# Functional Role of Infective Viral Particles on Metal Reduction

## Final Technical Report

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## 1.0 Abstract

A proposed strategy for the remediation of uranium (U) contaminated sites was based on the immobilization of U by reducing the oxidized soluble U, U(VI), to form a reduced insoluble end product, U(IV). Previous studies identified *Geobacter sp*., including *G. sulfurreducens* and *G. metallireducens,* as predominant U(VI)-reducing bacteria under acetate-oxidizing and U(VI)-reducing conditions. Examination of the finished genome sequence annotation of the canonical metal reducing species *Geobacter sulfurreducens* strain PCA and *G. metallireduceans* strain GS-15 as well as the draft genome sequence of *G. uraniumreducens* strain Rf4 identified phage related proteins. In addition, the completed genome for *Anaeromyxobacter dehalogenans* and the draft genome sequence of *Desulfovibrio desulfuricans* strain G20, two more model metal-reducing bacteria, also revealed phage related sequences. The presence of these gene sequences indicated that *Geobacter spp., Anaeromyxobacter spp., and Desulfovibrio spp*. are susceptible to viral infection. Furthermore, viral populations in soils and sedimentary environments in the order of $6.4\times10^{6}$–$2.7\times10^{10}$ VLP's $cm^{-3}$ have been observed. In some cases, viral populations exceed bacterial populations in these environments suggesting that a relationship may exist between viruses and bacteria. Our preliminary screens of samples collected from the ESR FRC indicated that viral like particles were observed in significant numbers (Fig. 1.1). The objective of this study was to investigate the potential functional role viruses play in metal reduction specifically Fe(III) and U(VI) reduction, the environmental parameters affecting viral infection of metal reducing bacteria, and the subsequent effects on U transport.

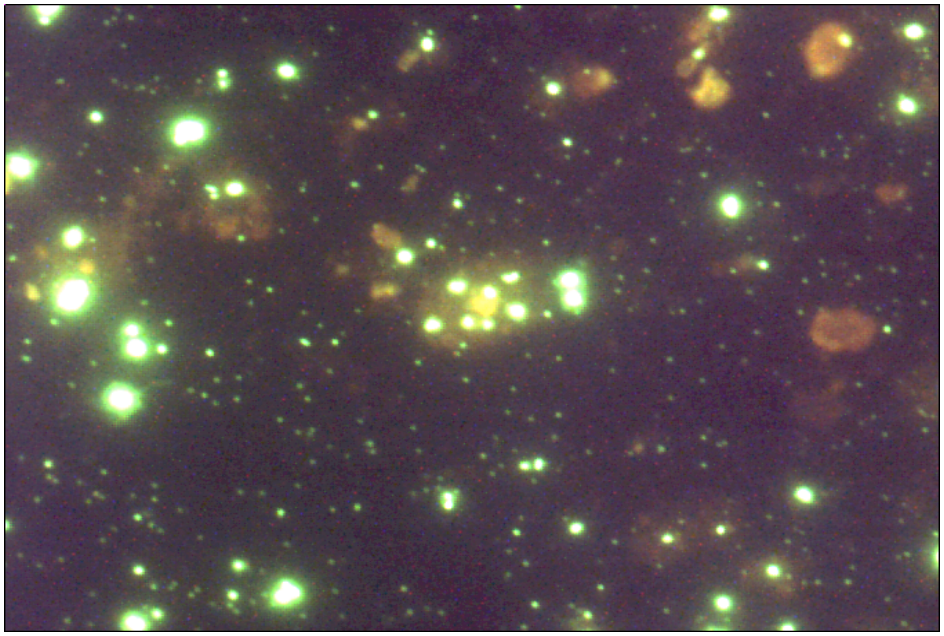

**Figure 1.1.** Epifluorescence micrograph of groundwater sample from ESR FRC site, February 2004, collected on a 0.02mM Anodisc filter stained with SYBR Green I (K.A. Weber unpublished data). Larger bright dots are indicative of bacterial cells and small pinpoint dots are indicative of viral like particles as described by Fuhrman (1999)(Fuhrman, 1999).

## 2.0 Project summary

The waste disposal practices employed during the processing of uranium ores as nuclear fuels and mining have resulted in high uranium (U) concentrations in environmental systems. A proposed strategy for the remediation of uranium (U) contaminated sites is based on the redox chemistry of U by immobilization of U by reducing the oxidized soluble U, U(VI), to form a reduced insoluble end product, U(IV). The production of an insoluble precipitate would not be transported through the subsurface ultimately leading to the immobilization of U. The recognition of microbially-mediated U(VI) reduction (Lovley et al., 1991) introduced a method of remediating contaminated environments *in situ* by stimulating the indigenous microbial community responsible for such a metabolism. The stimulation of microbially-mediated U(VI) reduction by the addition of organic matter has been demonstrated in both laboratory and field studies (Finneran et al., 2002a;Finneran et al., 2002b;Holmes et al., 2002;Anderson et al., 2003;Suzuki et al., 2003;Istok et al., 2004). *Geobacter* sp. have been identified as the predominant stimulated U(VI)-reducing community in freshwater environments with low sulfate concentrations (Holmes et al., 2002) including a stimulated environment at the ESR FRC site (Istok et al., 2004).

