Title: Soil composition and redox properties of infiltrating water are determinants of microbial communities at managed aquifer recharge sites

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# Abstract

In this study, we analyzed how water infiltration through shallow soils impacted the composition of microbial communities at three, pilot-scale field sites simulating managed aquifer recharge (MAR). We hypothesized this infiltration would lead to changes in subsurface microbial communities which could be related to biogeochemical processes relevant to water quality. While the three field sites differed in physical soil properties, infiltration significantly increased the relative abundance of *Proteobacteria* while reducing the relative abundance of *Abditibacteriota* and *Acidobacteriota* at all three locations. In plots amended with a carbon-rich permeable reactive barrier, we observed more nitrate removal and a decrease in genera capable of nitrification. Multivariate statistics, such as non-metric multidimensional scaling (NMDS) and constrained correspondence analysis (CCA), determined the main environmental drivers of microbial community composition. Grain size of the soil was the main driver of microbial communities, and analysis showed that the patterns of the most abundant genera corresponded to nitrate, iron, and manganese concentrations in the infiltrating water. These findings provide critical insights towards improving water quality by integrating microbial ecology and biogeochemistry in potential MAR systems.

# Introduction

Groundwater is the main water source for many populations worldwide. Aquifer overdraft occurs when more groundwater is extracted than is replenished (Aeschbach-Hertig and Gleeson, 2012). Overdraft is often more severe during drought periods, which are predicted to increase 25-100% in the next fifty years (Swain *et al.*, 2018). Worldwide, some governments are responding by implementing programs aimed at improved water storage and conveyance (Dillon *et al.*, 2009; California Department of Water Resources, 2018). One such management practice is managed aquifer recharge (MAR), the purposeful routing of surface water into an underlying aquifer using a variety of methods. Recharge is the largest source of inflow to most aquifers–it helps mitigate negative outcomes from overdraft, including reduced storage, disconnection from surface water systems, and saltwater intrusion. Moreover, during extreme drought conditions, some of the recharged water can be recovered and used for irrigation, drinking water, or other purposes (Dillon *et al.*, 2009). Stormwater (Schmidt *et al.*, 2011) and treated wastewater (Fournier *et al.*, 2016) are increasingly considered as sources of water input for MAR. The increasing reliance on non-traditional water sources has water-quality implications for MAR due to the variability of nutrients from these sources (Sheng, 2005; Fakhreddine *et al.*, 2015).

MAR can result in improved water quality through several mechanisms (Hartog and Stuyfzand, 2017). Infiltrating water may dilute polluted aquifers to acceptable contaminant levels (Stuyfzand *et al.*, 2017). Infiltration can also help to reduce virus and pathogen loads (Hartog and Stuyfzand, 2017) and facilitate the degradation of contaminants like nitrate (Schmidt *et al.*, 2011) and emerging organic compounds (Rauch-Williams *et al.*, 2010). The fate of many pollutants is determined by the pH, oxygen concentration, organic carbon availability, infiltration rate, microbial community, and the mineralogy of the soil in the subsurface during infiltration (Schmidt *et al.*, 2011; Casanova *et al.*, 2016; Gorski *et al.*, 2019). For example, adding dissolved organic carbon (DOC) to the input water can increase microbial attenuation of nitrate (Mariotti *et al.*, 1988; Starr and Gillham, 1993; Gorski *et al.*, 2019). Hence, understanding water-soil-microbial interactions during infiltration has the potential to influence the selection of MAR sites, as well as the design and operation of associated systems.

Microbial metabolism, the primary driver of geochemical reactions occurring during surface water infiltration (Stein *et al.*, 2010), can improve or worsen the quality of the infiltrating water. Microbes in the subsurface preferentially oxidize organic carbon compounds and reduce the most energetically favorable electron acceptor to obtain energy. This can involve specialized microbial populations that can take advantage of the most favorable pathways and processes under ambient conditions. For example, complex organic contaminants are more likely to be biodegraded in aerobic conditions (Valhondo *et al.*, 2014). Once available oxygen is depleted, nitrate is the next favorable electron acceptor, followed by metals such as manganese and iron. (Li *et al.*, 2012; Bayarsaikhan *et al.*, 2018). In these reducing conditions, metals such as arsenic can also be mobilized by desorption from soil minerals (Tufano *et al.*, 2008; Fakhreddine *et al.*, 2015). Understanding the microbial consortia present during infiltration will give insight into the geochemical cycling that is occurring.

