Bioresource Technology Reports 27 (2024) 101885

Contents lists available at ScienceDirect

Bioresource Technology Reports

journal homepage: www.sciencedirect.com/journal/bioresource-technology-reports

Microbial community dynamics in a two-stage treatment wetland: Insights from treating seasonal ski resort wastewater

Stephanie H. Ayotte [a,b,c], Sarah J. Wallace [d], Christopher R. Allen [a], Kela P. Weber [d], Otto R. Stein [a,b], Ellen G. Lauchnor [a,b,c,*]

[a] *Department of Civil Engineering, Montana State University, Bozeman, MT 59717, USA*
[b] *Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717, USA*
[c] *Thermal Biology Institute, Montana State University, Bozeman, MT 59717, USA*
[d] *Department of Chemistry and Chemical Engineering, Royal Military College of Canada, Ontario K7K 7B4, Canada*

ARTICLE INFO

Keywords:
16s rRNA
Constructed wetland
Metagenomics
Nitrification
Denitrification

ABSTRACT

Microbial community studies in treatment wetlands (TW) have not investigated cold-climate TW and have focused primarily on laboratory mesocosms. This study determined microbial community composition and dynamics using 16s rRNA sequencing in a partially-saturated two-stage, vertical-flow pilot TW treating ski resort wastewater from December–April. Indicator species analysis found organisms capable of the primary nitrogen transformations observed in each stage at 3 °C, with nitrifiers in the unsaturated second stage and heterotrophic denitrifiers in the first stage where recycled nitrate is removed. During seasonal TW operation, microbial communities developed significant differences between TW stages and α-diversity decreased. Microbial communities were compared to batch mesocosm TW communities, which were significantly different from the pilot. The study is the first to discern community dynamics and key microorganisms during seasonal loading of a cold-climate, field-scale TW. Results raise questions regarding comparability of mesocosms to pilot systems and indicate more field-scale TW investigations are needed.

1. Introduction

Treatment, or constructed, wetlands (TWs) are highly engineered systems designed for water pollution control and contaminant removal (Kadlec and Wallace, 2009). This growing technology has both complex and dynamic microbial communities due to the presence of aquatic macrophytes, the composition of the substratum, the unique nutrient composition of the wastewater, and environmental factors, such as temperature and oxidation-reduction potential (Faulwetter et al., 2009; Kadlec, 1999). Since the 1980s, the microorganisms that inhabit TWs have been studied in varying capacities to determine the activity, structure, enumeration, or function of their communities, with growing focus on structural and functional assessments (Weber, 2016).

The present study focused on a two-stage pilot TW operating at low temperatures (3–5 °C), where ammonia conversion to nitrate is observed in the second unsaturated (oxic) stage and removal of carbon and recycled nitrate from the second stage is observed in the saturated (anoxic) first stage (Ayotte et al., 2024). The system operates at these temperatures under seasonal loading with over 95 % removal of TOC and 70 % TN removal. Different saturation levels and influent water quality to each stage allow for analysis of how communities develop during seasonal operation and vary between the treatment stages.

Of the two primary nutrients (carbon and nitrogen) removed biologically in TW, carbon (C) is readily consumed as an energy source during heterotrophic respiration (Faulwetter et al., 2009) and nitrogen (N) removal occurs via the sequential processes of nitrification and denitrification. Nitrification is a 2-step aerobic process mediated by chemolithoautotrophic bacteria (Shammas, 1986) and consists of ammonia (NH_3) oxidation to nitrite (NO_2^-) catalyzed by ammonia oxidizing bacteria (AOB) or archaea (AOA) (Soliman and Eldyasti, 2018) followed by oxidation of NO_2^- to nitrate (NO_3^-) catalyzed by nitrite-oxidizing bacteria (NOB) (Shammas, 1986). Denitrification is a four-step anoxic heterotrophic process by facultative bacteria that reduce NO_3^- to nitrogen gas (N_2), with NO_2^-, nitric oxide (NO) and nitrous oxide (N_2O) as intermediates (Faulwetter et al., 2013). It is estimated that 87–96 % of total nitrogen removal is due to denitrifying bacteria in TW systems (Faulwetter et al., 2009; Zhang et al., 2022). The studied pilot TW is designed to utilize these two processes for total nitrogen removal

---

[*] Corresponding author at: Montana State University, 205 Cobleigh Hall, P.O. Box 173900, Bozeman, MT 59717-3900, USA.
E-mail address: ellen.lauchnor@montana.edu (E.G. Lauchnor).

https://doi.org/10.1016/j.biteb.2024.101885
Received 22 March 2024; Received in revised form 7 June 2024; Accepted 14 June 2024

by optimizing conditions for nitrification in one stage and denitrification in the other stage of the TW. Microbial community analysis can demonstrate whether optimizing operational conditions for these different processes leads to development of specialized microbial communities between the TW stages over a four-month operational season, providing insight to periodic TW performance.

The microorganisms that perform nitrification can be inhibited by low temperatures, leading to reduction of N removal. Nitrification rates in wastewater treatment systems have been found to decrease significantly at low temperatures, particularly below 5 °C (Zhou et al., 2018). In one full-scale sequencing batch reactor, ammonia monooxygenase copy numbers were shown to decrease significantly at temperatures below 15 °C, despite AOB maintaining their population abundance (Johnston et al., 2023). At 1 °C, a nitrifying moving bed biofilm reactor (MBBR) exhibited a significant reduction of nitrification rates to only 18 % of the initial ammonia removal observed at 20 °C; however, the communities returned to initial performance rates when temperatures increased back to 20 °C, indicating that reduction in activity did not result in cell death (Hoang et al., 2014). Comparatively, the TW studied here has operated at wastewater temperatures of 3–5 °C and exhibited efficient N removal, with over 98 % ammonia removal from high strength wastewater (170–200 mg L^{-1}) (Ayotte et al., 2024). Another pilot-scale MBBR has shown similar performance, with 75 to 89.5 % ammonia removal at 1 °C following a gradual decrease from summer water temperatures of 20 °C. Investigation of the microbial communities that drive these processes in cold-climate TWs is needed for better understanding of their capabilities across climates and operational schemes.

Fewer than 5 % of microbial community studies of TWs have been conducted on field-scale systems (Weber, 2016). Much of the focus on teasing out the dynamics and interactions of microbial communities in TW has been placed on mesocosm systems that have the benefit of laboratory-controlled climates, and artificial wastewater compositions (Weber, 2016). Evidence suggests that, although this research is meaningful in furthering our understanding of microbial functions, slight differences in operation between laboratory-operated systems and pilot or full-scale TWs can lead to significant differences in the community composition and potential functions (Aguilar et al., 2022; Bouali et al., 2014; Cydzik-Kwiatkowska and Zielińska, 2016; Faulwetter et al., 2013, Faulwetter et al., 2009; Silveira et al., 2021; Wei et al., 2021). Metagenomic structural studies of pilot-scale systems can further identify operational parameters that may aid or inhibit the establishment of vital taxa for nutrient removal that cannot be easily targeted or assessed in the idealized environmental conditions that are often present for mesoscale systems (Cydzik-Kwiatkowska and Zielińska, 2016). It is necessary to analyze microbial dynamics at multiple scales to understand whether studies of microbial communities in mesocosms can be conducted to accurately predict pilot- and full-scale TW behavior.

Studies have reported on the conditions in TW that influence microbial community development, including flow, wastewater composition, depth, plant species, and system age (Faulwetter et al., 2009; Silveira et al., 2021). Microbial community development may be partially correlated to turbidity, total organic carbon (TOC), total nitrogen (TN), distance along the flow path, and oxygen availability (Button et al., 2015; Silveira et al., 2022). However, questions remain regarding the interplay between these microbial communities, TW operational parameters and the overall efficiency of water treatment. Some studies have observed significant community differences due to feeding periods compared summer rest periods (Wang et al., 2020) and temporal shifts in nutrient loading (Ibekwe et al., 2016). Pavlou (2006) even noted that oscillating operating conditions enabled competitive organisms to coexist. However, seasonal operation and large fluctuations in nutrient loading at low temperatures have not been studied in TW thoroughly. To the best of our knowledge, this is the first investigation of a cold-climate intermittently loaded TW that operates only during the winter to better understand the interplay between shifts in nutrient loading and microbial community dynamics.

The results presented here answer questions regarding microbial structure and dynamics in TWs through the lens of combined long-term and seasonal start-up conditions in one of the most extreme TW environments yet studied. By considering a system operating under cold-climate environmental conditions, we garner a better understanding for the robustness and resilience of microbial communities in TWs. In this study, microbial communities in a two-stage, partially saturated pilot-scale vertical flow TW were sampled and sequenced using 16s ribosomal RNA (rRNA) amplicon sequencing with the following objectives: (1) to investigate the temporal shifts in microbial communities during seasonal shifts in loading; (2) to determine spatial dynamics of communities associated with low temperature conditions, including differences evident in the nitrification and denitrification stages; and (3) to compare microbial community structure between the pilot TW and mesocosms loaded with artificial wastewater.

2. Materials and methods

2.1. Experimental set up and pilot system operation

The study was conducted at a pilot-scale wastewater TW (45°49’01.0” N 110°54’21.8” W) during the 2020–2021 season. The high-strength domestic wastewater from the ski area's lower lodges is treated using a pilot-scale two-stage, sub-surface vertical flow TW system for carbon and total nitrogen removal (Fig. 1), similar to a modified Ludzak-Ettinger process. The system operates seasonally from early December to April with mean water temperatures between 2.5 and 5 °C. The first stage has a crushed gravel media treatment layer (nominal depth = 0.9 m, d_{50} = 5.3 mm) and is operated with a saturation depth of 0.71 m to facilitate anoxic conditions for denitrification below a shallow unsaturated zone (0.19 m). The second stage contains a washed concrete-sand treatment layer (nominal depth = 0.9 m; d_{50} = 0.53 mm) and is operated as unsaturated. Each stage has two cells in parallel (area per cell, A_C = 23.8 m^2).

