Microbial Community Responses to Organophosphate Substrate Additions in Contaminated Subsurface Sediments

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Abstract

Background: Radionuclide- and heavy metal-contaminated subsurface sediments remain a legacy of Cold War nuclear weapons research and recent nuclear power plant failures. Within such contaminated sediments, remediation activities are necessary to mitigate groundwater contamination. A promising approach makes use of extant microbial communities capable of hydrolyzing organophosphate substrates to promote mineralization of soluble contaminants within deep subsurface environments.

Methodology/Principal Findings: Uranium-contaminated sediments from the U.S. Department of Energy Oak Ridge Field Research Center (ORFRC) Area 2 site were used in slurry experiments to identify microbial communities involved in hydrolysis of 10 mM organophosphate amendments [i.e., glycerol-2-phosphate (G2P) or glycerol-3-phosphate (G3P)] in synthetic groundwater at pH 5.5 and pH 6.8. Following 36 day (G2P) and 20 day (G3P) amended treatments, maximum phosphate (PO₄³⁻) concentrations of 4.8 mM and 8.9 mM were measured, respectively. Use of the PhyloChip 16S rRNA microarray identified 2,120 archaeal and bacterial taxa representing 46 phyla, 66 classes, 110 orders, and 186 families among all treatments. Measures of archaeal and bacterial richness were lowest under G2P (pH 5.5) treatments and greatest with G3P (pH 6.8) treatments. Members of the phyla Crenarchaeota, Euryarchaeota, Bacteroidetes, and Proteobacteria demonstrated the greatest enrichment in response to organophosphate amendments and the OTUs that increased in relative abundance by 2-fold or greater accounted for 9%-50% and 3%-17% of total detected Archaea and Bacteria, respectively.

Conclusions/Significance: This work provided a characterization of the distinct ORFRC subsurface microbial communities that contributed to increased concentrations of extracellular phosphate via hydrolysis of organophosphate substrate amendments. Within subsurface environments that are not ideal for reductive precipitation of uranium, strategies that harness microbial phosphate metabolism to promote uranium phosphate precipitation could offer an alternative approach for in situ sequestration.

Introduction

Within sediments, the mobility of phosphate (PO₄³⁻) is controlled by pH, coprecipitation reactions with metals and radionuclides, adsorption/desorption, and ion-exchange reactions [1]. As a result of this poor mobility in subsurface environments, microorganisms release organic acids and/or express phosphatase enzymes [i.e., acid/alkaline phosphohydrolases] to enhance the solubility and cellular transport of phosphate [2–4]. Harnessing microbial phosphatases expressed by extant microbial communities within uranium (U)-contaminated environments represents an approach to leverage microbial phosphate acquisition phenotypes to promote in situ sequestration of U as insoluble phosphate minerals.

Alternative approaches for microbial mediated U immobilization have examined bioaccumulation, reductive precipitation, ligand-generated precipitation (e.g., carbonate and sulfide), and volatilization reactions to reduce contaminant solubility [5–8]. Microbial reductive precipitation of soluble U(VI) to insoluble U(IV) has been extensively examined in both laboratory and field studies where delivery of electron donor substrates, buffered at circumneutral pH, has proven effective as an immobilization strategy [9–12]. However, the limitations of U(VI) reduction are observed in environments that experience dynamic geochemical
conditions where low pH inhibits microbial U(VI) reduction [11,13] and reoxidation of U(IV) occurs in the presence of oxygen, nitrate, ferric iron, and humics [14–17].

Within U-contaminated sediments at the United States Department of Energy Oak Ridge Field Research Center (ORFRC), three distinct groundwater contaminant flow paths contribute to pH and co-contaminant heterogeneity (i.e., porewater pH ranging from 3.4–7.0 and [NO₃⁻] ranging from 29 mg L⁻¹ to 2300 mg L⁻¹), which inhibit or reverse microbial U(VI) reduction [12,18–20]. Alternatively, in situ precipitation of U(VI) as highly insoluble phosphate minerals (e.g., autunite) that remain stable across a broad pH range (Figure 1) offers an approach for U(VI) sequestration under both oxidizing and reducing conditions [21–23]. Autunite minerals have been identified in sediments at the U.S. Department of Energy (DOE) Fernald site, Hanford site, and Oak Ridge National Laboratory [24–26], suggesting that the U.S. Department of Energy (DOE) Fernald site, Hanford site, and Oak Ridge National Laboratory [24–26], suggesting that

The goal of this study was to utilize the 16S rRNA high-density microarray (PhyloChip), capable of detecting 8,741 archaeal and bacterial taxa [41], to characterize the extant prokaryotic community within ORFRC U-contaminated sediments that contributed to organophosphatase hydrolysis (i.e., G2P and G3P) hydrolysis. Due to the heterogeneity of geochemical parameters (pH, [U], [NO₃⁻], etc) present within the ORFRC subsurface, characterization of extant phosphate solubilizing microbial communities enriched under specific pH and organophosphate amendments can aid in development of strategies for in situ phosphate mineralization of U(VI).

