FINAL TECHNICAL REPORT
UNITED STATE DEPARTMENT OF ENERGY
(DE-SC0004113)

Exploratory Project
Viral Infection of Subsurface Microorganisms and Metal/Radionuclide Transport
July 1, 2010 – June 30, 2013

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NOTICE

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Executive Summary

Microbially mediated metabolisms have been identified as a significant factor either directly or indirectly impacting the fate and transport of heavy metal/radionuclide contaminants. To date microorganisms have been isolated from contaminated environments. Examination of annotated finished genome sequences of many of these subsurface isolates from DOE sites, revealed evidence of prior viral infection. To date the role that viruses play influencing microbial mortality and the resulting community structure which directly influences biogeochemical cycling in soils and sedimentary environments remains poorly understood. The objective of this exploratory study was to investigate the ***role of viral infection of subsurface bacteria and the formation of contaminant-bearing viral particles***. This objective was approached by examining the following working hypotheses: (*i*) subsurface microorganisms are susceptible to viral infections by the indigenous subsurface viral community, and (*ii*) viral surfaces will adsorb heavy metals and radionuclides. Our results have addressed basic research needed to accomplish the BER Long Term Measure to provide sufficient scientific understanding such that DOE sites would be able to incorporate coupled physical, chemical and biological processes into decision making for environmental remediation or natural attenuation and long-term stewardship by establishing viral-microbial relationships on the subsequent fate and transport of heavy metals and radionuclides.

Here we demonstrated that viruses play a significant role in microbial mortality and community structure in terrestrial subsurface sedimentary systems. The production of viral-like particles within subsurface sediments in response to biostimulation with dissolved organic carbon and a terminal electron acceptor resulted in the production of viral-like particles. Organic carbon alone did not result in significant viral production and required the addition of a terminal electron acceptor (nitrate), indicating that nutrients are not limiting viral production, but rather substrates that can be converted into energy for host metabolism. Our results also revealed that cell abundance was not correlated to the mineralization of organic carbon, but rather viruses were positively correlated with carbon mineralization. This is a result of viral-mediated cell lysis and demonstrates that viruses are sensitive indicators of microbial activity. Viruses as an indicator of microbial activity was not unique to batch culture studies as results obtained from an *in situ* field experiment conducted at the DOE Old Rifle Field site. This study revealed that viral abundance increased in response to the injection of oxygenated groundwater and influx of dissolved organic carbon whereas cell abundance changes were minimal. However, the extent to which viral-mediated cell lysis alters organic matter pools subsequently influencing microbial community structure and biogeochemical function remains a critical question in subsurface biogeochemical cycling.

The production of significant numbers of viruses in groundwater has implications for nanoparticulate metal as well as carbon transport in groundwater. We have demonstrated that the virus surface is reactive and will adsorb heavy metals. Thus viruses can promote colloidal contaminant mobility. Interestingly, the presence of heavy metals has a positive effect on infectivity of the phage, increasing phage infection which could lead to further production of viruses. Together, the results indicate that the sorption of metals to the surface of viruses could not only contribute to nanoparticulate metal as well as carbon transport but could also enhance infectivity further contributing to cell lysis which could subsequently influence biogeochemical cycling. As more viruses infect host microbial populations the high concentration of metals would enhance infection, resulting in cell lysis, and decreasing the metabolically active host population while yielding greater numbers of viruses capable of transporting contaminats. Additional studies will be necessary to further establish the potential relationship(s) between viruses, cells, carbon, and metals/radionuclides to provide sufficient scientific understanding to incorporate coupled physical, chemical, and biological processes into agent based and reactive transport models.

Viral Infection of Subsurface Microorganisms and Metal/Radionuclide Transport: Weber, Bender and Li (DE-SC0004113)
Technical Report

Microbially mediated metabolisms have been identified as significant factors either directly or indirectly impacting biogeochemical cycling. Bacterial communities are not immune to viral infection. However, the role that viruses play in subsurface microbial community dynamics and biogeochemistry in subsurface systems remains poorly understood. Viral infection could impede metal reduction rates and/or alter the microbial community potentially affecting metal reduction and thus metal mobility. Furthermore, the adsorption of metals/radionuclides on the viral surface could subsequently influence the fate and transport of contaminants. Therefore, efforts to predictably model remediation efforts require an understanding of the role that viral particles have on the fate and transport of contaminant heavy metals/radionuclides. The objective of this exploratory project was to investigate viral infection of subsurface bacteria and the formation of contaminant-bearing viral particles and approached by examining the following hypotheses: (H*i*) **subsurface microorganisms are susceptible to viral infections by the indigenous subsurface viral community**, and (H*ii*) **viral surfaces will adsorb heavy metals and radionuclides**.

This interdisciplinary project was conducted in collaboration between Drs. Karrie A. Weber and Yusong Li, University of Nebraska-Lincoln (UNL) and Dr. Kelly S. Bender at Southern Illinois University, Carbondale, Illinois (SIU). In an effort to link our experimental results to the demonstration of viral production in subsurface sediments and processes occurring *in situ* a collaboration between Dr. Kenneth H. Williams at the Old Rifle site, primary field study site of the Lawrence Berkeley National Laboratory's (LBNL) Science Focus Area (SFA) research program was established in 2012. Dr. Karrie A. Weber is an Assistant Professor in the School of Biological Sciences (joint appointment in Department of Earth and Atmospheric Sciences) with expertise in microbial metabolisms and viruses mediating soil/sedimentary carbon, nitrogen, and metal/radionuclide biogeochemical cycling. She was responsible for project oversight, experimental design, sample collection, basic bacteriophage isolation, adsorption experiments, and geochemical analyses. Dr. Bender is a microbial and viral molecular biologist at SIU and conducted molecular analyses of the viral community. Dr. Li is an Assistant Professor in the Department of Civil Engineering with an expertise in numerically simulating nanoparticle contaminant transport and multiphase flow. She conducted the numerical simulation of heavy metal/radionuclide virus interactions based on data generated in the Weber laboratory. Dr. Kenneth Williams is a geological scientist. He directs and coordinates all research conducted at the Old Rifle field site. His research is focused on constraining subsurface metabolic potential with biological, geochemical, and mineralogical datasets. He was responsible for sample collection and in-field geochemical analyses from an ongoing field experiment in conjunction with the LBNL SFA. In addition to the support of three early career female investigators, this project has supported the research of four graduate students at (1 Ph.D. and 1 M.S. in the School of Biological Sciences, UNL, 1 Ph.D. student Civil Engineering, UNL, and 1 M.S. student in Microbiology, SIU) and four undergraduate students (3 UNL and 1 SIU).

Completion of this project has resulted in one manuscript in review (Pan et al., *in review*) and an additional two manuscripts in preparation for submission (Pan et al., *in prep;* Tan et al., *in prep*). In addition to the annual DOE Subsurface Biogeochemistry PI meeting, results from this research project have been presented at local, regional, national and international conferences including the UNL Biology Graduate Student Symposium, Missouri Valley Branch American Society for Microbiology Meeting, American Society for Microbiology General Meeting, and the International Symposium on Microbial Ecology (16 presentations).

Two of the research projects presented at local, regional, national, and international meetings by one Ph.D. student in the Weber laboratory, Donald Pan, have received multiple awards: second place student poster award UNL SBS Graduate Student Symposium 2011, second place Outstanding Poster Presentation at the Missouri Valley Branch American Society for Microbiology Meeting in Lincoln, NE in 2011, an Outstanding Student Poster Award at the American Society for Microbiology 2012 General Meeting in San Francisco, CA, and a second place Poster Award at the 2013 Water for Food Global Conference, Lincoln, NE.

1.0 PROJECT OBJECTIVE

The objective of this exploratory study is to assess the potential biogeochemical role of viruses in subsurface environments. We proposed to (*i*) identify viruses commonly infecting subsurface microorganisms, specifically nitrate and metal reducing microorganisms, and (*ii*) investigate the capacity of viruses to adsorb contaminant heavy metals and radionuclides. Bacterial communities are not immune to viral infection. However, the role that viruses play in subsurface microbial community dynamics and biogeochemistry in subsurface systems remains poorly understood. Viral infection could impede metal reduction rates and/or alter the microbial community potentially affecting metal reduction and thus metal mobility. Furthermore, the adsorption of metals/radionuclides on the viral surface could subsequently influence the fate and transport of contaminants. Therefore, efforts to predictably model remediation efforts require an understanding of the role that viral particles have on the fate and transport of contaminant heavy metals/radionuclides. Here we outline research to investigate hypotheses addressing the basic research needed to determine the potential biogeochemical role of viruses in contaminated subsurface environments.

2.0 SUMMARY OF HYPOTHESES AND APPROACH

H*i*. *Subsurface microorganisms are susceptible to viral infections by the indigenous subsurface viral community.* Enrichments for viruses capable of infecting nitrate bacteria were initiated using subsurface environmental samples and a host nitrate or metal-reducing bacterium. In addition to cultivation of bacteriophage, a cultivation independent approach utilizing ^{13}C-acetate incorporation into the nucleic acids content (Stable Isotope Probing, SIP) of the microbial community and the infective viral community was conducted to identify infectious subsurface viruses.

H*ii*. *Viral surfaces will adsorb heavy metals and radionuclides.* Adsorption of a contaminant radionuclide or metal, ie. uranium, to the viral surface was tested in batch culture via chemical analyses. Experimental data was used as input parameters for the development of a numerical model and will serve as guide experimental design of future investigations.

3.0 BACKGROUND

The waste disposal practices employed during the processing of nuclear fuels has resulted in high heavy metal and radionuclide concentrations (including uranium, U) in the environment often with nitrate as a co-contaminant. In an oxidizing carbonate system above pH 5, U predominantly exists as soluble U(VI) complexes, such as $UO_2CO_3°$, $UO_2(CO_3)_2^{2-}$, or $UO_2(CO)_3^{4-}$, rendering U as a mobile contaminant(Brooks, 2001; Langmuir, 1997; Waite et al., 1994). Not only does the soluble nature of U(VI) promote mobility in the subsurface, but recent research has demonstrated the transport of actinides, including uranium, in

association with submicron organic colloids (Novikov et al., 2006). Microorganisms have also been identified as a factor that can either directly or indirectly impact the fate and transport of the heavy metal and radionuclide contaminants in soils and sedimentary environments. As such, much effort has been placed into understanding the microbiology, metabolisms, and microbe-mineral interactions influencing contaminant mobility. A diversity of microorganisms persists in these contaminated terrestrial soils and subsurface environments. To date, several microorganisms representing ubiquitous genera such as *Geobacter* (Holmes et al., 2007; Holmes et al., 2002; Istok et al., 2004), *Anaeromyxobacter* (North et al., 2004), *Desulfovibrio* (Wu et al., 2006), *Diaphorobacter* (Weber), and *Streptomyces* (Weber) have been isolated from these U contaminated environments. Examination of the completed genome sequences of these environmental isolates, *G. uraniumreducens* Rf4, *Geobacter* sp. FRC-32, and *Anaeromyxobacter* sp. Fw109-5, has revealed sequences for phage related proteins. In addition to these subsurface isolates, completed genome sequences of other model metal reducing bacteria, *G. metallireducens*, *G. sulfurreducens*, *A. dehalogenans*, and *Desulfovibrio vulgaris*, have also revealed several phage related sequences. Presence of these gene sequences indicates that subsurface bacteria are susceptible to viral infection. This is further supported with the cultivation of a temperate bacteriophage from *D. vulgaris* ssp. *vulgaris* strain Hildenborough (Walker et al., 2006).