The influent wastewater undergoes primary treatment in a series of sedimentation tanks before entering the system. The first stage is designed to remove COD while the second stage is designed to facilitate nitrification of influent ammonium. Effluent from the second stage containing nitrate is recycled and dosed onto the first stage independently from influent septic doses, facilitating total nitrogen removal. Septic doses of 2.5 cm wastewater were applied onto the first stage every

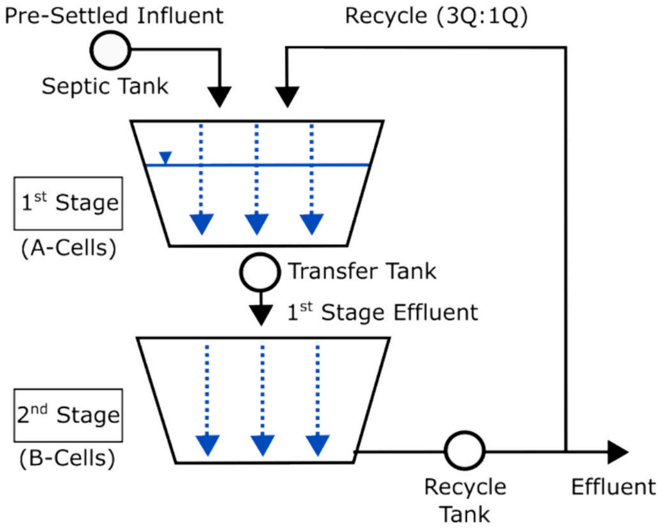

Fig. 1. System flow schematic of the pilot TW. Pre-settled influent (1Q) and final effluent (3Q) are loaded onto the partially saturated first stage. The first stage effluent (4Q) is loaded onto the second stage.

two hours and recycle doses of 1.3 cm were applied approximately every 40 min, alternating one septic dose with three recycle doses (S-R-R-R). The second stage was dosed with 5.0 cm of effluent from the first stage every 2 h. The TW treated approximately 7.2 m^3 d^{-1} (1900 gpd) of influent wastewater and operated at a 3:1 (recycle:influent) v:v ratio, for a total hydraulic loading of 28.8 m^3 d^{-1} (7600 gpd) of combined influent and recycle wastewater through the two stages. The system was planted with native sedge (*Carex utriculata*) and bulrush (*Schoenoplectus acutus*) in 2013.

2.2. Mesocosm operation

Mesocosms were constructed in 2020 to investigate the effects of nutrient loading and macrophyte selection and their root exudates on nitrogen removal. Polyvinyl chloride (PVC) columns (15 cm diameter, 40 cm depth) were filled with 30 cm of gravel (d_{10} = 2.38 mm to d_{60} = 4.76 mm) and cultivated at Montana State University's Plant Growth Center (PGC) in a randomized block design. The larger mesocosms compared to microcosm systems aimed to more closely simulate pilot- or full-scale TWs by reducing overgrowth of plants and root-binding that does not occur in large systems. However, growth and operation of mesocosms within the greenhouse enabled more controllable environmental conditions for targeted hypothesis testing. The selected plants, *Carex utriculata*, *Schoenoplectus acutus*, and *Phragmites americanus*, were selected based on prior studies (Taylor et al., 2011) and were grown following methods established by Allen et al. (2013) to determine plant species effects on water quality treatment. Temperature was maintained at 24 °C and diurnal cycles were artificially simulated with LED lights. In October 2021, artificial wastewater was supplied to the columns in two-week batch feeding cycles with the columns operated under saturated conditions to mimic horizontal subsurface-flow treatment wetlands. Columns were drained prior to feeding and re-filled with fresh medium to simulate draining and re-wetting of TW systems. Twenty-four columns were planted with one plant species per column (eight columns per plant). Half of the columns were fed with wastewater simulating primary settled domestic wastewater, with chemical oxygen demand (COD; 500 mg L^{-1} as sucrose) and ammonium (total NH_4^+; 40 mg L^{-1}). The second half of the columns were fed with wastewater simulating secondary treated wastewater with 120 mg L^{-1} of nitrate (NO_3^--N) and no supplemented carbon. Both solutions contained 10 mL L^{-1} of trace element solution SL-4 (Trace element solution SL-4; the Leibniz Institute DSMZ, Braunschweig GER).

2.3. Geochemical analysis

Water samples from the pilot system were collected from the influent, the combined effluent of the first stage, and the combined effluent of the second stage. Daily composite water samples were collected via autosampler three times a week and acidified to a pH less than two using 5 mL of 1.8 M sulfuric acid. Water samples from the mesocosms were collected from a tube located at the center of the gravel. After flushing the tube, 30 mL of water was sampled and filtered through a 0.2 μm nylon syringe filter and stored at 4 °C until analysis could be completed.

COD was measured using US EPA standard Method 410.4 with HACH® digestion vials (HACH® Co., Loveland, CO, USA). Anions were determined using the Metrohm Eco ion chromatograph with a Metrosep A Supp 5 150/4.0 column, Metrohm Suppressor Module and 3.20/1.00 mM sodium bicarbonate/sodium carbonate eluent at 0.7 mL min^{-1} (Metrohm USA, Riverview, FL, USA). Ammonium (NH_4^+-N) was determined using a modified Berthelot reaction scaled for 96-well plates (Rhine et al., 1998) and absorbances were read at 660 nm using a BioTek Synergy HTX Multimode Reader (Agilent Technologies, Inc., Santa Clara, CA, USA). Total inorganic nitrogen (TIN) was calculated by the summation of inorganic nitrogen species and determined to be representative of total nitrogen by comparison to TN results from a Shimadzu TOC-VSH (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) for total nitrogen.

2.4. Microbial sampling in the pilot Treatment Wetland

Media containing attached microbial communities were sampled from: (1) the saturated zone in the first stage below 30 cm, (2) the unsaturated zone in the first stage between 15 and 30 cm, and (3) the unsaturated second stage between 15 and 30 cm. Samples were collected three times over the course of the winter in December 2020, February 2021, and April 2021 (Fig. 2). December samples occurred prior to the beginning of the ski season to act as a baseline for the microbial communities. The two cells of each stage were treated as replicates. Two randomly selected locations (one per cell) per identified zone were sampled at each sampling time for paired temporal analysis (n = 18). Gravel and sand samples were collected by inserting a 30 cm-diameter cardboard tube into the surface of the wetland and excavating to the desired depth with a shovel sterilized with 70 % ethanol (Appendix Fig. A.1). Media samples were taken with autoclaved spoons and transferred to sterile 50 mL Falcon™ tubes (Fisher Scientific Inc., Waltham, MA USA). A minimum of 50 g of gravel or sand was collected at each sample location. All samples were stored on ice for transport and subsequently stored at −80 °C until DNA extraction was performed.

2.5. Microbial sampling from the meso-columns

Due to root establishment in the columns, the surface could not easily be accessed and was instead drilled to 8–10 cm depth using a drill with a 2 in metal coring bit sterilized with 70 % ethanol. Once at the desired depth, an autoclaved spoon was used to extend the hole beyond the contact location of the drill and then to collect gravel samples. Gravel samples were collected in sterile WhirlPack™ bags, stored on ice for transport and subsequently stored at −80 °C until DNA extraction was performed.

2.6. DNA extraction

DNA was extracted from approximately 0.5 g of sand or gravel samples using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, LLC., Santa Ana, CA, USA) following the manufacturer's instructions. No special methods were required for the extraction of DNA from gravel or sand samples to meet the required 5–10 ng μL^{-1} eluted DNA concentrations. DNA was quantified using the Invitrogen™ Qubit™ dsDNA Quantification Assay kit (ThermoFisher Scientific, Inc., Waltham, MA, USA). DNA was shipped on dry ice to the Royal Military College of Canada, in Ontario Canada for 16s rRNA sequencing.

2.7. High-throughput sequencing

For high-throughput sequencing, 16s rRNA sequence libraries were prepared by targeting the V3-V4 region from the extracted DNA (normalized to 5 ng μL^{-1}) using universal primers 341F 5′ -CCTACGGGNGGCWGCAG- 3′ and 785R 5′ -GACTACHVGGGTATCTAATCC-3′ (Klindworth et al., 2013) due to their broad taxonomic coverage of both *Bacteria* (~80 %) and *Archaea* (~50 %) following the Illumina 16S Metagenomic Sequencing Library Preparation guide (version B, Illumina Canada Ulc., Victoria, BC, Canada). Purified products were then normalized to 4 nM, denatured, and diluted to 2 pM for sequencing using the MiSeq™ platform (Illumina, Victoria, BC, CAN) following the standard operating procedure for generating 2 × 300 bp reads. A 10 % PhiX control library was added for quality control. The sequences were processed using QIIME2™, (version 2021.4) (Bolyen et al., 2019) on VirtualBox (v6.1.10). Sequences were denoised, demultiplexed, merged and chimeras removed using DADA2 (Callahan et al., 2016) for quality control. Subsequently, samples were clustered into operational taxonomic units (OTUs) at 0.03 (97 % similarity) and