Worldwide, nitrate (NO3-) is the most widespread non-point source groundwater contaminant (Spalding and Exner, 1993; Gurdak and Qi, 2012). The most well studied nitrate removal pathway is denitrification, where nitrate is reduced to inert nitrogen gas (NO3- 🡪 NO2- 🡪 NO 🡪 N2O 🡪 N2). This process is carried out in soil systems primarily by facultative heterotrophs once dissolved oxygen is depleted (Starr and Gillham, 1993; Rivett *et al.*, 2008). Incomplete denitrification is also of environmental concern due to the potential for production of nitrous oxide (N2O), a greenhouse gas (Starr and Gillham, 1993; Henry *et al.*, 2006; Gurdak and Qi, 2012). Complete microbial denitrification is promoted in wastewater treatment bioreactors and denitrification beds by increasing dissolved organic carbon (DOC) concentrations (Lu *et al.*, 2014). Similarly, the addition of a carbon-rich permeable reactive barrier (e.g. woodchips, compost) to soils at a MAR site stimulates microbial removal of nitrate (Beganskas *et al.*, 2018; Grau-Martínez *et al.*, 2018; Gorski *et al.*, 2019). While microbial ecology is a fundamental parameter in these wastewater treatment reactors (Chu and Wang, 2013), few studies have explored microbial communities in operating MAR systems (Regnery *et al.*, 2017). Barba *et al.*, 2019 used principal component analysis to determine statistical relationships between geochemical parameters and microbial community composition in the Llobregat MAR system (Spain), finding that certain genera correlated with carbon and nitrogen cycling. However, this study had limited resolution for taxa determination and only focused on one site. Next-generation sequencing methods should allow for more precise identification of the microorganisms within MAR systems.

The overall goal of this study was to identify how infiltration of water, which is simulating conditions during MAR, may influence microbial community composition and metabolism in soils, including soils augmented with a bio-available carbon source. We analyzed microbial communities and metabolisms from three sets of plot-scale field experiments conducted in the Pajaro Valley groundwater basin in southern Santa Cruz County, CA (Beganskas *et al.*, 2018; Gorski *et al.*, 2019, Pensky *et al*., submitted). The three field sites; Harkins Slough (HSP), Kelly Thompson Ranch (KTR), and Kitayama Ranch (KTYA); are active or planned locations of managed aquifer recharge. They are all located within a 15 km radius (Supplementary Information, Figure S-1). For each location, plots were dug to replicate the shallow soil conditions of a saturated MAR (Beganskas *et al.*, 2018). Some of the experiments were conducted in unamended native soils as a control, whereas others were completed with the addition of a carbon-rich, permeable reactive barrier (PRB) that released DOC into underlying soils. Microbial community composition in native soils and below the PRBs, before and after infiltration, was determined with next-generation sequencing data of the 16S rRNA gene and the clade I nitrous oxide reductase gene, *nosZ* (reduces nitrous oxide to nitrogen gas) (Henry *et al.*, 2006). The objectives of this study were to: (1) quantify the impact of infiltration on soil microbial communities in association with changes in water quality, and (2) document differences in microbial communities between native soils and in soils below a carbon rich PRB. We hypothesized that there would be common changes in soil microbial community compositions at the three sites induced by infiltration. In addition, we predict that the introduction of a bioavailable carbon from a woodchip PRB would result in a shift towards denitrifying microbes that correspond to geochemical gradients.

# Materials and Methods

This study combined newly acquired datasets with sediment, DNA sequencing, and water chemistry datasets from Beganskas, *et al.,* 2018; Gorski, *et al.,* 2019; and Pensky, *et al.*, submitted.

