = 25 mg/L
Volume to be treated = 3L /8 hr *24 hr *30.5 days * 3 months = 658.8
mg/L N-NO3 * volume = 16470 mg N-NO3
= 16.47 g N-NO3
g N-NO3 / 14 = 1.176 mol N
mol N * 20 = 23.528 mol of C

25 ppm N-NO3 (96 hr HRT)

WSC there is 42.1 % of carbon, or: 421.0 g/kg

n=m/M therefore n= 35.1 moles of C per 1 kg of WSC

For 4.084821 moles of carbon

m= 116.4 g of WSC in long HRT columns

Parameter	Value	Unit
N-NO3	25	mg/L
C:N	20:01	ratio
Volume	114.375	L
mg/L N-NO3 *Vol	2859.375	mg
Mg/1000	2.859375	g
g/ g/mol N	0.204241	mol
mol N * 20	4.084821	mol C

Target: 20 mol C per 1 mol N
Target [N-NO3] = 25 mg/L
Volume to be treated = 5L /8 hr *24 hr *30.5 days * 3 months = 114.37
mg/L N-NO3 * volume = 2859.375 mg N-NO3
= 2.859375 g N-NO3
g N-NO3 / 14 = 0.204241 mol N

APPENDIX III

Table 25 Flow rate calculations for Phase 4 column BR1

Column 1 (BR1) Cold, Fast																	
June 20 - started pump @ 15:30	(columns filled June 21st)					TARGET	Average				COL ID	In (mL)	Out (mL)	DIFFERENCE			
						Strange?	CHANGE IN				BR1	0.0	520935.1	-520935.1			
EFFLUENT	JAR WEIGHT	480.5	18.9L bucket			234 mL/hr	RPM		Target = 10 hr		EFFLUENT SAMPLING						
Week	Date	Effluent Jar (mL)	Jar Empty (g)	Effluent (mL)	Total Sampling	Days	Flow rates (mL/h)"	RPM	Volume (mL)	HRT (hr)	Week	Date	Sample Container +Sample	Container (g)	Sample (mL)		
0	Jun-20-23 03:30 PM	-	-	-	-	-		7.6	2295		0	Jun-20-23 03:30 PM	-	-			
1	Jun-23-23 02:30 PM	-	-	-	-	-		7.6	2295		1	Jun-23-23 02:30 PM	-	-			
1	Jun-25-23 09:40 AM	9066.5	480.5	8586	-	1.79861	198.903475	7.6	2295	11.5383	1	Jun-25-23 09:40 AM	-	-			
2	Jun-27-23 12:36 PM	7800	488	7312	265	2.12222	148.763089	7.6	2295	15.4272	2	Jun-27-23 10:20 AM	302	37	265		
2	Jun-29-23 01:13 PM	9548	489.5	9058.5	0	2.02569	186.324991	7.6	2295	12.3172	2	Jun-29-23 01:13 PM			0		
2	Jun-30-23 05:05 PM	3828.5	490	3338.5	263	1.16111	129.240431	7.6	2295	17.7576	2	Jun-30-23 01:52 PM	300	37	263		
3	Jul-02-23 11:33 AM	8728	491	8237	0	1.76944	193.963893	7.6	2295	11.8321	3	Jul-02-23 11:33 AM			0		
3	Jul-04-23 02:15 PM	8306.5	493.5	7813	208.5	2.1125	158.21499	7.6	2295	14.5056	3	Jul-04-23 11:51 AM	229.5	21	208.5		
3	Jul-06-23 02:19 PM	9843.5	492.5	9351	0	2.00278	194.542302	7.6	2295	11.7969	3	Jul-06-23 02:19 PM			0		
3	Jul-07-23 03:36 PM	4238.5	490.5	3748	280	1.05347	159.314436	7.6	2295	14.4055	3	Jul-07-23 11:14 AM	280		280		
4	Jul-09-23 09:57 PM	10461.5	494	9967.5	0	2.26458	183.394664	7.6	2295	12.514	4	Jul-09-23 09:57 PM			0		
4	Jul-11-23 03:51 PM	6607	488.5	6118.5	254	1.74583	152.088305	7.6	2295	15.0899	4	Jul-11-23 01:31 PM	275	21	254		
4	Jul-14-23 05:10 PM	13700	793	12907	250.5	3.05486	179.461241	7.6	2295	12.7883	4	Jul-14-23 01:12 PM	271.5	21	250.5		
5	Jul-18-23 02:42 PM	16500	793	15707	268	3.89722	170.794726	7.6	2295	13.4372	5	Jul-18-23 02:35 PM	289	21	268		
5	Jul-21-23 01:39 PM	12825.5	788.5	12037	265.5	2.95625	173.396758	7.6	2295	13.2355	5	Jul-21-23 12:01 PM	286	20.5	265.5		
6	Jul-25-23 01:07 PM	15600	799.5	14800.5	262	3.97778	157.777584	7.6	2295	14.5458	6	Jul-25-23 01:21 PM	282.5	20.5	262		
6	Jul-28-23 01:29 PM	13100	792	12308	260	3.01528	173.671119	7.6	2295	13.2146	6	Jul-28-23 12:05 PM	281	21	260		
7	Aug-01-23 03:10 PM	17300	796	16504	225.5	4.07014	171.262583	7.6	2295	13.4005	7	Aug-01-23 10:44 AM	246.5	21	225.5		
7	Aug-04-23 03:03 PM	12616.5	790	11826.5	266.5	2.99514	168.23093	7.6	2295	13.642	7	Aug-04-23 01:48 PM	287.5	21	266.5		
8	Aug-08-23 04:10 PM	16700	792.5	15907.5	0	4.04653	163.797838	7.6	2295	14.0112	8	############			0		
8	Aug-11-23 11:55 AM	10359	788.5	9570.5	248.5	2.82292	144.929889	7.6	2295	15.8352	8	Aug-11-23 09:42 AM	269	20.5	248.5		
9	Aug-15-23 04:07 PM	17000	798	16202	268	4.175	164.371257	7.6	2295	13.9623	9	Aug-15-23 03:48 PM	289	21	268		
9	Aug-18-23 02:59 PM	12384.5	794	11590.5	249	2.95278	167.067262	7.6	2295	13.737	9	Aug-18-23 01:35 PM	270	21	249		
10	Aug-22-23 12:25 PM	16300	788.5	15511.5	0	3.89306	166.016768	7.6	2295	13.8239	10	Aug-22-23 12:25 PM			0		
10	Aug-25-23 01:41 PM	12543.5	790	11753.5	245	3.05278	163.764786	7.6	2295	14.014	10	Aug-25-23 01:18 PM	265.5	20.5	245		
11	Aug-29-23 03:24 PM	16700	795.5	15904.5	0	4.07153	162.761385	7.6	2295	14.1004	11	Aug-29-23 01:41 PM			0		
11	Sep-01-23 12:44 PM	12047.5	798	11249.5	259.5	2.88889	165.995192	7.6	2295	13.8257	11	Sep-01-23 12:36 PM	280	20.5	259.5		
12	Sep-05-23 03:20 PM	16800	790.5	16009.5	0	4.10833	162.368154	7.6	2295	14.1345							
12	Sep-08-23 02:48 PM	12299.5	790	11509.5	237	2.97778	164.36334	7.6	2295	13.963	12	Sep-08-23 01:47 PM	257.5	20.5	237		
13	Sep-12-23 03:36 PM	16500	792	15708	0	4.03333	162.272727	7.6	2295	14.1429							
13	Sep-15-23 05:23 PM	12651	791.5	11859.5	266	3.07431	164.339282	7.6	2295	13.965	13	Sep-15-23 04:38 PM	286.5	20.5	266		
14	Sep-19-23 07:03 PM	16400	792	15608	0	4.06944	159.808874	7.6	2295	14.3609							
14	Sep-22-23 11:12 AM	11036	795	10241	242	2.67292	163.413874	7.6	2295	14.0441	14	Sep-22-23 10:36 AM	262.5	20.5	242		
15	Sep-26-23 02:33 PM	16700	790.5	15909.5	0	4.13958	160.135883	7.6	2295	14.3316							
15	Sep-29-23 08:37 AM	11268.5	789.5	10479	239	2.75278	162.230071	7.6	2295	14.1466	15	############	259	20	239		
16	Oct-03-23 02:27 PM	16500	795.5	15704.5	0	4.24306	154.217676	7.6	2295	14.8816							
6	Oct-06-23 11:44 AM	11866.5	796.5	11070	260	2.88681	163.531393	7.6	2295	14.034	16	Oct-06-23 11:12 AM	280.5	20.5	260		
17	Oct-10-23 03:02 PM	16500	794.5	15705.5	0	4.1375	158.162135	7.6	2295	14.5104							
17	Oct-13-23 10:05 AM	11600	792.5	10807.5	231.5	2.79375	164.63833	7.6	2295	13.9396	17	Oct-13-23 10:49 AM	252	20.5	231.5		
18	Oct-17-23 02:39 PM	16800	794	16006	0	4.19028	159.158104	7.6	2295	14.4196							
18	Oct-20-23 11:28 AM	11771.5	794	10977.5	234.5	2.86736	162.925648	7.6	2295	14.0862	18	Oct-20-23 10:41 AM	255	20.5	234.5		
19	Oct-24-23 04:05 PM	16100	797	15303	0	4.19236	152.092099	7.6	2295	15.0895							
19	Oct-27-23 11:42 AM	1131.6	793	338.6	222.5	2.81736	8.29824994	7.6	2295		19	Oct-27-23 09:02 AM	243	20.5	222.5		
	Oct-31-23 04:06 PM	13500	789	12711					Average	13.9702							