Viruses are submicron (20 - >200nm) particles consisting of single or double stranded DNA or RNA encapsulated by a protein coat (capsid) (Calander, 2006). Their growth and replication is dependent on the host cell and can often result in disease or death. Viruses have been estimated to be the most abundant biological entities; outnumbering all other organisms on Earth (Chibani-Chennoufi et al., 2004; Suttle, 2007; Weinbauer, 2004). The viruses infecting bacteria are referred to as bacteriophage, 'eaters of bacteria'(Weinbauer, 2004). It is the predatory nature of bacteriophage and their life cycles, lytic, lysogenic, psuedolysogenic, and chronic infection that can directly impact microbial communities (Weinbauer, 2004). Briefly, after infection (adsorption and entry into host cell) the bacteriophage nucleic acids are replicated and packaged subsequently lysing the host cell in the lytic cycle (2006). The lysogenic life cycle differs from the lytic cycle, as the bacteriophage incorporates its genome into the host chromosome, remains dormant, and becomes active only when induced (2006). Chronic infection occurs when the phage does not lyse the cell but rather is released from the cell via budding or extrusion (Weinbauer, 2004). In psuedolysogenic infections, bacteriophages multiply in a fraction of the population (2006).

Recent research focused in marine and freshwater pelagic ecosystems has recognized that viral communities play a significant role in the environment: controlling food webs and biogeochemical cycles (carbon, nitrogen, and phosphorus cycling), causing bacterial mortality, increasing microbial diversity by "killing the winner", and increasing microbial genetic diversity through horizontal gene transfer(Fuhrman, 1999; Suttle, 2007; Weinbauer, 2004; Wommack and Colwell, 2000). While a relationship between viruses and microbial communities in marine and freshwater pelagic systems has been established, the relationship between viruses and bacterial hosts in soils and sedimentary environments remains poorly understood(Kimura et al., 2008).

Viral like particles (VLPs) in soils and sedimentary environments have been demonstrated to be in the order of 6.4×10^{6}–2.7×10^{10} VLPs cm^{-3} (Ashelford et al., 2003; Danovaro et al., 2001; Danovaro et al., 2002a; Hewson et al., 2001a; Kyle et al., 2008b; Maranger and Bird, 1996; Williamson et al., 2005). A preliminary investigation of groundwater collected from the ORIFC site indicates that VLPs are present (Fig. 1). This is consistent with previous reports demonstrating virus occurrence transport in groundwater (Abbaszadegan et al., 2003; Drewry and Eliassen, 1968; Gerba, 1983; Ryan et al., 1999; Yates et al., 1985). Organic matter has been demonstrated to directly promote transport of

viral particles by inhibiting adsorption of viruses to the soil/sediment matrix (Gerba, 1984; Gerba et al., 1975). Another factor controlling viral transport is reactivity with solid surfaces such as Fe(III) oxides. A prior study demonstrated that the reductive dissolution of Fe(III) oxides results in the release and subsequent transport of viral particles (Ryan et al., 1999), as such organic carbon oxidation coupled to dissimilatory Fe(III) reduction would mobilize viral particles as the iron was microbially dissolved (Weber et al., 2006). Thus anthropogenic (organic carbon injections in bioremediation applications), allochthonous (organic carbon transported in a watershed and through the vadose zone), or autochthonous (lithoautotrophic metabolisms) organic carbon inputs could result in the direct or indirect mobilization of both infectious and non-infectious viral particles.

Biosorption of metal/radionuclide ions to bacterial and eukaryotic cell surfaces is a well-recognized process. A variety of organic molecules associated with biological surfaces such as carboxyl and amino acid groups, are responsible for binding these heavy metals/radionuclides (Beveridge and Murray, 1980; Fein, 2006). Similarly, viral capsids are protein (amino acid) structures that may display binding affinity for heavy metals/radionuclides. In fact, heavy metals and a radionuclide (uranium) have been used for decades as viral stains in transmission electron microscopic imaging (Arnott and Smith, 1968; Milne and de Zoeten, 1968). Thus, abundant and mobile viruses in a subsurface environment could promote contaminant mobility. Novikov and colleagues (Novikov et al., 2006) recently reported the transport of actinides, plutonium and uranium, associated with unknown Fe-organic submicron colloids (Novikov et al., 2006). The nature of the unknown Fe-organic submicron colloids could be viral particles, further emphasizing the necessity to understand the role that viruses may play in contaminant-viral-colloid formation and contaminant mobility.

Viral infection of indigenous subsurface bacteria, i.e. *Geobacter spp.*, *Anaeromyxobacter spp.*, and *Desulfovibrio spp.*, the presence of viral communities in soil and sedimentary environments including DOE research sites, and the potential for contaminant-viral-colloid formation, presents significant issues related to natural attenuation and/or bioremediation and the fate and transport of contaminants that have not been previously addressed. Some of these significant issues include but are not limited to:

1) Viral infection of the indigenous subsurface microbial community could influence metabolically active microorganisms, i.e. metal reduction, responsible for the mediation of contaminant mobility. This may not only alter microbial community structure but may also alter metabolic oxidation and reduction rates.
2) Adsorption and precipitation of metal/radionuclide and metal/radionuclide-bearing minerals on the viral surface could result in the formation of heavy metal/radionuclide-viral colloids transported through a porous matrix.

It is therefore necessary to identify the potential relationships between viruses and the indigenous subsurface microbial community, which could ultimately affect the fate and transport of heavy metals/radionuclides in contaminated environments. To date nothing is known about microbial and viral interactions in such environments. The research outlined in the following hypotheses will address basic research needed to establish metal-viral-microbial relationships in subsurface environments. Regardless of whether or not these hypotheses are supported, the data obtained will provide valuable information in beginning to understand the relationship between viruses and microorganisms and the potential fate and transport of contaminants in environments in which natural attenuation or bioremediation approaches are operational.

4.0 EXPERIMENTAL RESULTS

H*i*. Subsurface microorganisms are susceptible to viral infections by the indigenous subsurface viral community.

Microbial life in the terrestrial subsurface has been estimated to represent one-third of all microorganisms on Earth, with an estimated 22-215 Pg of C (Whitman et al., 1998). While such a significant proportion of life on Earth survives in these subsurface environments, we know very little about the interactions between the microorganisms, specifically the Bacteria and viruses that survive in this environment. Viruses have been estimated to be the most abundant biological entities on Earth with abundances typically an order of magnitude greater than cells (Srinivasiah et al., 2008). This is consistent with recent observations of viruses in shallow and deep subsurface environments ranging in abundance from 10^5/mL to 10^7/mL, (Eydal et al., 2009; Kyle et al., 2008a; Roudnew et al., 2012).

Viruses are submicron (20 - >200nm) particles consisting of single or double stranded DNA or RNA encapsulated by a protein coat (capsid) (Calander, 2006). Their growth and replication is dependent on the host cell and can often result in disease or death. Viruses have been estimated to be the most abundant biological entities; outnumbering all other organisms on Earth (Chibani-Chennoufi et al., 2004; Suttle, 2007; Weinbauer, 2004). The viruses infecting bacteria are referred to as bacteriophage, 'eaters of bacteria' (Weinbauer, 2004). It is the predatory nature of bacteriophage and their life cycles, lytic, lysogenic, psuedolysogenic, and chronic infection that can directly impact microbial communities (Weinbauer, 2004). Briefly, after infection (adsorption and entry into host cell) the bacteriophage nucleic acids are replicated and packaged subsequently lysing the host cell in the lytic cycle (2006). The lysogenic life cycle differs from the lytic cycle, as the bacteriophage incorporates its genome into the host chromosome, remains dormant, and becomes active only when induced (2006). Chronic infection occurs when the phage does not lyse the cell but rather is released from the cell via budding or extrusion (Weinbauer, 2004). In psuedolysogenic infections, bacteriophages multiply in a fraction of the population (2006). As such, viral infection results in significant bacterial mortality directly impacting the ecology and biogeochemistry of environmental systems (Furhman 1999; Suttle 2007). Thus leading to our hypothesis that *subsurface microorganisms are susceptible to viral infections by the indigenous subsurface viral community*. To test this hypothesis we combined (*a*) traditional bacteriophage enumeration and cultivation techniques with (*b*) a cultivation independent approach employing the addition of ^{13}C-aceate and use of molecular biological techniques in order to identify viruses infecting subsurface microorganisms, specifically nitrate reducers. Experiments were conducted in the laboratory in microcosms and *in situ*.

a. Batch culture stimulation of viral community (Pan et al., in review)

The addition of ^{13}C-labeled acetate and nitrate into subsurface sediment slurries containing collected from an alluvial aquifer in Nebraska bearing naturally occurring uranium (ca. 2000ppm) and groundwater contaminated with anthropogenic nitrate (30 ppb). The ^{13}C-acetate resulted in the production of cells and viral-like particles (VLPs). Cell and VLP production was concomitant with nitrate reduction and acetate oxidation in triplicate subsurface sediment slurries (25% w/v; 10mM acetate 10mM nitrate) (Fig. 1a,b,c). Bacterial cells increased to 5.7 x 10^5 cells mL^{-1} sediment slurry within 2 days of incubation in response to acetate oxidation and nitrate reduction to nitrite (Fig.1d). Increase in bacterial abundance was initially parallel with an increase in VLP abundance of 4.6x10^7 VLP mL^{-1} (Fig. 1e). However, fluctuations of the VLP to bacterial cell ratio (VCR)(Fig. 1f) were observed over the course of the study. The mean VCR increased from 0.33 to 5400 during this period (Fig. 1f), but variability in the lag phase and response between sediment slurry replicates lead to a high standard error measurement (±5200 (SE)). Bacterial abundance decreased by 4.8 x 10^5 cells mL^{-1} sediment slurry following an initial peak in viral abundance (Fig. 1d). While this decline may have been a result of cell lysis due to viral infection, it should be noted that acetate consumption ceased with the depletion of nitrate. During the 24 hour period between the exhaustion of nitrate and the start of nitrite reduction/acetate oxidation, VLP abundance rapidly decreased (Fig. 1e). Interestingly, resumption of acetate and nitrite consumption did not result in an increase in bacterial abundance but did result in VLP production (4.6 x 10^7 VLPs mL^{-1} sediment slurry) (Fig. 1a,b,d). While N_2 and N_2O were not measured in this study, it is assumed that N_2 or N_2O were end-products as nitrite was reduced and no significant ammonium production was observed over the course of the experiment (data not