Results

Microbial response to slurry incubations

Total DNA extractions from sediment slurry treatments were measured as a proxy for microbial growth in response to the different incubation conditions. Prior to treatments, ORFRC subsurface sediment DNA concentrations were 1.1 ± 0.1 µg g⁻¹ (Figure 2). Sediment slurries incubated at either pH 5.5 or pH 6.8 without organophosphate addition exhibited a 1.7-fold increase (1.8 ± 0.2 to 1.9 ± 0.4 µg g⁻¹) in DNA concentration after 36 days. DNA concentrations increased 20-fold (21.6 ± 6.8 to 23.9 ± 6.5 µg g⁻¹) after 36 days in G2P-amended treatments and 6-fold (7.2 ± 0.9 to 7.3 ± 1.1 µg g⁻¹) after 20 days in G3P-amended treatments at both pH values (Figure 2).

![Figure 1. Thermodynamic modeling of U(VI) in the presence of phosphate as a function of pH. ORFRC Area 2 groundwater concentrations of dissolved ions (GW-836 monitoring well), U(VI) = 4.5 µM, Ca²⁺ = 4.85 mM, (A) PO₄³⁻ = 500 µM and (B) PO₄³⁻ = 5 mM were used to model the distribution of U(VI) species. Dashed lines represent soluble species and solid lines represent insoluble species.](https://doi.org/10.1371/journal.pone.0100383.g001)
Chemical analyses of sediment slurry incubations

G2P, G3P, PO₄³⁻, NO₃⁻, NO₂⁻, and organic acids were measured at 96 h intervals over the course of all incubations. Average NO₃⁻ concentrations among all sediment slurry treatments did not decrease throughout the time course (data not shown) indicating that aerobic conditions were maintained during these incubations.

Phosphate concentrations in the G2P-amended slurries remained below 140 μM for 576 h then increased to 4.8 mM and 2.2 mM in the pH 5.5 and 6.8 incubations, respectively (Figures 3A and 3B). Combined concentrations of G2P and soluble phosphate in the pH 6.8 treatments exhibited that mass balance of PO₄³⁻ was respected throughout the entire time course (Figure 3B). Conversely, at pH 5.5, G2P was completely removed from solution without a proportional accumulation of soluble phosphate after 576 h (Figure 3A). In contrast to the G2P treatments, G3P was completely consumed within 300–400 h at both pH values (Figures 3A and 3B). Phosphate concentrations in G3P-amended slurries increased after 96 h (pH 5.5) and prior to the 96 h time point (pH 6.8), then accumulated over 4.7 mM phosphate by the 192 h time point. At the 480 h time point, soluble phosphate concentrations reached 8.9 mM and 8.7 mM phosphate in G3P (pH 5.5) and G3P (pH 6.8) treatments, respectively (Figures 3A and 3B).

Archaeal community structure

A total of 180 archaeal OTUs representing 3 phyla, 10 classes, 16 orders, and 25 families were detected amongst all pre-treatment and treatment samples (Table S1). The phyla Crenarchaeota and Euryarchaeota accounted for over 96% of the total archaeal richness within ORFRC sediments prior to treatments with the remainder comprised of unclassified Archaea (Figure 4A, Table S1). Following treatments, archaeal richness did not change significantly (p-value>0.05) regardless of the amendments (Figure 4B). NMDS ordination of archael community composition clustered the replicate samples into distinct groups based on treatment (Figure 4C), and MRPP tests confirmed that archael communities differed significantly (δₑ = 0.1255, δᵦ = 0.2798, p-value<0.001, A = 0.5516) amongst all treatments.

