Systems-based Approach to Understanding Technetium Immobilization in the Subsurface – 15326

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ABSTRACT

The purpose of this work is to identify the chemical and biological reactions that occur and potentially influence the mobility of Technetium (Tc-99) in the subsurface. Tc-99 is a by-product of nuclear fission processes and has a relatively long half-life (t1/2=212,000 years); additionally, it is highly soluble and mobile in groundwater and is most often remediated through pump and treat systems. The oxidized form of Tc-99 [Pertechnetate, $Tc(VII)O_4^-$] is highly soluble and very mobile, while the reduced form of Tc-99 [Technetium-oxide, $Tc(IV)O_2 \bullet nH_2O$] is insoluble. Historically, complex interactions within the subsurface have made clean-up efforts for Tc-99 challenging. It is thought that environmental bacteria play a role in reducing contaminants to less soluble forms but it is difficult to predict or estimate the interactions and impacts of the complex, mixed microbial communities found in the environment. We have set up controlled batch experiments to examine a selection of organisms (*Anaeromyxobacter dehalogenans* strain CP-C, *Geobacter sulfurreducens* strain PAC, *Geobacter daltonii* strain FRC-32, and *Shewanella oneidensis* strain MR1) that have known metal reducing capabilities, for their ability to reduce Tc-99 to less soluble forms. An additional organism, *Cellulomonas* strain ES6 was chosen based on its origin of isolation from the Hanford site. Organisms were evaluated under anaerobic conditions both as isolates and in mixed consortia with and without the presence of iron-bearing mineral substrate. Experiments were monitored using spectroscopy, qPCR, volatile organic acid (VOA) analysis, and inductively coupled plasma-optical emission spectroscopy (ICP-OES) and mass spectrometer techniques (ICP-MS). Initial data collected has shown that the presence and type of substrate may increase the reduction of Tc-99 to less mobile forms. Data collected from these experiments will allow for (a) a better understanding of the mobility of Tc-99 in the subsurface, (b) better predictions of contaminant fate and transport over time, and (c) the development of long term remediation strategies for Tc-99. Additionally, information collected will be used to develop a systems-based metabolic model with predictive capabilities.

INTRODUCTION

Tc-99 is a widespread contaminant throughout the world, considered one of the most problematic radionuclides in the environment, and is an important contaminant at several Department of Energy (DOE) sites, such as Hanford, WA; Oak Ridge, TN; Paducah, KY and Portsmouth, OH (1). At Hanford, over 500 Ci of Tc-99, as the pertechnetate anion (TcO_4^-), have been released to the vadose zone as part of past site operations. Tc-99 is mobile in predominantly oxidizing ground waters, with eventual discharge to the Columbia River, making it one of the site's major

risk-drivers (2). At the gaseous diffusion plants at Paducah, KY and Portsmouth, OH, Tc-99 levels of 1000-3000 pCi L^{-1} have been detected in the groundwater. In addition, residents near Paducah have been provided bottled drinking water as a consequence of spills and disposal operations that have resulted in the contamination of local aquifers and ultimately private drinking wells (U.S. DOE, 2008). At the field research center (FRC, Oak Ridge, TN), soils and groundwater are contaminated with concentrations of up to 40,000 pCi L^{-1} (0.02 µM)(3); while Hanford S-SX farm has had detections in groundwater of 10^8 pCi L^{-1} (54 µM).

The mobility of Tc-99 in the geologic medium is mainly a result of redox chemistry. Under oxidizing conditions, Tc-99 generally exists as TcO_4^{2-}, which has limited sorption to mineral surfaces and is therefore highly mobile (4-6). Under reducing conditions, TcO_4^- is reduced to the less soluble and therefore less mobile Tc(IV), which is strongly retained by geologic materials. Tc-99 immobilization can occur through direct enzymatic reduction to form hydrous TcO_2 or indirectly via abiotic electron transfer from reduced chemical species such as Fe(II) (7, 8). Direct enzymatic reduction of Tc-99 is typically associated with hydrogenase enzymes and is thought to be the predominant mechanisms for immobilization at relatively high concentrations of Tc-99 (9). At environmentally relevant concentrations (10^{-8} to 10^{-11} mol L^{-1}) (10, 11) reduction is thought to be mediated by abiotic reaction with reduction products, primarily Fe(II) (7, 12, 13), although some recent studies have shown enzymatic reduction at environmentally relevant conditions (6, 14).