Viral populations in soils and sedimentary environments have previously been demonstrated to be in the order of $6.4\times10^{6}$–$2.7\times10^{10}$ VLP's $cm^{-3}$ (Maranger and Bird, 1996;Steward et al., 1996;Danovaro and Serresi, 2000;Danovaro et al., 2001;Hewson et al., 2001;Danovaro et al., 2002;Ashelford et al., 2003). A preliminary investigation of groundwater collected from the ESR FRC site indicates that VLP's are present (Fig. 1). Viral abundance in sedimentary environments has been demonstrated to be significantly greater (9 to 1000x) than in the overlying water (Maranger and Bird, 1996;Steward et al., 1996;Hewson et al., 2001). In some cases, viral populations exceed bacterial populations in these environments (Drake et al., 1998;Danovaro and Serresi, 2000;Hewson et al., 2001). This suggests that viral-bacterial relationships in sedimentary environments may be similar to the relationships observed in marine and freshwater pelagic systems where viral populations ($10^{4}$-$10^{10}$ VPL's $L^{-1}$) have been recognized to play a primary role in the microbial ecology and biogeochemistry of these ecosystems (Fuhrman, 1999;Wommack and Colwell, 2000;Fuhrman et al., 2002). While a relationship between microbial populations in marine and freshwater pelagic systems has been established, the relationship between viruses and the bacterial hosts in soils and sedimentary environments is relatively unknown.

Virus occurrence and transport in groundwater is well-recognized (Drewry and Eliassen, 1968;Gerba, 1983;Yates et al., 1985;Ryan et al., 1999;Abbaszadegan et al., 2003). Temperature, geochemistry, clay minerals, metal oxides, and saturation primarily control viral transport in subsurface environments (Jin and Flury, 2002). Organic matter has also been demonstrated to promote transport of viral particles by inhibiting adsorption of viral particles to the soil/sediment matrix (Gerba et al., 1975;Gerba, 1984). The nature of the organic matter and the properties of the virus control this interaction, where an electrostatic interaction between the organic matter and virus promotes transport (Zhuang and Jin, 2003). Furthermore, reductive dissolution of Fe(III) oxides

results in the release and subsequent transport of viral particles (Ryan et al., 1999). The strategy employed to stimulate U(VI) reduction by the addition of organic matter into subsurface sediments typically results in the reduction of Fe(III) oxides (Finneran et al., 2002a;Holmes et al., 2002;Anderson et al., 2003;Istok et al., 2004). Thus suggesting that stimulated U(VI) reduction would lead to the release of adsorbed viral particles increasing potential infectivity and transport of the viral particle.

Given that *Geobacter spp., Anaeromyxobacter spp., and Desulfovibrio spp*. Like all organisms are putatively susceptible to viral infection and that viral populations are present in sedimentary environments including the ESR FRC site, it is not illogical to presume that a relationship between viruses and indigenous metal-reducing bacteria exist. Some significant issues regarding the potential functional role of viral particles in the U(VI) bio-reductive remediation strategy include:

1) Viral infection of the indigenous metal reducing bacterial population could ultimately alter the extent and rate of metal reduction.
2) Organic matter amendment would not only stimulate U(VI) reduction but would also lead to an increase in viral abundance and transport.
3) Precipitation of reduced, solid phase U, uraninite, on the viral surface could result in the subsequent transport of U.

It is therefore necessary to identify the potential relationships between viruses and metal reducing bacteria, which would affect the subsequent fate of a U(VI) reductive remediation strategy and the transport of U in contaminated environments. The objective of our research was to investigate the potential influence of viral particles on metal reducing bacteria. To date nothing is known about bacterial and viral interactions in such environments. The research outlined in the following paragraphs provides the basis for research needed to establish viral-bacterial relationships in anaerobic aquatic, soil, and sedimentary environments.

## 3.0 Genome analysis

A proposed strategy for the remediation of uranium (U) contaminated sites is based on the immobilization of U by microbial reduction of the oxidized soluble U, U(VI), to form a reduced insoluble end product, U(IV). Previous studies have identified *Geobacter sp*. as predominant U(VI)-reducing bacteria under acetate-oxidizing and