The combined influence of pH and organophosphate addition shaped the archael community by affecting OTU abundance relative to treatments at the same pH without organophosphate (Figures 5, Tables S1 and S2). Relative to total richness detected in sediments prior to treatments (134 OTUs), the richness of OTUs responding to treatment conditions decreased by 34-fold (6 OTUs), 4-fold (34 OTUs), 2-fold (85 OTUs), and 1.6-fold (85 OTUs) in G2P (pH 5.5), G3P (pH 5.5), G2P (pH 6.8), and G3P (pH 6.8) treatments, respectively (Figure 5). Archaea that demonstrated a relative increase in abundance of 2-fold or greater in G2P (pH 5.5) treatments consisted of two unclassified Crenarchaeota OTUs. In the G3P (pH 5.5) treatments, one Crenarchaeota OTU (unclassified at the class level) and two Euryarchaeota OTUs belonging to the classes Archaeoglobi and Methanobacteria demonstrated a 2-fold or greater increase in abundance (Figure 6A and Table S2). Class-level distribution with a 2-fold or greater increase in abundance in G2P (pH 6.8) treatments contained 13 OTUs composed of unclassified Crenarchaeota (15%), Methanobacteria (77%), and unclassified Archaea (8%). In the G3P (pH 6.8) treatments, 14 OTUs that increased in abundance by 2-fold or greater were composed of Thermoprotei (14%), unclassified Crenarchaeota (7%), Archaeoglobi (7%), Methanobacteria (7%), Methanomicrobia (14%), Thernoplasmat (21%), and unclassified Archaea (29%) (Figure 6A and Table S2).

Observed changes in relative abundance were identified in 4 OTUs [G2P (pH 5.5)], 34 OTUs [G3P (pH 5.5)], 65 OTUs [G2P (pH 6.8)], and 85 OTUs [G3P (pH 6.8)]. Four archeal OTUs belonging to Archaeoglobi, Methanobacteria, and two unclassified classes of Crenarchaeota (related to deep-sea sediment and landfill leachate environmental clones) were detected at a 2-fold or greater increase in relative abundance in multiple treatment conditions (Figure S1A and Table S3). The Crenarchaeota (landfill leachate related clone), Archaeoglobi, and Methanobacteria OTUs detected in multiple treatment conditions [i.e., G3P (pH 5.5), G2P (pH 6.8), and G3P (pH 6.8)] were most abundant in the G3P (pH 6.8) treatments, i.e., 29%–81% greater relative abundance when compared to the other treatments. The second Crenarchaeota OTU (deep-sea related clone) was 81% more abundant in treatment conditions with G2P (pH 6.8) relative to G3P (pH 5.5) treatments.

Bacterial community structure

A total of 1,940 bacterial OTUs, representing 43 phyla, 56 classes, 94 orders, and 161 families, were detected amongst all pre-treatment and treatment samples (Table S1). Prior to incubations, 1,540 OTUs representing 42 phyla were identified: 43% belonged to the phylum Proteobacteria, 20% belonged to 37 unique phyla, and the remaining 37% consisted of OTUs that belong to the Acidobacteria, Actinobacteria, Bacteroidetes, and Firmicutes (Figure 4D, Table S1). G3P treatments at pH 5.5 and pH 6.8 were the only conditions in which a significant decrease (p-value<0.05) in total bacterial richness was observed relative to pre-treatment sediments (Figure 4E). NMDS ordination and MRPP tests confirmed bacterial communities differed significantly (δₑ = 0.04862, δᵦ = 0.137, p-value<0.001, A = 0.6451) based on pre- and post-treatment conditions (Figure 4F). Observed changes in relative abundance were identified in 672 OTUs [G2P (pH 5.5)], 983 OTUs [G3P (pH 5.5)], 788 OTUs [G2P (pH 6.8)], and 1120 OTUs [G3P (pH 6.8)] (Figure 5). Within these treatments, only 3%–17% of detected OTUs increased in relative abundance by 2-fold or greater.

Within the pH 5.5 treatments, the phylum Proteobacteria accounted for 94%–99% of the 29 OTUs detected with a 2-fold or greater increase in relative abundance (Figure 6B and Table S2). In treatments with G2P (pH 5.5), z-proteobacteria was the dominant class. The orders Caulobacteriales and Rhizobiales account-
ed for 14% and 24%, respectively, of all proteobacterial OTUs that increased in abundance by as much as 7-fold. Only one OTU from the family Hyphomicrobiaceae was enriched in this treatment and demonstrated the greatest increase in relative abundance (17-fold). The remaining β-, δ-, and γ-proteobacteria classes were composed of OTUs from the orders Burkholderiales, Rhodocyclales, Desulfovibrio, Myxococcales, Chromatiales, Enterobacteriales, Pseudomonadales, Thiotrichales, and Xanthomonadales.