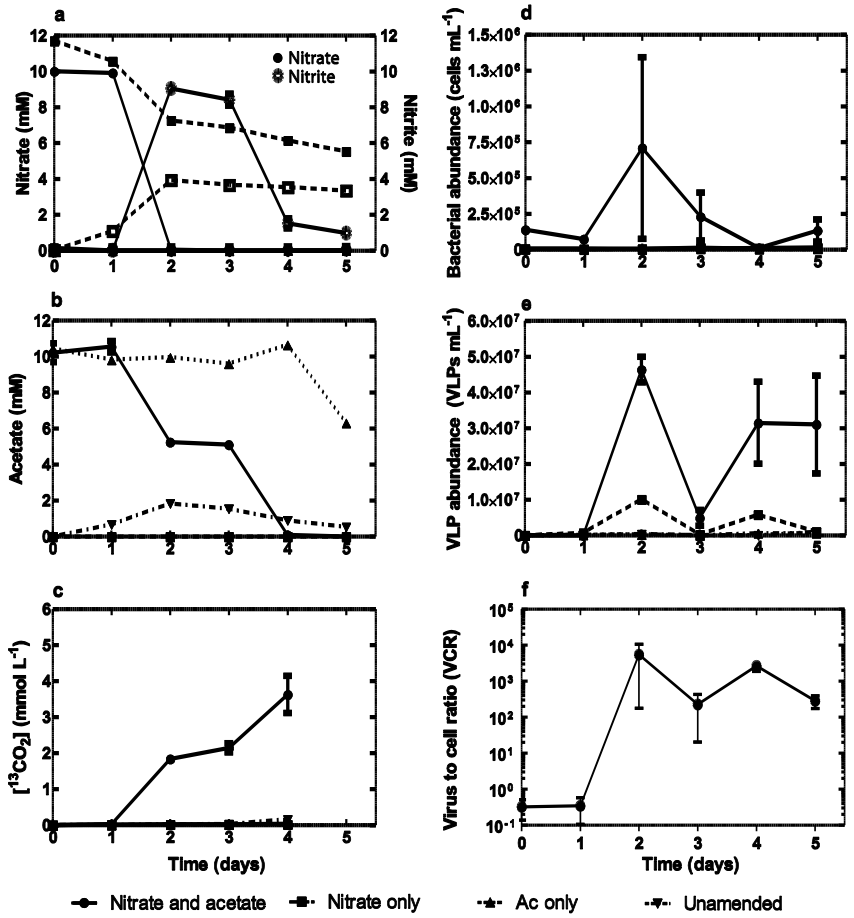

Fig. 1. Nitrate and subsequent nitrite reduction as well as the production of VLPs occurred following inoculation (25% w/v) of subsurface sediment. Experimental treatments conducted in triplicate were amended with 10mM ^{13}C-acetate and 10mM nitrate and compared to control treatments amended with 10mM nitrate only, 10mM ^{13}C-acetate only, or without any electron donor or acceptor. **a.)** Changes in nitrate and nitrite, **b.)** acetate concentration, **c.)** $^{13}CO_2$ production , **d.)** cell abundance, VLP abundance, and virus to cell ratio (VCR). Symbols represent the average of treatments. Error bars denote standard error of measure.

shown). Sulfate concentrations remained constant over the course of the study, indicating that sulfate reduction did not occur in the experimental treatment. Methane was not detected in the headspace of slurry incubations.

Slurries in which acetate or both acetate and nitrate were omitted did not result in significant increases in VLP abundance or increases in bacterial cell abundance (Fig. 1d,e). However, naturally occurring organic carbon did stimulate nitrate reduction (4.4mM decrease) to nitrite (3.9mM increase) (Fig. 1a) in controls in which acetate was omitted. Oxidation of naturally occurring organic carbon is supported by the production of $^{12}CO_2$ (data not shown). This resulted in a slight increase in both cell (9.7×10^3 cells mL^{-1} sediment slurry) and VLP abundance (9.4×10^6 VLP mL^{-1} sediment slurry, Fig.A1d). Nitrate (3.9mM) was reduced in cultures in which acetate was omitted indicating that at least 0.98 milli reducing equivalents L^{-1} sediment slurry contributed to this process. VLP production was not significant in acetate amended/nitrate omitted cultures due the unavailability of an electron acceptor.

Analysis of the production of VLPs was positively correlated with acetate consumption (r^2=0.6252, p=0.0004) and $^{13}CO_2$ production (r^2=0.6572, p=0.0014; Fig.2a,b) in each replicate slurry amended with acetate and nitrate. Interestingly, changes in bacterial abundance were not correlated to acetate consumption (r^2=0.07023, p=0.3398) nor $^{13}CO_2$ production (r^2=0.07400, p=0.3924; Fig. 2c,d). No significant $^{13}CO_2$ production was observed when unlabeled acetate was added or acetate was omitted from sediment slurries, further supporting uptake of the ^{13}C-acetate and respiration of cell material rather than the indigenous ^{13}C-organic carbon. These data reflect that periods of viral production during the incubation are associated with periods of high carbon mineralization within the sediment slurry, while periods of low carbon transformation are associated with decreases in viral abundance. The same observation is not reflected in bacterial abundance due to the likely consequence of viral induced cell lysis.

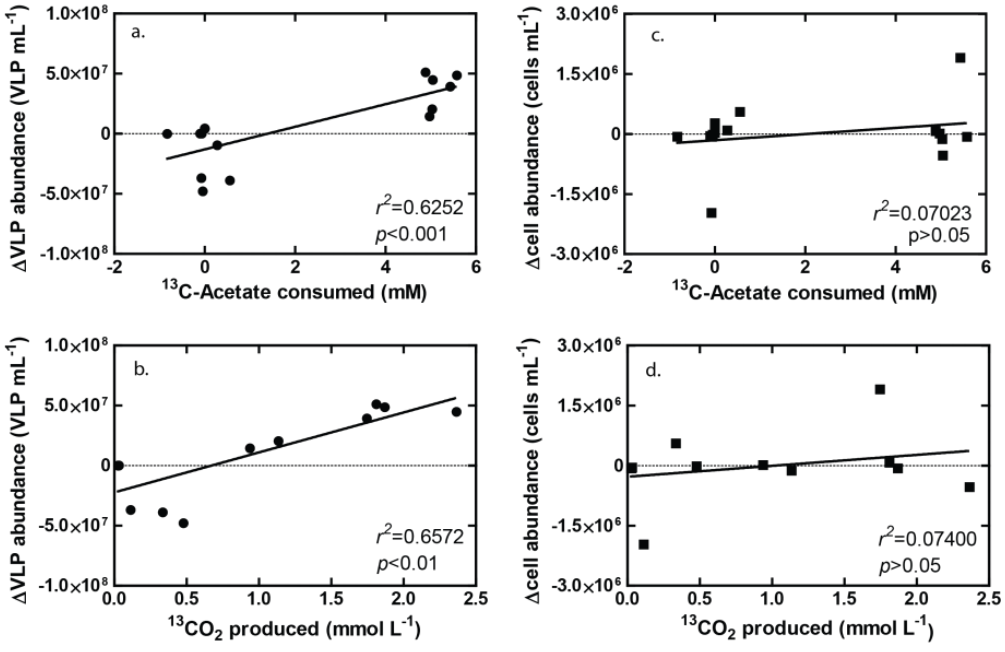

Fig. 2. Linear regression of changes in VLP abundance and acetate consumed (r^2=0.6252, P=0.0004) to acetate b.) Linear regression of changes in VLP production and CO_2 produced (r^2=0.6572, P=0.0014) c.) Linear regression of changes in prokaryotic cell abundance and acetate consumed. Correlation is not significant (P=0.3398). d.) Linear regression of changes in prokaryotic cell abundance and CO_2 produced. Correlation is not significant (P=0.3924).

Bacterial community composition over the course of the study was followed using culture independent techniques specifically the V5 hypervariable region of the 16S rRNA gene was amplified and sequenced via Illumina HiSeq2000 analyzer as previously described (Lazarevic et al., 2009). Replicate samples of the indigenous shallow subsurface sediment bacterial community was dominated by the Betaproteobacteria (62% of paired-end reads, Fig.3a), specifically unclassified members of the Oxalobacteraceae (57%) and Commonadacae, *Curvibacter* (2%) (data not shown). After the first day of incubation, the slurry bacterial community composition shifted to a community dominated by the Gammaproteobacteria (74% of paired-end reads, Fig. 3a), specifically *Pseudomonas* spp. (69%). *Pseudomonas* represented 11% of the microbial community at the beginning of the experiment. Although the Gammaproteobacteria were abundant, a transient increase (12%) in members of the Clostridia (*Desulfotomaculum* sp., 9%; *Acetobacterium* sp. 2%; *Desulfosporosinus*, 1%; Table 1) were also observed. However, after this initial increase after 3 days of incubation, members of the Clostridia decrease in relative abundance while members of the Gammaproteobacteria continue to dominate and increase in relative abundance (Fig. 3a). By day 3, *Pseudomonas* spp. accounted for over 82% of all sequences in the bacterial community. The relative abundance of the *Pseudomonas* spp. continued to increase and represented 88% of the microbial community by the end of the experiment (Fig. 3a). The relative abundance of the Betaproteobacteria decreased representing only 1% of the microbial community at the end of the experiment. The most abundant organisms resulting from the enrichment were cloned and sequenced confirming dominant taxa resulting from the incubation as *Pseudomonas* spp. Of the 48 clones sequenced, only 2 non *Pseudomonas* spp. clones were present, both classified as *Dechloromonas* spp. (data not shown).