APPENDIX IV

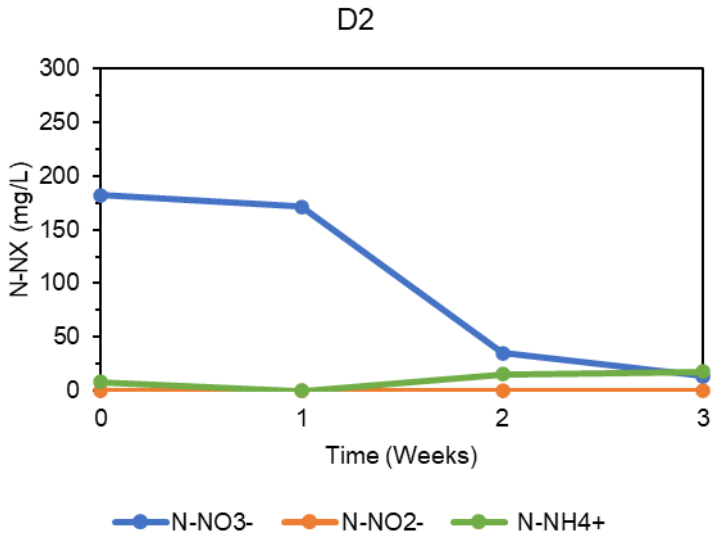
Table 26 General chemistry results from Phase 1 BRs