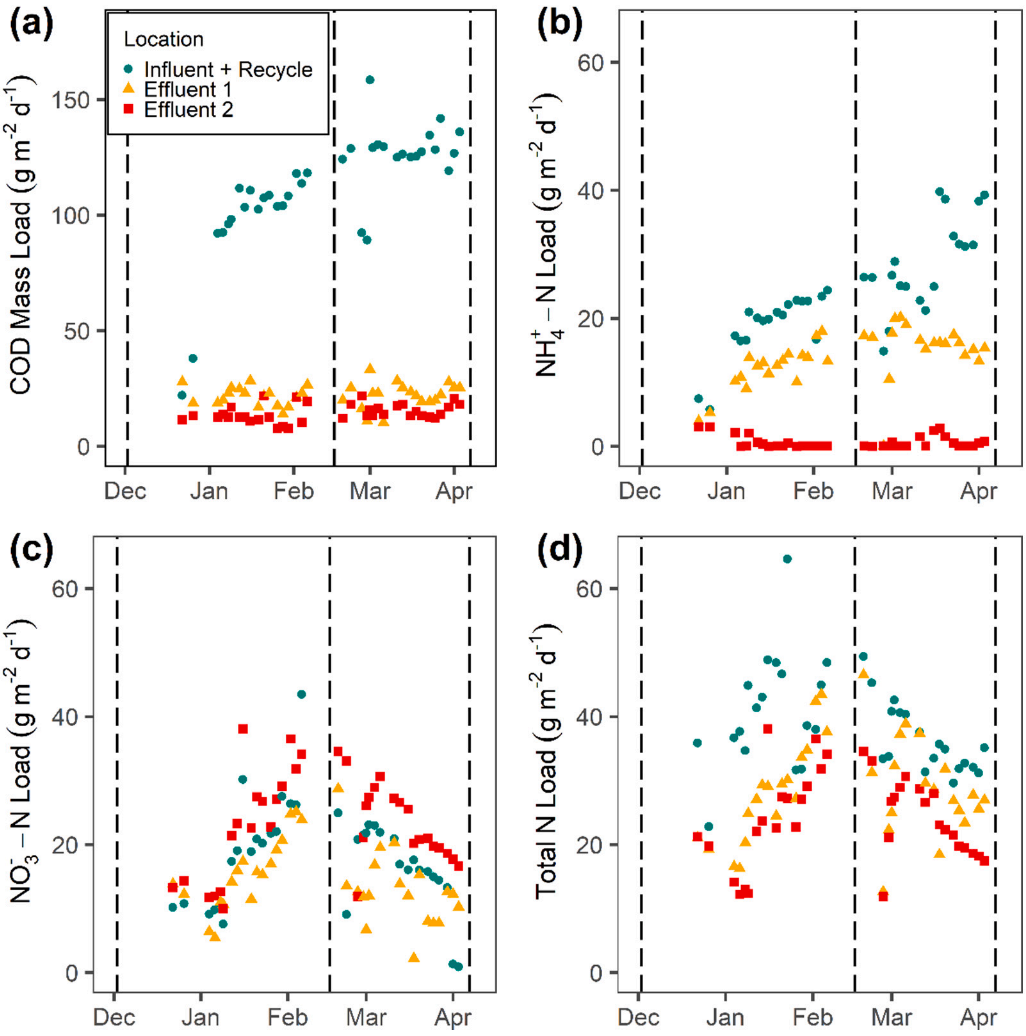

Fig. 2. Mass loading of carbon (COD), total inorganic nitrogen (TIN), total ammonium (NH_4^+) and nitrate (NO_3^-) from the influent to the first stage (Influent), effluent of the first stage (Effluent 1), and effluent of the second stage (Effluent 2). Influent includes mass loading from both septic and recycle doses. Vertical dashed lines indicate when microbial sampling occurred. Data shows removal of COD, TIN, and NH_4^+ across the system, with a production of NO_3^- in the second stage.

classified using the pre-trained classifier (Bokulich et al., 2018) of the SILVA database (version 138 99 % OTUs full length sequences, Quast et al., 2013) to the genus level. Sequences and features related to mitochondrial, or chloroplasts, were removed. For all samples, the median number of reads after quality control was 32,880 (min: 7,164-max: 55,891) reads; the number of sequences were rarified to the minimum number of reads (7,164) for each sample for the diversity analysis of both the greenhouse and the pilot TW samples. When assessing the diversity of pilot TW samples independently, the samples were each rarefied to 17,161 reads (min reads for the TW).

2.8. Statistical analysis

Analysis of 16S rRNA data was performed in R version 4.3.1 (R Core Team, 2022) using the vegan (v2.6.4) package (Oksanen et al., 2022). Alpha diversity assessed the complexity of microbial diversity for each sample, using observed OTUs to measure observed species richness (number of unique species per sample), diversity by the Shannon index (H), and sample evenness by the Shannon Equitability Index (E_H). Differences in alpha diversity were assessed for significance due to sample stage and month of sampling using two-way ANOVA and differences between treatment means were assessed using Tukey's post-hoc test at a significance level of $p < 0.05$. Indicator species analysis of the microbial communities was assessed using the indicspecies (v1.7.14) package (De Caceres and Legendre, 2009) in R studio with the multipatt function where a multilevel pattern analysis identified key organisms associated with each system stage. For this analysis only organisms with a genus level relative abundance >2 % were assessed. Beta diversity, or dissimilarity of samples from different regions, was assessed using Principal Coordinate Analysis (PCoA) with the Bray-Curtis distance metric ordination. The categorical variables assessed in the PCoA ordinations (month, system stage, and depth) were analyzed for significance using permutational multivariate analysis of variance (PERMANOVA) using distance matrices (ADONIS) based on 999 permutations. All parameters were tested for significance ($p < 0.05$).

3. Results and Discussion

3.1. Water quality and system performance

The 2020–2021 ski season observed an increase in nutrient loading of COD from 47 g m^{-2} d^{-1} to over 100 g m^{-2} d^{-1} from December to January, before stabilizing at 120 g m^{-2} d^{-1} in mid-February for the remainder of the season (Fig. 2). The system undergoes a seasonal start-up as influent concentrations increased due to increasing skier visits during the first few weeks of the ski season (Fig. 2). Areal mass loading rates (calculated with recycle) indicate influent levels of COD and NH_4^+ increase to approximate steady state conditions by mid-January to early

February (Fig. 2a & b). Mean mass loadings of COD, TIN, and NH_4^+ to the first stage were 111.0 ± 26.6 g m^{-2} d^{-1}, 38.8 ± 8.2 g N m^{-2} d^{-1}, and 23.3 ± 7.8 g N m^{-2} d^{-1}, respectively. The first stage effluent, which is subsequently loaded onto the second stage, had average seasonal mass loads of 21.8 ± 4.7 g m^{-2} d^{-1}, 28.7 ± 8.4 g N m^{-2} d^{-1}, and 13.4 ± 4.8 g N m^{-2} d^{-1} for COD, TIN, and NH_4^+, respectively. Final effluent average mass loads had a 33.0 % additional reduction of COD, 14.3 % reduction of TIN and a 95.5 % reduction of NH_4^+ from the second stage influent. Average final effluent mass loadings were 14.6 ± 3.7 g m^{-2} d^{-1}, 24.6 ± 8.7 g N m^{-2} d^{-1}, and 0.6 ± 0.9 g N m^{-2} d^{-1} for COD, TIN and NH_4^+, respectively. Outside of the operational season, the system is recirculated with treated wastewater and supplemented with surface water from a proximal creek to maintain plant health. Since nitrate is not completely removed during treatment, the remaining nitrogen continues to circulate in the TW. Prior to the operational season, concentrations of nitrogen were predominantly in the form of NO_3^- at 26.5 ± 0.7 mg NO_3^--N L^{-1}, (October to December).

Average COD, NH_4^+ and TIN removal across the system was 95.5 ± 3.7 %, 98.77 ± 2.8 % and 68.9 ± 15.5 %, respectively during the 2020–21 season (Table A.1). Areal mass loading figures include addition of both septic and recycled effluent onto the first stage, resulting in high nitrate and COD loading on the first stage. COD was removed primarily in this first stage (Fig. 2a) and little additional COD was removed in the second stage. The TIN load across both stages was reduced by 20–40 % in a single pass through the system, with most TIN removal occurring in the first stage and 12–18 % across the second stage (Fig. 2d). However, the higher TIN removal (70 %) is achieved with recirculation of treated effluent at three times the influent flow rate.

Some removal of NH_4^+-N (13.3–52.0 %) was observed across the first stage. The mechanism driving this first stage NH_4^+-N removal has not been investigated, but could be due to microbial assimilation or ammonia oxidation in the shallow unsaturated zone. Removal of ammonia in the aerobic second stage often exceeded 97 % (Fig. 2b). Daily NH_4^+ loading to the second stage was on average 13.4 ± 4.8 g N m^{-2} d^{-1} and the increase in nitrate across the second stage was 9.1 ± 6.2 g NO_3^--N m^{-2} d^{-1} (Fig. 2b & c), indicating that approximately two-thirds of the influent mass load of ammonia was converted to nitrate on a conservation basis. The additional third of NH_4^+ could have been consumed by other microbial N transformations, assimilated for microbial growth or sorbed to the mineral media (Kadlec and Wallace, 2009). Recycled NO_3^- from the second stage effluent was removed concurrently with COD in the first stage (Fig. 2).

The pilot TW system has been in operation and monitored for water quality since 2013, with consistent water quality monitoring beginning in 2015 (Table A.1). With water temperatures ranging from 2.5 to 5.5 °C and influent wastewater containing 767 (188) mg L^{-1} COD and 151 (44) mg L^{-1} TN, the system has removed 97.4 % of influent NH_4^+-N (93.3 % to 99.6 %), 95.0 % of COD (91.6 % to 96.7 %), and 67.2 % of total inorganic nitrogen (53.2 % to 76.4 %) from 2015 to 2023 (Table A.1). The studied pilot performs on par with many other cold-climate TWs for both total inorganic nitrogen and carbon removal (Varma et al., 2021; Yan and Xu, 2014) and consistently outperforms the published performance data for ammonium removal in other wastewater systems (Hoang et al., 2014; Ji et al., 2020; Murphy et al., 2016; Young et al., 2016). The studied TW has additionally outperformed a multistage mechanical system with rotating biological contactor that operated at higher average temperatures of 6–18 °C (Zha et al., 2020).

The mesocosms showed near complete removal of both COD and NO_3^- (Fig. A.2) within 72 h of dosing in the first stage and second stage operated columns, respectively. This suggests efficient and potentially similar nutrient removal processes were established in the mesocosms and the pilot TW.

3.2. Spatial and temporal analysis of microbial communities

3.2.1. Microbial α-diversity: sample richness and evenness

The coverage of the samples was evaluated using rarefaction analysis (Fig. A.3). All samples showed asymptotic curves at the rarefaction point, indicating that the sampling was adequate to capture the overall diversity. Samples collected in December prior to wastewater loading enabled comparison of microbial community diversity before and after high-strength wastewater treatment in the TW.

The Shannon index, indicating sample diversity, decreased in the pilot TW from December to April across both the first and second stages, suggesting that fewer species with a higher abundance dominated over the duration of the operational season ($p = 0.03$, Fig. 3). Additionally, the Shannon Equitability index, indicating sample evenness (Table A.2), decreased slightly in the first stage of the pilot from December to April although no significant decrease was detected ($p > 0.05$). Averaged over the operational season, the second stage had only a slightly higher Shannon index of 4.84 compared to 4.44 in the first stage, although this difference was not significant ($p = 0.09$). Other WWTPs have observed significant community differences across different operational and nutrient loading parameters despite minimal changes of overall diversity, evenness or numeration (Al-Rashdi et al., 2024; Fernandes et al., 2015; Gao et al., 2022). Thus, the specific bacteria present in various TW samples may be different despite similar α-diversity metrics.