Treatments amended with G3P (pH 5.5) contained 164 OTUs that increased by 2-fold or greater relative to unamended (control) treatments and were dominated by the class γ-proteobacteria (Figure 6B and Table S2). The orders Enterobacteriales and Pseudomonadales accounted for 39% and 22%, respectively, of all proteobacterial OTUs with a 2-fold or greater increase in relative abundance. Less than 5% of the OTUs from this treatment increased in relative abundance (increases ranged from 13- to 406-fold) and belonged to the genera Arsenophonus, Pseudomonas, Pectobacterium, Rhahnella, Photorhabdus, Ochsenbacterium, and Brenneria. The remaining α-, β-, and γ-proteobacteria classes were composed of OTUs (with relative abundance increases between 2- and 4-fold) from the orders Rhizobiales, Rhodobacterales, Rhodospirillales, Burkholderiales, Hydrogenophilales, Aeromonadales, Alteromonadales, Chromatiales, Oceanospirillales, SAR86, Thiotricha, uncultured γ-proteobacteria, Vibrionales, and Xanthomonadales.

Within the pH 6.8 treatments, a 40%-60% increase in phylum-level richness was detected for OTUs with a 2-fold or greater increase in abundance relative to pH 5.5 treatments. The dominant phyla under growth conditions at pH 6.8 were Bacteroidetes and Proteobacteria, and accounted for 71%-79% of all OTUs with a 2-fold or greater increase in relative abundance (Figure 6B and Table S2). In treatments amended with G2P, the phylum Bacteroidetes was composed of three orders: Bacteroidales (38%), Cytophagales (25%), and Sphingobacteriales (38%). The distribution of Proteobacteria consisted of the orders: Rhizobiales (78%), Sphingomonadales (11%), and Enterobacteriales (11%). An OTU from the family Enterobacteriales demonstrated the greatest increase in relative abundance (16-fold) under these treatment conditions and members of the Prevotellaceae, unclassified Bacteroidetes, and one unclassified Bacteria were shown to increase in abundance by as much as 6-fold.

The G3P (pH 6.8) treatment exhibited the greatest number of phyla that had a 2-fold or greater increase in relative abundance (Figure 6B and Table S2). The phylum Bacteroidetes was composed of the Bacteroidales (10%), Flavobacteriales (20%), Sphingobacteriales (65%), and unclassified Bacteroidetes (5%). The two dominant proteobacterial orders were Pseudomonadales (50%) and Enterobacteriales (17%). The following orders comprised 11% or less of the remaining proteobacterial richness: Alteromonadales, Rickettsiales, Myxococcales, and Vibrionales. Four OTUs from the order Sphingobacteriales (uncultivated family-level) and one Enterobacteriaceae OTU demonstrated the greatest increase in relative abundance in this treatment (11- to 50-fold).

Further analysis of all treatment conditions identified 400 bacterial OTUs that were previously below the limit of detection in sediments prior to any treatments (Table S1). Under all treatment conditions, a subset of the previously undetected OTUs (i.e., 125 OTUs representing 3 phyla, 7 classes, 20 orders, and 22 families) were shown to increase in relative abundance by 2-fold or greater (Table S2). A total of 36 OTUs were detected in two or more treatment conditions at a 2-fold or greater increase in abundance relative to unamended treatments, 17 of the 36 OTUs were undetected in sediments prior to any treatments (Table S1). Ten families within the phylum Proteobacteria accounted for 75% of all OTUs detected in multiple treatment conditions. The dominant proteobacterial families OTUs detected in multiple treatment conditions, accounting for over 70% of Proteobacteria, belonged to Enterobacteriaceae, Pseudomonadaceae, and Rhizobaceae. Two OTUs from the families Phyllobacteriaceae and
**Pseudomonadaceae** were detected in three of the four organophosphate-amended treatments with a 2-fold or greater increase in relative abundance.

**Discussion**

Within U-contaminated subsurface environments, *in situ* sequestration approaches that minimize contaminant transport under dynamic hydrogeological conditions (i.e., pH, O$_2$, and co-
Figure 5. Dynamic archaeal and bacterial OTUs within sediment slurry treatments. Total detected archaeal (left column) and bacterial (right column) OTUs compiled from replicate treatments that significantly increased or decreased relative to incubations lacking organophosphate. Treatment conditions and total number of taxa plotted: (A) G2P (pH 5.5), (B) G3P (pH 5.5), (C) G2P (pH 6.8), and (D) G3P (pH 6.8). OTUs with a 2-fold or greater decrease in fluorescence were not detected.