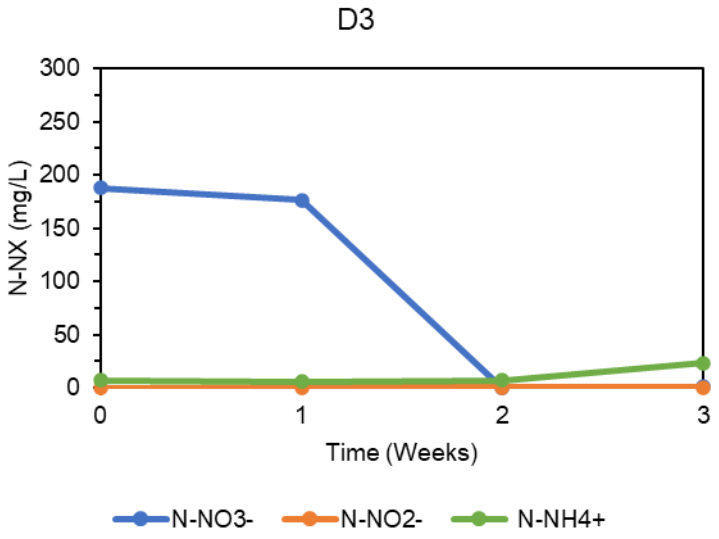
	pH						ORP (mV)				
BR	Initial	Day 0	Day 7	Day 14	Day 21	BR	Initial	Day 0	Day 7	Day 14	Day 21
N1	7.27	6.94	7.15	7.37	8.15	N1	155.4	36.5	16.7	-55.1	49.1
N2	7.54	7.02	7.14	7.66	7.85	N2	157	35	4.3	-85.6	42.3
N3	7.02	6.99	7.25	8.4	7.41	N3	158	39.8	-85.7	-5.7	14.9
N4	6.89	7.8	7.16	7.77	8.48	N4	12.3	-16.1	-35.6	-109.3	16.7
D1	7.55	6.8	7.91	7.06	7.35	D1	17.7	0.9	41.8	-109.1	-121
D2	6.93	6.67	7.83	6.98	7.51	D2	-189.6	-129.5	-117.2	-21.7	-22.1
D3	7.13	6.85	7.85	7.08	7.32	D3	-205.9	-142.3	-88.7	-123.5	-149.4
D4	7.48	6.82	7.55	7.08	7.72	D4	87.1	38.2	68.7	-15	-72.1
D5	8.11	6.85	6.8	6.96	7.49	D5	194.3	72.3	66.4	77.1	-105
D6	7.46	6.5	6.56	6.81	7.34	D6	171.7	-91.8	93.6	64.6	-133.7
D7	7.45	6.43	6.62	7.19	7.4	D7	192.2	-124.3	-16.1	-125.6	-172.9
D8	8.16	6.94	6.88	6.87	6.87	D8	168.2	64.9	112.3	62.2	28.5
D9	7.3	6.74	6.81	7	7.62	D9	183.1	-115	-37.2	-44	-131.3
D10	7.55	6.88	7.12	7.59	7.67	D10	-94.9	-180.1	-38.7	-20.3	-16.8
D11	7.5	6.74	6.86	6.96	7.3	D11	-167.6	-186.8	-14.1	-46.1	-73.3
D12	5.87	6.78	6.83	7.3	7.72	D12	90.1	-99.6	25.6	-71.4	-102.8
D13	7.35	6.66	6.84	7.65	7.85	D13	59.6	-32.2	-21.3	-132.1	-190.6
D14	7.15	6.47	6.65	7.33	7.54	D14	89.2	-131.1	-26.6	-124.8	-165.4
D15	7.95	6.92	6.9	7.47	7.72	D15	101.2	39.8	59.5	-119.3	-110.6
D16	7.22	6.88	6.93	7.42	8.44	D16	99.3	15.5	67.7	-46.8	-113.6
	DO (%)						Conducitvity (µS/cm)				
BR	Initial	Day 0	Day 7	Day 14	Day 21	BR	Initial	Day 0	Day 7	Day 14	Day 21
N1	86	65.1	49.3	0	62.2	N1	0.5517	9.595	8.78	10.28	10.91
N2	89.4	61.8	58	0	68.2	N2	0.36	9.511	7.71	9.997	10.96
N3	81.7	63.1	2.1	42.8	63.2	N3	23.53	9.544	9.87	10.18	11.67
N4	34.8	46.8	55.8	0	45.9	N4	31	9.957	10.67	11.42	11.9
D1	48.1	49.8	3.3	7.6	0	D1	0.8471	5.035	8.731	3.803	6.887
D2	9.8	0	0	22.3	12.1	D2	0.8475	4.723	8.369	3.825	6.747
D3	15.4	0	0	3.5	0	D3	1.008	5.351	8.645	3.795	6.8
D4	79.6	36.5	9.6	20.8	8.3	D4	0.6792	5.624	9.549	4.18	7.331
D5	103.2	59.2	88.5	40.8	5	D5	1.513	14.02	13.25	11.59	9.904
D6	88.6	5.6	69.7	22.8	0	D6	0.3836	11.76	11.71	10.27	8.935
D7	66.9	2.5	13.2	0	0	D7	0.6389	11.54	11.05	9.619	8.695
D8	108.3	50.2	70.7	68.8	27.4	D8	0.3474	13.26	12.5	11.19	9.028

D9	83.2	32	4.5	11	2.5	D9	0.5462	5.734	12.38	11.42	9.899
D10	47.5	16.3	4.3	12.3	14.8	D10	0.9152	5.995	12.55	11.54	9.172
D11	34.3	17.5	20.6	13.4	9.3	D11	0.9405	6.034	12.56	11.45	10.13
D12	101.7	35.4	16.7	7.4	2.9	D12	0.2876	5.73	11.92	11.12	8.074
D13	28.8	32.7	18.9	0	1.6	D13	1.134	13.2	12.91	11.47	10.66
D14	85.1	0	21	0	3.8	D14	0.9943	11.8	12.14	10.47	11.35
D15	68.9	60.7	44.3	0	8.7	D15	0.9033	14.73	14.63	12.68	11.57
D16	70.1	52.4	50.5	8.9	8.9	D16	0.2627	14.05	13.9	12.36	11.21

Table 27 General chemistry results from Phase 2 BRs

BR	Week	pH	DO (%)	DO (mg/L)	ORP	Conductivity
D17a	0	7.41	73.1	6.76	-4.9	9.997
	1	7.69	7.8	0.71	-67.5	11.52
	2	8.22	17.1	1.52	-135.2	6.148
	3	7.38	7.3	0.65	-72.3	9.869
D17b	0	7.44	74.9	7.01	-5.3	8.952
	1	8.34	4.8	0.42	-109.6	10.71
	2	7.37	22.7	2.14	-122.3	6.637
	3	7.51	2.7	0.17	-140.3	7.802


D2
300
250
200
150
100
50
0
N-NX (mg/L)
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+


D3
300
250
200
150
100
50
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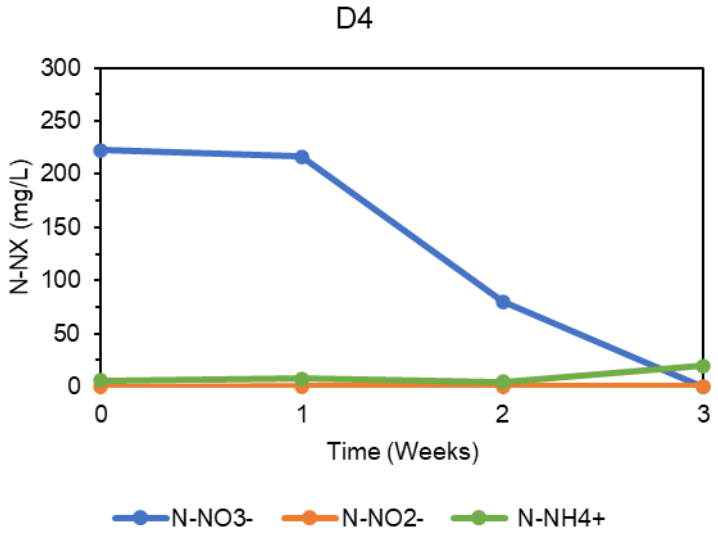
N-NX (mg/L)
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+


D4
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150
100
50
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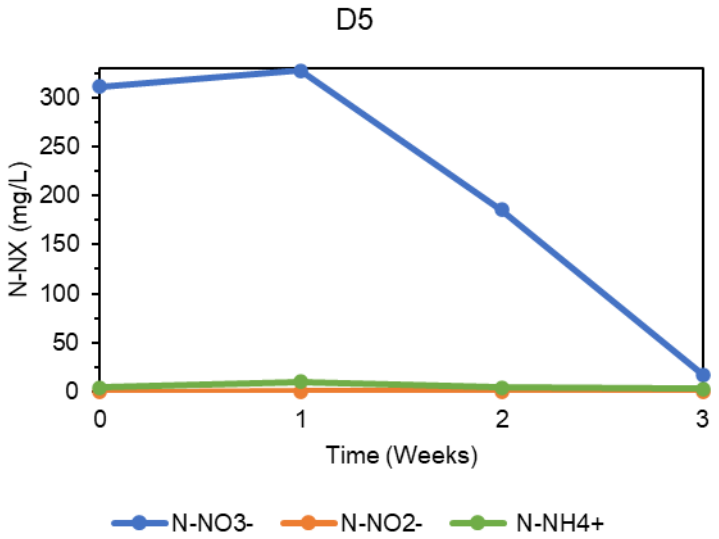
N-NX (mg/L)
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+