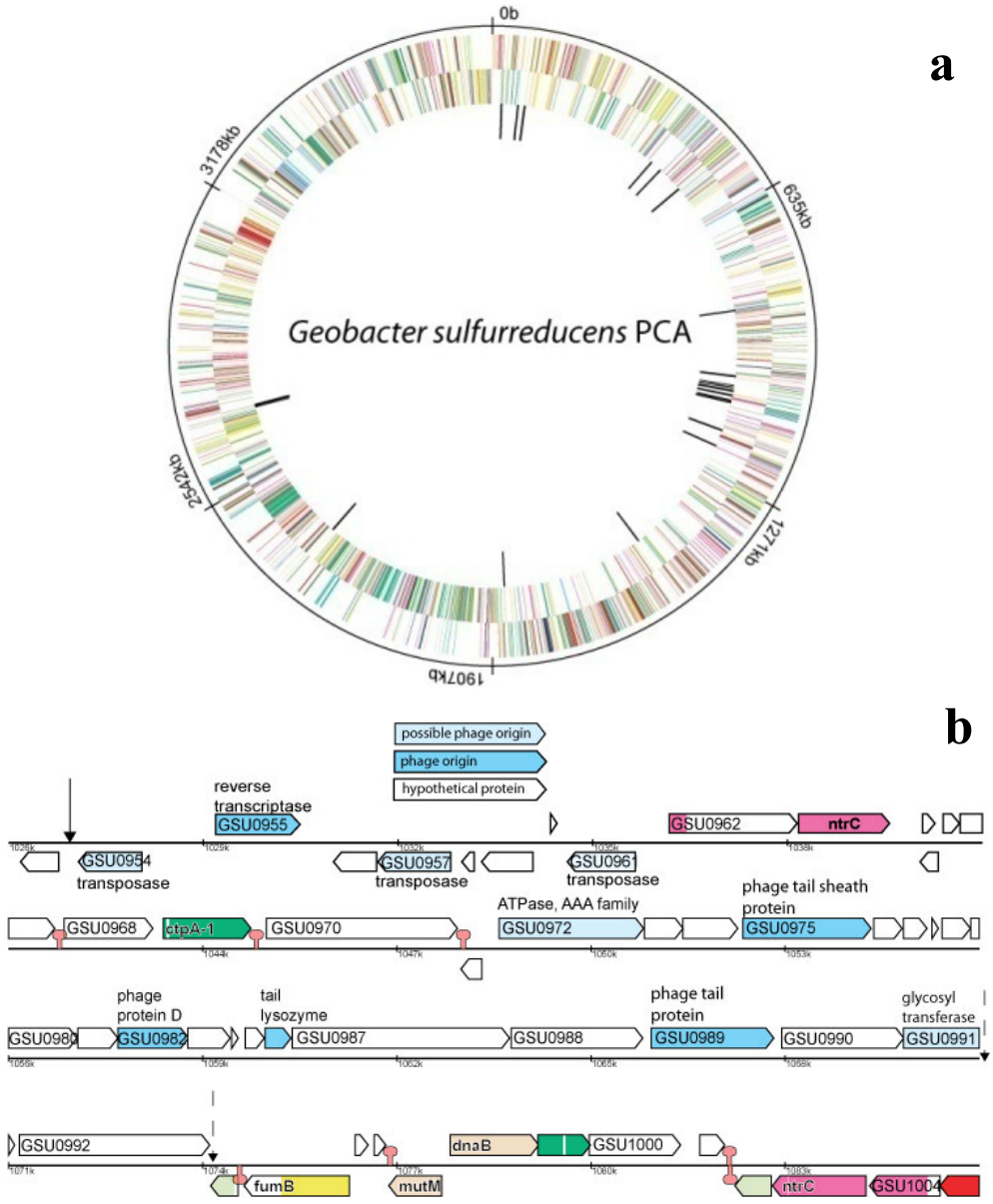


**Figure 3.2.** ***Geobacter sulfurreducens*** **PCA chromosome.** (A) Black lines in the inner circle depict gene sequences consistent with bacteriophage origin. Image generated with Microbial Genome Viewer (http://www.cmbi.ru.nl/MGV/). (B) Chromosomal region within the *G. sulfurreducens* PCA genome predicted to encode a prophage genome. The region was selected based on annotation and similarity searches. This region contains genes annotated to encode: 1) phage structural proteins; 2) proteins with functions previously identified in a range of phage isolates; and 3) an abundance of hypothetical proteins. Similarity searches also indicated that most of the genes in this region are either unique to *Geobacter* sp. or originated from bacterial groups not closely related to *Geobacter* sp..

U(VI)-reducing conditions in subsurface environments contaminated with U. Our examination of the annotated finished genome sequence of *Geobacter sulfurreducens* strain PCA (Fig. 3.2A and B), *G. uraniumreducens* strain Rf4, *G. metallireduceans* strain GS-15, and *Geobacter sp*. strain FRC-32 has identified phage related proteins. Presence of these gene sequences indicates that model metal reducing bacteria, *Geobacter spp*., are susceptible to viral infection. Analysis of the completed genome sequences of *G. metallireducens* strain GS-15, *G. sulfurreducens* strain PCA, and *A. dehalogenans* contained sequences for phage related proteins in loci Gmet0772, Gmet0793, Gmet1128, Gmet1137, Gmet1139, Gmet1142; GSU0014, GSU0398, GSU0492, GSU0975,