## Field Sites

Experimental plots were established at three field sites in the Pajaro Valley, central coastal California (SI, Figure S1), as described in earlier studies (Beganskas *et al.*, 2018; Gorski *et al.*, 2019). The field sites are adjacent to active agricultural fields and active or planned locations of managed aquifer recharge. Most freshwater demand in the Pajaro Valley is satisfied by extraction of groundwater, and groundwater in many locations has nitrate concentrations that are elevated relative to pre-development values, with some areas exceeding the federally mandated, maximum contaminant level of 10 mg/L NO3-N (714 µmol/kg) (Pajaro Valley Water Management Agency Salt and Nutrient Management Plan , 2016). Experimental plots were square in plain view (1 m x 1 m area), were hand excavated to 0.6 to 1 m depth, and were instrumented with thermal probes (to measure flow rates using heat as a tracer), piezometers (shallow, subsurface fluid samplers), and a continuous infiltration system. Fiberglass walls were inserted in the plots and backed with water-activated bentonite to limit lateral flow. As part of tests at each field site, 1-2 plots were established as "native sediment" controls, and one plot was modified with a 30-40 cm layer of redwood chips acquired from a local landscape supply as a source of bioavailable carbon. The details of each experiment, including water sources, test duration, and hydrologic analyses are described in papers explaining studies of Harkins Slough (HSP, Beganskas et al., 2018), Kelly Thompson Ranch (KTR, Gorski. *et al.,* 2019), and Kitayama Ranch (KTYA, Pensky *et al*., 2021, *submitted*).

## Fluid and Sediment Sampling

Infiltration tests lasted ~10-16 days, and fluid samples were collected from piezometers installed below the plots every 1-2 days. These samples were filtered and analyzed colorimetrically using a Lachat QuickChem to measure NO3-, NO2-, and NH4+ or a Shimadzu TOC Analyzer to measure DOC. A day before the plots were disassembled, samples were filtered and sent to a professional water quality laboratory (Monterey Bay Analytical Services, MBAS) and subjected to a drinking water panel, including analyses of NO3-, Mn, Fe, and DOC. MBAS data were used in the CCA plots. Values are listed in Supplementary Information Table 2.

Sediments were collected during plot construction and after experiments were complete, to determine conditions before and after infiltration. The pre-infiltration samples were analyzed for grain size distribution using a Beckman Coulter LS 13320 Particle Size Analyzer. Total carbon and total nitrogen were analyzed using a Thermo Fisher Flash 2000. Sediment samples for DNA sequencing were collected before and after infiltration using sterile technique, transported in liquid nitrogen, and stored at -80°C. Samples are listed in Supplementary Information Figure Table 1.

## DNA extraction and Processing

Soil DNA was extracted using Qiagen PowerSoil DNA Isolation Kit according to the manufacturer’s instructions. The V4 and V5 regions of the 16S rRNA gene were amplified using 515F-Y and 926R primers that had an attached MiSeq adapter (Table 1)(Parada *et al.*, 2016). Each reaction had 0.2 mM dNTP (New England Biolabs), 5 µL 10X Titanium Taq buffer, 5 µL 10X MasterAmp PCR enhancer (Illumina), 0.2 µM of each forward and reverse primer, 1µL Titanium Taq polymerase, 3 ng of DNA, and DEPC-treated water up to 50 µL. After the initial amplicons were produced, the Illumina MiSeq Platform Protocol was used as the pipeline for 16S rRNA samples (Illumina). The pooled 16S library was sequenced on the Illumina MiSeq (600 cycles v3 PE300 flow cell kit) at the University of California, Davis Genome Center. KTR and KTYA samples from 30 cm below the plot were sequenced for *nosZ* using the same 50 µL reactions using nosZ2-F and nosZ2-R (Henry *et al.*, 2006) as primers (Table 1). After an initial cleanup with AMPure XP beads, amplicons were sent to Genewiz to be sequenced. The HSP raw reads can be found National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession number: SRP151895). The KTR reads are at accession number PRJNA523645, KTYA at PRJNA787642 and *nosZ* reads at PRJNA777280.

Sequenced reads were filtered and grouped into Amplicon Sequencing Variants (ASVs) using the Divisive Amplicon Denoising Algorithm (DADA2) (Callahan *et al.*, 2016) in R. 16S ASVs were assigned taxonomy using the SILVA reference database version 138 (Quast *et al.*, 2013), while *nosZ* taxonomy was based on a custom curated database created using sequences from Fungene (Fish *et al.*, 2013) and taxonomy from NCBI(National Center for Biotechnology Information, 2010). Sequenced reads from different field sites were combined and stored in a Phyloseq object (McMurdie and Holmes, 2013). A maximum likelihood tree for *nosZ* sequences was created using the phylogeny.fr program from the Laboratoire d’Informatique, de Robotique et de Microélectronique de Montpellier (LIRMM) (Dereeper *et al.*, 2008). Data transformation and visualizations were conducted using R packages tidyverse (Wickham *et al.*, 2019), ggplot2 (Wickham, 2016), tidytree (Yu *et al.*, 2021), ggtree (Yu, 2020), ggvegan (Simpson, 2019), ggpubr (Kassambara, 2020), and ggtext (Wilke, 2020).