D5
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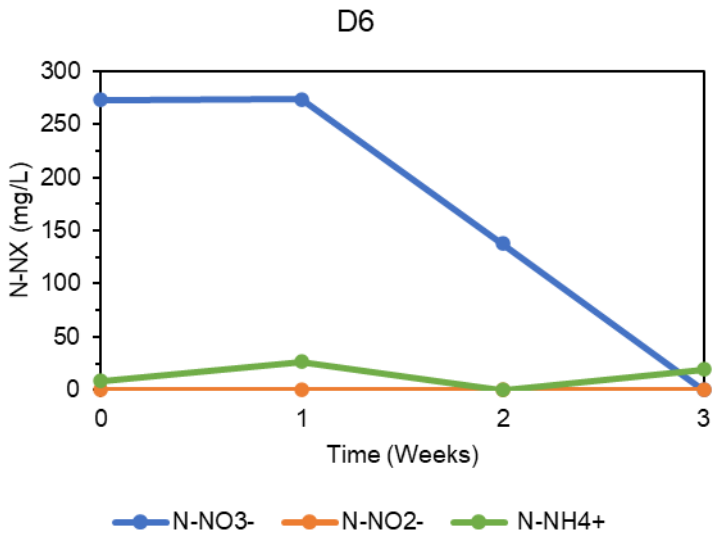
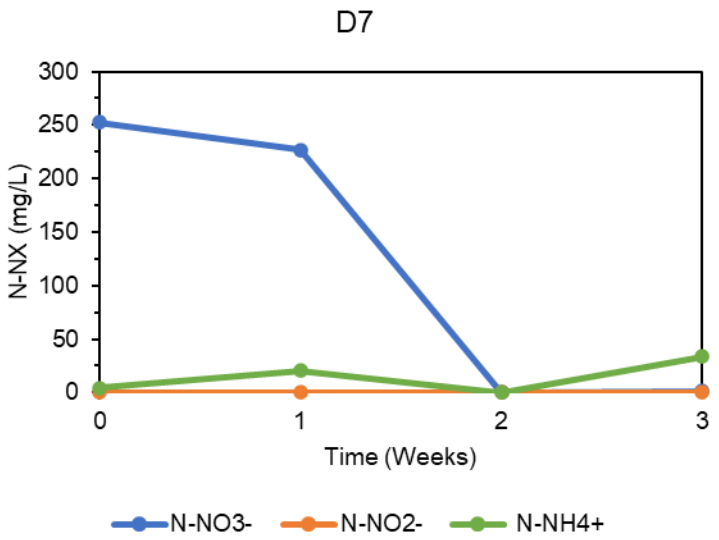
250
200
150
100
50
0
N-NX (mg/L)
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+


D6
300
250
200
150
100
50
0
N-NX (mg/L)
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+


D7
300
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150
100
50
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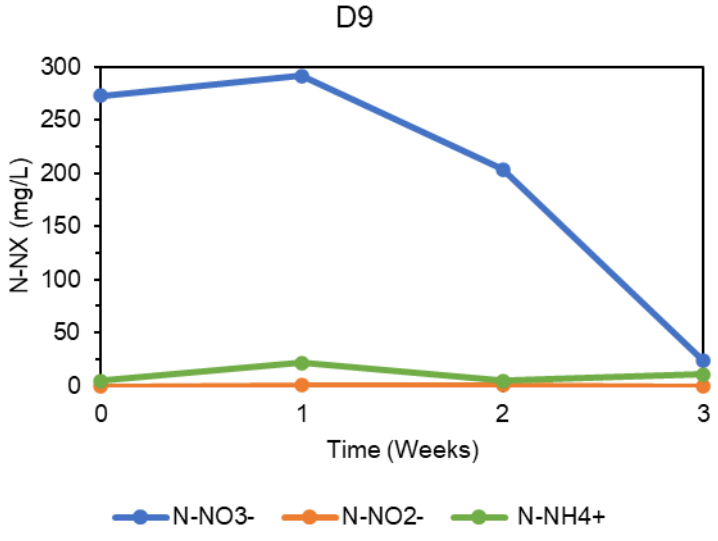
N-NX (mg/L)
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+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50
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1
2
3
Time (Weeks)
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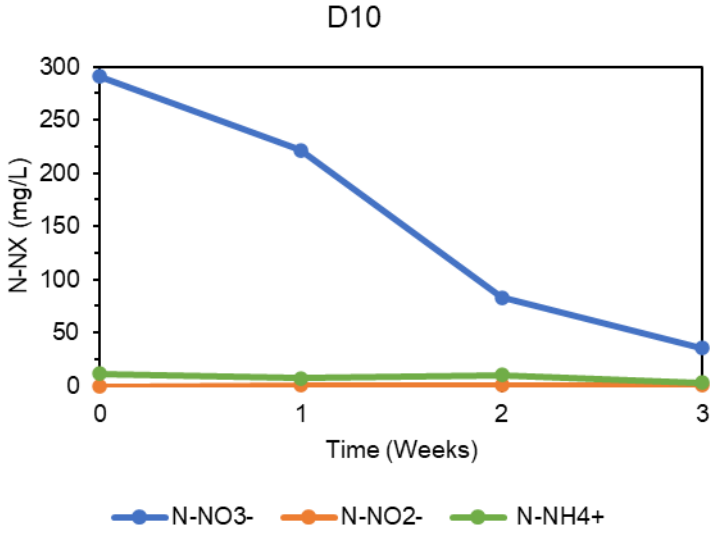
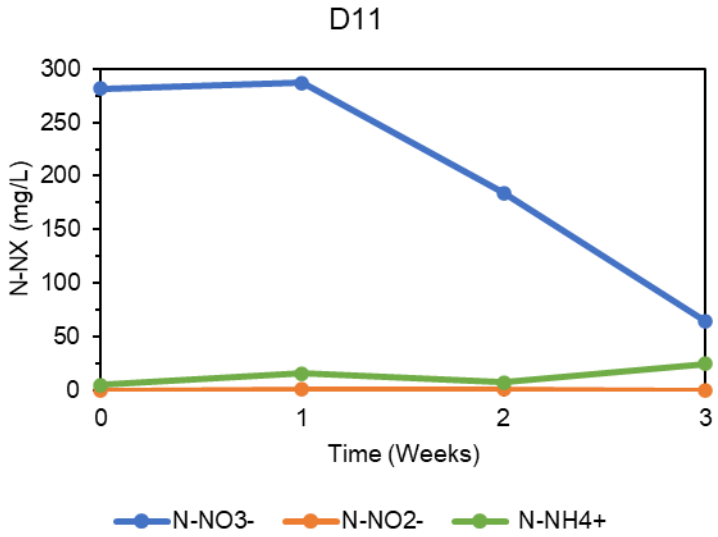
N-NO2-
N-NH4+