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Conversely, the greater concentrations of total extracted DNA microbial enzymes that can utilize G3P over G2P as a substrate. 36-day treatment (Figure 3) likely reflects the predominance of extracellular phosphate. The rapid hydrolysis of approximately microbial activity that contributed to increased accumulation of phosphate treatments (Figure 2) was used as a proxy for increased dynamic prokaryotic communities that contribute to organophosphate hydrolysis with a concomitant increase in extracellular phosphate concentration is essential that contributes to organophosphate assimilation rather than rapid hydrolysis. Characterization of the extant subsurface archaeal and bacterial community as well as the dynamic OTUs responding to growth treatments was determined via PhyloChip 16S rRNA microarray hybridization. Although direct measure of population abundance is not possible with this method, the capability of detecting $10^2$-$10^3$ 16S rRNA gene copies [42] supported our goal in characterizing OTUs most responsive (i.e., OTUs that increased 2-fold or greater in relative abundance were designated as responsive) to organophosphate amendments. Archaeal community characterization within Oak Ridge National Laboratory U-contaminated sediments is currently limited to examination of U- and Hg-contaminated river sediments shown to be dominated by acetate- and hydrogen-dependent methanogens [43] and the enrichment of hydrogen-dependent methanogens following Area 2 subsurface injection of emulsified vegetable oil [44]. While our studies maintained oxic growth conditions, OTUs related to hydrogen-dependent methanogens increased in relative abundance for all treatments except G2P pH 5.5 (Figures 5 and 6) represent 9%-50% of total archaeal richness detected in ORFRC sediment slurry treatments. Observations of dynamic archaeal taxa within ORFRC sediments highlight the need for future studies that examine functional contributions under oxic growth conditions. Of the total observed bacterial richness detected in ORFRC sediment slurry treatments, only 3%-17% demonstrated an increase in relative abundance by 2-fold or greater (Figures 5 and 6). Within the pH 5.5 treatments, the phyla Proteobacteria represented 94% (G2P) and 98% (G3P) of the enriched OTUs. Alternatively, Proteobacteria and Bacteroidetes dominated the pH 6.8 treatments, which combined represented 71% (G2P) and 79% (G3P) of enriched OTUs. From culture-dependent studies, isolates belonging to the phyla Bacteroidetes, Firmicutes, and Proteobacteria have been shown to enhance phosphate solubility within the rhizosphere [51,52]. Use of the PhyloChip has provided an expanded view of bacterial taxa that can contribute to phosphate-cycling within ORFRC sediments. The lack of mass balance between organophosphate and phosphate concentrations was observed in the G2P (pH 5.5) treatments and the most dynamic OTU (17-fold increase in relative abundance) was related to the genus Hyphomicrobium. Members of the family Hyphomicrobiaceae are capable of C$_1$ metabolism, denitrification, and polyphosphate accumulation [53,54]. Interestingly, previous work examining ORFRC subsurface microbial communities capable of denitrification have identified Hyphomicrobium spp. as an abundant member of Area 2 ORFRC sediments [55], the dominant denitrifying species within Area 1, 2, and 3 ORFRC groundwater [56], and a readily culturable species from ethanol amended Area 2 sediment enrichments [57]. These observations suggest that in addition to the important role of denitrification within ORFRC sediments, Hyphomicrobium species could play a role in sequestering extracel-

**Figure 6. Class-level distribution of enriched OTUs.** Compiled OTUs enriched (i.e., 2-fold or greater increase in relative abundance) in replicate treatments representing the most responsive (A) archaeal and (B) bacterial classes from treatments amended with organophosphates at pH 5.5 and 6.8.

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lular phosphate via intracellular polyphosphate accumulation. Although polyphosphate accumulation could reduce extracellular phosphate concentrations, this physiological response is essential in controlling the cytotoxicity of metals and radionuclides which ultimately can aid in continued denitrification processes.

The γ-proteobacteria were shown to be the most dynamic class within G3P (pH 5.5) treatments where OTUs related to the genera *Arsenophonus*, *Brenneria*, *Pseudomonas*, *Ochrobactrum*, *Pectobacterium*, *Rahnella*, and *Photorhabdus* increased from 13-fold to 106-fold. The enrichment of OTUs related to *Pseudomonas* and *Rahnella* is likely due to the previously described phosphate solubilizing activities of related genera isolated from rhizosphere and U-contaminated sediments [22,31,51]. The *Ochrobactrum* related OTU has not been described as a common phosphate solubilizing isolate but characterization of an encoded phytase in *Ochrobactrum* proteins suggests related strains may be capable of organophosphate hydrolysis [58]. The genera *Arsenophonus*, *Photorhabdus*, *Brenneria*, and *Pectobacterium* contain species that have been described as symbionts or plant pathogens but to date have not been shown to enhance phosphate solubilization [59–62].

