THE EFFECTS OF REDUCED PHOSPHORUS COMPOUNDS ON BACTERIAL POPULATIONS IN THE SOIL

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in

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by

Raj Patel

Fall 2015

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P	Phosphorus. Refers to any molecule containing phosphorus.
Pi	Phosphate. Inorganic phosphorus compound. Oxidation state +5.
Pt	Phosphite. Inorganic reduced phosphorus compound. Oxidation state +3.
PH_3	Phosphine. Inorganic reduced phosphorus compound. Oxidation state -3.
*Ptx*D	Gene responsible for the oxidation of phosphite to phosphate.
MOPS	3-(N-morpholino) propanesulfonic acid buffer with glucose added.
Km	Kanamycin. Bactericidal antibiotic.
Cm	Chloramphenicol. Bactericidal antibiotic.
PCR	Polymerase Chain Reaction. Technology used to amplify small amounts of DNA to greater magnitudes.
DGGE	Denaturing Gradient Gel Electrophoresis. It is a fingerprinting methodology that studies microbial interactions between themselves and their environment.
OTU	Operational Taxonomic Unit. Defining term for a species or group of species when only DNA is available.
GC	Guanine-Cytosine. Two nucleotides that are held together by 3 hydrogen bonds. They have a strong bond that is more resistant to chemical and temperature degradation.

ABSTRACT

THE EFFECTS OF REDUCED PHOSPHORUS COMPOUNDS ON BACTERIAL POPULATIONS IN THE SOIL

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The characterization of bacterial metabolic pathways oxidizing phosphite have been characterized. However, bacterial oxidation of phosphite in the environment and its impact on the biogeochemistry has been largely ignored. In this study I attempted to answer 1) do excessive phosphite concentrations in soil significantly alter bacterial populations in soil; and 2) to what extent do these changes affect the overall bacterial community. To answer these questions we performed a soil competition assay, utilizing isogenic reporter bacteria (1 oxidizer and 1 non-oxidizer of phosphite) that were inoculated into an intact soil environment and recovered them from the same soil environment, and tracked their population decreases and increases over time in relation to different phosphorus treatments, including phosphite. We saw a significant change in our reporter bacteria in the phosphite treatment, and saw potential phosphate recycling occur

via phosphite oxidizing bacteria utilizing phosphite as a sole phosphorus source and subsequently recycling the phosphate into the soil environment for phosphite non-oxidizers to use as a sole phosphorus source. Denaturing Gradient Gel Electrophoresis also displayed significant change in bacterial populations over time in the soil sample used for the soil competition assay. My results indicate that excessive dumping of phosphite into soil can lead to major bacterial population change, the exact effects from this change still need to be investigated by future researchers, which directly will contribute to the little known phosphorus cycle.

CHAPTER I

INTRODUCTION

Phosphate (Pi), is critical in molecules such as ATP, DNA, RNA, and phospholipids. In many environments, these essential molecules cannot be continuously produced since Pi is often a limiting nutrient in the environment, thus organisms frequently endure Pi starvation. Due to the high biological demand for Pi, and the scarcity of soluble forms that can be used by most organisms, bacteria have developed strategies to scavenge phosphorus (P) compounds may be present, including compounds in which P is in a more reduced state relative to Pi. The ability of numerous bacteria to do this has challenged the general perception that Pi is the only biologically relevant P compound available in the environment. There are an increasing number of reports that demonstrate the significant role of reduced P compounds as a P source for bacteria in the environment (1-16).