## Statistical Analysis

For all these analyses, there was no rarefaction. Non-metric multidimensional scaling, constrained correspondence analysis, Shannon-Weaver index, and analysis of variance were calculated using the *vegan* (v 2.5.7) package within R (Oksanen *et al.*, 2020). Environmental and taxa data were extracted from the Phyloseq object. For the CCA plots, constraint formulas were calculated to increase significance and reduce collinearity. The CCA of the initial communities used the formula Sand + Clay + Silt + C +N and was scaled to show relationships between samples and the constraints. The CCA formula using water chemistry data was DOC + NO3 + Mn + Fe and was scaled to show species’ relationships to constraints. Before differential abundance was calculated, taxa were filtered so only reads representing 0.1% in a quarter of the samples were included. Differential analysis was conducted using Analysis of Composition of Microbiomes with Bias Correction (ANCOM-BC) (Lin and Peddada, 2020).

## Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) was used to calculate the number of *nosZ* reads for KTR and KTYA samples from 30 cm below the plot. Table 1 shows the primers and cycling used for *nosZ* as well as 16S rRNA, the housekeeping gene. Each sample was done in triplicate and fluorescence was recorded during the annealing step. Each reaction had 10 µL of 2X SYBR Green Master Mix (QuantaBio), 3 ng of DNA, 0.5 µM forward and reverse primers, and water up to 20 µL. Changes in *nosZ* between pre- and post- infiltration were calculated using the Pfaffl method (Pfaffl, 2001). Significance was calculated using a two-tailed t-test.

# Results

## Summary of Field Sites Geochemistry

The three field sites, Harkins Slough (HSP), Kelly Thompson Ranch (KTR), and Kitayama Ranch (KTYA), were previously analyzed for geochemical parameters and soil properties. The fraction of dissolved nitrogen species (NO3-, NO2-, and NH4+) was calculated by dividing the difference of the surface water and corresponding deepest piezometer concentrations by the total dissolved N of the surface water. While the dissolved N remained unchanged or increased in the native soil plots, the woodchip-amended plots show removal of dissolved nitrogen (Figure 1). Overall, the addition of a PRB significantly increased the fraction N removed compared to the native soil. KTR had the largest removal fraction between the treated and untreated plots, while HSP and KTYA had similar differences between treatments.

We first assessed how the three field sites differed from each other with respect to soil physical-chemical properties that could influence how subsurface microbial communities respond to infiltration. Non-metric multidimensional scaling (NMDS) was used to visualize these differences. Total carbon (TC), total nitrogen (TN) and sand, silt, and clay were used as inputs. NMDS analysis (Figure 2) shows that samples from the three sites are significantly grouped by location (Permutational multivariate analysis of variance (PERMANOVA) < 0.001), with Kelly-Thompson Ranch (KTR) samples being especially distinct from Kitayama Ranch (KTYA) and Harkins Slough (HSP) samples. HSP and KTYA field samples were composed mainly of sand (93% and 88%, respectively) and were relatively uniform to ~110 cm below the plots, whereas KTR sediments averaged 46% sand, 36% silt, and 17% clay (Supplementary Information, Figure S-2). Conditions at the KTR site were also more heterogeneous; one plot was dominated by sandy layers near 30 and 60 cm depth, and silty layers at 10, 40, 80 and 90 cm depth. The other two KTR plots were a mixture of silt and clay. These physical properties should impact the microbial community makeup. KTR samples had significantly higher TC and TN than did samples from the other sites. TC and TN at KTR averaged 0.64% and 0.06% (by weight), compared to 0.04% and 0.004% at HSP and 0.09% and 0.01% at KTYA, respectively (Supplementary Information, Figure S-3). Additional insights into the hydrology can be found at Beganskas, *et al.,* 2018 for HSP; Gorski, *et al.,* 2019 for KTR; and Pensky, *et al.*, submitted for KTYA.