D10
300
250
200
150
100
50
0
N-NX (mg/L)
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+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200
150
100
50
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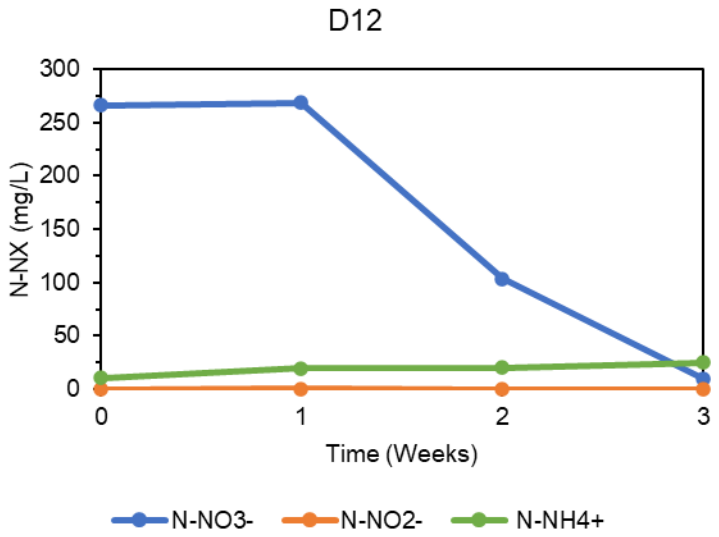
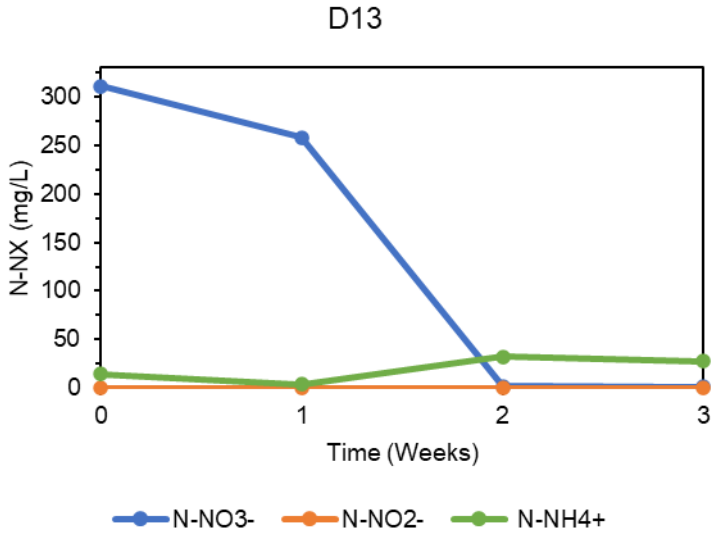
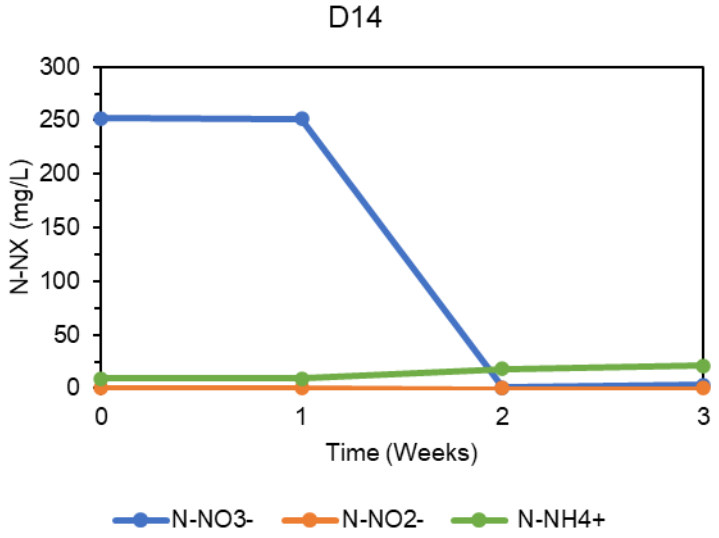
N-NX (mg/L)
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+


D12
300
250
200
150
100
50
0
N-NX (mg/L)
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+


D13
300
250
200
150
100
50
0
N-NX (mg/L)
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+


D14
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250
200
150
100
50
0
N-NX (mg/L)
0
1
2
3
Time (Weeks)
N-NO3-
N-NO2-
N-NH4+

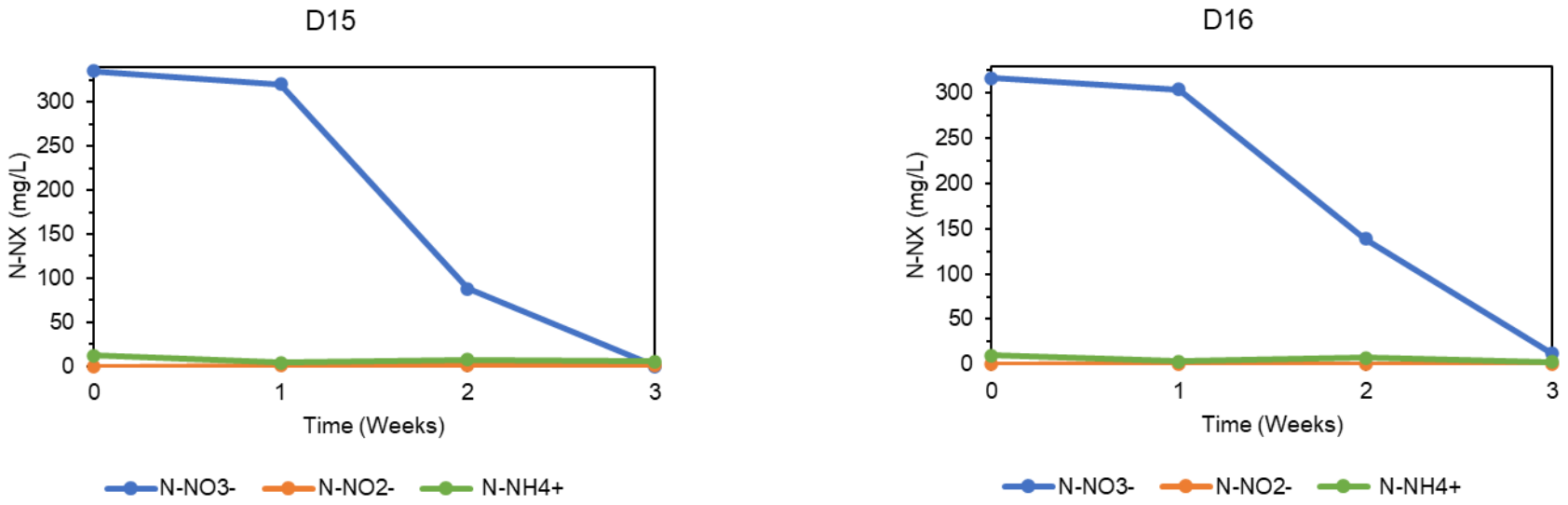

Figure 28 Nitrogen compound concentrations in Phase 1 bioreactors.