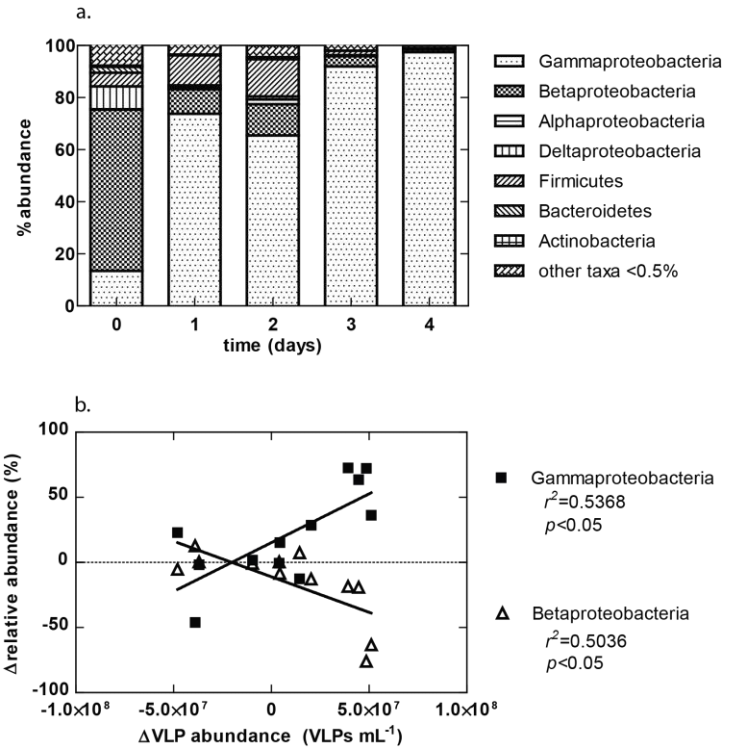

Fig. 3. a) Taxonomic composition of bacteria within the enrichment. Enrichments were performed in triplicate. Abundances were averaged across all three replicates. b**.)** Gammaproteobacteria and Betaproteobacteria follow VLP abundances with a lag of 1 day (r^2=0.5368 and r^2=0.5036; *P*=0.0067 and *P*=0.0097, respectively).

Rarefaction curves of the bacterial communities at the beginning of the experiment did not reach an asymptote, suggesting that the identity of all community members was not recovered (data not shown). However, as few as 33 OTUs were sufficient to describe over 90% of the microbial community for all samples. OTUs that composed fewer than 0.5% of the bacterial community (181-189 taxa) represented only 6.7% of the microbial community. It should be noted that we are concerned with major changes to community structure and

composition, not rare members. The bacterial communities had a higher richness (by Chao1 estimator) than the final enriched bacterial community (data not shown).

The change in bacterial community structure is correlated to the change in the abundance of VLPs. Changes in VLP abundance are correlated to changes in the relative abundance of Gammaproteobacteria (r^2=0.5368, P=0.0067) (Fig. 3b), while changes in VLP abundance are negatively correlated to changes in Betaproteobacteria with a 1 day delay (r^2=0.5036, P=0.0097) (Fig. 3b). Without the 1 day lag in response, VLP abundance is not correlated to Betaproteobacteria (P=0.41) and Gammaproteobacteria (P=0.14). No statistically significant correlation to VLP abundance was observed for the other groups. One *Pseudomonas* OTU was the only Gammaproteobacteria to be correlated to changes in viral abundance (r^2=0.3551, P=0.041). Initially, the most abundant OTU (ca. 3-66% of all sequences) was classified as an unclassified Oxalobacteraceae. This OTU was negatively correlated to changes in viral abundance (r^2=0.3664, P=0.037). Two other Betaproteobacteria OTUs, both also classified as unclassified Oxalobacteraceae, were also correlated to changes in viral abundance (r^2=0.4201, P=0.023) (r^2=0.3341, P=0.049).

Samples were collected for the analyses of the viral communtiy using culture independent methods based on methods derived at the start of the project. We had initially proposed to follow the viral community using a Stable-Isotope-Probing technique. While preliminary measurements made on the collected VLPs demonstrated that viruses were enriched in ^{13}C-carbon, the concentration of DNA extracted was not sufficient from the large samples collected in order to conduct SIP. This method poses a challenge to batch culture studies conducted in the laboratory but could be amenable to large volume (<20L) or field scale experiments. We additionally tried an alternative to the SIP approach using a RAPD-PCR technique in order to fingerprint the viral community. RAPD-PCR (Winget and Wommack, 2008) was selected as an alternative approach to produce molecular 'viral fingerprints' from experiments using different electron acceptors and induced and un-induced conditions. This methodology also provides amplicons for characterization of the infective viruses through DNA sequence analysis. The DNA extract from a 15 g sediment sample only resulted in the 26 ng of DNA. Unfortunately a RAPD-PCR profile was never obtained despite the use of fluctuating concentrations of $MgCl_2$, different polymerases, numerous random primers, and various reagents to circumvent PCR inhibitors that may have remained in the DNA sample. The DNA sample was also concentrated using Amicon 100K filters, but no amplicons were ever produced, thus suggesting larger initial sediment samples may be necessary to yield the adequate amount of template DNA for RAPD-PCR analysis. Subsequent to the analyses additional studies using culture-dependent techniques suggest that ssDNA phage may have also been important in this viral community (described below). While the primers would have amplified the ssDNA within the extract, visualization of the ssDNA product on the agarose gels would have required the use of SYBR Gold or another stain designed for ssDNA as well as dsDNA instead of the ethidium bromide used in our prior analyses. In the future, studies investigating viral communities should take into consideration not only dsDNA phage, but also ssDNA and possibly RNA phage when working with communities of unknown viral community composition, especially during an enrichment process.

In an effort to begin to look at the viral community using culture-dependent techniques we initiated the production of VLPs using a chemical known to induce lysogens and subsequently isolate VLPs using trational plaque assays. Production of VLPs (3.1×10^7 VLP mL^{-1} sediment slurry) resulted from the addition of mitomycin C after pre-incubation of the slurries with nitrate (final concentration 10 mM) and acetate (6.25mM) for 48 hours (Fig. 4a). Cell abundances following mitomycin C induction did not change compared to an uninduced control (Fig. 4b). When mitomycin C was omitted, triplicate pre-incubated sediment slurries did not result in a change in VLP abundance, indicating that the indigenous

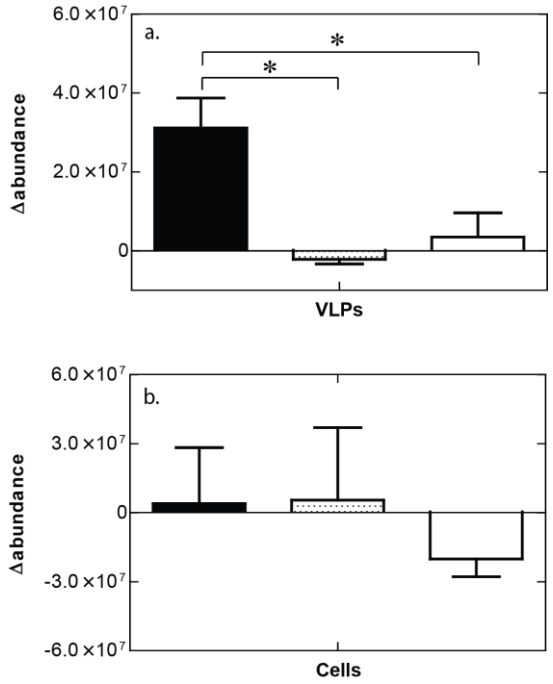

Fig. 4. **a.)** Increase in VLP abundance following induction of sediment slurry with 1.0µg/mL mitomycin C (■) compared to control replicates without mitomycin C (▭) and control replicates without nitrate and acetate (□). **b)** Changes in prokaryotic cell abundances following mitomycin C induction. * represents a treatment that is significantly different. Bars represent results from triplicate cultures. Error bars denote standard error of measure.

nitrate reducing cells harbor inducible prophage. Furthermore, results demonstrate that mitomycin C alone was not sufficient to induce viral production, as pre-incubation with acetate and nitrate was also necessary to observe significant production of VLPs, suggesting that viral production require metabolic stimulation of the host community. Sediment slurries containing acetate and nitrate, but not pre-incubated for 48 hours prior to mitomycin C induction, had high variation in viral production which was not significantly different from a control (data not shown).

In an effort to specifically identify a virus that may have been infecting a predominant nitrate reducing bacterium isolated from these sediments plaque assays were initiated. The 16S rRNA gene sequence of Alda10, the bacterium isolated from nitrate reducing MPN enrichments (data not shown), was classified as a *Pseudomonas* species by the GreenGenes classifier. *Pseudomonas* sp. Alda10. Alda10 has a 98% 16S rRNA gene sequence identity with the *Pseudomonas stutzeri* clones identified in this study. No plaques were visible. However, when treated with mytomycin C (1.0µg/mL), the production of VLPs (an increase of 1.9 x 10^6 VLPs/mL) was also observed. Cultures in which mitomycin C was omitted produced substantially lower numbers of VLPs (5.1 x 10^4 VLPs/mL culture) (Fig. 4a). This suggested the presence of a lysogenic phage. Plaque assays were then set up using other *Pseudomonas* spp. closely related to Alda10 and similarly did not result in the production of plaque forming units. Chloroform lysis of cells grown under similar conditions without mitomycin C resulted in VLP production similar to culture amended with mytomycin C (data not shown); thus indicating that mitomycin C is not inducing a lysogen but rather increasing the release of VLPs from the cell. Upon examination by transmission electron microscopy, mitomycin C treated cell-free cultures contained putative VLPs morphologically similar to filamentous bacteriophage (Fig.5b), thus suggesting that *Pseudomonas* sp. Alda 10 harbors a chronic bacteriophage infection that may not necessarily lyse the cells. The nitrate-reducing *Pseudomonas* sp. Alda10 isolated in this study is infected with a filamentous bacteriophage and the predominance of *Pseudomonas* sp. at the conclusion (88%) may be a result of infection with the filamentous phage. Thus infection with a filamentous phage may confer a competitive advantage to the host as cell lysis would not necessarily occur (Berngruber et al., 2010; Cheng et al., 1999; Kimsey and Waldor, 2004).