The oxidized and mobile form of Tc-99 [Tc(VII)] undergoes microbially-mediated reductive transformation that decreases its solubility and thus mobility in groundwater, a process known as direct, (enzymatic) immobilization. Enzymatic Tc-99 reduction and associated immobilization via precipitation by metal reducing bacteria has been examined in both laboratory and field experiments (3, 6, 8, 11, 15-27). These studies have shown that metal and radionuclide reduction is catalyzed by a variety of phylogenetically diverse metal-reducing bacteria, typically as a product of electron flow through respiratory processes following the addition of an oxidizable carbon source or hydrogen. Although the organisms with capacity to reduce radionuclides are ubiquitous in the subsurface (28), their activity is often limited by the availability of suitable electron donors, inhibition by competing electron acceptors [Mn(IV), NO_3^-, SO_4^-] or other inhibitory conditions (e.g., low pH). The ability to enzymatically reduce Tc(VII) is found in several groups of proteobacteria, including members of the genera *Shewanella*, *Geobacter*, *Deinococcus*, *Escherichia* and *Desulfovibrio* (7, 9, 15, 16, 25, 27, 29-31). Tc(VII) is also reduced under acidic conditions by *Thiobacillus thiooxidans* (32), under alkaline conditions by *Halomonas* strain Mono (21) and at high temperature by *Pyrobaculum islandicum* (33).

Although much has been learned about the physiology and metabolic potential of single microbial species (pure cultures) that immobilize Tc-99, major gaps exist in our understanding of the functioning of these and other microorganisms in natural and contaminated ecosystems. The purpose of this research is to develop a genome-enabled systems biology approach to model and predict mechanisms of Tc-99 immobilization in the subsurface using a defined metal reducing microbial community and mineral phases. This research includes the use of advanced molecular techniques to evaluate the transfer of energy and metabolites within a defined Tc-99 reducing community and their environment (mineral and liquid phases), and the development of metabolic

network models for predicting microbially-mediated contaminant fate and transport. Together these methods will be used to predict Tc-99 fate and transport. The chosen radionuclide Tc-99 is a critical risk driver for DOE contaminated sites, and little success has been achieved in applying in situ techniques for immobilizing and/or remediating this contaminant. Thus, this research focuses on Tc-99, but will provide an approach with significant implications for metal and radionuclide remediation strategies as well as organic contaminant plumes, common across the DOE complex.

MATERIALS AND METHODS

Strain and Growth Conditions

The organisms used in this study included *Anaeromyxobacter dehalogenans* strain CP-C, *Cellulomonas* sp. strain ES6, *Geobacter sulfurreducens* strain PAC, *Geobacter daltonii* strain FRC-32, and *Shewenella oneidensis* strain MR1. Cultures were initiated from glycerol stocks and seed cultures for batch experiments were cultivated in TSB media for strains ES6 and MR1, SAG media for strains PAC and FRC-32, and R2A supplemented with 10 mM sodium fumarate for strain CP-C. Strains were cultivated at 30 °C under anaerobic conditions.

Medium Preparation and Batch Cultivations

A minimal medium (SAG) was used for analysis of Tc-99 reduction, and was formulated from three media types used in the cultivation of *Anaeromyxobacter*, *Shewanella*, and *Geobacter*. SAG medium constituents contained, per g/L^{-1}, Na_2HCO_3 (2.5), NH_4Cl (1.5), NaH_2PO_4 (0.6), $MgCl_2 \cdot 6H_20$ (0.5), NaCl (0.5), KCl (0.1), $Na_2WO_4 \cdot 2H_20$ (0.25 mg), Fe(III) citrate (25mM), acetate (12 mM), Wolfes vitamin solution (10 ml), and Trace elements (10 ml). MR1 utilized 10 mM lactate as oppose to acetate in SAG medium, and ES6 had 0.2% xylose supplemented into the SAG media.