APPENDIX V

Table 28 General chemistry results from Phase 3.1 BRs

BR	Time	pH	ORP	Conductivity	DO (mg/L)	DO (%)	BR	Time	pH	ORP	Conductivity	DO (mg/L)	DO (%)
D20a	0	7.20	37.8	5.117	3.34	41.2	D24a	0	7.11	31.9	4.153	3.57	41.5
Foxtail barley	1	7.28	-65.6	7.654	0.51	5.7	Sweet clover	1	7.19	-106.1	5.22	0.11	1.4
	2	7.09	-81.4	4.386	0.49	5.5		2	7.14	-97.5	47.65	0.35	3.9
	3	7.41	-75.9	7.23	0.41	4.6		3	7.07	-95.2	6.387	0.15	1.7
D20b	0	7.20	40.4	7.72	3.24	40.2	D24b	0	7.18	29	4.141	3.85	44.6
Foxtail barley	1	7.11	-46.6	7.77	0.88	10	Sweet clover	1	7.04	-70.6	5.84	0.3	3.4
	2	7.11	-101.4	4.224	0.3	3.5		2	7.16	-94	4.327	0.22	2.5
	3	7.34	-71.5	6.577	0.5	5.7		3	7.21	-69.8	4.205	0.35	4
D21a	0	7.19	10.6	7.852	2.44	30.4	D25a	0	7.22	35.8	4.137	3.3	39
Compost	1	7.15	2.4	7.553	2.24	25	Wood chips	1	7.18	37	6.774	2.23	24.5
	2	7.18	-19.6	4.385	1.46	16.2		2	7.22	42	7.217	2.04	22.7
	3	7.46	27.8	4.283	3.78	41.7		3	7.29	50.7	5.202	3.62	40.5
D21b	0	7.17	12.4	4.323	3.03	37.3	D25b	0	7.18	39.8	4.124	3.69	43.3
Compost	1	7.28	6.8	8.007	2.17	24.2	Wood chips	1	7.20	40.9	4.537	2.71	29.7
	2	7.19	-23.5	5.643	1.34	14.9		2	7.26	52.1	5.312	2.56	28.6
	3	7.44	37.4	5.448	3.4	37.5		3	7.36	62.9	4.191	4.43	49.5
D22a	0	7.17	59.5	7.67	3.44	41.7	D26a	0	7.27	20.7	5.268	4.37	48.3
Brewery residue	1	7.31	-91.9	5.657	0.26	3.1	Sodium acetate	1	7.49	-60.9	4.761	0.92	10.1
	2	7.10	-90.6	5.997	0.22	2.5		2	7.18	-92.7	4.736	0.25	2.9
	3	7.30	-114.5	6.524	0.05	0.6		3	7.36	-120.2	8.536	0.08	0.9
D22b	0	7.10	49.5	4.23	3.34	40	D26b	0	7.33	17.8	8.105	4.58	50.7
Brewery residue	1	7.15	-124.7	5.663	0	0.1	Sodium acetate	1	7.34	-106.6	4.785	0.1	1.1
	2	6.98	-104	4.259	0	0		2	7.26	-117.6	5.188	0.14	1.7
	3	7.26	-87.2	5.912	0.1	1.2		3	7.39	-72.5	4.503	0.51	5.6
D23a	0	7.12	55.4	7.976	2.98	36.7	D27a	0	7.14	-25.8	7.658	2.6	29.4
Wood shavings	1	7.36	37.5	6.387	2.29	24.9	Molasses	1	6.88	-151	4.347	0.09	1
	2	7.17	55.4	4.401	2.8	30.4		2	6.79	-160.7	4.439	0.06	0.8
	3	7.33	58.6	6.083	4.02	45		3	6.92	-136.6	5.533	0.11	1.1
D23b	0	7.12	56.3	7.957	3.25	40	D27b	0	7.09	-28.8	7.203	2.78	31.4
Wood shavings	1	7.38	33.9	7.35	2.32	25.4	Molasses	1	6.95	-142.9	4.314	0.13	1.3
	2	7.07	75.4	5.817	2.91	31.5		2	6.88	-150.6	5.972	0.01	0.2
	3	7.37	69.5	5.155	4.23	47.4		3	7.00	-146.2	5.195	0.13	1.5

BR	Time	pH	ORP	Conductivity	DO (mg/L)	DO (%)	BR	Time	pH	ORP	Conductivity	DO (mg/L)	DO (%)
D20a	0	7.20	37.8	5.117	3.34	41.2	D24a	0	7.11	31.9	4.153	3.57	41.5
Foxtail barley	1	7.28	-65.6	7.654	0.51	5.7	Sweet clover	1	7.19	-106.1	5.22	0.11	1.4
	2	7.09	-81.4	4.386	0.49	5.5		2	7.14	-97.5	47.65	0.35	3.9
	3	7.41	-75.9	7.23	0.41	4.6		3	7.07	-95.2	6.387	0.15	1.7
D20b	0	7.20	40.4	7.72	3.24	40.2	D24b	0	7.18	29	4.141	3.85	44.6
Foxtail barley	1	7.11	-46.6	7.77	0.88	10	Sweet clover	1	7.04	-70.6	5.84	0.3	3.4
	2	7.11	-101.4	4.224	0.3	3.5		2	7.16	-94	4.327	0.22	2.5
	3	7.34	-71.5	6.577	0.5	5.7		3	7.21	-69.8	4.205	0.35	4
D21a	0	7.19	10.6	7.852	2.44	30.4	D25a	0	7.22	35.8	4.137	3.3	39
Compost	1	7.15	2.4	7.553	2.24	25	Wood chips	1	7.18	37	6.774	2.23	24.5
	2	7.18	-19.6	4.385	1.46	16.2		2	7.22	42	7.217	2.04	22.7
	3	7.46	27.8	4.283	3.78	41.7		3	7.29	50.7	5.202	3.62	40.5
D21b	0	7.17	12.4	4.323	3.03	37.3	D25b	0	7.18	39.8	4.124	3.69	43.3
Compost	1	7.28	6.8	8.007	2.17	24.2	Wood chips	1	7.20	40.9	4.537	2.71	29.7
	2	7.19	-23.5	5.643	1.34	14.9		2	7.26	52.1	5.312	2.56	28.6
	3	7.44	37.4	5.448	3.4	37.5		3	7.36	62.9	4.191	4.43	49.5
D22a	0	7.17	59.5	7.67	3.44	41.7	D26a	0	7.27	20.7	5.268	4.37	48.3
Brewery residue	1	7.31	-91.9	5.657	0.26	3.1	Sodium acetate	1	7.49	-60.9	4.761	0.92	10.1
	2	7.10	-90.6	5.997	0.22	2.5		2	7.18	-92.7	4.736	0.25	2.9
	3	7.30	-114.5	6.524	0.05	0.6		3	7.36	-120.2	8.536	0.08	0.9
D22b	0	7.10	49.5	4.23	3.34	40	D26b	0	7.33	17.8	8.105	4.58	50.7
Brewery residue	1	7.15	-124.7	5.663	0	0.1	Sodium acetate	1	7.34	-106.6	4.785	0.1	1.1
	2	6.98	-104	4.259	0	0		2	7.26	-117.6	5.188	0.14	1.7
	3	7.26	-87.2	5.912	0.1	1.2		3	7.39	-72.5	4.503	0.51	5.6
D23a	0	7.12	55.4	7.976	2.98	36.7	D27a	0	7.14	-25.8	7.658	2.6	29.4
Wood shavings	1	7.36	37.5	6.387	2.29	24.9	Molasses	1	6.88	-151	4.347	0.09	1
	2	7.17	55.4	4.401	2.8	30.4		2	6.79	-160.7	4.439	0.06	0.8
	3	7.33	58.6	6.083	4.02	45		3	6.92	-136.6	5.533	0.11	1.1
D23b	0	7.12	56.3	7.957	3.25	40	D27b	0	7.09	-28.8	7.203	2.78	31.4
Wood shavings	1	7.38	33.9	7.35	2.32	25.4	Molasses	1	6.95	-142.9	4.314	0.13	1.3
	2	7.07	75.4	5.817	2.91	31.5		2	6.88	-150.6	5.972	0.01	0.2
	3	7.37	69.5	5.155	4.23	47.4		3	7.00	-146.2	5.195	0.13	1.5