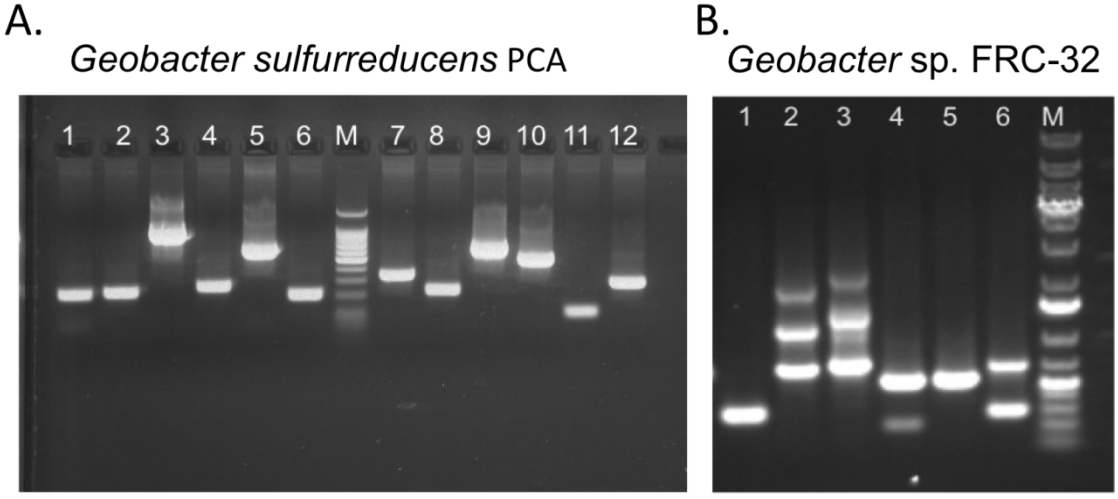


**Figure 4.3. PCR confirmation of phage-like DNA sequences in the genomes of *Geobacter sulfurreducens* PCA and *Geobacter* sp. FRC-32.** (A) *Geobacter sulfurreducens* PCA. Lane 1- GSU0063, lane 2- GSU0529, lane 3- GSU0975, lane 4- GSU0979, lane 5- GSU0982, lane 6- GSU0986, lane 7- GSU0989, lane 8- GSU1126, lane 9- GSU2118, lane 10- GSU2119, lane 11- GSU2174, and lane 12- GSU3324. (B) *Geobacter* sp. FRC-32. Lane 1- Geob_2405, lane 2- Geob_3736 (bottom band), lane 3- Geob_3742 (bottom band), lane 4- Geob_3748, lane 5- Geob_3755, and lane 6- Geob_0530 (bottom band). Gene numbering based on genome annotations. No products were visible in the no-template controls (data not shown).

GSU1126; and Adeh1801, Adeh2871, Adeh1844, Adeh3343, Adeh4132; respectively. In addition the unfinished genome of *G. uraniumreducens* strain Rf4 and *D. desulfuricans* strain G20, two other model metal reducing bacteria, also revealed several phage related sequences. Presence of these gene sequences supports that model metal reducing bacteria are susceptible to viral infection although currently nothing is known about the nature of these phages.

## 4.0 Viral gene confirmation

Polymerase chain reaction (PCR) primer sets were designed to confirm the presence of viral-like sequences in the genomic DNA of laboratory cultivated *Geobacter sulfurreducens* PCA and *Geobacter* strain FRC-32. Twelve of the 25 annotated viral like