Organic Reduced Phosphorus Compounds in The Environment

In aerobic environments, nearly all of the P present can be found in compounds in which P is in its highest oxidation state (+5 oxidation state) as it is found in Pi or phosphate-esters. However, more sensitive detection methods that have been recently developed have exposed a variety of additional and naturally occurring P compounds in which P is in a more reduced state. For example, phosphonate (Pn) and

phosphinate compounds have been shown to be abundant in a variety of environments (Fig. 1) (6, 17, 18). These biologically produced molecules possess a carbon-phosphorous (C-P) bond, a bond which is more stable than the carbon-oxygen-phosphorous which is more stable than the carbon-oxygen-phosphorous (C-O-P) bond found in phosphate-esters (6). For example, 20-30% of the available P in the world's oceans are composed of phosphonic acids, and soil dwelling *Steptomyces* sp. produce numerous reduced P compounds in addition to Pn compounds (17). In 1959, a naturally produced Pn (2-aminoethane Phosphonic Acid) was isolated from rumen protozoa (17, 18). More so, Aminoethylphosphonate (AEpn), is the most common Pn, it is found in transferase enzymes and is crucial in the formation of Pn macromolecules (lipids and exopolysaccharides, found in membranes) (17). Pn presence can be observed in prokaryotes and eukaryotes, ranging from microbes to humans. While vertebrates seem to obtain Pn compounds through diet rather than *de novo* synthesis, bacteria can produce them (6, 17). For example, a majority of the natural Pn antibiotics such as phosphonotrixin, fosfomycin, plumbemycin, and fosmidomycin are created by Actinobacteria, with studies showing some *Bacillus* and *Pseudomonas* species producing these products as well (6, 17). While many soil dwelling bacteria have the ability to produce these compounds, other bacteria are very adept at breaking them down and oxidizing them for use as a P source. To date, four pathways for phosphonate and phosphinate oxidation are known and these have been identified in numerous γ-proteobacteria species (6, 19). In most cases, the intermediates produced during the oxidation of Pn are inorganic reduced P compounds. Cumulatively, these reports strongly suggest an important role for naturally produced Pn in the environment.