Table 29 General chemistry results for Phase 3.2 BRs

BR	Time	pH	ORP (mV)	Conductivity	DO (mg/L)	DO (%)	BR	Time	pH	ORP (mV)	Conductivity	DO (mg/L)	DO (%)
D28a	0	7.05	0	6.814	4.11	46.1	D32a	0	6.94	-8.2	8.695	3.97	44.9
Foxtail	1	6.77	-43.7	7.497	0.85	9.6	Sweet clover	1	6.58	-49.6	8.787	0.93	10.5
	2	6.96	28.6	7.831	1.82	20.6		2	6.68	-34.2	8.869	0.93	10.5
	3	7.05	-39.6	7.949	1.07	11.9		3	6.59	-16.5	8.627	1.22	13.7
D28b	0	7.04	-3.2	9.584	3.91	43.6	D32b	0	6.88	-11.7	9.054	3.94	44.6
Foxtail	1	6.73	-81.2	8.169	0.48	5.5	Sweet clover	1	6.5	-45	8.164	0.62	7
	2	6.88	-50.7	8.458	0.69	8		2	6.52	-46.1	8.869	0.72	8.1
	3	6.98	-40.8	8.199	1.35	15		3	6.48	2.5	8.57	1.54	17.3
D29a	0	6.98	-30.6	9.525	1.48	16.7	D33a	0	7.06	6.2	9.164	3.6	40.8
Compost	1	7.03	-30.8	8.295	1.26	14.2	Wood chips	1	7.03	27.7	8.806	1.61	18.1
	2	7.08	-24.4	8.069	1.4	15.9		2	7.1	40.9	8.864	3.01	33.9
	3	7.16	-9.5	8.049	1.73	19.3		3	6.99	37.7	8.582	2.41	26.9
D29b	0	7.03	-23.6	9.578	2.14	24.2	D33b	0	7.03	5.6	8.655	4.04	45.6
Compost	1	7.03	-29.4	8.176	1.13	12.8	Wood chips	1	7.07	32.4	9.087	1.99	22.4
	2	7.13	-23.1	8.261	1.74	19.8		2	7.07	52.2	8.815	2.79	31.3
	3	7.1	-14	8.317	1.69	18.8		3	7	27.3	8.84	2.59	28.8
D30a	0	7	-13.7	6.315	2.83	32	D34a	0	7.05	208.7	11.27	4.09	45.7
Brewery waste	1	6.84	-126.8	8.726	0.17	2	Sodium acetate	1	7.22	-66.5	11.6	0.59	6.7
	2	6.88	-133.8	8.064	0.06	0.7		2	7.24	-98.2	11.73	0.13	1.5
	3	6.66	-146	8.253	0.04	0.6		3	7.22	-69.2	12.38	0.3	3.5
D30b	0	6.95	-19.7	5.659	3.07	34.4	D34b	0	7.14	178	12.05	3.87	43.2
Brewery waste	1	6.78	-129.4	4.626	0.11	1.3	Sodium acetate	1	7.15	-65	11.95	0.45	5.1
	2	6.75	-146.7	8.115	0	0		2	7.44	-131.6	12.3	0.06	0.8
	3	6.66	-146.5	8.298	0	0		3	7.38	-106.4	12.31	0	0
D31a	0	6.96	7.4	4.87	2.85	32.9	D35a	0	7.06	-47.3	8.842	1.56	17.9
Wood shavings	1	7.17	32.5	9.033	1.74	19.2	Molasses	1	6.06	-133.4	9.012	0.29	3.4
	2	7.09	3.2	8.564	2.3	25.7		2	6.1	-168.7	8.169	0.56	6.3
	3	6.92	11.8	8.586	2.73	30.9		3	6.17	-125.7	9.135	0.19	2.1

D31b	0	7.01	3.9	4.793	2.88	33	D35b	0	7.06	-46.4	4.89	1.42	16.5
Wood shavings	1	7.13	51.5	8.873	1.72	19.1	Molasses	1	5.91	-101.7	9.176	0.27	3.1
	2	7.08	24.4	8.787	2.42	27.1		2	6.04	-136.4	9.225	0.42	4.7
	3	6.95	1.8	8.504	1.7	18.9		3	6.24	-119.7	8.99	0.21	2.4
							C100a	0	6.99	21.3	4.481	4.28	48.2
							blank	1	7.07	169.2	4.383	5.91	65.6
								2	7.05	200.4	4.442	6.6	73.8
								3	6.88	148.3	4.699	6.35	70.3
							C100b	0	6.99	20.8	4.388	4.7	52.7
							blank	1	7.03	135	4.332	5.95	66.6
								2	7.11	77.8	4.319	6.7	75.5
								3	6.86	27.5	4.283	2.85	31.6

APPENDIX VI

Table 30 Complete results from sorption tests conducted with complex carbon sources