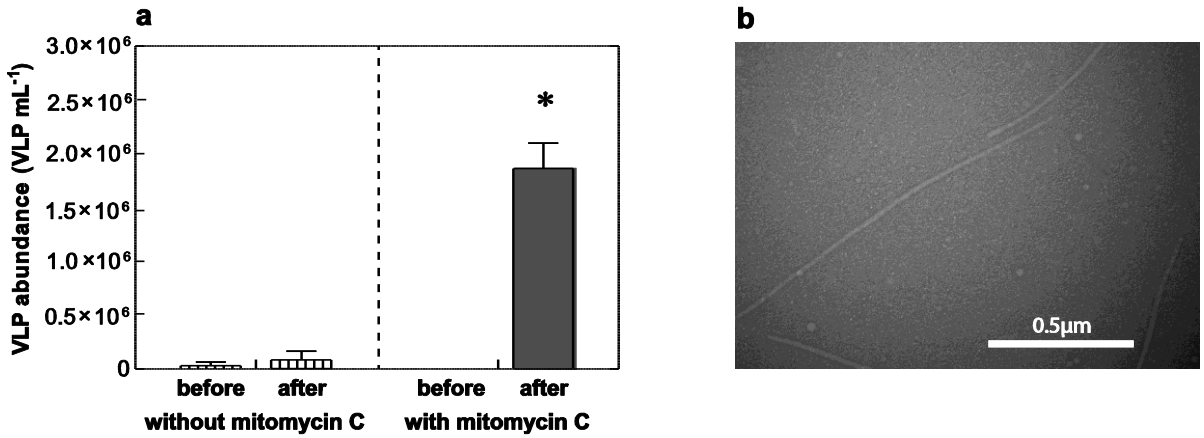

Fig. 5. Mitomycin C amendment (1.0µg/mL) of isolate *Pseudomonas* sp. Alda10 resulted in (**a**) enhanced VLP production by compared to control cultures without mitomycin C. The * indicates statistical significance (two-tailed unpaired t-test $P<0.05$). Columns represent average of triplicates and error bars denote standard error of measure. (**b**) Transmission electron micrograph of *Pseudomonas* sp Alda10 supernatant revealing VLPs with a filamentous morphology.

Summary batch culture experiments. Here we have demonstrated that viruses play a significant role in microbial mortality and community structure in terrestrial subsurface sedimentary systems. The production of VLPs within an indigenous shallow subsurface microbial community was stimulated with the addition of nitrate and ^{13}C-labeled acetate. Interestingly, this stimulation led to VLP production positively correlated with oxidation of dissolved organic carbon (^{13}C-labeled acetate) and $^{13}CO_2$ production; whereas changes in bacterial abundance were not correlated with acetate consumption nor $^{13}CO_2$ production. These results indicate that viruses are contributing to microbial cell lysis. Lysis in the subsurface not only can influence community structure from the top-down but also has implications for bottom up control of populations by releasing organic carbon substrates that can be utilized by other members of the microbial community (Lennon and Martiny, 2008; Middelboe et al., 2003; Noble and Fuhrman, 1999). In contrast to surface environments, subsurface microbial communities frequently experience energy limitations that directly impact cell survival and maintenance. Carbon in subsurface environments may be highly recalcitrant (Stevens, 1997), thus viral induced microbial cell lysis could release labile dissolved organic carbon and particulate organic carbon (Middelboe and Jørgensen, 2006). The release of labile carbon from infected cells may relieve carbon limitation and stimulate metabolism. This is especially important within subsurface environments where microbial communities are carbon and energy limited. In deep sea benthic sediments, release of 0.37-0.63 Gt C / year from lysed cells is estimated to occur, contributing to 35% of total benthic prokaryotic metabolism (Danovaro et al., 2008). Based on the observation of viral production following stimulation of the host community, it would not be unreasonable to infer that viral induced cell mortality would have a similar role in terrestrial subsurface sediments.

The production of viral-like particles in response to carbon and nitrate addition is consistent with previous studies demonstrating that amendments of organic carbon and/or inorganic nutrients stimulated viral production in pelagic systems (Hewson et al., 2001b; Motegi and Nagata, 2007; Noble and Fuhrman, 1999; Tuomi et al., 1995; Weinbauer et al., 2003). However, in our studies organic carbon alone did not result in significant viral production and required the addition of a terminal electron acceptor (nitrate). This would indicate that nutrients are not limiting viral production, but rather substrates that can be

converted into energy for host metabolism. These results are consistent with *in situ* observations reporting higher viral abundance in productive pelagic environments whereas oligotrophic environments report lower viral abundance (De Corte et al., 2012; Hewson et al., 2001b; Weinbauer et al., 1993). Within deep sea sediments, viral abundance was the lowest where low bacterial growth rates were observed, leading Danovaro and colleagues to hypothesize that growth may play a role in limiting virus development (Danovaro et al., 2002b). Bacterial growth as a factor controlling virus development is supported by experiments within model bacteriophage. When *E.coli* host cells are grown in a nutrient-rich complex medium relative to cells grown in a nutrient-limited defined medium, production of virions is greater (Clark et al., 1986). This result is dependent on levels of cAMP, an indicator of host cell energy conditions (Rolfe et al., 1973), demonstrating that viruses may be highly sensitive to the energy availability to the host.

Viral production was correlated to the decrease of specific microbial taxa, (Betaproteobacteria), inferring that cell lysis could be controlling taxa from the top-down as influenced by predator-prey dynamics occurring between viruses and microbial community members. However, microbial community structure may also have shifted in response to changes in organic matter pools as a result of viral-mediated cell lysis, in effect altering microbial community structure from the bottom-up. This study did not attempt to distinguish between top-down and bottom-up controls. Associations between total viral abundance and certain OTUs have been observed in microbial association network studies in natural, unmanipulated marine systems using local similarity analysis (Fuhrman and Steele, 2008; Ruan et al., 2006; Steele et al., 2011), but viral abundances were relatively invariant. By manipulating our experimental system with acetate and nitrate, we induced viral production and thus discovered significant correlations with changes in microbial community structure. This effect would be smaller in unmanipulated natural conditions closer to steady-state conditions. Although our use of 16S Illumina tags are not likely to completely capture the strain and subspecies level dynamics predicted by the "kill the winner" hypothesis, they are sufficient to observe general associations. In a natural environment with more evenness in the microbial community, high resolution methods delineating species or subspecies will be necessary to observe associations with viral production.

Interestingly, positive correlation between change in the relative abundance of Gammaproteobacteria and change in VLP abundance is likely a result of infection by a filamentous virus, which do not typically lyse hosts but are rather extruded from the cell without significant cell lysis (Bradley, 1973; Stanisich, 1974). The nitrate-reducing *Pseudomonas* sp. Alda10 isolated in this study is infected with a filamentous bacteriophage and the predominance of *Pseudomonas* sp. at the conclusion (88%) may be a result of infection with the filamentous phage. Thus infection with a filamentous phage may confer a competitive advantage to the host as cell lysis would not necessarily occur (Berngruber et al., 2010; Cheng et al., 1999; Kimsey and Waldor, 2004).

Together, these results indicate a link between viral abundance and carbon biogeochemistry in the terrestrial subsurface. VLP productivity was significantly correlated to acetate consumption and CO_2 production whereas cell abundance was not, which was likely due to viral-mediated cell lysis. As environmental conditions (availability of electron acceptors) alter host metabolic activity and growth, those changes are reflected in the virus to cell ratio (VCR). Furthermore, the consequences of viral lysis on cell abundance and specific microbial populations may also explain why cell abundance in a metaanalysis of different biogeochemical processes failed to predict microbially mediated biogeochemical fluxes (Röling, 2007). Thus, these rapid changes in VCR can potentially serve as indicators of microbial activity in environmental systems when cell abundance fails. Viral-mediated cell lysis also influences bacterial community structure within a short period of time (~24 hours).

Thus, viral lysis may cause misleading interpretations of community dynamics monitored in the laboratory or field when sampling is infrequent. Viral productivity may be especially important in environments where there are fluctuating conditions such as redox boundary zones or in environments where bioremediation by biostimulation or bioaugmentation is performed. However, the extent to which viral-mediated cell lysis alters organic matter pools subsequently influencing microbial community structure and function from the bottom up remains a critical question in subsurface microbial ecology and carbon dynamics. Further studies are necessary to establish these relationships as well as relationship between the indigenous microbial and viral community *in situ*.

b. In situ stimulation of viral community (Pan et al., in prep)

Our prior research conducted in batch cultures demonstrated that viruses within the subsurface have the potential influence microbial community structure and biogeochemistry (Pan et al., *in review*). Furthermore, viral abundance was a better predictor of metabolic activity than cell abundance alone (Pan et al., *in review*). The dynamics of viral abundance, cell abundance and biogeochemistry *in situ* in an alluvial aquifer at the Old Rifle site (Fig. 6), primary field study site of the Lawrence Berkeley National Laboratory's (LBNL) Science Focus Area (SFA) research program was investigated in 2012. Groundwater was pumped out of the aquifer and sparged with molecular oxygen (O_2) and reinjected into the experimental well-field, Exp. Plot C (Fig. 6), which had been stimulated in 2010 with acetate.

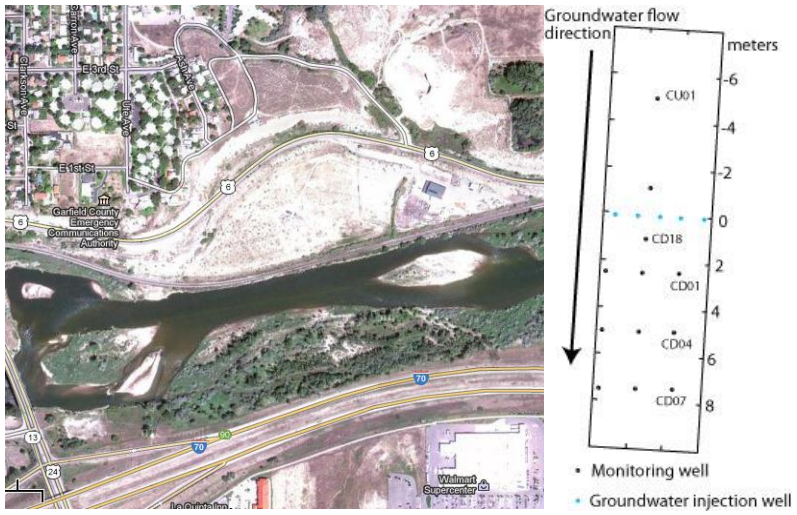

Fig 6. Layout of experimental plot at Old Rifle. CU01 denotes the upstream (background monitoring well) and CD01 denotes the monitoring well receiving stimulant.