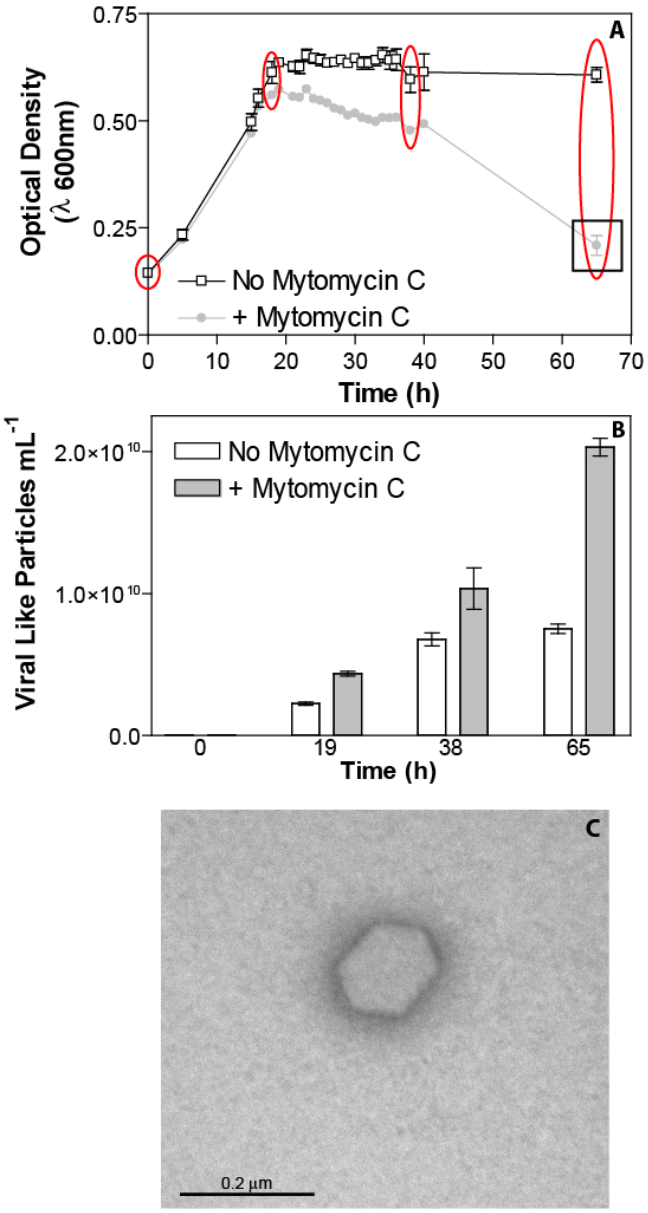


**Figure 5.4. *Geobacter sulfurreducens* PCA prophage induction with mytomycin C growing with fumarate as an electron acceptor.** (**A**) Cultures of *G. sulfurreducens* PCA with (●) and without (□) the addition of 1 µg $mL^{-1}$ mytomycin C. Red circles denote direct counts for VLPs. Box at the end of the growth phase denotes samples collected for the enumeration and identification VLPs. (**B**) Epifluorescent enumeration of VLPs in the 0.02-0.22 µm fraction. (**C**) Transmission electron micrograph of a VLP stained with PTA collected from a culture of *G. sulfurreducens* PCA exposed to mytomycin C. Error bars represent standard error of triplicate cultures.

sequences in *G. sulfurreducens* were tested. The primers were successful in amplifying viral-like genes in *G. sulfurreducens* (Fig. 4.3A) associated with GSU0063 (prophage type transcription regulator), GSU0529 (phage-induced endoribonuclease IV), GSU0975 (phage tail sheath), GSU0979 (phage tail protein), GSU0982 (phage protein D), GSU0986 (phage base plate protein), GSU0989 (phage tail protein), GSU1126 (phage DNA polymerase), GSU2118-2119 (integrase), GSU2174 (phage transcriptional regulator), and GSU3324 (Cro-like transcription regulator). Six putative viral genes within the genome of *Geobacter* sp. FRC-32 were also targeted via PCR. Products were obtained corresponding to Geob_2405 (putative phage protein), Geob_3736 (viron core protein), Geob_3742 (phage-related), Geob_3748 (tail lysozyme), Geob_3755 (phage tail sheath), and Geob_0530 (phage related) (Fig. 4.3B). Continued efforts in this regard will focus on PCR amplification of the remaining viral-like sequences within these microorganisms as well as *G. uraniumreducens* to identify target genes and primer sets for further analysis of active infections by following mRNA expression under metal reducing conditions within these pure cultures.

**5.0 Viral gene expression.**

The incubation of a pure culture of *G. sulfurreducens* PCA with 1 mg $mL^{-1}$ mytomycin C (a potent mutagen used to induce prophage (Paul and Jiang, 2001)) during mid-log phase resulted in significant cell lysis relative to cultures that remained unamended (Fig. 5.4A). Repeated experiments resulted in similar results (data not shown). Samples collected following the lytic event were filtered through a 0.22 mm sterile polyvinylidene fluoride (pvdf) filter. Samples were then collected on a 0.02 mm aluminum oxide filter, stained with SYBR Gold and enumerated via epifluorescence microscopy as previously described (Wen et al., 2004). Icosohedral viral like particles were also identified via transmission electron microscopy of phosphotungstic acid (PTA) stained samples. Together both epifluorescent microscopy and TEM revealed the presence of viral like particles in the culture exposed to mytomycin C (Fig. 5.4B and C). Expression of the phage tail genes were identified under both treatments. The expression of viral-like genes following the control treatment is expected since VLPs increased in the control treatment in a previous study (Fig. 5.5A and B). However, band intensity is greater in cultures amended with mytomycin C suggesting increased expression. qPCR analysis is ongoing in order to determine differences in gene expression. Together these results indicate the infection of *G. sulfurreducens* PCA with a prophage.