The highest diversity and richness of communities in the pilot were noted in the second stage in December (Fig. 3, Table A.2), which corresponded to early season operation when nutrient concentrations were significantly lower. This finding differs from one TW undergoing initial start-up where a rapid increase in microbial abundance and diversity was observed (Oopkaup et al., 2016); however, our TW's seasonal start-up is likely occurring with an already established TW community. We hypothesize that increased microbial diversity may occur when nutrients are depleted during the fall months when wastewater is not actively applied to the TW. Oligotrophic conditions have been observed to establish more diverse microbial metabolisms as bacteria compete for limited nutrients, and sharp increases in nutrient availability, such as nitrogen, can drastically reduce bacterial diversity (Liu et al., 2020;

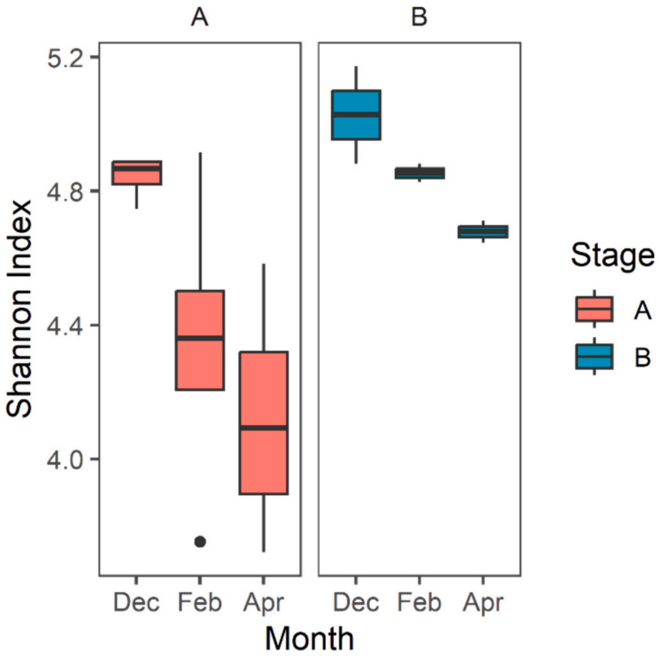

Fig. 3. Shannon index of samples over December, February and April separated by stage. Higher alpha diversity existed in the second stage throughout the ski season, but both stages had observed decreased in alpha diversity as the season progressed.

Wang et al., 2022). Similar trends were noted by Silveira et al. (2022) with more diverse and richer communities observed at locations where nutrient availability was depleted along a horizontal flow TW. On the contrary, Button et al. (2015) reported a marked decrease in richness along the horizontal flow path of a TW, proposing that more specialized communities developed where nutrients were depleted. The same study, however, found no significant change in diversity with depth in a vertical flow TW (Button et al., 2015). Despite mixed findings in previous studies depending on TW type and application, in the present study, diversity was not significantly different with location, though temporal changes were observed.

3.2.2. *Microbial indicators in the first stage*

Temporal sampling prior to loading wastewater onto the system (December) and over the operational season (February, April) allowed for analysis of microbial community development between resting and operational phases of the TW, as well as across stages. Indicator species analysis identified three genera in the first stage that served as a significant representation of the unique environmental conditions that existed within those locations (Table A.3).

The genera *Dokdonella* ($p = 0.003$), *Dechloromonas* ($p = 0.033$), and *Rhodanobacter* ($p = 0.034$) were indicator organisms identified in the first stage. *Dechloromonas* was the most abundant genus across the pilot (Fig. 4, Table A.3). *Dechloromonas* increased in relative abundance from December to February, starting at <2 % relative abundance and increasing to over 30 % at some locations. The *Dechloromonas* genus is classified as facultative anaerobes (Achenbach et al., 2001) and have been measured at higher abundances in one low temperature anaerobic/aerobic/anoxic wastewater reactor (Gao et al., 2022). Specific species of *Dechloromonas* have been assessed for denitrifying capabilities, of which both *D. denitrificans* and *D. aromatica* have been identified as heterotrophic denitrifying bacteria (Salinero et al., 2009; Yoon et al., 2016).

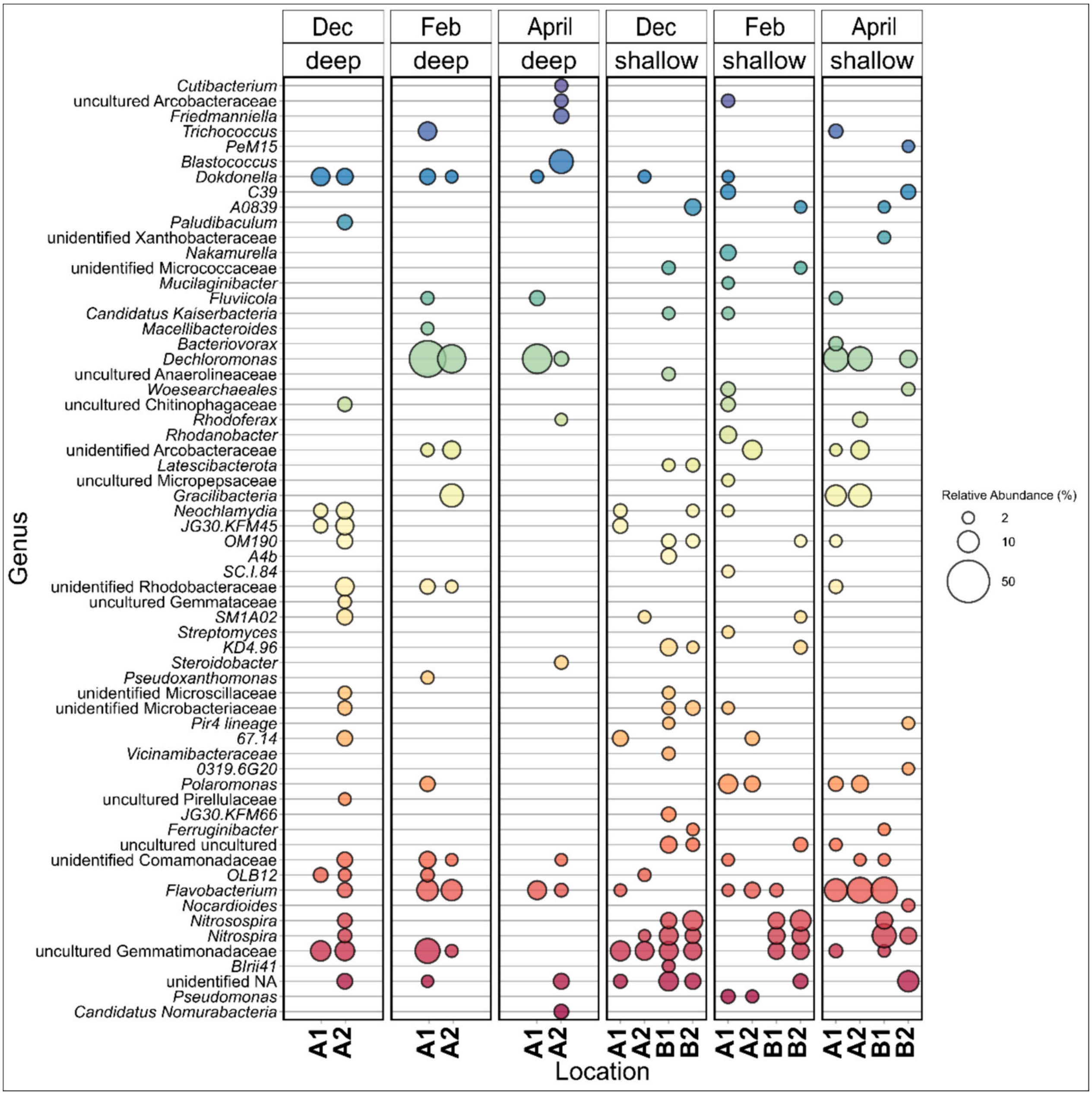

Fig. 4. Relative abundance of genera (> 2 %) grouped by sampling depth, month of sampling and system stage (A or B) and cell (1 or 2). Uncultured or unidentified (NA) genera are denoted by family names.

Interestingly, the genus, *Dokdonella*, was observed to decrease in abundance as the ski season progressed, with <2 % abundance observed in the first stage in February. *Dokdonella* are strictly aerobic gram-negative bacteria, and it is unclear whether they are capable of reducing NO_3^- (Pishgar et al., 2019). *Dokdonella* consists of six identified species, primarily isolated from soils and activated sludge (Lee and Weinstein, 2014). The genus *Rhodanobacter* is also capable of denitrification, and has been found to dominate contaminated subsurface nuclear waste sites (Green et al., 2012). In our system, *Rhodanobacter* typically existed at low relative abundances (< 2 %).

Other organisms, such as *Flavobacterium* and *Polaromonas*, were not identified as indicator organisms in the first stage but had high relative abundances throughout the ski season. *Flavobacterium* maintained a relative abundance between 3 and 10 % (Fig. 4) at depths >30 cm in the first stage for the entirety of the ski season. The relative abundance increased at shallower depths of 15–30 cm from just over 2 % in December to nearly 30 % by April. In other TW systems, *Flavobacterium* were found to decrease with depth and increase during periods of feeding (Silveira et al., 2021). Species of *Flavobacterium* are either strict aerobes or facultative and capable of growing by denitrification or nitrification in mineral media (Fu et al., 2018; Horn et al., 2005; Silveira et al., 2021). Some have hypothesized that *Flavobacterium* may also be capable of aerobic denitrification (Horn et al., 2005).

Polaromonas was identified to have high relative abundances compared to other organisms and was observed in greater relative abundance in the first stage at depths 15–30 cm in February and April (3.9–7.3 %; Fig. 4). These psychrophilic bacteria are most abundant on glacial surfaces, but have additionally been found to have high relative abundance throughout cold-climate wastewater treatment systems (Gawor et al., 2016; Gil-Pulido et al., 2018; Jankowski et al., 2022). The distribution and function of these isolates globally and in wastewater systems is currently unclear. However, this genus is thought to play a large role in biogeochemical cycling in cold climates, potentially including biological nutrient removal (Gil-Pulido et al., 2018). Some *Polaromonas* species may thrive under aerobic conditions, as one intermittently aerated sequencing batch reactor observed a reduction in *Polaromonas* relative abundance from over 51 % to 11 % after aeration was reduced (Gil-Pulido et al., 2018). This finding corresponds to the higher relative abundances of *Polaromonas* observed in the more aerobic shallow depths of the pilot TW's first stage.

The identification of these heterotrophic bacteria as indicator species in the first stage is consistent with the observed removal of NO_3^- and COD (Fig. 2) and indicates the likelihood of denitrification as the dominant mechanism of NO_3^- removal in that stage. Results from this structural analysis confirm the presence of relevant and appropriate functional groups that have been observed in various other studies (Engida et al., 2021; Huang et al., 2017; Wang et al., 2016). From these organisms and water quality analysis, the first stage can generally be characterized by an oxidation-reduction potential that likely varies from oxic to anoxic conditions, low temperatures, and potential metabolic capabilities for NO_3^- reduction.