Batch experiments were set up in 25 ml serum vials. Each serum vial contained 2.0 grams (dry weight) Hanford fine sands (HFS). Additionally, 18 mls of minimal media (SAG) was dispensed aseptically into each serum vial. All cultivations were conducted at room temperature at pH 7 in anoxic conditions by sparging the media with O_2 free N_2 for 15 minutes. Ammonium pertechnate (Tc-99) was added to achieve a final concentration of 10 ppm. Following addition of Tc-99 stock, vials were mixed thoroughly and allowed to sit overnight at room temperature. Organisms used for inoculation were grown up to mid-log phase, centrifuged at 10K g for 15 minutes, and the cell pellet was washed three times anaerobically with SAG medium without electron donor or electron acceptor. Cultivations were initiated by inoculating $1 \cdot 10^8$ cells along with the appropriate electron donor.

Anaeromyxobacter dehalogenans strain CP-C, *Cellulomonas* sp. strain ES6 , *Geobacter sulfurreducens* strain PAC, *Geobacter daltonii* strain FRC-32, and Shewenella *oneidensis* strain MR1 were evaluated both as axenic cultures and as mixed consortias to determine the feasibility of symbiotic Tc-99 reduction. Additionally, both a cell free control and heat killed control (using MR1) were tested. The mixed consortias were grouped as follows: (3) *Anaeromyxobacter dehalogenans*, *Geobacter sulfurreducens*, Shewnella *oneidensis* strain MR1, (4) *Anaeromyxobacter dehalogenans*, *Cellulomonas* sp. strain ES6, *Geobacter sulfurreducens*,

Shewnella oneidensis strain MR1 and (5) *Anaeromyxobacter dehalogenans*, *Cellulomonas* sp. strain ES6, *Geobacter daltonii* strain FRC-32, *Geobacter sulfurreducens*, *Shewnella oneidensis* strain MR1 (Table 1). Cell densities for the mixed consortias were divided equally among the consortia within the 10% inoculum volume. Both time t-0 and t-final samples were run in triplicate; t-0 vials were harvested following inoculation and t-final vials harvested under anaerobic conditions 15 days following inoculation. For the heat killed controls, MR1 was heated to 100 °C for one hour prior to inoculation. The complete set of t-0 samples was inoculated, mixed thoroughly and the substrate was allowed to settle. Vials were removed from the anaerobic chamber and subsequently sampled for the analyses shown in Table 2.

Table 1. Consortiums and carbon sources used to analyze Tc-99 reduction.

Bacteria	**Substrate**	**Media**	**Conditions**	**Inoculum Volume (mls, 10%)**	**Inoculum Cell Density**	**Carbon Source**
***Cellulomonas* sp. strain ES6**	HFS	SAG	Anaerobic, Room Temperature	2.0	$1 \cdot 10^8$	Xylose- 0.2%
***Shewenella oneidensis* strain MR1**	HFS	SAG	Anaerobic, Room Temperature	2.0	$1 \cdot 10^8$	Lactate- 10mM
***Anaeromyxobacter dehalogenans* strain CP-C**	HFS	SAG	Anaerobic, Room Temperature	2.0	$1 \cdot 10^8$	Acetate- 10mM
Geobacter sulfurreducens	HFS	SAG	Anaerobic, Room Temperature	2.0	$1 \cdot 10^8$	Acetate- 10mM
Geobacter daltonii	HFS	SAG	Anaerobic, Room Temperature	2.0	$1 \cdot 10^8$	Acetate- 10mM
Consortium (3) ***Anaeromyxobacter dehalogenans*** ***Geobacter sulfurreducens*** ***Shewnella oneidensis* strain MR1**	HFS	SAG	Anaerobic , Room Temperature	2.0	$1 \cdot 10^8$	Lactate- 10mM
Consortium (4) ***Anaeromyxobacter dehalogenans*** ***Cellulomonas* sp. strain ES6** ***Geobacter sulfurreducens*** **Shewnella *oneidensis* strain MR1**	HFS	SAG	Anaerobic , Room Temperature	2.0	$1 \cdot 10^8$	Lactate- 10mM
Consortium (5) ***Anaeromyxobacter dehalogenans*** ***Cellulomonas* sp. strain ES6** ***Geobacter daltonii* strain FRC-32** ***Geobacter sulfurreducens*** ***Shewnella oneidensis* strain MR1**	HFS	SAG	Anaerobic , Room Temperature	2.0	$1 \cdot 10^8$	Lactate- 10mM
Cell Free Control	HFS	SAG	Anaerobic , Room Temperature	2.0	$1 \cdot 10^8$	Lactate- 10mM
Heat Killed Control (MR1)	HFS	SAG	Anaerobic , Room Temperature	2.0	$1 \cdot 10^8$	Lactate- 10mM

Table 2. Sample analysis preformed and Preservation of archive samples.