Samples collected for the enumeration of viruses and bacteria were obtained from groundwater. Bacteria (and large viruses) greater than 0.1μm were passed through a 0.45μm filter, placed on ice and immediately taken into the lab on site for processing as described below. Viruses were functionally defined as particles less than 0.1μm. This functional definition is a direct result of the identification of bacterial cells at this field site that are less than 0.2μm but greater than 0.1μm (Wrighton et al., 2012). Preliminary enumeration of VLPs from a fraction <0.1μm ($4.19x10^6$ VLPs/mL) and <0.2μm ($4.38x10^5$ VLPs/mL) did not result in a significant difference in total VLP abundance ($p>0.05$, data not shown). This result indicates that most of the viruses are less than 0.1μm. Immediately after collection, bacterial cell and VLP samples were treated with DNAse, fixed with electron microscopy (EM)-grade glutaraldehyde (0.5% final concentration) for 15-30 min at 4ºC, and flash frozen in liquid N_2 prior to storage at -80°C at the field site (Brussaard, 2009; Pan et al., *in review*). Samples were stored at -80°C and shipped to the Weber laboratory on ice. VLPs in environmental samples were enumerated by thawing the frozen samples in TE buffer (10mM Tris 1mM EDTA, pH8.0) and collected on a 0.1μm black polycarbonate filters (prokaryotic cells) or 0.02μm Anodisc filters (viruses). Samples were stained with SYBR Green I (1:80 dilution) as

previously described (Suttle and Fuhrman, 2010). Groundwater samples were collected periodically from monitoring wells for the measurement of geochemical parameters, ie., dissolved oxygen (DO), oxidation and reduction potential (ORP), DH_2O, pH, anions, cations, Fe(II), Mn(II), H_2S, acetate, DOC, and DIC.

Following the injection of oxygenated groundwater changes in viral abundance were observed in response to the injection (Fig. 7). Following the injection of oxygenated groundwater an increase in VLPs was initially observed in both the background ($9.5 \times 10^5 – 3.1 \times 10^6$ in CU01) and a stimulated wells ($2.2 \times 10^6 – 4.5 \times 10^6$, CD01 and CD18)(Fig. 7). This increase in VLP abundance occurs concurrent with the drop in the oxidation reduction potential (ORP) within the wells. The decrease in oxidation reduction potential is counterintuitive as we would expect the addition of oxygenated groundwater to increase the oxidation reduction potential. This result suggests that O_2 was reduced as a result of reducing equivalents that either entered the well-field from natural groundwater flow or were directly injected into the well-field by the treatment. The dissolved organic carbon (DOC) data that was collected over the course of the study does reveal an increase in DOC concentrations within the experimental wells (Fig. 8), which would have provided reducing equivalents. The increase in DOC corresponds to the decrease in the ORP. Thus suggesting utilization of the DOC by the indegenous microbial community coupled to the reduction of terminal electron acceptors, ie. O_2. However, only minimal changes in the abundance of cells was observed over the same time period while significant increases in viral abundance was observed during this period (Fig. 7). The increase in viral abundance can be attributed to microbial activity as

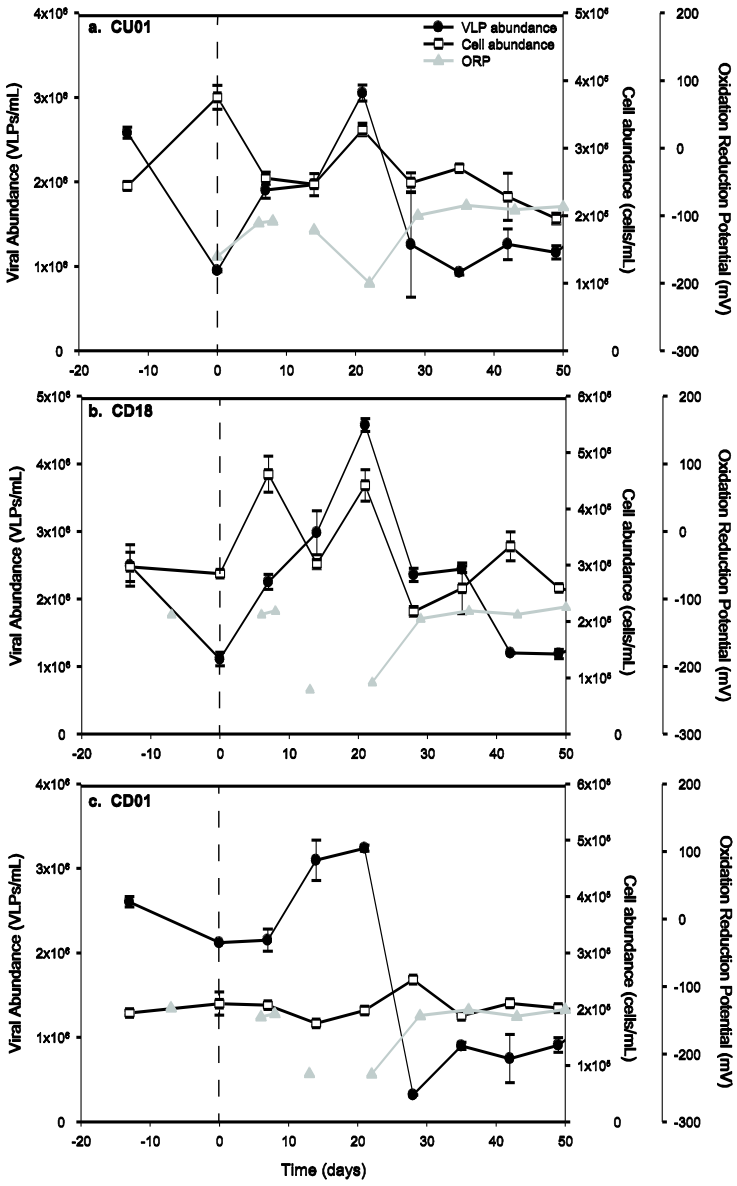

Fig. 7. A drop in the oxidation reduction potential is observed despite the injection of oxygenated groundwater. The decrease results in increases in viral like particle (VLP) abundance with minimal changes in cell abundance. Dashed line indicates time in which oxygenated groundwater was injected into the well field. Time is reported in reference to date of injection. (**a**) control well that did not receive amendment, (**b**) well nearest to injection field receiving amendment, (**c**) well next in series receiving amendment

we previously demonstrated (Pan et al., *in review*). An alternative explanation to the increase in viral abundance could be explained if significant iron reduction was observed and VLPs associated with mineral surfaces were relesed as a result of reductive dissolution of iron minerals (Ryan et al., 1999), as such organic carbon oxidation coupled to dissimilatory Fe(III) reduction would mobilize viral particles as the iron was microbially dissolved (Weber et al., 2006). However Fe(II) concentratons in the groundwater decreased relative the start of the experiment (data not shown).

Viral abundance was observed to increase (Fig. 7) until a rapid decrease in DOC, pH values and Fe(II) occurred (data not shown). The loss of Fe(II) was likely due to Fe(II) oxidation. The presence of iron oxides precipitating was confirmed by visual inspection of the monitoring well (data not shown). The precipitation of iron oxides could likely have had an effect on virus enumeration as the iron oxide would provide surfaces for virus adsorption or viruses could serve as nucleation sites for mineral formation (Daughney et al., 2004). Thus removing VLPs from the groundwater and resulting in an underestimation of VLPs during this phase of the experiment.

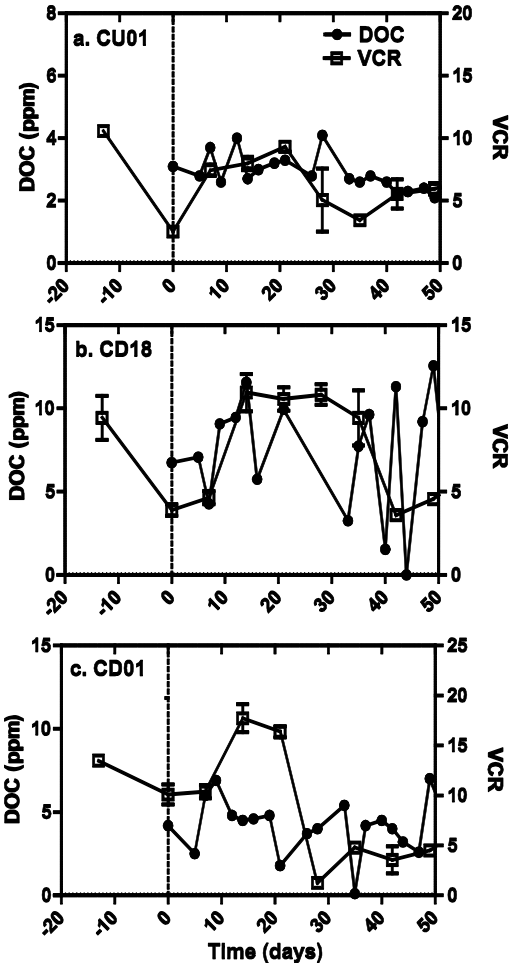

Fig. 8. Increase in dissolved organic carbon (DOC) in upstream precede the increase in the virus to cell ratio (VCR). Dashed line indicates time in which oxygenated groundwater was injected into the well field. Time is reported in reference to date of injection. (**a**) control well that did not receive amendment, (**b**) well nearest to injection field receiving amendment, (**c**) well next in series receiving amendment

Summary in situ experiment. In summary, viral abundance fluctuated with geochemical changes; whereas cell abundance did not similarly respond. This result is consistent our prior laboratory experiments demonstrating a correlation between carbon consumption/CO_2 production and viral abundance, but not carbon consumption/CO_2 production and cell abundance (Pan et al., *in review*). Together with our prior results (H*i*.a; (Pan et al., *in review*) indicate that the virus-to-cell ratio and viral abundance are better indicators of biological activity than cell abundance alone. Viral mediated cell lysis does contribute to changes in microbial community structure. As such, cells within the microbial community that are subject to viral infection may be missed not only in cell counts but also metagenomic samples. Thus, samples specifically for the viral community should be collected over time in order to sample the genetic diversity of any natural system. Furthermore, viruses are sensitive to changes in the environment. Further research is necessary in order to understand the effect of environmental conditions on virus decay and infectivity. These changes are a part of a system's natural responses to disturbances. Thus, changes in viral abundance may also reflect changes in geochemistry and water quality.

H*ii*. Viral surfaces will adsorb heavy metals and radionuclides

Biosorption of metal/radionuclide ions to bacterial and eukaryotic cell surfaces is a well-recognized process. A variety of organic molecules associated with biological surfaces such as carboxyl and amino acid groups, are responsible for binding these heavy metals/radionuclides (Beveridge and Murray, 1980; Fein, 2006). Similarly, viral capsids are protein (amino acid) structures that may display binding affinity for heavy metals/radionuclides. In fact, heavy metals and a radionuclide (uranium) have been used for decades as viral stains in transmission electron microscopic imaging (Arnott and Smith, 1968; Milne and de Zoeten, 1968). Thus, abundant and mobile viruses in a subsurface environment could promote contaminant mobility. Novikov and colleagues (Novikov et al., 2006) recently reported the transport of actinides, plutonium and uranium, associated with unknown Fe-organic submicron colloids (Novikov et al., 2006). The nature of the unknown Fe-organic submicron colloids could be viral particles, further emphasizing the necessity to understand the role that viruses may play in contaminant-viral-colloid formation and contaminant mobility.