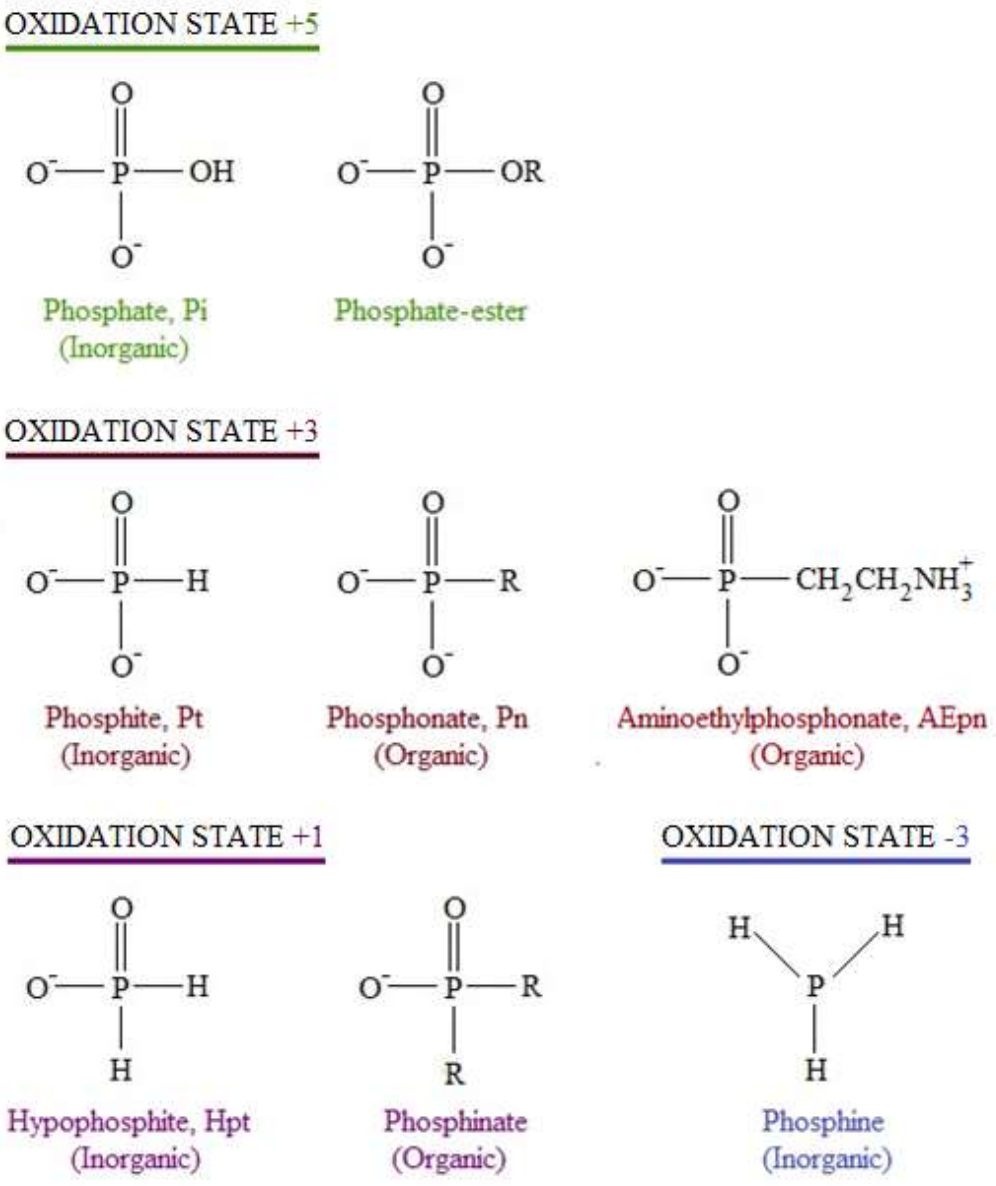

FIG. 1. Different organic and inorganic structures of P compounds. All pertinent structures, oxidation states, and abbreviations are given.

Pn compounds are also commonly introduced into the environment through human activity. The most prevalent of these is through the widespread use of Roundup®, which contains glyphosate, an herbicide containing Pn (20). Furthermore, human

urbanization has caused destruction of forests and grasslands which inadvertently accelerate erosion and runoff, releasing reservoirs of Pn into the environment (21). More so, activities such as recycling manure for farming, and human waste in urban sewage systems, are also intensifying Pn levels in the environment (21). Although, examining contributions made by human activity is an important factor for determining the levels of Pn in the environment, determining the natural concentrations of Pn in the environment is important for understanding the impact these compounds have on individual bacteria, and the overall P cycle.

Inorganic Reduced Phosphorus Compounds In the Environment

Pi concentration levels in the environment have been commonly investigated and reported. However, in 2003, the validity of these Pi level reports were questioned since the standard P detection protocol failed to address how reduced P compounds (if present) were quantified during the assay (22). Previous environmental samples were analyzed using a colorimetric agent which binds to the reactive P in the sample, mainly the +5 oxidation state P (22). However, Morton *et al.* observed that only 5-16% of the reduced P compounds in their samples were reactive to the colorimetric agent (22). Furthermore, it was believed that soluble phosphite (Pt, oxidation state +3) and hypophosphite (Hpt, oxidation state +1) (both reduced P compounds) were previously incorrectly categorized as either polyphosphates or organic phosphates (22). This study suggests that inorganic P compounds are present in the environment, but have been historically overlooked due to the lack of adequately sensitive and accurate detection methods. Using more modern detection methods has resulted in the detection of both

compounds in variety of environments including hotels, restaurants, hospitals, factories, and refineries throughout the United States (23).