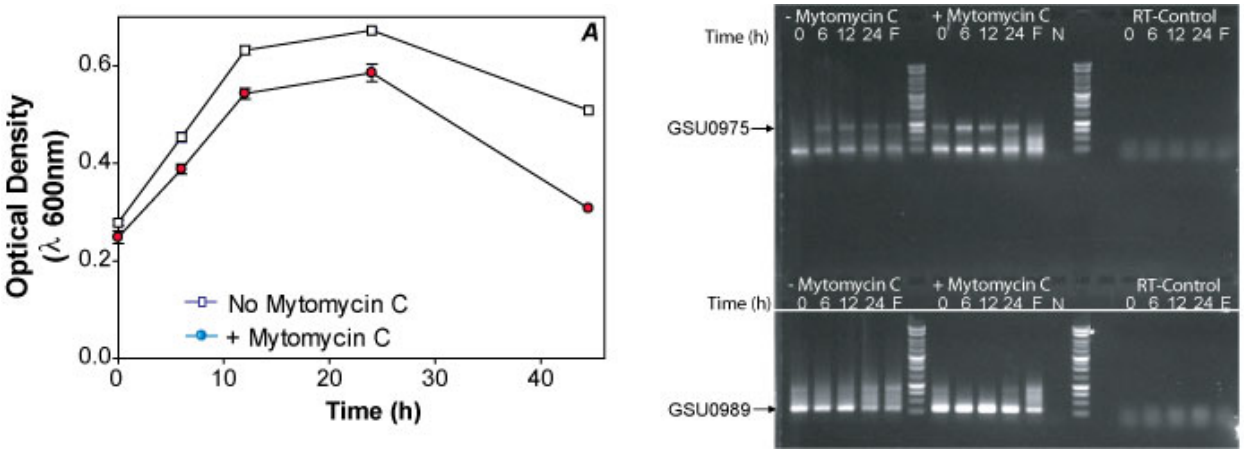


**Figure 5.5. Expression of viral-like genes in *G. sulfurreducens* PCA.** (A) The incubation of a pure culture of *G. sulfurreducens* PCA with 1 μg $mL^{-1}$ mytomycin C (●) and without (□) during mid-log phase resulted in cell lysis relative to cultures that remained unamended similar to the experiment described above. (B) Reverse transcription of mRNA from viral-like gene sequences (phage tail) identified in *G. sulfurreducens* PCA from uninduced and mytomycin C cultures. F denotes the final time point and N denotes PCR reaction in which the template has been omitted.

Attempts to continuously cultivate the bacteriophage via plaque formation with *G. sulfurreducens* PCA as the host bacterium were unsuccessful. This result could suggest that *G. sulfurreducens* PCA may have an acquired defense against the induced prophage (Barrangou et al., 2007). Clustered regularly interspaced palindromic repeats (CRISPRs), a proposed bacteriophage resistance mechanism (Barrangou et al., 2007), have been identified in the *G. sulfurreducens* PCA genome (data not shown). Together these CRISPR regions contain 180 spacer sequences between them. It is currently unknown if

the homology between the gene sequence of the CRISPR region is similar to the genes contained within the induced prophage. Future testing would focus on determining whether or not other *Geobacter* spp. may serve as alternative host strains for the continuous cultivation of the induced prophage.

## 6.0 Viral decomposition as a nutrient source.

Viruses are composed of the same carbon-based biological macromolecules that make up and are metabolized by all cells, and in turn must be a part of the carbon (and other biogeochemical) cycle(s). While viruses promote the cycling of carbon by lysing

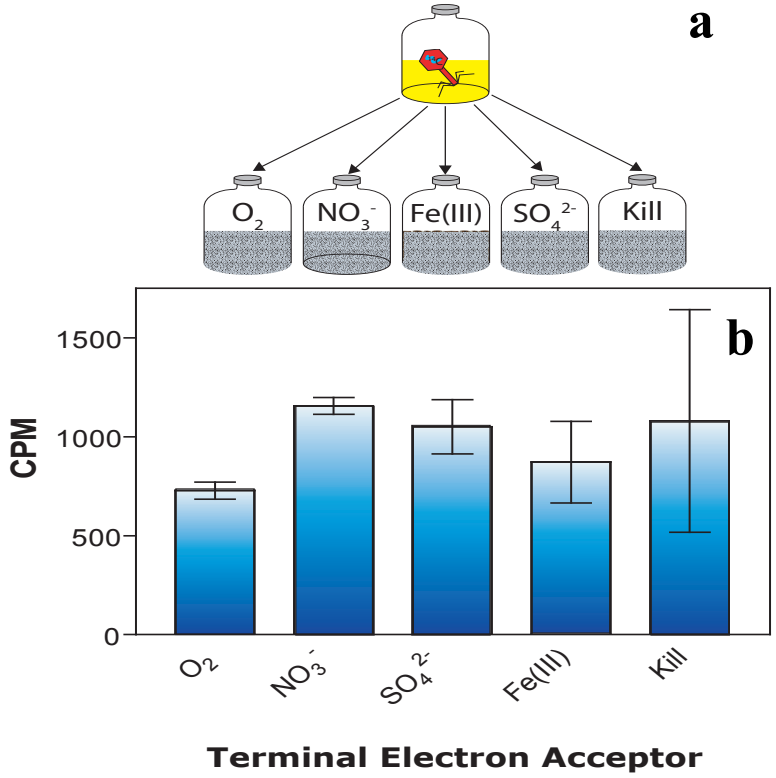


**Figure 6.6. Bacteriophage as a carbon source.** Viruses are submicron (20-200nm) particles consisting of single or double stranded DNA or RNA encapsulated by a protein coat (capsid). As such, viruses may be a source of organic carbon contributing to the DOM and POM pool that could be utilized by indegnous microbiota. a) Schematic for wastewater sludge preincubated under various terminal electron accepting processes and amended with radiolabeled (1.6% $^{14}C$:$^{12}C$) bacteriophage T4, a lytic phage infecting *Escherichia coli*. b) Radiolabeled carbon was not completely respired to $^{14}CO_2$ after one month of incubation as signficant losses of the radiolabeled carbon were not observed (p=0.406). This result is supported by headspace $CO_2$ analysis; $^{14}CO_2$ was not detectable. It is currently unknown if the indigenous microbial community incorporated the $^{14}C$ organic carbon into biomass. Further studies are necessary to determine if bacteriophage degradation was enhanced in live cultures.

cells, the production of progeny virions during infection also interrupts the carbon cycle by removing organic matter from the living organic pool. For example, the estimated $10^{31}$

viral particles found in the Earth's ocean would represent approximately 200 Mt of carbon. Unless viruses are decomposed, these viral particles would represent a terminal point in the carbon/nutrient cycle.