Correspondence of Initial Microbial Communities to Geochemical Data

We next assessed how much the microbial community composition at the three sites differed prior to initiating infiltration. The V4-V5 region of the 16S rRNA gene sequence data was filtered by quality, binned into Amplicon Sequence Variants (ASVs) using the DADA2 algorithm (Callahan *et al.*, 2016). We performed constrained correspondence analysis (CCA) to relate the initial ASVs of the field sites to the inputs used in the NMDS in Figure 1 (TC, TN, Percent Sand, Silt, and Clay). CCA is an ordination method that only displays the variance in population that can be explained by environmental inputs. The constrained axes of the model explain 31.2% of the variance among the community (Figure 3). Including the ASV data allowed for greater sample separation and grouping by location compared to environmental data alone (PERMANOVA(Location)=9.99 x 10-5). The field locations themselves, as well as their initial microbial communities, are different from one another.

## Microbial Community Characterization

To characterize microbial communities and their changes due to infiltration, ASVs were assigned taxonomy using the SILVA database. At least 90% of the reads per sample fell into 10 phyla: *Acidobacteriota*, *Actinobateriota*, *Bacteroidota*, *Crenarchaeota*, *Firmicutes*, *Myxococcota*, *Nitrospirota*, *Planctomycetota*, *Proteobacteria*, and *Verrucomicobiota* (Figure 4a). At all three locations regardless of treatment, there was an increase in *Proteobacteria* after infiltration compared to samples collected before infiltration. *Proteobacteria* was the most abundant phylum in all samples, making up on average 41.9% of the relative abundance among all samples. Shannon-Weaver indices were used to calculate sample diversity before and after infiltration (Figure 4b). At KTYA and HSP there was a significant decrease between before and after samples in diversity due to infiltration, whereas KTR showed no significant change. NMDS analysis of the microbial communities shows that samples from KTR and HSP are especially well grouped by location, whereas the KTYA samples encompass the two other groups (Figure 4c).

## Impacts of Infiltration on Microbial Community

Infiltration through shallow soils resulted in measurable changes in hydrologic and geochemical conditions, and consequent impacts on microbial communities. A PERMANOVA analysis showed that the microbial communities did not significantly separate into before and after infiltration groups(p=0.05459). Therefore, we pooled the native soil samples and compared after infiltration samples with before using Analysis of Composition of Microbiomes with Bias Correction (ANCOM-BC). To reduce the impact of low abundance species, taxa with less than 10 reads in 25% of the samples were removed. Differentially abundant taxa with adjusted *p* values greater than 0.05 were also removed. This analysis demonstrates that 23 genera were differentially abundant in samples after infiltration relative to samples taken before infiltration (Figure 5). Only three genera (*Abditibacterium*, *Vicinamibacter*, and *Candidatus Alysiosphaera*) decreased in samples after infiltration. These genera are from the phyla *Abditibacteriota, Acidobacteriota*, and *Proteobacteria* respectively. In contrast, most genera that had increased abundance following infiltration (15 of 20 genera) were *Proteobacteria*. *Candidatus Nitrosotenius* from the *Crenarchaeota* phylum was the most differentially represented, with >5 log 2-fold abundance increase.

## Impact of a Permeable Reactive Barrier on Microbial Communities

As part of infiltration experiments at all three field sites, a carbon rich PRB composed of wood chips was added to one of the test plots. We expected the differences in nitrogen cycling associated with the addition of a carbon rich PRB (Figure 1), will also be expressed in the makeup of associated microbial communities. For this part of the analysis, we focus on KTR and KTYA samples, because HSP lacked sufficient water chemistry data. Post-infiltration samples were filtered to remove scarce ASVs, and ANCOM-BC analysis was performed to show which genera were significantly abundant across the experimental conditions. Figure 6 shows the 27 genera that differed in abundance after infiltration in the PRB treatment compared to the after-infiltration samples through just native soil. Sixteen genera were less abundant in the PRB samples, while 11 were more abundant. Three genera had increased differential abundance due to infiltration in the native soil plots but had lower abundance following infiltration through a woodchip PRB. *Verrucomicrobiota* was the phylum that had the most genera with higher abundance in PRB samples. The 3 genera with the largest abundance difference (*Sphingomonas, Novosphingobium,* and *Tropicimonas*) all belong to the *Proteobacteria* phylum. Curiously, the majority of *Proteobacteria* genera (6 of 10 with significant changes) were less abundant after infiltration through a carbon rich PRB.