3.2.3. *Microbial indicators in the second stage*

The indicator species that statistically significantly represented the second stage included three genera well-known to be involved with nitrification, *Nitrosospira* ($p = 0.002$), *Nitrospira* ($p = 0.001$) and *Candidatus Nitrotoga* ($p = 0.008$,) (Daims et al., 2016; Huang et al., 2017). In the second stage, NH_4^+-N (~40 mg L^{-1}) from the first stage effluent is converted to NO_3^- at an efficiency of 97 %, providing supporting evidence that nitrifying bacteria are active in the second stage. Overall, seven genera were identified as indicators in the second stage (Table A.3). Other genera identified as indicators, including KD94.96, SWB02, and *Latescibacterota*, have been observed in both natural and contaminated soil or mine environments with influence over bioremediation and nutrient cycling in those systems (Kujala et al., 2018; Lhoste et al., 2023; Oliveira et al., 2023); whereas the genus A0839 has yet to be cultivated (Kim et al., 2021).

The sole AOB genus identified in the TW, *Nitrosospira*, was observed in relative abundances from 2.5 to 7 % in most samples from the second stage for the entire ski season (Fig. 4). Studies of nitrifying communities have identified *Nitrosospira* as important AOB in cold-adapted communities and have also characterized its members as psychrotolerant (Sanders et al., 2019). While other studies on wastewater treatment claim that nitrification is significantly inhibited at temperatures <15 °C (Hoang et al., 2014; Johnston et al., 2023; Zhou et al., 2018), the studied pilot TW operates between 2.5 and 5 °C and putatively oxidizes up to 55 g m^{-2} d^{-1} NH_4^+-N.

The genera *Nitrospira* was observed in relative abundances from 3 to 7 % in the second stage throughout the ski season. *Nitrospira* are facultative chemolithoautotrophic, gram-negative NOB (Daims et al., 2015). These bacteria are K-strategic, meaning they adapt well to low NO_2^- and O_2 conditions (Daims et al., 2016). In conventional wastewater treatment plants, *Nitrospira* have been found to contribute to nitrite oxidation and are typically found in high relative abundances (2–6 %) (Hoang et al., 2014; Jankowski et al., 2022). *Nitrospira* are one of the most diverse nitrifier groups and are also capable of complete ammonia oxidation to nitrate (COMAMMOX) (Koch et al., 2019; Silveira et al., 2021). *Nitrospira* have been found in greater abundance than AOB in TW and have been observed to increase in relative abundance at low temperatures (Tan et al., 2020), which was observed in our system. Additional analyses were not performed to determine whether *Nitrospira* were performing COMAMMOX in the studied TW. The other indicator organism, *Nitrotoga*, grows abundantly at low temperatures and has been observed to be the most abundant NOB in engineered systems (Daims et al., 2015). In this study it was present only up to 1.8 % relative abundance and is therefore not shown in Fig. 4.

Indicator species analysis paired with mean mass removal of 21.1 ± 8.5 g of NH_4^+-N per day across the season strongly indicated the presence of nitrification processes occurring within the second stage. The second stage additionally supported a greater number of indicator organisms and on average a higher Shannon diversity index compared to the first stage, which has been observed in other aerobically operated TWs (Bouali et al., 2014; Silveira et al., 2022). Further, our analysis indicates that the studied TW supported statistically unique compositions of microbial communities due to the present saturation conditions of the stages and the nutrient composition of the wastewater.

3.2.4. *Microbial β-diversity between system stages and months of operation*

PCoA and PERMANOVA were used to assess the β-diversity, or dissimilarity, of microbial communities across space and time. Analysis of bacterial communities across the system stages was noted by statistically distinct groupings ($p = 0.001$) between the first and second stages (Fig. 5a), likely due to influent nutrients, saturation depth or redox conditions in the water entering each stage.

Similar to our pilot study, He et al. (2018) observed significant differences between stages of sequencing batch fed TWs, with high abundances of heterotrophic denitrifiers associated with the stage receiving high C and NO_3^- and nitrifying bacteria associated with the stage receiving synthetic wastewater with low C and high NH_4^+. Ibekwe et al. (2016) observed that approximately 54 % of the microbial community differences in their TW were explained by the combined NH_4^+ and PO_4^{3-} concentrations. Average influent mass loads of COD and NH_4^+ to the pilot TW stages in this study differed by 80.3 % and 42.5 %, respectively, and likely contributed to the structural community differences observed. Furthermore, Yu et al. (2024) found that intermittent aeration improved microbial diversity, which supports the hypothesis that the elevated diversity observed in the second stage of our pilot TW may be due to more aerobic conditions.

Statistically significant groupings ($p = 0.003$) by month (Fig. 5b) displayed a shift in the community structure over the course of the ski season. Fig. 5b shows three groupings, in which the community structure, prior to the operating season in December, was distinctly different

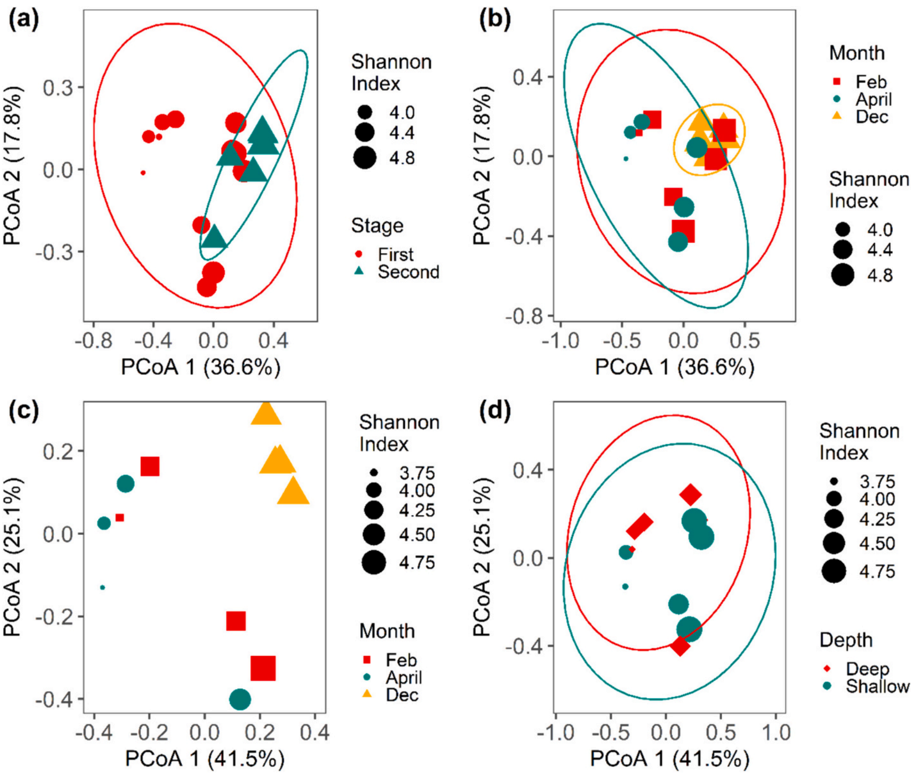

Fig. 5. PCoA plot using the Bray-Curtis distance matrix of microbial communities from pilot-scale TW sampled across the operational season and grouped via statistical ellipses according to categorical variables. (a) system stage, (b) month, (c) month in only the first stage, and (d) depth in the first stage. Data in (c) are not grouped due to limited replication per condition. PERMANOVA analysis confirmed statistically significant differences between system stage and month of sampling; however, depth was deemed non-significant.

from April. Comparatively, February was relatively diverse, as noted by the large spread of its ordination. In PCoA of only the first stage (Fig. 5c), the February and April groupings were significantly different ($p \leq 0.05$) from December, indicating a shift in the community structure over the course of the ski season, which could have been related to shifts in water composition, redox conditions and seasonal operation similar to observations in other wastewater treatment systems (Tan et al., 2020; Wang et al., 2022). The second stage in Fig. 5a had a tighter overall ordination, indicating that the second stage experienced less change in the microbial composition over the course of the operating season. This suggests a more stable microbial community in the second stage, which has been observed in a TW undergoing enhanced aeration (Tan et al., 2020).

Previous research has additionally observed significant differences between winter and summer feeding periods compared to summer rest periods (Wang et al., 2020), suggesting that communities shifted due to nutrition deficiency. Ibekwe et al. (2016) also hypothesized that observed shifts in TW and lagoon microbial communities were due to changes in nutrient loading over time to the systems. This finding may suggest that temporal differences in our TW microbial community structure are strongly correlated to shifts in influent nutrient loading over the course of the season.

The saturation in the first stage, assessed via sampling above and below the saturation level did not establish significantly different microbial communities ($p > 0.05$; Fig. 5d). Thus, the different sampling depths may not have represented significantly different nutrient and redox conditions.

The pilot TW was designed to promote COD removal and anoxic denitrification of recycled nitrate in the first stage, followed by nitrification in the second, unsaturated stage. The different microbial communities observed nitrogen transformations across each stage, and the identified indicator species support the hypothesis that the system design is sustaining the microbial populations and metabolisms required to perform the intended transformations for TIN removal (Engida et al., 2021; Oopkaup et al., 2016; Tan et al., 2020).

3.3. *Microbial communities in the mesocosms*

The microbial communities established in the pilot TW and the mesocosms were assessed using PCoA ordination and PERMANOVA analysis. A correlation test assessed the goodness of fit of the Bray-Curtis dissimilarity matrix ordination and was determined to have adequate fit (87 %; Fig. A.4). Distinct groupings between the laboratory and pilot systems were noted (Fig. 5), indicating a significant difference ($p \leq 0.05$) of the microorganisms that populate each individual system. Highly reduced conditions in the mesocosms, due to fully saturated conditions, were evidenced by the presence of the methanogenic archaeal genera, *Methanobacterium*, an obligate anaerobe (Fu et al., 2023). Methanogens or genera requiring specifically anaerobic conditions were not detected in the pilot system, indicating a potentially less reduced environment, even in the saturated portion of the first stage.