One potential way for viral particles to re-enter the carbon cycle is through microbially-mediated decomposition. Due to their small size, viruses have historically been included in the pool of dead or dissolved organic matter with regards to the aquatic food web. Previous research in marine sediments has demonstrated that a small percentage of

radiolabeled high molecular weight dissolved organic matter released from viral induced cell lysis (which therefore includes viral particles as well as cell debris) will be converted into microbial biomass. Furthermore, studies have also demonstrated that *Escherichia coli* is capable of utilizing either amino acids or DNA, the basal components of viruses, as a sole carbon and energy source. We therefore set out to test for microbially-mediated decay of viral particles, both in a pure culture system and in a natural microbial community. Cell lysis originating from viral infection liberates organic matter into POM and DOM. The organic matter is then converted and nutrients enter into the inorganic nutrient pool. DOM denotes dissolved organic matter. POM denotes particulate organic matter. Similar to aquatic environments, viral induced host cell lysis in terrestrial oligotrophic subsurface ecosystems could also play a significant role in nutrient cycling. However, the role that viruses play in nutrient cycling in subsurface environments remains unknown. Given that metal-reducing environmental isolates such as *G. sulfurreducens* PCA are susceptible to viral infection, host cell lysis under these conditions could release organic matter and nutrients back into subsurface soils and groundwater. To test the biodegradability of phage particles, 10ml of anaerobic mesophilic sludge collected from San Francisco East Bay Municipal Utility District (EBMUD) wastewater treatment digestor was incubated for two months in anoxic sealed vials under an $N_2$ headspace (Fig. 6.6a). T4, a well-studied *E. coli* phage, was added as the sole electron donor to each vial in addition to one of 10 mM oxygen, nitrate, sulfate, or ferric iron (in the form of hydrous ferric iron oxide) as potential terminal electron acceptors. After one month, the vials were re-amended with phage and electron acceptor. After a second month of incubation, 2 ml of sludge was removed for analysis and determination of the remaining electron acceptor concentration. The vials were subsequently amended with electron acceptor and T4, along with 8 nCi of $^{14}$C-labeled T4 (approximately 1.6% $^{14}$C:$^{12}$C) prior to incubation for 30 days after which the sludge and headspace of each vial were tested for the presence of $^{14}$C. If $^{14}$C-labeled phage were being completely metabolized, we would have expected $^{14}$C-$CO_2$ to evolve into the headspace and a decrease in the sludge activity. Unexpectedly radiolabeled carbon was not completely respired to $^{14}$C-CO after more than 30 days incubation under any of the conditions tested relative to the heat-killed control. It is currently unknown if the indigenous microbial community incorporated the $^{14}$C organic carbon into biomass but this seems unlikely without the production of $^{14}$C-$CO_2$. Further studies are necessary to determine if bacteriophage degradation was enhanced in live cultures relative to the heat-killed controls in these environmental microcosms or if the result was simply an anomaly of the experimental design.