We used CCA and water chemistry data to infer which parameters were associated with these shifts in bacterial communities. Due to the increased nitrate removal in the plots with a PRB, we first looked at how the 20 most abundant genera related to redox sensitive constituents in the infiltrating water (Figure 7). The CCA model was based on NO3-, Fe, Mn, and DOC concentrations. Nitrite was not included in our analysis as its concentration was negligible and sulfate was not included because it was collinear with nitrate. Overall, the constrained model explains 48.3% of the variance and was significant using PERMANOVA. *Nitrospira*, *Nitrosomondaceae* (*MND1* and *IS-44*), and *Anaerolineaceae* were found in samples that had high nitrate concentrations. *Pyrinomonadaceae RB41* and *Candidatus Nitrososphaera* were more likely to be found in samples with increased dissolved iron. Most *Proteobacteria* (e.g., *Sphingobium*, *Novosphingobium*, and *Pseudomonas*) correlated with lower concentrations of NO3-, Fe, Mn, and DOC. These correlations help to explain how individual environmental variables stimulates activity within specific microbial communities.

## Metabolism of Microbial Communities

To further understand how the microbial ecology of denitrification was impacted by infiltration and the presence or absence of a carbon rich PRB, we examined the relative abundance and diversity of the clade I nitrous oxide reductase gene, *nosZ*. This gene encodes for the enzyme responsible for reducing nitrous oxide to nitrogen gas in the last step of denitrification. Samples were available for this analysis from KTR and KTYA, but not HSP. We performed quantitative PCR (qPCR) and analyzed fold-changes using the Pfaffl method. The number of *nosZ* genes did not significantly change due to infiltration (Supplementary Information, Figure S-4). The addition of a carbon rich PRB also did not change the number of *nosZ* genes.

We determined the sequence diversity of the *nosZ* gene as a proxy for which taxonomic group(s) are functionally active within each infiltration system. The partial *nosZ* sequencing reads were classified into 17 genera, all of which belonged to the *Proteobacteria* phylum. Most of the *nosZ* sequences are classified into one clade containing an unclassified *Alphaproteobacteria*, an unclassified *Rhodobacteraceae*, *Cereibacter*, and *Ensifer* (Figure 8). *Cereibacter* was the most abundant, comprising on average 38% of the reads per sample. An unclassified ASV from the order *Hyphomicrobiales* was phyletically separate from the clade with the most abundant genera, but accounts for almost a tenth of the reads per sample. The phylogenetic tree shows that the taxonomy assigned to the ASVs are closely related to the taxonomy of the reference sequences. None of the genera were significantly differentially abundant because of PRB treatment or infiltration (based on ANCOM-BC analysis).

# Discussion

In this study we related microbial communities with soil and water chemistry, but the main driver for the initial populations was grain size. Grain size has been used as a constraint in predicting microbial communities in environments ranging from eutrophic lagoons (Highton *et al.*, 2016) to the Namib Dessert (Gombeer *et al.*, 2015). The two field sites rich in sand (Figure 2) (HSP and KTYA), had similar pre-infiltration microbial communities (Figure 3). In contrast, KTR samples are finer grained, with a higher percentage of silt and clay, and had the most distinct initial community. KTR also had a more significant increase fraction of dissolved nitrogen removed (Figure 1) in woodchip-amended plots than HSP or KTYA, indicating that grain size may also be connected to microbial nutrient cycling. The addition of initial microbial communities into the CCA model improved the statistical separation of samples by location. Even though the three sites were located less than 15 km from one another, they provide unique soil profiles and starting microbial communities.