	Analysis	Approximate Sample Volume (mls)	Sample Preservation
1	Volatile Organic Acids	5.0 mls	0.2 µm Filter; Store @-20°C.
2	Total Tc-99	2.0 mls	0.2 µm Filter; Store @-20°C.
3	Liquid for Tc-99 Speciation	5.0 mls	0.2 µm Filter; Store @-20°C.
4	Liquid DNA Extraction & qPCR	1.0 ml	1.0 ml sample + 500 µl 25% Glycerol; Store @-20°C.
5	Solid DNA Extraction , qPCR &Tc-99 Speciation	2.0 grams	Add 500 µl 25%; Store @-20°C

Serum vials were incubated at room temperature in the anaerobic chamber for 15 days and subsequently sampled and preserved under anaerobic conditions for the analyses shown in Table 2. Care was taken to ensure that the vials were not disturbed significantly during sample processing. At a later date preserved substrate samples divided into two 0.5 gram (wet weight) aliquots to be used for analyzing total Tc-99 present in the soil as well and completing DNA extractions and qPCR. Remaining substrate was left in original serum vial and recapped and is designated for Tc-99 speciation.

DNA Extraction and qPCR

Cell releasing buffer was added to the samples and they were sonicated for 20 minutes. DNA was then extracted using the MoBio Power Soil DNA Isolation kit according to the manufacturer's instructions.

Microwave Digestion

The microwave digestions were prepared by adding 9 mL of concentrated nitric acid, 2 mL of concentrated hydrochloric acid, and 2 mL of hydrofluoric acid to approximately 0.05 to 1 gram of sediment. The EasyPrep vessels were sealed and loaded into a CEM MARS Xpress microwave and ran at 210°C for one hour with a 30 minute ramp time. Once cooled, 20 mL of a 5% boric acid (by weight) solution was added (to neutralize the hydrofluoric acid), and the vessels sealed and returned to the microwave for digestion at 180°C for 25 minutes with a 25 minute ramp time. The digested sample was filtered (passed through a 0.45 µm PVDF membrane) and analyzed by inductively coupled plasma-mass spectrometry ICP-MS) and/or inductively coupled plasma-optical emission spectroscopy (ICP-OES).

Cations and Trace Metals

Major cation analysis was performed using a PerkinElmer 8300DV ICP-OES unit using high-purity single element calibration standards to generate calibration curves and a separate standard source to verify continuing calibration during the analytical run. This method is similar to Environmental Protection Agency (EPA) Method 6010B (EPA 2000b). The second instrument

used to analyze technetium-99 and uranium-238 was a PerkinElmer ELAN DRC-II ICP-MS using a Pacific Northwest National Laboratory procedure similar to EPA Method 6020 (EPA 2000c).

Speciation

A Biotek EON plate reader was used to measure absorbance over a range of wavelengths to determine Tc-99 speciation as shown in Figure 1.

RESULTS AND DISCUSSION

Cultures of dissimilatory metal reducing bacteria (DMRB) isolated from Oak Ridge FRC sediments (*Geobacter sulfurreducens* strain PCA, *G. daltonii* strain FRC-32, and *Anaeromyxobacter dehalogenans* sp. strain 2CP-C), from Hanford subsurface sediments (*Cellulomonas* sp. strain ES6), and from freshwater lake sediments (*S. oneidensis* MR-1), were obtained and cultured. These specific bacteria were chosen because they have been shown to reduce metals and radionuclides (such as Tc-99) chemically. In addition, genome sequence information used to create the metabolic models is available for all species with the exception of *Cellulomonas* sp. strain ES6. During this study the genome of *Cellulomonas* sp. strain ES6 was successfully sequenced. Currently the genome is being assembled and annotated. Information from the annotation will be used to complement and enhance our metabolic model of Tc-99 reduction.