While the natural occurrence of Hpt and Pt in the environment has only recently been established, the abundance of PH_3 (-3 oxidation state) has been well documented for decades. Furthermore, it has been recently shown that PH_3 acts as a natural reservoir for Hpt and Pt production. Numerous studies have shown PH_3 production during waste processing (sewage sludge, landfills, etc.), in anaerobic sediments, in marsh gases, and in digestive tracks of swine, cattle, and other livestock (12, 22-29). Concentrations of PH_3 have been detected in adult human feces (81 ng/kg^{-1}), swine colon (103 ng/kg^{-1}), and cattle rumen (2.9 ng/kg^{-1}) (30). Also, PH_3 concentrations are prevalent in the high troposphere across the North-Atlantic at concentrations of 0.39-2.45 ng/m^3 (31). Finally, PH_3 concentrations have also been measured in the lower terrestrial troposphere at both urban and rural sites. A German rural site has detectable PH_3 levels at 2030 pg/m^{-3}, while a German urban site had detectable PH_3 levels at 4630 pg/m-3, suggesting a correlation between human activity and PH_3 production (32). In most cases, the production of PH_3 is believed to be due to microbial reduction of Pi to PH_3, perhaps through Hpt and Pt intermediates. However, this has not been reliably demonstrated and the proposal remains contentious due to the unfavorable energetics of this reaction (6, 7).

PH_3 is known to rapidly oxidize to Pi in the presence of oxygen. An investigation took way in 2004 in which researchers used ion chromatography to detect signal intensities for Hpt, Pt, and Pi over time after all three compounds were injected into columns at time 0 (33). Hpt was detected first at 3 minutes, followed by Pt at 10

minutes, and finally Pi at 14 minutes (33). The three peaks observed in the ion chromatography confirm that the compounds elute in this order, suggest the intermediate pattern from PH_3 to Pi is first through Hpt followed by Pt, and it is in agreement with the common belief that Hpt and Pt are intermediate compounds of PH_3 oxidation to Pi (Fig. 2A). This study revealed the direct evolution of Pt and Hpt from PH_3 using ion chromatography. In addition, Robinson and Bond discovered Hpt and Pt to be radioactive residues of $^{32}PH_3$ fumigated wheat storage facilities (34). Two years later it was found that when PH_3 was introduced to a soil environment, only 70% of the initial amount was traceable as Pi after 40 days (35). This leaves the remainder 30% as reduced P compounds; the authors concluded that Hpt and Pt should compose some percentage of the untraceable Pi (35). Finally, PH_3 levels have consistently been observed to deplete over time when added to different soil environments, with faster rates of depletion in the presence of Fe (III). However, it is unknown if the PH_3 oxidizes to Hpt or to Pt or all the way to Pi in these conditions (25).

In addition to the apparent natural production of PH_3, Pt, and Hpt, these compounds are regularly introduced into the environment through human activities such as fumigation (PH_3), manufacturing of metal plates and aviation (Hpt), and as herbicides, pesticides, fertilizers, and in sudden oak death treatment (Pt) (36-38). Thus, while these human practices have clearly shown to form a significant portion of the total P available in the environment, the effects of these alternative P compounds on environmental microbial populations have gone unexamined.