Samples from HSP and KTYA both showed a decrease in Shannon-Weaver Index after infiltration (Figure 4b). An infrequent large disturbance, like the saturation of soil, promotes metabolically versatile populations and is likely to reduce diversity (Connell, 1978). Previous studies show a loss of diversity due to MAR-induced infiltration (Barba, Folch, Sanchez-Vila, *et al.*, 2019; Fillinger *et al.*, 2021). In both HSP and KTYA, there is an increase in the relative abundance of *Proteobacteria* due to infiltration (Figure 4a), which is similar to the results found in the samples from the Llobregat MAR basin (Barba, Folch, Gaju, *et al.*, 2019). This likely means that during infiltration, *Proteobacteria* are less perturbed by the disturbance of water and can gain dominance. Diversity indices at KTR remained stable pre-and post-infiltration (Figure 4b) as did the relative abundance of *Proteobacteria* (Figure 4a). *Proteobacteria* preferentially colonize and form biofilms on larger grains (Santmire and Leff, 2015). Therefore, the *Proteobacteria* in the clay and silty KTR samples may not have had as much of a trophic benefit to establish dominance during infiltration. Future work focused on grain size and biofilm formation could shed light on the competitive benefit of *Proteobacteria* during infiltration.

Infiltration impacted the relative abundance of specific microbial taxa. We consider infiltration a large disturbance; the soil went from dry conditions to fully saturated within a day. This infiltrating water also brings in more metabolites for microbes to consume (Ginige *et al.*, 2013). A previous study (Lennon *et al.*, 2012) found most Gram-negative bacteria preferred soil with higher moisture content. *Proteobacteria* preferred the wettest conditions out of all the phyla tested. This was consistent with our findings, where 15 genera that were more abundant in the post-infiltration samples were *Proteobacteria*. Two *Crenarcheota* genera (*Candidatus Nirosotenuis* and *Nitrosarchaeum*) also demonstrated greater changes in post-infiltration samples differential abundance. *Crenarcheota* are a common microbe in many aquatic settings. They are also known to oxidize ammonia and fix carbon (Hu *et al.*, 2011). *Abditibacterium*, the genus with the most dramatic decrease, is an obligate aerobe that has few cultured species. *Abditibacterium utsteinense*, isolated from Arctic soil, is a slow growing, oligotrophic bacteria (Tahon *et al.*, 2018). Infiltration most likely created suboxic to anoxic conditions, especially when there was bioavailable carbon released by the PRB, that were unfavorable to *Abditibacterium* growth. *Vicinamibacter*, a member of the *Acidobacteriota* phylum, also showed a considerable decrease in abundance. *Vicinamibacter* is a rarely cultured genus and little is known about its physiology in soils (Huber and Overmann, 2019). Yet, *Acidobacteriota*, was the only Gram-negative phylum in the Lennon et. al (2021) study that preferred dry soil conditions. These significant changes in genera gives us insights on the impact infiltration had on the microbial community.

Earlier studies have shown that a carbon rich PRB releases DOC (Qian *et al.*, 2011; Grau-Martínez *et al.*, 2017; Gorski *et al.*, 2019). The microbial nitrogen cycle is highly influenced by the C:N ratio; a large change in the ratio can favor microbes with different metabolisms (Kraft *et al.*, 2011). Furthermore, isotope analysis at KTR indicated nitrate removal under a PRB was primarily driven by microbes (Gorski *et al.*, 2019). Therefore, we predicted changes due to carbon influx by a PRB during infiltration could alter microbial community composition and ecophysiology. Interestingly, the three most differentially abundant genera (*Sphingoibium*, *Novosphingoibium*, and *Tropicimonas*) in samples collected from PRB-treated plots are all known to be aerobic complex carbon degraders (White *et al.*, 1996; Harwati *et al.*, 2009; Wang *et al.*, 2018). One explanation for this is that the organic carbon leached from the woodchip PRB was degraded by these genera, while there was sufficient oxygen available, producing breakdown products that were subsequently used to fuel denitrification (Figure 6). These three genera also correspond to low nitrate and DOC concentrations in the CCA plot (Figure 7), which supports this hypothesis. In contrast, genera capable of aerobic ammonia oxidation (*Nitrosarchaeum* and *Nitrosomonas*) (He *et al.*, 2018) exhibited the largest decrease in relative abundance within PRB-treated plots. Interestingly, these potential nitrifiers exhibited increases of relative abundance when comparing pre- and post- infiltration native soil samples (Figure 5). During infiltration, these genera may have added nitrate to the system through ammonium and nitrite oxidation pathways but were less dominant in the environment under the PRB. We observed a close relationship between aerobic ammonia-oxidizing genera and high nitrate concentration (Figure 7), further supporting their potential function towards nitrification. While anaerobic ammonia oxidizers (Anammox) from the phylum *Planctomycetes* existed in the plots, they were neither differentially abundant between the PRB-amended and native soil plots nor pre- and post- infiltration samples.