Given the potential reactivity of proteins composing viral capsids, these surfaces may display an affinity for the adsorption of heavy metals/radionuclides. The mobility of viral particles in saturated systems is well recognized. Together the mobility of viruses in soil/sedimentary environments and the abundance of VLPs (6.4×10^{6}–2.7×10^{10} VLPs cm^{-3}) in natural soil and sedimentary systems including the DOE sites suggest that viruses in subsurface environments represent abundant colloids. These submicron particles could potentially have reactive surfaces capable of adsorbing contaminant metals and facilitating contaminant transport. Various interactions between metal, viral surfaces, and the soil/sediment surface are anticipated in an environmental system. Heavy metals/radionuclides such as Fe or U, can be dissolved in the aqueous phase or adsorbed to or precipitate on a particle surface. Similarly, viruses will either be transported in the fluid matrix or adsorbed to the surface of a soil particle. Therefore, if viral infections of subsurface microbiota are prevalent and viruses serve as nucleation sites for heavy metal/radionuclide adsorption, then the continuous generation of viral particles in the subsurface could be a significant factor potentially influencing particle-facilitated contaminant mobility. As the first step, we must establish that viral surfaces adsorb heavy-metals and radionuclides. Here proposed to test the following hypothesis: *viral surfaces will adsorb heavy metals and radionuclides***.** To test this hypothesis we proposed to investigate the adsorption of metals and radionuclides to the surface of bacteriophage.

Adsorption of metals and radionuclides to the surface of bacteriophage (Tan et al., in prep)

The production of VLP's in groundwater has implications for nanoparticulate carbon or metal transport. Here we selected *Escherichia coli* phage T4 as the model bacteriophage simply due to the ability to cultivate the phage T4 to a high abundance within a short period of time which was required in order to develop techniques to test this hypothesis. In addition to the rapid cultivation of this bacteriophage, phage T4 is a model bacterial virus in which the capsid and tail structure are known thus would allow us to predict potential adsorption sites. We had initially proposed to use the plate lysate method (Suttle and Chen, 1992) to cultivate and harvest bacteriophage, where the lysed bacterial lawn is harvested by scraping off the soft agar layer and resuspended in a low nutrient aqueous matrix. Agar and other particulates were to be removed by slow speed centrifugation at 2,000 × *g* for 10 min at 28°C. However, this method did not yield enough bacteriophage for replicated treatments across a broad range of conditions required by the experimental design. As an alternative, we cultivated phage T4

in liquid culture with the host *E. coli*. Bacteriophage T4 was then harvested from lysed cultures of *E.coli* grown to mid-log phase by centrifugation (10,000 x *g*, 20 minutes) to separate cell debris from the bacteriophage. The supernatant was decanted from cell and debris pellet. The supernatant was then filtered through a 0.2µm PVDF filter and finally concentrated and washed with PIPES buffer (20 mM, pH 7.0) using a 100kDa molecular weight cutoff filter (Amicon® Ultra-15 Centrifugal Filters). Bacteriophage were immediately enumerated using epifluorusence microcospy (Noble and Fuhrman, 1998) in order to amend each replicate with 10^{10} VLPs mL^{-1}. The number of bacteriophage added to each replicate was quantified at the end of the experiment using flow cytometry (Brussaard, 2009). A dissolved heavy metal or radionuclide, aqueous Fe(II), Zn^{2+}, and U(VI) was be added to the bacteriophage suspension to achieve a final concentration ranging from 10-300µmole kg^{-1}. Metal-virus suspensions were placed horizontally on a rotary shaker and agitated (750 rpm) continuously for 18 hours at 28°C. Tubes in which the bacteriophage were been omitted served as negative controls. Samples were collected and filtered through a 0.025µm filtered in order to determine the concentration of metal remaining in solution via ICP-MS. Total metal concentrations were measured on unfiltered samples. Total metal minus the filtered fractions was used to determine the mass of metal adsorbed to the bacteriophage. Significant adsorption of Fe(II) and U(IV) by filters used to separate the bacteriophage from aqueous solution was observed. As such we proceeded with Zn^{2+} as the model metal cation due to no significant adsorption to mixed cellulose ester membrane filter (0.025µm). Given the low biomass of bacteriophage (10^{10} VLPs mL^{-1} = 3.3 µg mL^{-1}) data are reported as metal adsorbed/10^{10} VLPs instead of metal adsorped/g biomass.

A series of adsorption expeirments demonstrated that metals (Zn^{2+}) will adsorb to the surface of phage T4 (Fig. 9). The raw adsorption data generated from laboratory adsorption experiments were fitted with sorption isotherm models to quantitatively describe the adsorption of Zn^{2+} onto the surface of phage T4. A Freundlich isotherm was able to fit the sorption data. MATLAB 2013b Curve Fitting Toolbox 3.4 (The MathWorks, Inc) was used to conduct the curve fitting. Trust Region algorithm for nonlinear least squares optimization was app*l*ied to minimize the least absolute residuals. Table 1 provides the fitted parameters, their 95% confidence interval, and the goodness of fit. The Freundlich isotherm reasonably describes the sorption data, with an R-square of 0.97. Here, the value of n is less than 1. This indicates that the sorption sites on the surface of phage T4 are heterogeneous, with varied sorption energies for Zinc. This is supported by the identification of two different proteins, Hoc and Soc, on the outer surface of the capsid. The Hoc and Soc capsid proteins of phage T4 possess negatively charged binding sites, which are the C-terminus for the Soc protein and the N-terminus for the Hoc protein. These two sites have been proven to be biologically active in studies and that they are able to bind certain proteins and antibodies (Ren and Black, 1998). Thus, it is likely that these negatively charged binding sites adsorb positively charged metal cations. Further studies are

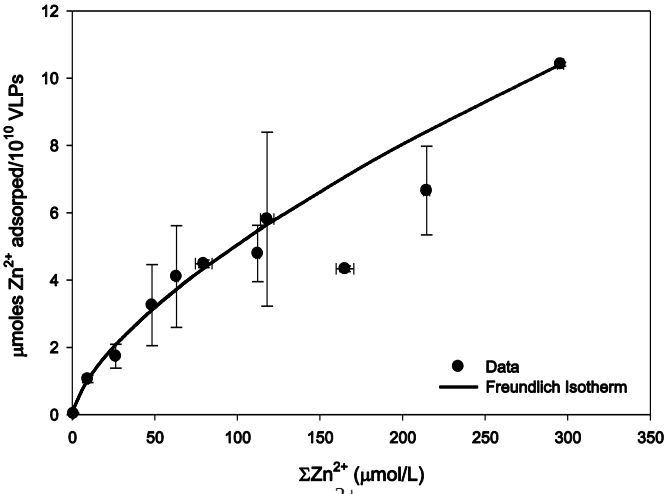

Fig. 9. Adsorption of Zn^{2+} to the surface of *E. coli* phage T4 modeled using a Freundlich isotherm.

necessary in order to verify if these proteins serve as adsorptive sites on the phage capsid surface as well as identify other sorptive sites on the surface of the bacteriophage.

Table 1. Fit Parameters of Freundlich isotherm

Freundlich Isotherm: $S=K_f C^n$
Coefficients (with 95% confidence bounds):
K_f = 0.239 (0.1138, 0.3641)
n = 0.6636 (0.5622, 0.765)
Goodness of fit:
SSE: 2.036
R-square: 0.9752
Adjusted R-square: 0.9724
RMSE: 0.4756

In an effort to investigate the surface charge of phage T4 we initially investigated methods to estimate the surface charge using potentiometric titrations. However, current techniques require the use of 1g of biomass. It is not physically feasible to produce (ca. 100L of culture), separate, purify, concentrate, and wash the biomass of viruses to conduct one titration experiment before significant phage decay has occurred (24 hours) using standard methods. In lieu of potentiometric analyses we have conducted zeta potential analysis as a proxy for cell surface potential (Wilson et al., 2001) using a ZetaPALS analyzer (Brookhaven Instruments Corporation, Holtsville, NY). Zeta potential analysis demonstrated phage T4 (10^{10} VLPs mL^{-1}) not exposed to zinc at pH 7.0 to be approximately -11.48 ± 1.16 mV. These results demonstrate the surface of phage T4 is naturally electronegative, which supports the capability of the surface of *E. coli* bacteriophage T4 to adsorb positively charged ions (cations). This was subsequently demonstrated when the zeta potential shifted to -2.96 ± 1.60mV at pH 7.0 and exposure of 10^{10} VLPs mL^{-1} to 150µM Zn^{2+}, which suggests that adsorption of Zn^{2+} ions onto the phage resulted in the neutralization of negative charges on the phage surface.

Phage decay has the potential to either increase or decrease the available surface area for metal adsorption. We conducted a series of experiments in order to determine the effect of metal adsorption on phage decay. Briefly, phage titers were conducted to assess the number of remaining bacteriophage post metal exposure. Infection of the host depends on an intact tail and capsid and would thus reveal if the number of bacteriophage were lost as a result of metal exposure. Briefly, the host bacteria, *E. coli* was be grown to mid log phase in order to prepare a soft agar overly where the inoculated with phage T4 post metal exposure. Following a 2 hour incubation plaque forming units (PFUs) were visible and counted relative to with a negative control (T4 no metal exposure) revealing the number of infective viruses in the sample.

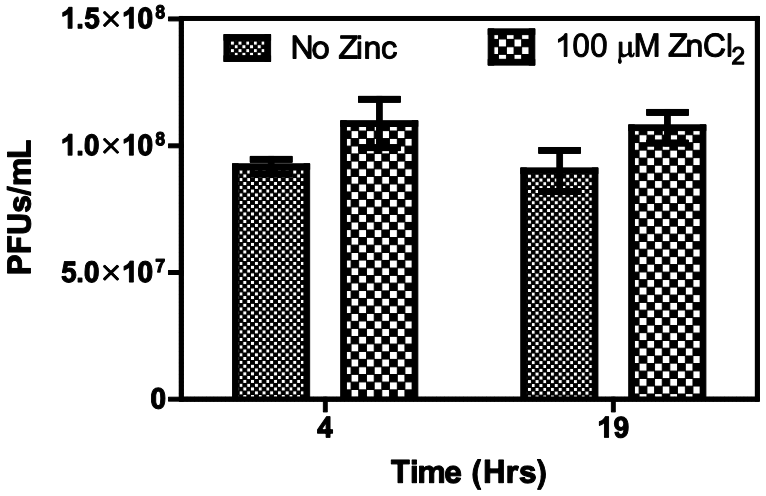

Fig. 10. Infectivity of *E. coli* phage T4 after exposure to Zn at 4 hours and 19 hours relative to Zn-free controls.