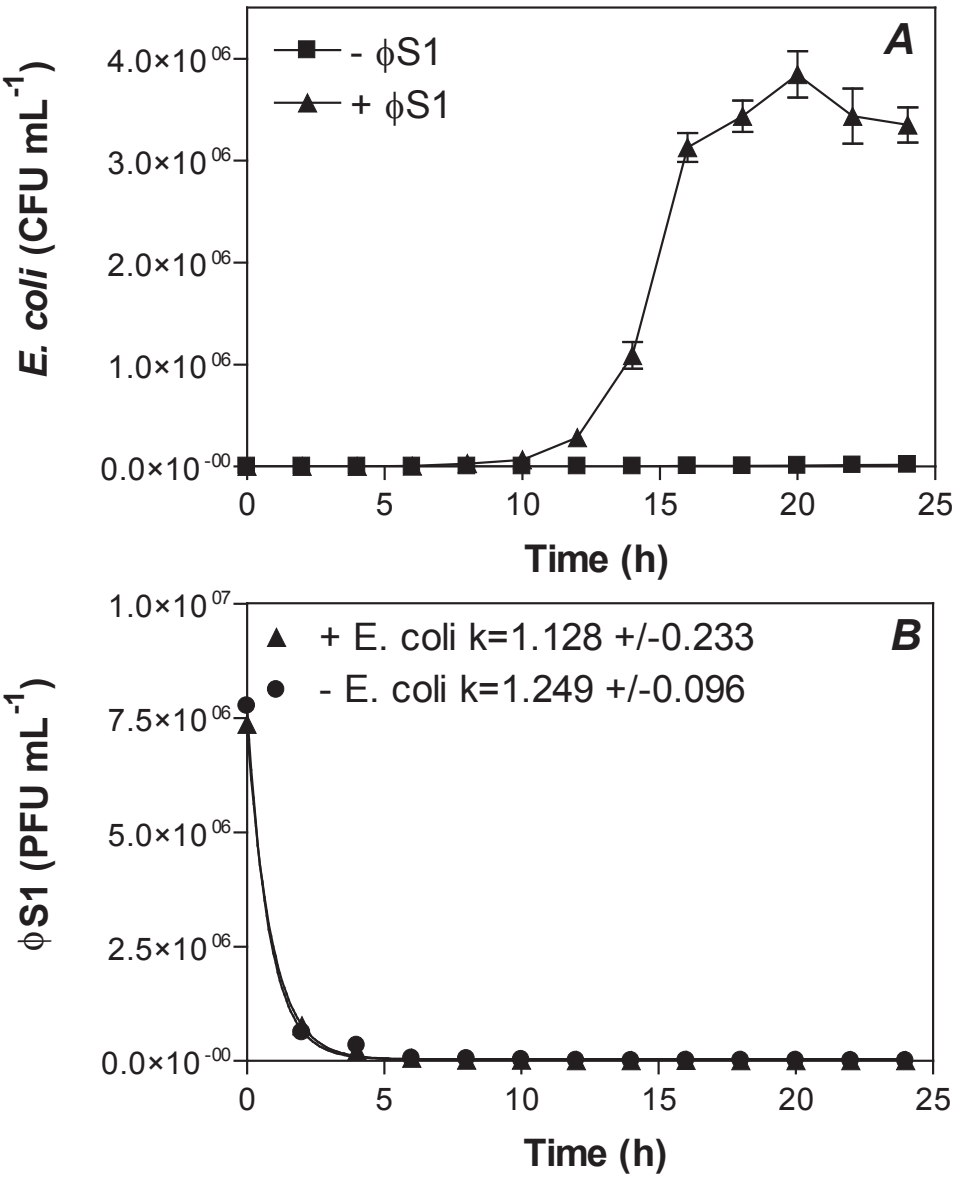


**Figure 6.7. Bacteriophage as a carbon source.** While T4 may not have been subject to complete degradation *E. coli* was capable of growing in minimal medium amended with CsCl purified S1, a lytic bacteriophage infecting *Pseudomonas fluorescens* as the sole carbon source (a). The rate of S1 decay was not enhanced by the presence of *E. coli* (b), thus suggesting the *E. coli* is not mediating decay but rather utilizing the decay products of S1. This preliminary evidence suggests that viral particles can contribute to the DOM/POM pool potentially utilized by an indegenous microbial community.

As a continuation of these studies we determined the ability of *E. coli* to grow using virions as a sole carbon source by performing growth curves in basal phosphate media at 37 ºC with either no carbon source, lactate as a positive control, or with purified ϕS1, a bacteriophage that infects *Pseudomonas fluorescens*. Prior to use the ϕS1 was purified through a CsCl density gradient to remove any other organic matter, and the CsCl was then removed by trapping and recovering the phage off of a 50 kD molecular weight cutoff filter. *E. coli* titers were determined by plate counts while ϕS1 titers were determined as plaque forming units on a lawn of *P. fluorescens*. The results obtained

indicated that after a lag phase of some 10 hours *E. coli* rapidly grew using ϕS1 as the sole carbon and energy source relative to the unamended control (Fig. 6.7a). Interestingly, monitoring of the ϕS1 titers indicated that the titer dropped off precipitously during the initial 6 hours of incubation and the rate of ϕS1 decay was unaffected by the presence of the *E. coli* strain (Fig. 6.7b). This results suggests that while *E. coli* can satisfy its nutritional needs using the decay products of ϕS1, it does not initiate or enhance a decay of the viral particle itself.

## 7.0 Conclusions.

The objective of this study was to investigate the interaction between viral particles and metal reducing bacteria. Efforts to predictably model remediation efforts require an understanding of the role that viral particles have on the growth and metabolism of metal reducing bacteria and thus metal (specifically Fe and U) biogeochemical cycling. The research outlined in the following sections represented a first attempt to address basic research needed to establish viral-metal-reducing bacterial relationships in anaerobic subsurface sedimentary environments. The data obtained provides valuable information in beginning to understand the relationship of viral and bacterial populations and the effect of viral particles on microbial communities in the environment and their potential impact on immobilization of U via U(VI) reduction. The presence of viral gene sequences within the genomes of model metal-reducing bacteria, specifically *Geobacter* spp., suggests these microorganisms are susceptible to viral infection. This is further supported by the induction of prophage incorporated into the *G. sulfurreducens* PCA genome. Viral infection of the metal reducing microbial community could influence the metabolic activity of these 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. In addition to the generation of carbon based viral particles, host cell lysis of indigenous microbiota, including metal-reducing bacteria, would result in the release organic matter and nutrients back into subsurface soils and groundwater. It is therefore important to understand potential factors controlling microbial metabolism in order to optimize bioremediatation strategies or to model and predict the transport of contaminants under natural attenuation scenarios.

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