In the G2P (pH 6.8) treatments, the two dominant classes were *Sphingobacteria* and γ-proteobacteria but an OTU from the family *Enterobacteriaceae* demonstrated the greatest increase in relative abundance (over 16-fold). The *Bacteroidetes* OTUs that were enriched in this treatment were most closely related to rumen and soil isolates capable of phytase activity [63–65]. In addition to phytate hydrolysis, the phytase enzyme has been shown to hydrolyze various organophosphate substrates, including G2P [66]. Thus, enrichment of *Bacteroidetes*-related OTUs may also contribute to the hydrolysis of G2P as well as other organophosphate substrates within the ORFRC subsurface.

Enrichment of *Sphingobacteria* and γ-proteobacteria dominated the treatments at pH 6.8 amended with G3P. The same *Enterobacteriaceae* OTU that exhibited the enrichment in the G2P (pH 6.8) treatment also increased 16-fold in relative abundance. *Sphingobacterales* OTUs that demonstrated the greatest increases in abundance were related to *Bacteroidetes* clones from soil, river, and wastewater samples [67–69]. Within G3P (pH 6.8) treatments, a *Sphingobacteriales* OTU that exhibited the greatest increase in relative abundance (50-fold) was related to a clone associated with polyhydroxalkanoate (PHA)- and polyphosphate (polyP)-accumulating communities from a biological phosphorus removal reactor.

Additional taxa that have not been described as phosphate-solubilizing bacteria were enriched under all amended treatments and may suggest additional ecological functions within sediments that include organophosphate turnover. Within both G2P treatments, the enrichment of OTUs related to *Chloroflexi*, *Deferribacteres*, *Nitrospira*, and *Planctomycetes* were detected. Analysis of the *Candidateus Nitrospira* delnuvi and *Isosphaera pallida* genomes reveal that both encode a putative Class C acid phosphatase that could, in theory, contribute to G2P hydrolysis by related *Nitrospira* and *Planctomycetes* OTUs. The lack of studies that examine *Chloroflexi* and *Deferribacteres* organophosphate utilization underlines the need for future studies to determine the physiological capacities of related OTUs. The G3P (pH 6.8) treatments were shown to enrich *Cyanoacteria* and *Chlamydiaceae* OTUs related to *Euglena* chloroplast symbionts and pathogens harbored by and *Acanthamoeba* spp., respectively [70,71]. Due to the fact that all incubations where conducted in the dark, it is unlikely that photosynthetic algae where enriched but further studies are required to determine if these OTUs were enriched as a result of protozoan-association. Within both pH treatments amended with G3P, six OTUs from the family *Vibrionaceae* were enriched.

Although this finding has not been reported in previous ORFRC sediment diversity studies, members of this family have been detected in other terrestrial and freshwater environments but their ecological function remains unknown [72,73].

Within environments such as the ORFRC that are defined by acidic-to-circumneutral subsurface regions, thermodynamic modeling of PO$_4^{3-}$ species in ORFRC groundwater containing two different concentrations of P (e.g., 500 μM and 5 mM) demonstrates the formation of hydroxyapatite across a wide pH range (Figure S2A and S2B), resulting in a secondary path for remediation by providing mineral surface sites for the adsorption of metals and radionuclides. This additional path for phosphate mineral sequestration of U(VI) has been described in a recent study examining microbial hydrolysis of G3P in ORFRC Area 2 synthetic groundwater containing U(VI) and a calcium concentration of 4 mM that resulted in U(VI) coprecipitation with hydroxyapatite [33]. Furthermore, Ca concentrations greater than 1 mM (Figure S2C and S2D) that have been shown to enhance U(VI) transport as well as decrease U(VI) reduction rates [12,74–76]. Thus, harnessing P metabolic capabilities within the ORFRC subsurface that sequester Ca as a mineral phosphate could augment in situ U reduction processes. Within this study, the PhyloChip microarray rapidly identified relative abundance changes of prokaryotes with previously characterized P-solubilizing phenotypes as well as several archael and bacterial taxa that have yet to be described influences on terrestrial phosphate-cycling. The rapid assessment of microbial community dynamics provided by microarray analyses represents an approach that can provide insight into the diversity of prokaryotes that contribute to terrestrial phosphate-cycling and the influence these taxa could have on the cycling of metals and radionuclides within subsurface environments.

**Materials and Methods**

**Ethics Statement**

All sediment samples from the U.S. Department of Energy Oak Ridge Field Research Center were requested and obtained from David Watson, Oak Ridge National Laboratory Field Research Manager. This work did not involve field studies nor did it require specific permits.