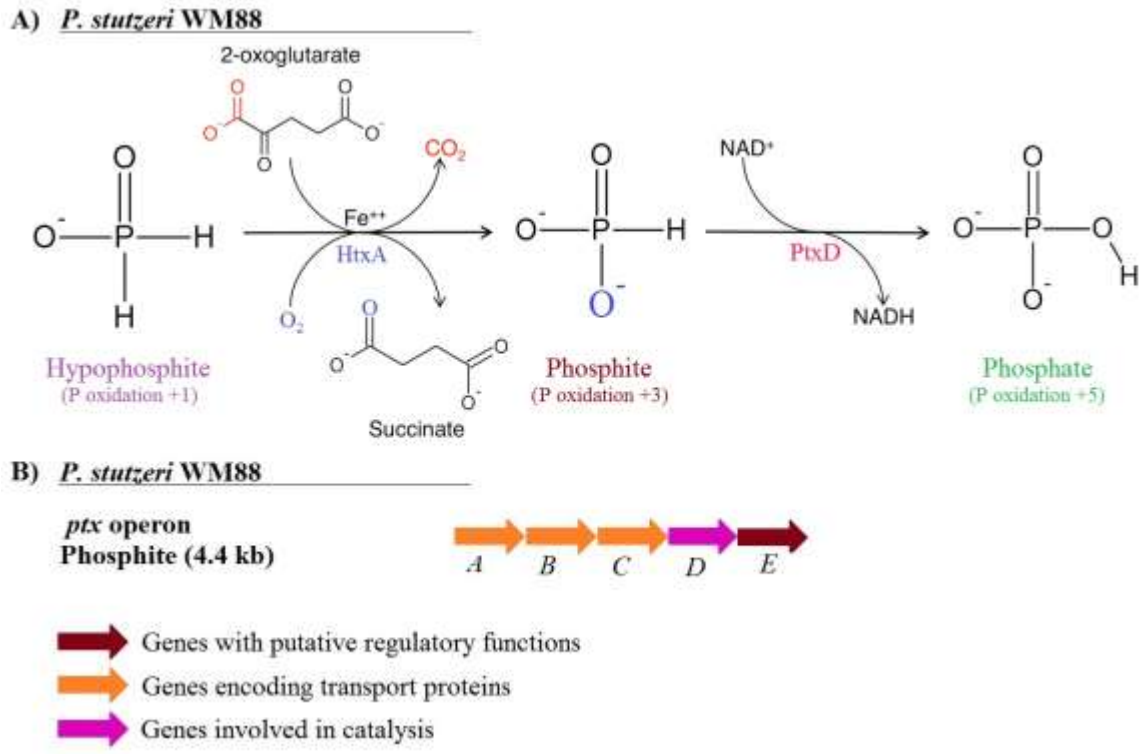

FIG. 2. A) Hpt and Pt oxidation pathway via genes *htx*A and *ptx*D, respectively. B) The *ptx* operon which is involved in the oxidation of Pt. Colored arrows show genes involved in putative regulatory functions, transport, and catalysis. Both models are completely relevant in *P. stutzeri* WM88.

Phosphite Oxidation of Bacteria

While Pi is believed to be the preferred source of P for organisms, many studies have shown that when Pi starvation does occur, numerous bacteria are capable of utilizing reduced P compounds as the sole P source in place of Pi (10, 13, 39-41), a beneficial strategy that bacteria can employ in the environment.

Adams and Conrad conducted the first bacterial Pt oxidation study in 1953 (39). They isolated soil bacteria that oxidized Pt to Pi and used the Pi as a source of P for growth, an ability which they believed many other microbes possessed. More so, they determined that Pt oxidation occurred via a metabolic pathway possessed by the bacteria

process. Seven years later, Casida reinforced these findings with nearly identical results to those of Adams and Conrad (41). Some of the genera found to oxidize Pt in these studies include: *Pseudomonas*, *Erwinia*, *Aerobacter*, *Agrobacterium*, *Rhizobium*, *Azotobacter*, *Staphylococcus*, *Bacillus*, and *Xanthomonas* (39, 41). The next two decades revealed anaerobic utilization of Hpt and Pt by a *Bacillus sp.*, and more specifically a partially purified hypophosphite oxidase enzyme was isolated from *Bacillus caldolyticus* (10, 11). A cascade of inquiries emerged as a result of these studies, focusing on both the ecological and molecular aspects of P compound redox reactions in bacteria (1-3, 6, 11-14, 17, 25, 29, 30, 42, 43).

The oxidation pathway of Pt to Pi has been characterized in *Pseudomonas stutzeri* WM88 and involves five genes in the *ptx* operon, *ptx*ABCDE (Fig. 2B) (3, 8). The gene *ptx*D (NAD-dependent phosphite dehydrogenase) is known to be solely responsible for the oxidation of Pt to Pi (3, 8). Refer to Fig. 2 for the functions of all *ptx* genes. These studies have established biochemical pathways of reduced P oxidation in bacteria, and more generally a solid foundation for further investigations concerning bacteria and reduced P compound interactions.

In 2002, *Desulfotignum phosphitoxidans* FiPS-3 was isolated from marine sediment (15). This strict anaerobic bacterium can utilize Pt as the sole electron donor, and can oxidize Pt along with glutamate, fumarate, acetate, and hydrogen (15). This organism's unique metabolic capability to use Pt as its sole energy source, rather than a P source, for growth, suggests significant quantities of Pt in its environment. This phenomenon remarkably changes our understanding of the P cycle, and knowledge about reduced P compound redox reactions carried out by bacteria.