Interestingly, the presence of Zn^{2+} significantly increased (ca.100 µM) infectivity relative to unamended controls (Fig. 8; ANOVA $p<0.05$). Thus, indicating that Zn^{2+} enhances the infectivity of phage T4. Metal cations such as Mn^{2+}, Co^{2+}, Zn^{2+} have been proposed to stabilize the structures of bacteriophage by forming a complex between the phage structure and the metal ion resulting in a decrease in the rate of phage inactivation (Adams, 1949). Given that we did not see a significant decrease in infection between 4 and 19 hours suggest that over the period of time we investigated omission of metals did not appear to affect infectivity with time. Rather we saw the addition of metals have positive effect on infection, suggesting that infection is enhanced with metal adsorption. This is consistent some previous studies that have demonstrated that adsorption of the phage to the hose is enhanced by the presence of the divalent cation Ca^{2+} (Shafia and Thompson, 1964; Suarez et al., 2008), divalent metal cations could similarly influence infection.

H*ii*. Summary

We had originally considered subsurface viruses as vectors of nanoparticlate contaminant transport, moving the contaminants as submicron organic colloids. We have demonstrated that the virus surface is reactive and will adsorb heavy metals. Thus viruses can promote colloidal contaminant mobility and could perhaps provide identification of the submicron unknown organic colloids identified by Novikov and colleagues (Novikov et al., 2006) that were transporting actinides. As we continued our research in an effort to understand the role metals on the infectivity of the bacteriophage, we demonstrated that the presence of heavy metals has a positive effect on infectivity of the phage. Together, the results suggest that the sorption of metals to the surface of viruses could not only contribute to nanoparticulate carbon and metal transport but could also enhance infectivity further contributing to cell lysis. These factors could have significant implications to subsurface biogeochemistry and bioremediation efforts. As more viruses infect host microbial populations enriched in subsurface systems through bioremediation efforts, the high concentration of metals could enhance infection, thus decreasing the metabolically active host population. Greater numbers of viruses would be produced resulting in higher amounts of the contaminant transported in association with viruses. It is therefore necessary to establish potential relationship(s) between viruses, cells, carbon, and metals/radionuclides to provide sufficient scientific understanding to incorporate coupled physical, chemical, and biological processes into agent based and reactive transport models.

5.0 PROJECT SUMMARY

Our results demonstrated that viruses do play a significant role in microbial mortality and community structure in terrestrial subsurface sedimentary systems. The production of viral-like particles within subsurface sediments in response to biostimulation with dissolved organic carbon and/or a terminal electron acceptor resulted in the production of viral-like particles. Organic carbon alone did not result in significant viral production and required the addition of a terminal electron acceptor (nitrate). This would indicate that nutrients are not limiting viral production, but rather terminal electron acceptors that can be converted into energy for host metabolism. Our results also revealed that cell abundance was not correlated to the mineralization of organic carbon, but rather viruses were positively correlated with carbon mineralization. This is likely a result of viral-mediated cell lysis and demonstrates that viruses are sensitive indicators of microbial activity. Viruses as an indicator of microbial activity was not unique to the batch culture studies as results obtained from an *in situ* field experiment conducted at the DOE Old Rifle Field site revealed that viral abundance increased in response to the injection of oxygenated groundwater and influx of dissolved organic

carbon whereas cell abundance changes were minimal. Together these results indicate that viruses are mediating cell lysis subsurface systems. Cell lysis has implications not only to metal biogeochemical cycling but also carbon cycling. However, the extent to which viral-mediated cell lysis alters organic matter pools subsequently influencing microbial community structure and biogeochemical function from the bottom up remains a critical question in subsurface microbial ecology and biogeochemical cycling.

The production of significant numbers of viruses in groundwater has implications for nanoparticulate metal as well as carbon transport in groundwater. We have demonstrated that the virus surface is reactive and will adsorb heavy metals. Thus viruses can promote colloidal contaminant mobility. Interestingly, the presence of heavy metals has a positive effect on infectivity of the phage, increasing phage infection which could lead to further production of viruses. Together, the results suggest that the sorption of metals to the surface of viruses could not only contribute to nanoparticulate metal as well as carbon transport but could also enhance infectivity further contributing to cell lysis which could subsequently influence biogeochemical cycling. As more viruses infect host microbial populations enriched in subsurface systems through bioremediation efforts, the high concentration of metals could enhance infection, thus decreasing the metabolically active host population. Greater numbers of viruses would be produced resulting in higher amounts of the contaminant transported in association with viruses. Additional studies will be necessary to further establish the potential relationship(s) between viruses, cells, carbon, and metals/radionuclides to provide sufficient scientific understanding to incorporate coupled physical, chemical, and biological processes into agent based and reactive transport models.

6.0 PUBLICATIONS

‡Undergraduate/Post-baccalaureate Student Contributing Author
§Graduate Student Contributing Author

Pan, D. §, Watson, R. ‡, Wang, D. ‡, Tan, Z.H. ‡, Snow, D., Weber, K.A., *in review*. Correlation between viral production and carbon mineralization under nitrate-reducing conditions in aquifer sediment. *ISME J.*

Pan, D. §, Robbins, M., Williams, K. H., and Weber, K. A., *in prep. In situ* production of Viral Like Particles in Groundwater.

Tan, Z. §, Kananizadeh, N. §, Li, Y., Weber, K.A., *in prep*. Zinc Ion Biosorption by *Escherichia coli* Phage T4 and the Potential for Nanoparticulate Contaminant Transport.

7.0 CONFERENCE PROCEEDINGS

*Presenting Author
‡Undergraduate/Post-baccalaureate Student Contributing Author
§Graduate Student Contributing Author

2013

Pan, D. *§, Robbins, M., Williams, K. H., and Weber, K. A. Production of Viral Like Particles (VLPs) following *in situ* stimulation of a subsurface microbial community. American Society for Microbiology General Meeting, Denver, CO, May 18-22, 2013.

Tan, Z. *§ and Weber, K.A., Biosorption of Heavy Metals by Bacteriophage T4. American Society for Microbiology General Meeting, Denver, CO, May 18-22, 2013.

Pan, D. *§, Robbins, M., Williams, K. H., and Weber, K. A. Production of Viral Like Particles (VLPs) following *in situ* stimulation of a microbial community. Water for Food Global Conference, Lincoln, NE, May 5-8, 2013. ***(2nd Place Student Poster Award, D. Pan)***

Pan, D. *§, Robbins, M., Williams, K. H., and Weber, K. A. Production of Viral Like Particles (VLPs) following *in situ* stimulation of a subsurface microbial community. School of Biological Sciences Annual Graduate Student Symposium. April 2013.

Tan, Z. *§ and Weber, K.A., Biosorption of Heavy Metals by Bacteriophage T4. School of Biological Sciences Annual Graduate Student Symposium. April 2013.

Weber, K. A.*, Pan, D.§, Z. H. Tan§, K.H. Williams., M. Robbins, K. S. Bender, Y. Li. Viral Infection of Subsurface Microbiota: Implications for Carbon Cycling and Metal Transport. Joint DOE Terrestrial Ecosystems/DOE Subsurface Biogeochemistry PI Meeting, Potomac, M.D., May 14-15, 2013.

2012

Pan, D.*§, R. Watson‡, Z. H. Tan‡, D. Snow, K. A. Weber. Induction of a viral community infecting nitrate reducing bacteria within a nitrate and uranium contaminated aquifer. 14th International Symposium on Microbial Ecology, Copenhagen, Denmark, August 19-24, 2012.

Pan, D.*§, R. Watson‡, Z. H. Tan‡, D. Snow, K. A. Weber. Production of Viral Like Particles Following the Stimulation of Heterotrophic Nitrate Reduction in Continental, Shallow Subsurface Sediment. American Society for Microbiology General Meeting, San Francisco, CA, June 16-19, 2012. **(*Outstanding Student Poster Award, D. Pan*)**

Pan, D.*§, Z. H. Tan‡, D. Snow, K. A. Weber. Production of Viral Like Particles Following the Stimulation of Heterotrophic Nitrate Reduction in Continental, Shallow Subsurface Sediment. Water for Food Global Conference, Lincoln, NE, May 30 -June 1, 2012.

Weber, K. A.*, Pan, D.§, Z. H. Tan§, K. S. Bender, Y. Li. Viral infection of subsurface microorganisms and metal/radionuclide transport. DOE Subsurface Biogeochemistry PI Workshop, Washington, D. C. April 30- May 2, 2012.

Z.H. Tan*§, D. Pan§, K. A. Weber. Adsorption of Zn^{2+} to the surface of bacteriophage: Implications for nanoparticulate metal transport. School of Biological Sciences Annual Graduate Student Symposium. April 2012.

Pan, D.*§, R. Watson, Z. H. Tan‡, D. Snow, K. A. Weber. Induction of a Viral Community Infecting Nitrate Reducing Bacteria within a Nitrate and Uranium Contaminated Aquifer. School of Biological Sciences Annual Graduate Student Symposium. Lincoln, NE. April 2012.

Watson, R.*‡, D. Pan§, K. A. Weber. Characterization of *Pseudomonas frederiksbergensis* Alda10, a Nitrate Reducing Subsurface Bacterium Isolated from an Alluvial Aquifer near Alda, Nebraska. UNL Research Fair. Lincoln, NE. March 2012.

2011

Weber, K. A.*, Pan, D.*§, Z. H. Tan‡, D. Snow, K. S. Bender, Y. Li. Viral infection of subsurface microorganisms and metal/radionuclide transport. DOE Subsurface Biogeochemistry PI Workshop, Washington, D. C. April 26-28, 2011.

Pan, D.*§, Z. H. Tan‡, D. Snow, K. A. Weber. Production of Viral Like Particles Following the Stimulation of Heterotrophic Nitrate Reduction in Continental, Shallow Subsurface Sediment. Biology Graduate Student Symposium, Lincoln, NE, April 8, 2011. ***(2nd Place, Graduate Student Poster, D. Pan)***

Pan, D. *§, Z. H. Tan‡, D. Snow, K. A. Weber. Production of Viral Like Particles Following the Stimulation of Heterotrophic Nitrate Reduction in Continental, Shallow Subsurface Sediment. Missouri Valley Branch ASM Meeting, Lincoln, NE, March 31 – April 2, 2011. ***(2nd Place, Graduate Student Poster, D. Pan)***

8.0 COLLABORATIONS FOSTERED

Lawrence Berkeley National Laboratory's (LBNL) Science Focus Area (SFA), Dr. Kenneth H. Williams, geological scientist. We collected bacterial and viral abundance data from the 2012 field experiment. Results were presented to the Rifle Science Community Telecon on September 10, 2013 and the manuscript reporting this data is currently in preparation with an anticipated submission of December-January.

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