The two groups of mesocosms fed with either high ammonia or high nitrate artificial wastewater differed significantly in microbial community composition (Fig. 6). However, both groups of mesocosms differed to a much higher degree from the pilot system communities, regardless of the stage and wastewater composition (Fig. 6). Thus, the type of experimental system (mesocosm vs. pilot) accounted for most of the differences in microbial communities, while the different wastewater compositions accounted for much smaller differences. Specifically, the use of artificial wastewater likely impacted the microbial community that developed in the columns compared to the pilot system which was fed with high-strength domestic wastewater from the ski resort. In addition to scale and use of artificial wastewater, other key variables between the systems exist, such as temperature and different loading rates. Any differences between laboratory and full-scale systems should

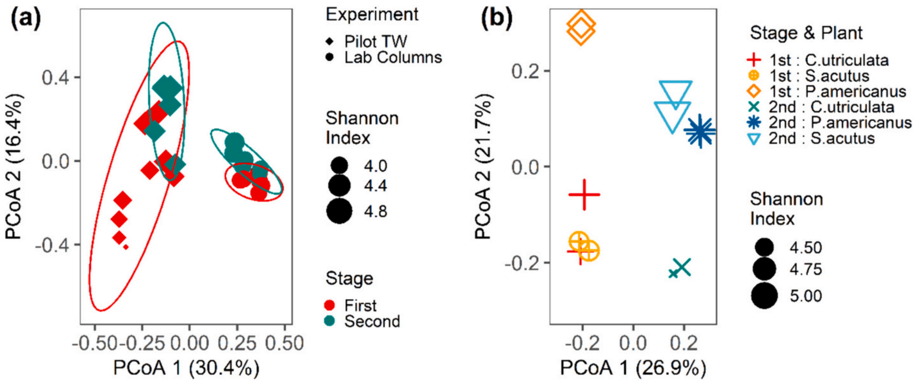

Fig. 6. PCoA using the Bray-Curtis distance matrix of microbial communities from (a) both the pilot-scale TW and the laboratory mesocosms and (b) the greenhouse columns alone. In plot (a) data point shapes were determined according to operational scale (pilot- or meso-scale) and colored according to nutrient-type loading (first stage or high COD, NH_4^+-N, and second stage or high NO_3^--N). Statistically significant groupings were noted for both nutrient loading (system stage) as well as operational scale (meso- or pilot-). In plot (b) shapes and colors of data points were factored according to plant species (*S. acutus*, *C. utriculata*, and *P. americanus*). Statistically significant differences between microbial community structure and plant type were observed ($p < 0.05$).

be assumed to potentially influence the microbial communities.

Each column in the laboratory system was planted with one of three plant species (*C. utriculata*, *P. americanus*, *S. acutus*). Significant differences were found between the microbial communities based on plant species ($p = 0.01$) (Fig. A.2). This is a similar finding to a previous study that specifically analyzed microbial communities on plant roots in TW mesocosms and found that plant species and season had a significant impact on relative abundance of AOB and sulfate-reducing bacteria (Faulwetter et al., 2013). Microbial communities attached to roots and surrounding TW media may differ due to microscale gradients of aqueous nutrients and the impact of plant root exudates (Faulwetter et al., 2009; Wu et al., 2017). However, another study found that microbial community function, measured by metabolism of various carbon compounds, in TW mesocosms planted with *Phragmites* and *Phalaris* were not significantly different (Button et al., 2016). While these results indicate that TW plant species can impact microbial community composition, the previous studies suggest that plant-microbe interactions are complex and require more investigation.

3.4. Implications for TW design and functions

Low temperatures often result in declining or poor wastewater treatment performance due to slowed microbial metabolism that occur with decreasing temperatures, particularly for nitrogen removal (Varma et al., 2021; Yan and Xu, 2014). Many wastewater treatment facilities observe a notable decline in performance during the winter months, requiring operational changes to maintain sufficient nutrient removal (Gao et al., 2022; Varma et al., 2021; Yan and Xu, 2014). AOB reportedly lack resilience to dynamic environmental conditions and have been documented to become less efficient or die off due to shock loading or changes of temperature, resulting in elevated NH_3 in wastewater effluent (Johnston et al., 2023). Some studies have reported a decline in nitrification performance when wastewater temperatures drop below 10 °C, with significant reductions in performance at temperatures <5 °C (Zhou et al., 2018).

Community shifts and the growth of psychrophiles may be key to preventing this decrease in performance (Yan and Xu, 2014), as observed in the studied pilot TW. Other researchers agree, stating that microbial communities adapt metabolic functions with temperature changes to maintain their activity at low temperatures (Cookson et al., 2002; Sundberg et al., 2007).

Potential adaptations due to the eight to nine month rest periods in the studied TW may contribute to the unique development of these psychrophilic bacterial communities, indicating the presence of preferential conditions that differ from systems that operate year-round. Oscillating or sinusoidal operating conditions have been observed to establish a coexistence of competitive organisms (Pavlou, 2006). The extended oscillation of long rest periods followed by seasonal wastewater operation may allow coexistence of fast growing heterotrophic bacteria with the often-slow growing psychrophiles and nitrifiers throughout the year. This may contribute to survival at comparable relative abundances throughout the summer and fall months, followed by proliferation in the winter. Psychrophilic bacteria, such as *Polaromonas*, have been found on and within glacial ice indicating the ability of psychrophiles to thrive in extremely nutrient limited environments (Gawor et al., 2016), therefore long periods without wastewater feeding may enable these bacteria to maintain higher relative abundances. Engineered systems that run continuously cannot be temporarily paused in operation, thus designing two system trains that alternate in operation may encourage better winter performance, as well as adding redundancy to operations.

The pilot system is unique in that wetland plants only grow in the TW outside of the winter ski season and when wastewater is not being fed to the system. During system operation in the winter, the plants are primarily dormant. Plant inputs and root growth during the periods without nutrient input from wastewater may also contribute to the robustness of the microbial community. While this was beyond the scope of the present study, future work should include analysis of the microbial community dynamics outside of the TW operating season and during macrophyte proliferation.

It should be noted that bacterial abundances do not always correlate to activity, and rare species can be the most active organisms within a system (Kurm et al., 2017). For example, while nitrifying organisms were strong indicators of the second stage and correlated with conversion of ammonia to nitrate, each of the indicator OTUs were present at 7 % or less relative abundance. Despite this caveat, the assumed metabolic capabilities of indicator organisms correlated with the observed nitrogen species transformations in each stage. Further research is required to directly measure metabolisms and substrate consumption rates of the bacteria in the studied TW.

4. Conclusion

1. The TW performed efficient nutrient removal of COD, NH_4^+, NO_3^- and TIN at extremely low water temperatures (<3 °C); performance was complemented by high relative abundances of psychrophiles or psychrotolerant genera, such as *Polaromonas* and *Dechloromonas*, that indicate robust microbial community adaptation to low temperatures.

2. Spatial differences across stages indicated that the first and second stages supported distinct microbial communities and indicator organisms with denitrification and nitrification capabilities, respectively. Proof of denitrification and nitrification processes was further evidenced by consistent removal of NO_3^- and COD on a mass basis across the first stage and NH_4^+ in the second stage.
3. Temporal shifts in the microbial communities suggest that the seasonal TW operation paired with long, low-nutrient rest periods influenced the community development and enable diverse species to remain viable throughout the year despite extended periods without nutrient input. Future studies should more thoroughly investigate the impact of temporary operation compared to continuous operation to determine whether this feature may play a key role in the robustness of the observed microbial communities.
4. Mesocosms with similar nutrient loading to pilot TW garnered similar results based on final effluent water quality, indicating a likelihood for mesocosms to act as potential functional proxies of pilot scale systems. However, mesocosms were not accurate representations of the microorganisms that colonized the pilot TW, indicating the prevailing experimental conditions must mimic the true system conditions to the closest degree possible to avoid preferential selection.
5. Temporary pauses in operation may influence microbial community development in a way that allows for the observed robust seasonal operation at low temperatures. More analyses during the plant growth season should be performed to determine the impact of plant inputs on community dynamics during periods of system rest and lack of wastewater input.

CRediT authorship contribution statement

Stephanie H. Ayotte: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Sarah J. Wallace:** Writing – review & editing, Validation, Software, Methodology, Data curation. **Christopher R. Allen:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Kela P. Weber:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Otto R. Stein:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Ellen G. Lauchnor:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by the Bridger Bowl Foundation; the National Science Foundation Research Traineeship [award no. 2125748]; the Benjamin Fellowship through Montana State University's Norm Asbjornson College of Engineering and the Montana Department of Environmental Quality under the State Revolving Fund (SRF) [contract no. 221031]. KPW gratefully acknowledges funding in the form of a NSERC Discovery Grant [RGPIN-2018-04886]. Funding sources were not involved in the study design, collection, analysis and interpretation of data, in the writing of the report or in the decision to submit the article for publication. We thank the following entities and individuals for their support of this project: the Montana Department of Environmental Quality, especially Paul Lavigne; Bridger Bowl Inc., especially Erik Pidgeon; Montana State University's Environmental Analytical Lab; graduate students Tucker Bowman and Paul Karcher; and undergraduate Nina Denny.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2024.101885.

References

Achenbach, L.A., Michaelidou, U., Bruce, R.A., Fryman, J., Coates, J.D., 2001. Dechloromonas agitata gen. nov., sp. nov. and Dechlorosoma suillum gen. nov., sp. nov., two novel environmentally dominant (per)chlorate-reducing bacteria and their phylogenetic position. Int. J. Syst. Evol. Microbiol. 51, 527–533. https://doi.org/10.1099/00207713-51-2-527.

Aguilar, L., Pérez, L.M., Gallegos, Á., Fores, E., Arias, C.A., Bosch, C., Verdum, M., Jove, P., De Pablo, J., Morató, J., 2022. Effect of aeration on nitrogen removal-associated microbial community in an innovative vertical cork-based constructed wetland for winery wastewater treatment. Ecol. Eng. 185, 106781 https://doi.org/10.1016/j.ecoleng.2022.106781.

Allen, C.R., Stein, O.R., Hook, P.B., Burr, M.D., Parker, A.E., Hafla, E.C., 2013. Temperature, plant species and residence time effects on nitrogen removal in model treatment wetlands. Water Sci. Technol. 68, 2337–2343. https://doi.org/10.2166/wst.2013.482.

Al-Rashdi, T., Blackburn, D.M., Ahmed, M., 2024. Sludge treatment reed wetland microbiome communities under increasing loading rates. Soil & Environmental Health 100083. https://doi.org/10.1016/j.seh.2024.100083.