Environmental Studies of Bacteria Utilizing Hypophosphite, Phosphite, and Aminoethylphosphonate

Due to the important role reduced P compounds can play for soil microbes during Pi starvation, further investigations are required to better understand how bacteria oxidize and exploit these compounds metabolically during such conditions in the environment. Furthermore, research efforts need to be focused on improving an overall picture of the P cycle and how environmental bacteria contribute to this. In an effort to understand the ecological significance of these processes, Stone and White determined the concentration and diversity of bacteria that could oxidize at least one (or more) of the following reduced compounds: Hpt, Pt, and AEpn in different environments (14). In these studies, through quantification and statistical validation two crucial patterns were observed: 1) Regardless of season, type of sample (sediment, water, soil, etc.), or sample site (pristine vs. urban) the most probable number of P oxidizing bacteria per gram (MPN/g) showed bacterial growth on Hpt, Pt, or AEpn to be comparable to bacterial growth on Pi (1e+6 to 1e+7 cells/gram) (14): and 2) A broad range of reduced P oxidizing bacteria were isolated, most of them common soil bacteria (44). When compared to the findings of Adams *et al.* and Casida, Stone's examination revealed two novel reduced P oxidizing genera: *Varivorax* and *Micrococcus*, as well as three novel reduced P oxidizing species of *Pseudomonas*: *P. paradoxus*, *P. mendocina*, and *P. reinekei* (14, 39, 41).

These findings suggest that bacterial reduced P oxidation cannot be a new phenomenon, primarily indicated by the lack of difference in the concentrations of bacteria from pristine environments compared to the concentration of bacteria in human

influenced environments. Clearly, reduced P oxidation cannot be a recently acquired bacterial trait caused from human activity (farm, run-off, etc.), solidified by the observation that no differences were noted when comparing bacterial cell concentrations between disrupted human environment samples and pristine environment samples. This study's discovery of novel genera and species associated with reduced P compound oxidation should urge researchers to consider this ability to be much more prevalent than previously assumed, both in the environment and within the bacteria domain.

Denaturing Gradient Gel Electrophoresis

This study will focus on the effects of Pt on bacterial populations when Pt is introduced into the environment through human activities, such as fertilizing. A tool that can be used for investigating bacterial population changes in the soil is Denaturing Gradient Gel Electrophoresis (DGGE). DGGE was solidified as a molecular approach in 1993, and it stimulated a new area of microbial research that allowed in situ analysis of sediment bound microbes and microbes that do not proliferate with traditional laboratory selective enrichment cultures (45). Numerous studies have taken advantage of DGGE as an effective tool for examining microbial populations in various environments including, hot springs (46), Antarctica coastal waters (47), rhizospheres (48, 49), paddy fields (44), wastewater (50), high temperature petroleum reservoirs (51), food (52, 53), and soil (54, 55).

DGGE works with DNA that is directly extracted from an sample (e.g. soil) and has undergone polymerase chain reaction (PCR), typically to amplify a variable region of all 16S rRNA genes present in the sampled environment. These identical sized

DNA fragments are then run down a polyacrylamide gel and the fragments are separated based on Guanine-Cytosine (GC) content of each band, thus allowing separation of distinct 16S rRNA genes based on sequence. More so, it is thought that denaturing also occurs in areas that are rich in Adenine-Tyrosine (A-T) content more than the rich GC areas, thus there are different migration patterns down the DGGE gel even with a single base pair difference between sequences. Each band ideally should represent a single organism, also known as Operational Taxonomic Units (OTUs), and each OTU should migrate different distances down the gel. However, there is no exact way to predict ahead of time the patterns that will form before the experiment is conducted. Theoretically, the number of bands present in the gel should represent the number of OTUs present in the sample, and the band intensity should represent the abundance of that taxon in the sample. In practice, both of these factors can be complicated with differing rRNA and due to micro-variations within a taxon. More so, PCR bias and chimeras can result in false bands shown on the gels, thus a low diversity sample (not soil) would be ideal for DGGE. However, with limitations, this study will use this powerful tool to examine total bacterial population changes over time when different P compounds are added to an intact soil environment.