Initial experiments have been performed to look at metal reduction using ferric iron [Fe(III)] as a surrogate. All five metal reducing bacterium mentioned above were shown to reduce Fe(III) in SAG media. Batch experiments were conducted using axenic cultures of each organism to monitor Tc-99 reduction as individuals. Additionally three consortia experiments that utilized Hanford fine sands were designed to provide insight into community interactions during microbial Tc-99 reduction in the subsurface. Data from these batch experiments show biotic and/or abiotic Tc-99 reduction when implementing the microbial consortias as well as cell free controls when compared to negative controls without Hanford fine sands. Spectrophotometric data show that Tc(VII) was reduced completely in all axenic culture, mixed communities, and in the cell free controls with HFS. These data indicate that a potential abiotic reduction occurred from reduced metals, such as Fe(II) in the substrate (HFS). Additionally, positive controls with media and Tc(VII) showed absorbance at both 244nm and 288 nm. See Figure 1 for Tc absorbance based on speciation.

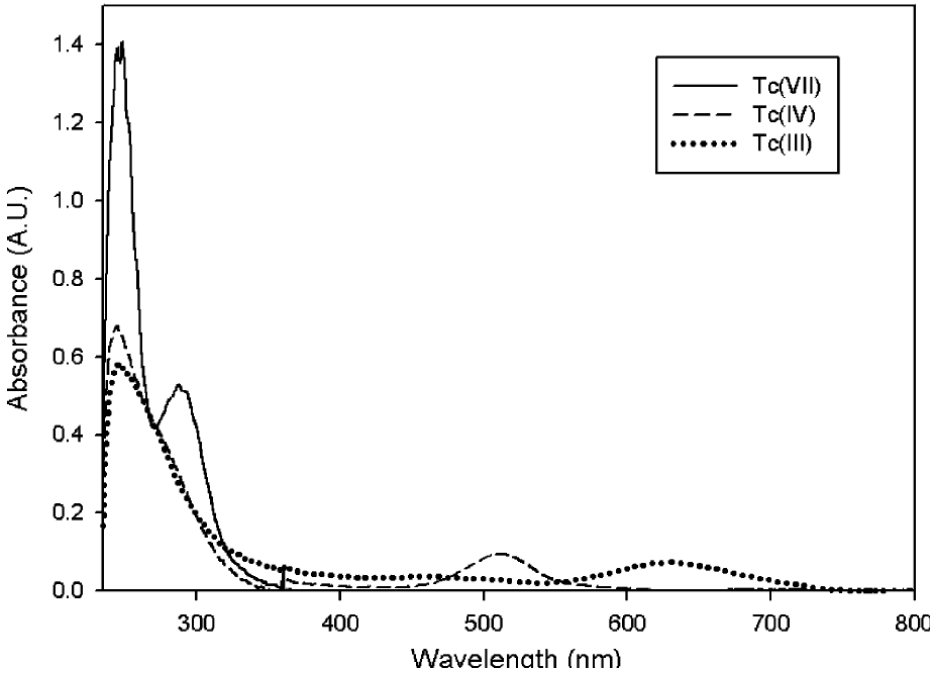

Figure 1. UV-visible absorption spectra for Tc-99 species. Taken from (34).

Finally, metabolic models for individual species have been initiated using genome information available for the above cultures in DOE's Joint Genome Institute. A community level metabolic model, which will provide knowledge on carbon and electron flow within the community and how that affects Tc-99 reduction, has also been initiated. In addition, growth information from the literature and experiments performed has been used to estimate biomass production rates. This model is based on elementary mode analysis and different methods of combining each species metabolism are being performed. We are currently setting up advanced hardware and software for developing these metabolic models.

While other metabolic models have been tested and validated, some for contaminated subsurface environments, the proposed approach, stoichiometric metabolic modeling, simplifies the microbial community into key or central metabolisms (Figure 2), and is not based on surrogates or well characterized single microbial members. This type of modeling has been used successfully to analyze natural and engineered microbial systems on both a pure culture and community level, and defines a network's metabolic potential based on a complete listing of the simplest, non-divisible pathways. That is, the proposed metabolic model (a) evaluates key, dominant members of the community, (b) assumes the most beneficial pathways for growth and energy for those community members, and (c) maintains the fewest metabolic pathways possible for a stable community. This type of approach allows for more real time prediction of metabolic microbial activities in natural systems through relatively simple straightforward measurements of a few key metabolisms. Understanding the microbial community at this level, allows for better model integration and evaluations across disciplines and more importantly when incorporating microbial rates/activities in site conceptual models for understanding complex contaminant fate and transport.