At all three sites, there was an increased in DOC below the plots amended with a woodchip PRB (Beganskas *et al*., 2018, Gorski *et al.*, 2019, Pensky *et al*., submitted). We assume that the DOC can be oxidized by the soil microbes. One study of four soil ecosystems found that two genera were responsible for nearly half of the carbon flow by respiration even though they comprised less than 20% of total sequencing reads (Stone *et al.*, 2021). Both genera identified in the Stone study, *Bradyrhizobium* and *RB-41* (member of the *Pyrinomonadaceae* family), were highly abundant in the soil samples. However, *Bradyrhizobium* corresponded with lower DOC levels whereas *RB-41* was more associated with higher levels of DOC (Figure 7). *RB-41* is known for its ability to hydrolyze polymers (Pascual *et al.*, 2018) and therefore could contribute to increasing DOC concentrations as a consequence of its metabolic activities.

While carbon can drive denitrification, DOC input may also influence metal cycling during infiltration. *Flavobacterium*, which oxides aqueous manganese (II) to manganese oxide precipitates (Akob *et al.*, 2014), corresponds to low Mn concentrations in the input water (Figure 6). Further investigation into the microbial Fe/Mn cycle could reveal important trends in how DOC could impact metal release during infiltration for MAR. In soil there are numerous complex metabolisms interacting with one another. To better understand how the microbes are utilizing the carbon leached by the PRB material, techniques such as quantitative stable isotope probing would give an indication of what populations are increasing productivity with the addition of a PRB. Additionally, characterizing the carbon leached off the PRB could further aid in predicting the trajectory of a microbial community towards a certain eco-physiological outcome such as denitrification, nitrification, and/or metal release.

In this study, we investigated clade I nitrous oxide reductase (*nosZ*) gene relative abundance and diversity to gain insights into changes in nitrogen cycling during infiltration. A study of denitrifying genes in agricultural soils found *nosZ* was stable in abundance and community makeup after irrigation with different water sources while other nitrogen cycling genes had changes in response to input water (Zhou *et al.*, 2011). Relative abundances and diversity of clade I *nosZ* looked similar at both plots in our study regardless of field site, treatment, or infiltration (Figure 8 and Supplementary Information, Figure S-4). The genera identified by the *nosZ* sequences were not among those taxa displaying significantly abundant 16S rRNA-gene classifications (Figure 5 and 6). The taxa associations of the *nosZ* sequences made up less than 1% of the total 16S rRNA sequencing reads. However, all the sequences belong to *Alpha-*, *Beta-*, and *Gammaproteobacteria*, which make up around half of the 16S reads. Previous studies also found that *nosZ* reads from sediments and soil are around 5% of the 16S reads (Mounler *et al.*, 2004; Zhou *et al.*, 2011; Bellini *et al.*, 2013). The primers we used are selective for clade I *nosZ* genes which is usually made up by *Alpha-, Beta-, and Gammaproteobacteria*. We only looked at clade I due to the high levels of 16S reads from those classes as well as clade I *nosZ* being >1000 times more abundant than clade II *nosZ* in coastal sediments and nitrate-rich bioreactors (Hallin *et al*., 2018). However, we may have underestimated *nosZ* abundance and diversity by not including clade II *nosZ*. A metagenomic analysis will give a more comprehensive understanding of how microbial metabolism was affected by infiltration.

In this study, we identified grain size was the most important predictor for soil microbial communities in shallow soils through which water was infiltrated. We found that infiltration promoted the dominance of *Proteobacteria* while decreasing slow-growing bacteria that prefer dry conditions such as *Abditibacterium* (Lennon *et al.*, 2012; Tahon *et al.*, 2018). Addition of carbon rich PRB reduced populations capable of nitrification and promoted populations known for complex carbon degradation. Although more nitrate was removed during infiltration in soils below a PRB, the quantity of the clade I nitrous oxide reductase gene did not change due to infiltration or addition of a PRB. This suggests that other genes and pathways may play an important role in enhancing denitrification during infiltration for MAR. This study connects the physical-chemical properties, microbial communities, with observed trends to find key genera that may help improve water quality as part of groundwater resource management.

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