**Sampling site**

Contaminated sediments were collected from the ORFRC (Area 2) located within the Oak Ridge National Laboratory Reservation in Oak Ridge, Tennessee. The contaminated sediments are located adjacent to three former waste ponds (S-3 ponds) used during decades of nuclear weapons production. The ponds and surrounding sediments received uranium, other radionuclides, heavy metals, organic solvents, and nitric acid waste (DOE Subsurface Biogeochemical Research website; http://esd.lbl.gov/research/projects/ersp/). Sediment cores (5 cm internal diameter with an average length of 168 cm) were collected aseptically and preserved under an argon atmosphere. Sediment samples from borehole FB107-04-00 at a depth of 7 meters below ground surface were obtained from the saturated zone where groundwater is approximately 4.5 meters below ground surface (http://public.ornl.gov/oric/site/narrative.cfm#Anchor12). Sediments from borehole FB107-04-00 were used for all incubations. Sediment from 7 meters below ground surface was aseptically subsampled, placed in a sterile plastic bag and homogenized. All subsequent analyses and slurry treatments utilized subsampled homogenized FB107-04-00 sediments. Sediment porewater was pH 6.8, measured with an Orion Dual Star digital meter and
calibrated electrode (Thermo Scientific, Beverly, MA). Prior to treatments, carbon (C) content was measured before and after acidification with a Leco CNS 2000 analyzer (Leco Corporation, St. Joseph, MI) at the University of Georgia College of Agricultural and Environmental Sciences Laboratories. Total C, organic C, and inorganic C content was 1077, 93, and 993 ppm, respectively.

Sediment slurry incubations and DNA extractions

Sediment slurry treatments were conducted in acid washed 1 L glass Erlenmeyer flasks containing 4 g sediment and synthetic groundwater in a final volume of 250 mL. Synthetic groundwater consisted of: 2 μM FeSO₄, 5 μM MnCl₂, 8 μM Na₂MoO₄, 0.8 mM MgSO₄, 7.5 mM NaNO₃, 0.4 mM KCl, 7.5 mM KNO₃, and 0.2 mM Ca(NO₃)₂. Sediment slurry pH 5.5 treatments were buffered with 50 mM 2-(N-Morpholino) ethanesulfonic acid (Sigma Aldrich, St. Louis, MO) and pH 6.8 treatments were unbuffered. Either G2P or G3P (Sigma Aldrich, St. Louis, MO) were added to sediment slurries as the sole C and P amendment at a final concentration of 10 mM. Control sediment slurry treatments were conducted at pH 5.5 and 6.8 without organophosphate additions. The combinations of pH and organophosphate amendments yielded six different treatment conditions: (1) unamended control (pH 5.5), (2) G2P (pH 5.5), (3) G3P (pH 5.5), (4) unamended control (pH 6.8), (5) G2P (pH 6.8), and (6) G3P (pH 6.8). To maintain oxic growth conditions, sediment slurries were constantly mixed in the dark with a magnetic stir bar at 200 rpm on a Variomag Multipoint 15 magnetic stirrer (Thermo Scientific, Beverly, MA) at 22°C. Aseptic techniques were followed during assembly and sub-sampling of all treatments. All sediment slurry treatments were conducted in triplicate and all subsequent analyses utilized all replicates from each respective treatment.

Once incubations were completed, all replicate sediment slurries were centrifuged at 10,000 g for 10 min, supernatant decanted. MP Biomedicals FastDNA spin kit for soils (MP Biomedicals, Solon, OH) was utilized according to manufacturer’s protocol to extract genomic DNA from 500 mg of homogenized sediment prior to treatment (subsampled in triplicate) as well as pelleted sediment sample prior to treatment were analyzed on 42 separate microarrays. Microarray sample preparation, hybridization, and normalization were performed as previously described [55]. The threshold for identifying an operational taxonomic unit (OTU) present in a sample was a positive fraction (pF) ≥0.9, indicating that over 90% of perfect match probes from the entire probe set of a given OTU were positive. Total richness each for each sample was determined by summation of all OTUs with a pF ≥0.9. The fold change in community richness of a treatment at a given pH was determined by dividing the average richness of the replicate unamended control treatments by the average richness of the replicate amended treatments. Student t-test was performed and p-value of ≤0.05 was used as cutoff for OTUs with significantly increasing or decreasing abundance based on treatment.