Carbon source	Carbon source mass (g)	Nitrate (ppm) initial (theoretical)	NOx (ppm) initial (actual)	NOx (ppm) after 2 hours	Removal (%)	Qt (mg/g)	Average removal (%)	Qt Average (mg/g)
Foxtail Barley	0.4802	25	23.7	22.1	6.8	1582.67	3.9	935.65
	0.4927	100	84	83.7	0.4	289.22		
	1.016	50	42.9	40.9	4.7	935.04		
White Sweet Clover	0.4807	25	23.7	22.1	6.8	1581.03	2.1	-637.43
	0.4873	100	84	88.6	-5.5	-4483.89		
	1.007	50	42.9	40.8	4.9	990.57		
Wood chips	0.4562	25	23.7	21.8	8.0	1978.30	4.0	818.43
	0.439	100	84	84.4	-0.5	-432.80		
	0.992	50	42.9	41	4.4	909.78		
Brewery residue	0.4562	25	23.7	22.5	5.1	1249.45	3.9	1275.08
	0.4523	100	84	82.5	1.8	1575.28		
	0.997	50	42.9	40.8	4.9	1000.50		
Wood shavings	0.445	25	23.7	23	3.0	747.19	5.1	1858.21
	0.4303	100	84	81.3	3.2	2980.48		
	1.003	50	42.9	39	9.1	1846.96		
Compost	0.7286	25	23.7	23.7	0.0	0.00	2.8	827.39
	0.718	100	84	82.1	2.3	1256.96		
	1.008	50	42.9	40.3	6.1	1225.20		

APPENDIX VII

Supplemental results from DNA characterization.

Table 31 UV-Vis results from DNA extracted from select bioreactors

Sample ID	Nucleic Acids (ng/µL)	260/280	260/230
D17a_1	20.8	1.73	1.6
D17a_2	25.1	1.74	0.19
D17a_3	14.8	1.79	0.7
D17a_4	22.8	1.8	0.08
D17a_5	29.6	1.82	0.69
D17a_6	19.2	1.74	0.89
D17b_1	25.8	1.74	1
D17b_2	23.8	1.87	0.49
D17b_3	18.7	1.75	0.1
D17b_4	16.9	1.76	0.45
D17b_5	19.1	1.8	0.09
D17b_6	17.6	1.75	1.71
D24a_1	4.6	1.68	0.21
D24a_2	4.6	1.7	0.04
D24a_3	5.3	1.59	0.04
D24a_4	2.1	1.74	0.38
D24b_1	4.1	1.65	0.04
D24b_2	4.2	1.92	0.03
D24b_3	4.4	1.61	0.08
D24b_4	3.2	1.65	0.18
D32a_1	7	1.93	0.1
D32a_2	4.2	1.51	0.23
D32a_3	2.2	2.32	0.11
D32a_4	2	2.58	0.03
D32b_1	5	1.79	0.05
D32b_2	3	1.23	0.05
D32b_3	4.6	2.16	0.03
D32b_4	2.7	1.71	0.03

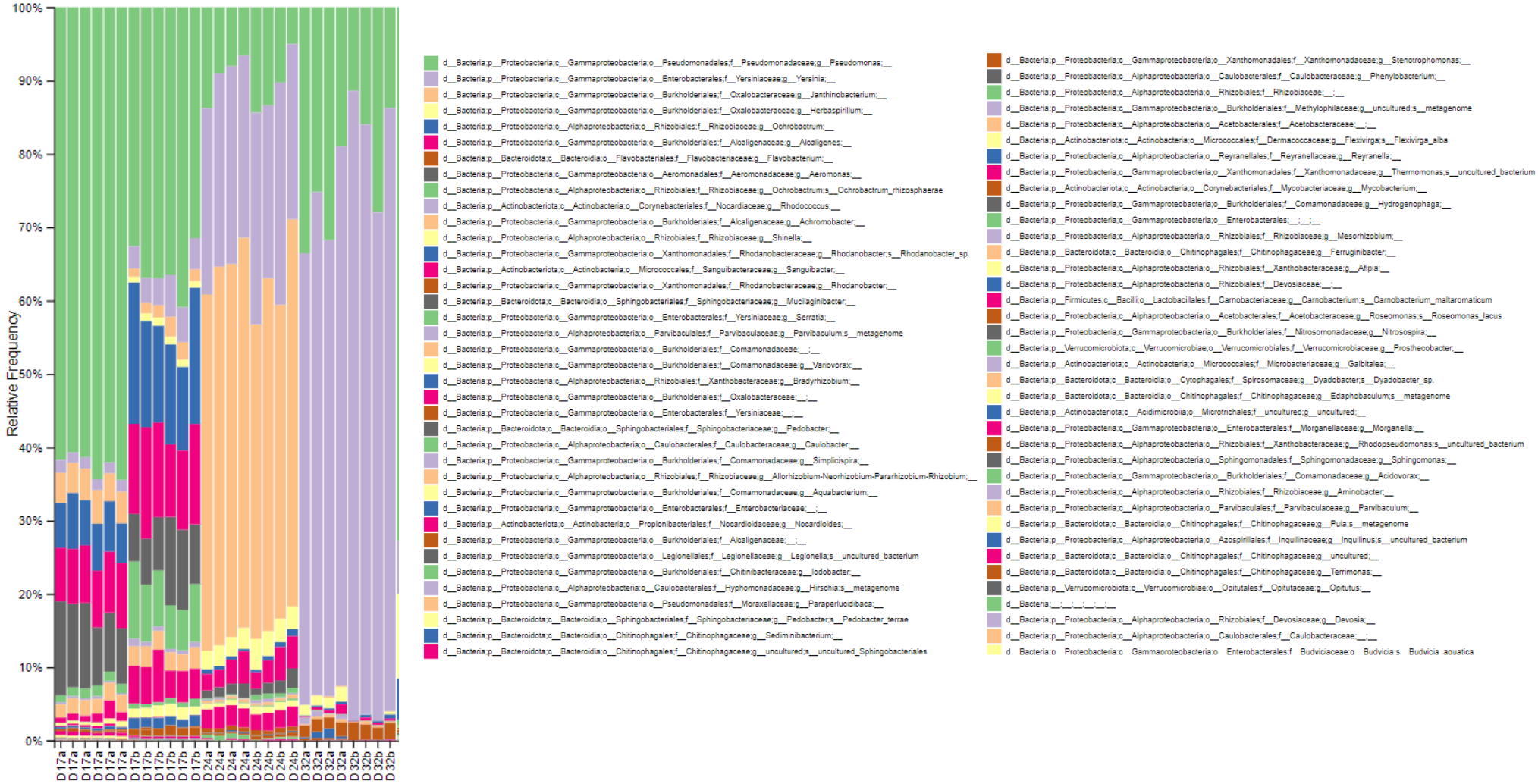

Figure 29 Relative abundance of most dominant taxa in replicate samples of select BRs.