Ayotte, S.H., Allen, C.R., Parker, A., Stein, O.R., Lauchnor, E.G., 2024. Greenhouse gas production from an intermittently dosed cold-climate wastewater treatment wetland. Sci. Total Environ. 924, 171484 https://doi.org/10.1016/j.scitotenv.2024.171484.

Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G.A., Gregory Caporaso, J., 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6, 90. https://doi.org/10.1186/s40168-018-0470-z.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., Van Der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., Von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Bouali, M., Zrafi, I., Bakhrouf, A., Chaussonnerie, S., Sghir, A., 2014. Bacterial structure and spatiotemporal distribution in a horizontal subsurface flow constructed wetland. Appl. Microbiol. Biotechnol. 98, 3191–3203. https://doi.org/10.1007/s00253-013-5341-8.

Button, M., Nivala, J., Weber, K.P., Aubron, T., Müller, R.A., 2015. Microbial community metabolic function in subsurface flow constructed wetlands of different designs. Ecol. Eng. 80, 162–171. https://doi.org/10.1016/j.ecoleng.2014.09.073.

Button, M., Rodriguez, M., Brisson, J., Weber, K.P., 2016. Use of two spatially separated plant species alters microbial community function in horizontal subsurface flow constructed wetlands. Ecol. Eng. 92, 18–27. https://doi.org/10.1016/j.ecoleng.2016.03.044.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Cookson, W.R., Cornforth, I.S., Rowarth, J.S., 2002. Winter soil temperature (2–15°C) effects on nitrogen transformations in clover green manure amended or unamended soils; a laboratory and field study. Soil Biol. Biochem. 34, 1401–1415. https://doi.org/10.1016/S0038-0717(02)00083-4.

Cydzik-Kwiatkowska, A., Zielińska, M., 2016. Bacterial communities in full-scale wastewater treatment systems. World J. Microbiol. Biotechnol. 32, 66. https://doi.org/10.1007/s11274-016-2012-9.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R.H., Von Bergen, M., Rattei, T., Bendinger, B., Nielsen, P.H., Wagner, M., 2015. Complete nitrification by Nitrospira bacteria. Nature 528, 504–509. https://doi.org/10.1038/nature16461.

Daims, H., Lücker, S., Wagner, M., 2016. A new perspective on microbes formerly known as nitrite-oxidizing bacteria. Trends Microbiol. 24, 699–712. https://doi.org/10.1016/j.tim.2016.05.004.

De Caceres, M., Legendre, P., 2009. Associations between species and groups of sites: indices and statistical inference. Ecology 90, 3566–3574. https://doi.org/10.1890/08-1823.1.

Engida, T., Alemu, T., Wu, J., Xu, D., Zhou, Q., Wu, Z., 2021. Microbial community dynamics and activity in constructed wetland treating agro-industrial and domestic wastewater: a review. Appl. Ecol. Environ. Res. 19, 2667–2687. https://doi.org/10.15666/aeer/1904_26672687.

Faulwetter, J.L., Gagnon, V., Sundberg, C., Chazarenc, F., Burr, M.D., Brisson, J., Camper, A.K., Stein, O.R., 2009. Microbial processes influencing performance of treatment wetlands: a review. Ecol. Eng. 35, 987–1004. https://doi.org/10.1016/j.ecoleng.2008.12.030.

Faulwetter, J.L., Burr, M.D., Parker, A.E., Stein, O.R., Camper, A.K., 2013. Influence of season and plant species on the abundance and diversity of sulfate reducing bacteria and ammonia oxidizing bacteria in constructed wetland microcosms. Microb. Ecol. 65, 111–127. https://doi.org/10.1007/s00248-012-0114-y.

Fernandes, J.P., Almeida, C.M.R., Pereira, A.C., Ribeiro, I.L., Reis, I., Carvalho, P., Basto, M.C.P., Mucha, A.P., 2015. Microbial community dynamics associated with veterinary antibiotics removal in constructed wetlands microcosms. Bioresour. Technol. 182, 26–33. https://doi.org/10.1016/j.biortech.2015.01.096.

Fu, G., Yu, T., Huangshen, L., Han, J., 2018. The influence of complex fermentation broth on denitrification of saline sewage in constructed wetlands by heterotrophic nitrifying/aerobic denitrifying bacterial communities. Bioresour. Technol. 250, 290–298. https://doi.org/10.1016/j.biortech.2017.11.057.

Fu, J., Yan, B., Gui, S., Fu, Y., Xia, S., 2023. Anaerobic co-digestion of thermo-alkaline pretreated microalgae and sewage sludge: methane potential and microbial community. J. Environ. Sci. 127, 133–142. https://doi.org/10.1016/j.jes.2022.04.020.

Gao, X., Xu, Z., Peng, Y., Zhang, L., Ding, J., 2022. The nitrification recovery capacity is the key to enhancing nitrogen removal in the AOA system at low temperatures. Sci. Total Environ. 818, 151674 https://doi.org/10.1016/j.scitotenv.2021.151674.

Gawor, J., Grzesiak, J., Sasin-Kurowska, J., Borsuk, P., Gromadka, R., Górniak, D., Świątecki, A., Aleksandrzak-Piekarczyk, T., Zdanowski, M.K., 2016. Evidence of adaptation, niche separation and microevolution within the genus Polaromonas on Arctic and Antarctic glacial surfaces. Extremophiles 20, 403–413. https://doi.org/10.1007/s00792-016-0831-0.

Gil-Pulido, B., Tarpey, E., Finnegan, W., Zhan, X., Dobson, A.D., O'Leary, N., 2018. Dominance of the genus *Polaromonas* in the microbial ecology of an Intermittently Aerated Sequencing Batch Reactor (IASBR) treating dairy processing wastewater under varying aeration rates. J. Dairy Res. 85, 388–390. https://doi.org/10.1017/S0022029918000572.

Green, S.J., Prakash, O., Jasrotia, P., Overholt, W.A., Cardenas, E., Hubbard, D., Tiedje, J. M., Watson, D.B., Schadt, C.W., Brooks, S.C., Kostka, J.E., 2012. Denitrifying bacteria from the genus Rhodanobacter dominate bacterial communities in the highly contaminated subsurface of a nuclear legacy waste site. Appl. Environ. Microbiol. 78, 1039–1047. https://doi.org/10.1128/AEM.06435-11.

He, S., Wang, Y., Li, C., Li, Y., Zhou, J., 2018. The nitrogen removal performance and microbial communities in a two-stage deep sequencing constructed wetland for advanced treatment of secondary effluent. Bioresour. Technol. 248, 82–88. https://doi.org/10.1016/j.biortech.2017.06.150.

Hoang, V., Delatolla, R., Abujamel, T., Mottawea, W., Gadbois, A., Laflamme, E., Stintzi, A., 2014. Nitrifying moving bed biofilm reactor (MBBR) biofilm and biomass response to long term exposure to 1 °C. Water Res. 49, 215–224. https://doi.org/10.1016/j.watres.2013.11.018.

Horn, M.A., Ihssen, J., Matthies, C., Schramm, A., Acker, G., Drake, H.L., 2005. Dechloromonas denitrificans sp. nov., Flavobacterium denitrificans sp. nov., Paenibacillus anaericanus sp. nov. and Paenibacillus terrae strain MH72, N2O-producing bacteria isolated from the gut of the earthworm Aporrectodea caliginosa. Int. J. Syst. Evol. Microbiol. 55, 1255–1265. https://doi.org/10.1099/ijs.0.63484-0.

Huang, X., Zheng, J., Liu, C., Liu, L., Liu, Y., Fan, H., Zhang, T., 2017. Performance and bacterial community dynamics of vertical flow constructed wetlands during the treatment of antibiotics-enriched swine wastewater. Chem. Eng. J. 316, 727–735. https://doi.org/10.1016/j.cej.2017.02.029.

Ibekwe, A.M., Ma, J., Murinda, S., Reddy, G.B., 2016. Bacterial community dynamics in surface flow constructed wetlands for the treatment of swine waste. Sci. Total Environ. 544, 68–76. https://doi.org/10.1016/j.scitotenv.2015.11.139.

Jankowski, P., Gan, J., Le, T., McKennitt, M., Garcia, A., Yanaç, K., Yuan, Q., Uyaguari-Diaz, M., 2022. Metagenomic community composition and resistome analysis in a full-scale cold climate wastewater treatment plant. Environmental Microbiome 17, 3. https://doi.org/10.1186/s40793-022-00398-1.

Ji, B., Zhao, Y., Vymazal, J., Qiao, S., Wei, T., Li, J., Mander, Ü., 2020. Can subsurface flow constructed wetlands be applied in cold climate regions? A review of the current knowledge. Ecol. Eng. 157, 105992 https://doi.org/10.1016/j.ecoleng.2020.105992.

Johnston, J., Du, Z., Behrens, S., 2023. Ammonia-oxidizing bacteria maintain abundance but lower amoA gene expression during cold temperature nitrification failure in a full-scale municipal wastewater treatment plant. Microbiol Spectr 11. https://doi.org/10.1128/spectrum.02571-22 e02571-22.

Kadlec, R.H., 1999. Chemical, physical and biological cycles in treatment wetlands. Water Sci. Technol. 40, 37–44 doi:S0273-1223(99)00417-5.

Kadlec, R.H., Wallace, S.D., 2009. Treatment Wetlands, 2nd ed. CRC Press, Boca Raton, FL.

Kim, S., Islam, Md.R., Kang, I., Cho, J.-C., 2021. Cultivation of dominant freshwater bacterioplankton lineages using a high-throughput dilution-to-extinction culturing approach over a 1-year period. Front. Microbiol. 12, 700637 https://doi.org/10.3389/fmicb.2021.700637.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41 (1), e1. https://doi.org/10.1093/nar/gks808.

Koch, H., Van Kessel, M.A.H.J., Lücker, S., 2019. Complete nitrification: insights into the ecophysiology of comammox Nitrospira. Appl. Microbiol. Biotechnol. 103, 177–189. https://doi.org/10.1007/s00253-018-9486-3.

Kujala, K., Mikkonen, A., Saravesi, K., Ronkanen, A.-K., Tiirola, M., 2018. Microbial diversity along a gradient in peatlands treating mining-affected waters. FEMS Microbiol. Ecol. 94 https://doi.org/10.1093/femsec/fiy145.

Kurm, V., Van Der Putten, W.H., De Boer, W., Naus-Wiezer, S., Hol, W.H.G., 2017. Low abundant soil bacteria can be metabolically versatile and fast growing. Ecology 98, 555–564. https://doi.org/10.1002/ecy.1670.