Sediment slurry treatments were analyzed by inductively-coupled plasma mass spectrometry (ICP-MS) with a Agilent 7500a Series system. Blanks, calibration check standards (95–105% recovery), and River Water Certified Reference Material for Trace Metals (SLRS-4, National Research Council Canada, Ottawa, Canada) were analyzed for quality controls. The analytical error on replicate samples was <3% relative standard deviation (RSD). Sediments (2 g) were digested in 10 mL of 2% nitric acid (trace metal grade, Fisher Scientific, Pittsburgh, PA) for 1 h at room temperature under constant agitation with a cell mixer (New Brunswick Scientific, Edison, NJ). Samples were filtered through a 0.2 μm polyethersulfone membrane (Millipore, Billerica, MA) and nutrients, metal and radionucleide measurements

Nutrient measurements (nitrate, nitrite, phosphate, G2P, and G3P) were measured with an ICP-2000 ion chromatography system with an AS-DV automated sampler ( Dionex, Sunnyvale, CA) equipped with a degasser, a KOH eluent generator with a continuously regenerating anion trap column, AS11-HC (4×250 mm) anion exchange column, AG11-HC guard column (4×250 mm), ARS 300 4 mm anion regenerating suppressor (164 mA current setting), and Chromelon 6.8 software. A 25 μL sample loop was used for all samples. The KOH eluent delivered at a flow rate of 1.25 mL min⁻¹ as follows: 0–4 min isocratic (10 mM); 5–20 min gradient (10 mM to 45 mM); 20–23 min isocratic (45 mM); 23–24 min gradient (45 mM to 10 mM). The samples were filtered through a 0.2 μm polyethersulfone membrane (Millipore, Billerica, MA) before analyses. Prior to sediment slurry incubations, nitrate was extracted from 2 g sediment with 2 mL water (18.2 MΩ) by constant agitation with a cell mixer (New Brunswick Scientific, Edison, NJ) for 1 h at room temperature. Nitrate concentration was 39 mg kg⁻¹ and nitrite was not detected.

Total dissolved uranium was measured by inductively-coupled plasma mass spectrometry (ICP-MS) with an Agilent 7500a Series system. Blanks, calibration check standards (95–105% recovery), and River Water Certified Reference Material for Trace Metals (SLRS-4, National Research Council Canada, Ottawa, Canada) were analyzed for quality controls. The analytical error on replicate samples was <3% relative standard deviation (RSD). Sediments (2 g) were digested in 10 mL of 2% nitric acid (trace metal grade, Fisher Scientific, Pittsburgh, PA) for 1 h at room temperature under constant agitation with a cell mixer (New Brunswick Scientific, Edison, NJ). Samples were filtered through a 0.2 μm polyethersulfone membrane (Millipore, Billerica, MA) and
diluted in 18.2 MΩ water (Nanopure; Barnstead International, Dubuque, IA). Sediment uranium concentration was 19 mg kg⁻¹.

**Thermodynamic modeling**

Thermodynamic equilibrium modeling of ORFRC groundwater was conducted using MINEQL+ v. 4.5 [82] updated with the Nuclear Energy Agency’s thermodynamic database for uranium [93]. The equilibrium model was developed using the average concentrations of dissolved ions from GW-836 including calcium (4.5 mM), uranium (4.21 μM), carbonate (5 mM), and phosphate (500 μM and 5 mM). GW-836 is the closest groundwater monitoring well proximal to borehole FB107-04-00 (http://public.ornl.gov/oric/history.cfm?Location = ‘GW-836’).

**Supporting Information**

Figure S1 Venn diagram of OTUs enriched in multiple treatments. (A) Archaeal and (B) bacterial OTUs detected in one or more of the organophosphate-amended treatments. Only OTUs that had a 2-fold or greater increase in fluoride for each respective treatment were used for comparisons. (PPTX)

Figure S2 Thermodynamic modeling of P and Ca in the absence of U(VI) as a function of pH. ORFRC Area 2 groundwater concentrations of dissolved ions (GW-836 monitoring well), U(VI) = 4.5 μM, and Ca²⁺ = 4.85 mM were used to model the distribution of PO₄³⁻ species with (A) PO₄³⁻ = 500 μM, (B) PO₄³⁻ = 5 mM as well as the distribution of Ca²⁺ species with (C) PO₄³⁻ = 500 μM and (D) PO₄³⁻ = 5 mM. Dashed lines represent soluble species and solid lines represent insoluble species. (PPTX)

**Table S1** Total archaeal and bacterial OTUs detected in ORFRC sediments prior to treatment and following each of the six treatment conditions. Positive fraction and normalized fluorescence values are reported for each of the triplicate treatments. (XLS)

**Table S2** OTUs with a 2-fold or greater relative increase in fluoride intensity following sediment slurry treatments. (DOC)

**Table S3** OTUs with a 2-fold or greater relative increase in fluoride intensity detected in two or more treatments. (DOC)

**Author Contributions**

Conceived and designed the experiments: RJM CHW MJB MT PAS. Performed the experiments: RJM CHW MJB. Analyzed the data: RJM CHW MJB MT PAS GLA MEC TCH. Contributed reagents/materials/analysis tools: GLA MEC TCH. Wrote the paper: RJM CHW MJB MT PAS.

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