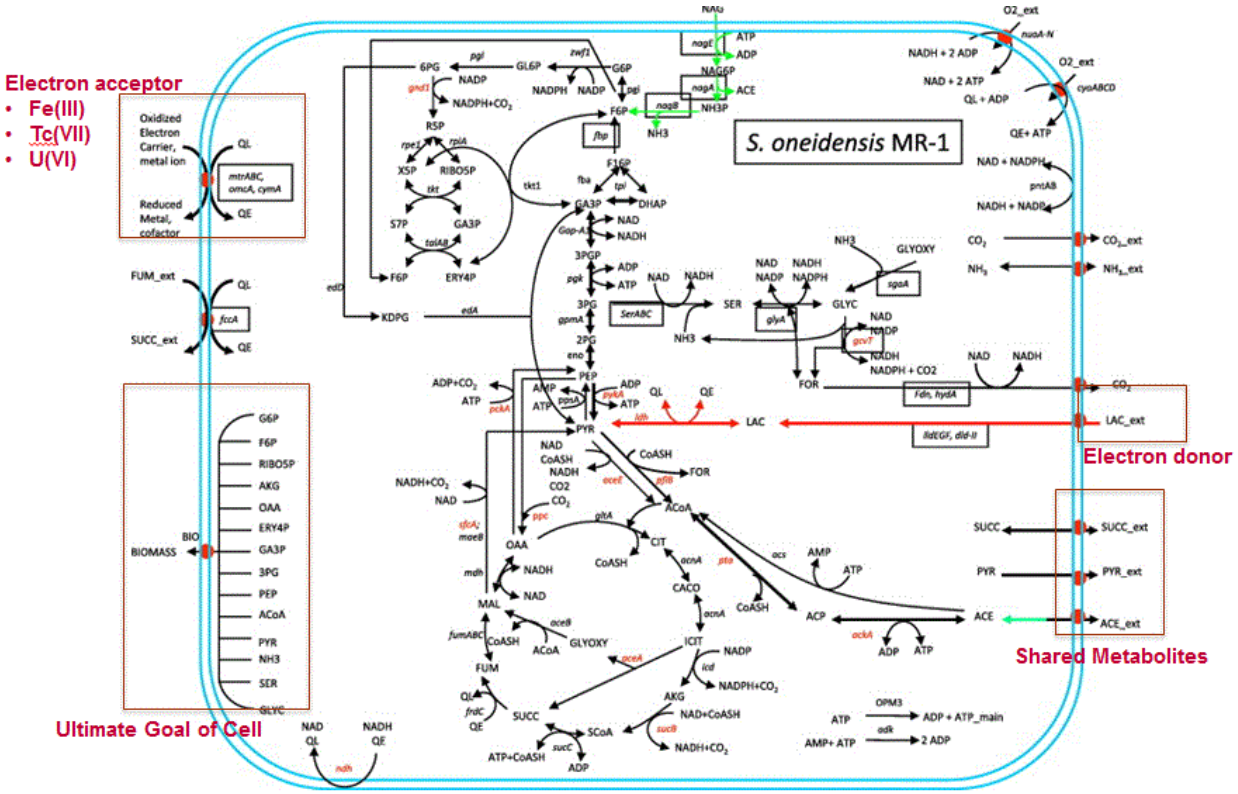

Figure 2. *S. oneidensis* central metabolism, a basis for our metabolic model to provide context to understanding and predicting contaminant plum migration and evaluate potential remedial strategies. Modified from (35).

CONCLUSIONS

Reduction of Tc-99 in the environment is highly complex and dependent on environmental, geological, and microbiological conditions of the contamination area. We have been able to show that under controlled conditions that the organisms examined (*Geobacter sulfurreducens* strain PCA, *G. daltonii* strain FRC-32, *Anaeromyxobacter dehalogenans* sp. strain 2CP-C, *Cellulomonas* sp. strain ES6, and *S. oneidensis* MR-1) have the ability to reduce Tc-99 to more insoluble forms. Further metabolic modeling will enable us to better understand the interactions that occur in complex microbial environments regarding Tc-99 reduction. Additionally, further molecular analyses (e.g. qPCR) is being performed to enhance our understanding of these microbial community interactions and more, to develop methods and tools to monitor and better understand the implications for these interactions in real time.

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