Lee, B., Weinstein, M.R., 2014. *Dokdonella Koreensis* bacteremia: a case report and review of the literature. Can. J. Infect. Dis. Med. Microbiol. 25, 255–256. https://doi.org/10.1155/2014/810917.

Lhoste, E., Comte, F., Brown, K., Delisle, A., Jaclin, D., Ponsin, V., Rosabal, M., Lazar, C. S., 2023. Bacterial, archaeal, and eukaryote diversity in planktonic and sessile communities inside an abandoned and flooded iron mine (Quebec, Canada). Appl. Microbiol. 3, 45–63. https://doi.org/10.3390/applmicrobiol3010004.

Liu, W., Jiang, L., Yang, S., Wang, Z., Tian, R., Peng, Z., Chen, Y., Zhang, X., Kuang, J., Ling, N., Wang, S., Liu, L., 2020. Critical transition of soil bacterial diversity and composition triggered by nitrogen enrichment. Ecology 101, e03053. https://doi.org/10.1002/ecy.3053.

Murphy, C., Rajabzadeh, A.R., Weber, K.P., Nivala, J., Wallace, S.D., Cooper, D.J., 2016. Nitrification cessation and recovery in an aerated saturated vertical subsurface flow treatment wetland: field studies and microscale biofilm modeling. Bioresour. Technol. 209, 125–132. https://doi.org/10.1016/j.biortech.2016.02.065.

Oksanen, J.F., Simpson, G.L., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H.B.A., FitsJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M.O., Lahti, L., McGlinn, D., Ouellette, M.-H., Cunha, E.R., Smith, T., Stier, A., Braak, C.J.F.T., Weedon, J., 2022. Vegan: Community Ecology Package. R Package Version 2.4-6.

Oliveira, R.S., Pinto, O.H.B., Quirino, B.F., De Freitas, M.A.M., Thompson, F.L., Thompson, C., Kruger, R.H., 2023. Genome-resolved metagenomic analysis of Great Amazon Reef System sponge-associated Latescibacterota bacteria and their potential contributions to the host sponge and reef. Front. Microbiomes 2, 1206961. https://doi.org/10.3389/frmbi.2023.1206961.

Oopkaup, K., Truu, M., Nõlvak, H., Ligi, T., Preem, J.-K., Mander, Ü., Truu, J., 2016. Dynamics of bacterial community abundance and structure in horizontal subsurface flow wetland mesocosms treating municipal wastewater. Water 8, 457. https://doi.org/10.3390/w8100457.

Pavlou, S., 2006. Microbial competition in bioreactors. Chem. Ind. Chem. Eng. Q. 12, 71–81. https://doi.org/10.2298/CICEQ0601071P.

Pishgar, R., Dominic, J.A., Sheng, Z., Tay, J.H., 2019. Denitrification performance and microbial versatility in response to different selection pressures. Bioresour. Technol. 281, 72–83. https://doi.org/10.1016/j.biortech.2019.02.061.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219.

R Core Team, 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org.

Rhine, E.D., Mulvaney, R.L., Pratt, E.J., Sims, G.K., 1998. Improving the Berthelot reaction for determining ammonium in soil extracts and water. Soil Sci. Soc. Am. J. 62, 473. https://doi.org/10.2136/sssaj1998.03615995006200020026x.

Salinero, K., Keller, K., Feil, W.S., Feil, H., Trong, S., Di Bartolo, G., Lapidus, A., 2009. Metabolic analysis of the soil microbe Dechloromonas aromatica str. RCB: indications of a surprisingly complex life-style and cryptic anaerobic pathways for aromatic degradation. BMC Genomics 10, 351. https://doi.org/10.1186/1471-2164-10-351.

Sanders, T., Fiencke, C., Hüpeden, J., Pfeiffer, E.M., Spieck, E., 2019. Cold adapted Nitrosospira sp.: a potential crucial contributor of ammonia oxidation in cryosols of permafrost-affected landscapes in Northeast Siberia. Microorganisms 7, 699. https://doi.org/10.3390/microorganisms7120699.

Shammas, N.K., 1986. Interactions of Temperature, pH, and Biomass on the Nitrification Process, 58. Wiley, pp. 52–59.

Silveira, D.D., Filho, P.B., Philippi, L.S., Cantão, M.E., Foulquier, A., Bayle, S., Delforno, T.P., Molle, P., 2021. In-depth assessment of microbial communities in the full-scale vertical flow treatment wetlands fed with raw domestic wastewater. Environmental Technology (United Kingdom) 0, 1–16. https://doi.org/10.1080/09593330.2020.1723709.

Silveira, D.D., Farooq, A.J., Wallace, S.J., Lapolli, F.R., Nivala, J., Weber, K.P., 2022. Structural and functional spatial dynamics of microbial communities in aerated and non-aerated horizontal flow treatment wetlands. Sci. Total Environ. 838, 156600 https://doi.org/10.1016/j.scitotenv.2022.156600.

Soliman, M., Eldyasti, A., 2018. Ammonia-Oxidizing Bacteria (AOB): opportunities and applications—a review. Rev. Environ. Sci. Biotechnol. 17, 285–321. https://doi.org/10.1007/s11157-018-9463-4.

Sundberg, C.K., Stendahl, J.S., Tonderski, K., Lindgren, P.-E., 2007. Overland flow systems for treatment of landfill leachates—potential nitrification and structure of the ammonia-oxidising bacterial community during a growing season. Soil Biol. Biochem. 39, 127–138. https://doi.org/10.1016/j.soilbio.2006.06.016.

Tan, X., Yang, Y.-L., Li, X., Zhou, Z.-W., Liu, C.-J., Liu, Y.-W., Yin, W.-C., Fan, X.-Y., 2020. Intensified nitrogen removal by heterotrophic nitrification aerobic denitrification bacteria in two pilot-scale tidal flow constructed wetlands: influence of influent C/N ratios and tidal strategies. Bioresour. Technol. 302, 122803 https://doi.org/10.1016/j.biortech.2020.122803.

Taylor, C.R., Hook, P.B., Stein, O.R., Zabinski, C.A., 2011. Seasonal effects of 19 plant species on COD removal in subsurface treatment wetland microcosms. Ecol. Eng. 37, 703–710. https://doi.org/10.1016/j.ecoleng.2010.05.007.

Varma, M., Gupta, A.K., Ghosal, P.S., Majumder, A., 2021. A review on performance of constructed wetlands in tropical and cold climate: Insights of mechanism, role of influencing factors, and system modification in low temperature. Sci. Total Environ. 755, 142540 https://doi.org/10.1016/j.scitotenv.2020.142540.

Wang, P., Zhang, H., Zuo, J., Zhao, D., Zou, X., Zhu, Z., Jeelani, N., Leng, X., An, S., 2016. A hardy plant facilitates nitrogen removal via microbial communities in subsurface flow constructed wetlands in winter. Sci. Rep. 6, 1–11. https://doi.org/10.1038/srep33600.

Wang, S., Cui, Y., Li, A., Zhang, W., Wang, D., Chen, Z., Liang, J., 2020. Deciphering of organic matter and nutrient removal and bacterial community in three sludge treatment wetlands under different operating conditions. J. Environ. Manag. 260, 110159 https://doi.org/10.1016/j.jenvman.2020.110159.

Wang, J., Long, Y., Yu, G., Wang, G., Zhou, Z., Li, P., Zhang, Y., Yang, K., Wang, S., 2022. A review on microorganisms in constructed wetlands for typical pollutant removal: species, function, and diversity. Front. Microbiol. 13, 845725 https://doi.org/10.3389/fmicb.2022.845725.

Weber, K., 2016. Microbial community assessment in wetlands for water pollution control: past, present, and future outlook. Water 8, 503. https://doi.org/10.3390/w8110503.

Wei, J., Cui, L., Li, Wei, Ping, Y., Li, Wan, 2021. Denitrifying bacterial communities in surface-flow constructed wetlands during different seasons: characteristics and relationships with environment factors. Sci. Rep. 11, 4918. https://doi.org/10.1038/s41598-021-82438-3.

Wu, H., Wang, X., He, X., Zhang, S., Liang, R., Shen, J., 2017. Effects of root exudates on denitrifier gene abundance, community structure and activity in a micro-polluted constructed wetland. Sci. Total Environ. 598, 697–703. https://doi.org/10.1016/j.scitotenv.2017.04.150.

Yan, Y., Xu, J., 2014. Improving winter performance of constructed wetlands for wastewater treatment in Northern China: a review. Wetlands 34, 243–253. https://doi.org/10.1007/s13157-013-0444-7.

Yoon, S., Nissen, S., Park, D., Sanford, R.A., Löffler, E., 2016. Clade I NosZ from those harboring clade II NosZ. Applied and Envioronmental Microbiology 82, 3793–3800. https://doi.org/10.1128/AEM.00409-16.Editor.

Young, B., Delatolla, R., Ren, B., Kennedy, K., Laflamme, E., Stintzi, A., 2016. Pilot-scale tertiary MBBR nitrification at 1°C: characterization of ammonia removal rate, solids settleability and biofilm characteristics. Environ. Technol. 37, 2124–2132. https://doi.org/10.1080/09593330.2016.1143037.

Yu, G., Chen, H., Chen, J., Chen, S., Long, Y., Huang, J., Wang, Y., He, S., 2024. Enhanced nitrogen removal through aerobic denitrifying bacteria in horizontal subsurface flow constructed wetlands: Influencing factors and microbial community structure. Chem. Eng. J. 481, 148654 https://doi.org/10.1016/j.cej.2024.148654.

Zha, X., Ma, J., Lu, X., 2020. Use of a low-cost and energy-efficient device for treating low-strength wastewater at low temperatures focusing on nitrogen removal and microbial community. Sci. Total Environ. 722, 137916 https://doi.org/10.1016/j.scitotenv.2020.137916.

Zhang, Y., Dong, W., Yan, G., Wang, Haiyan, Wang, Huan, Chang, Y., Yu, S., Chu, Z., Ling, Y., Li, C., 2022. Plant carbon sources for denitrification enhancement and its mechanism in constructed wetlands: a review. Sustainability 14, 12545. https://doi.org/10.3390/su141912545.

Zhou, H., Li, X., Xu, G., Yu, H., 2018. Overview of strategies for enhanced treatment of municipal/domestic wastewater at low temperature. Sci. Total Environ. 643, 225–237. https://doi.org/10.1016/j.scitotenv.2018.06.100.