Aims and Questions for this Study

As a limited and non-renewable resource, it is an invaluable obligation for microbiologists to assess reduced P impacts and its recycling occurrences in the environment, via bacterial metabolic processes. Recently, great progress has been made over the past 40 years in our understanding of bacterial metabolic pathways that oxidize

reduced P compounds, and of the sources these compounds in the environment. However, this new information has brought to light how little we actually know about P cycling in the environment relative to other elemental nutrient cycles and has led to an increased appreciation for the complex roles microbes have in this process. Yet, some very fundamental questions as to the environmental significance of microbial reduced P conversions and the effect of reduced P compounds on the ecology of soil microbe remain. Understanding the fate of Pt and its effects on microbial communities in the environment is of particular importance due to the increased application of this compound agriculturally as a fertilizer and in forest management as a fungicide to treat sudden oak death. Given the introduction of large amounts of Pt into the environment by human activity, the known ability of many known soil bacteria to convert this into Pi, and the crucial role of Pi to all organisms in the environment, it is essential to understand how bacterial populations (both reduced P oxidizers and reduced P non-oxidizers) might be altered by Pt influx, and how this affects Pi availability in the environment.

Pt has a half-life of 90-120 days in the soil, during this time it is oxidized to Pi (38). What happens to microbial communities after Pt treatments of soils during this time has not been examined. Specifically, how do bacterial communities change over time through the addition of Pt in soil? Do these bacterial community changes affect any aspect of the P cycle? To address these questions two approaches will be used:

1) A bacterial competition assay which consists of tracking the bacterial growth overtime in soil, both before and after the addition of Pt, Pi, and No P in different soil samples.

2) Denaturing Gradient Gel Electrophoresis (DGGE), to allow a qualitative analysis of the total bacterial community changes in soil over time from part 1.

From these two experimental approaches, we hope to learn how Pt affects the populations of reduced P oxidizing bacteria, relative to non-oxidizers, and how this compound may induce larger scale changes in the bacterial compositions of the exposed soil microbial communities. We hypothesize that when soil is treated with Pt, the populations of Pt non-oxidizing bacteria will decrease significantly while the populations of Pt oxidizing bacteria will increase significantly.

CHAPTER II

METHODS AND MATERIALS

The soil competition assay was used to quantify changes in reporter bacterial populations added into soil. These organisms are added to an intact soil environment and the soil samples are treated with either Pt, Pi (control), or No P (control). Pt oxidizing and Pt non-oxidizing isogenic strains of *Pseudomonas stutzeri* are the reporter organisms used in this study. These two organisms are Tn5 transposon mutant bacteria containing two distinct antibiotic markers for simple selection conditions. The reporter bacteria were previously isolated from soil, and will easily adapt to a soil environment when added back into soil. More importantly, the strains used have been well characterized for their ability to oxidize Pt.

Media

Unless stated, all media and buffers used in this study were prepared as previously described (56). Luria-Bertani (LB) medium contained 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter. Minimal Media (GluMOPS) contained in final concentration of 0.2% glucose in 1x "M" MOPS (3-[N-morpholino] propanesulfonic acid) buffer. Unless specified, all added P source compounds for media were present at a 1 mM final concentration. Antibiotics were used at the following concentrations: kanamycin (Km), 50 µg/ml; ampicillin (Amp), 50 µg/ml; and chloramphenicol (Cm), 12 µg/ml. Agar solidified medium contained 16 g of agar per liter.

Construction of Reporter Bacteria

P. stutzeri strains WM88 and WM3617 were the starting strains used in the construction of the reporter bacteria; both strains have been previously described (3, 7). *P. stutzeri* WM88 is a wild-type soil isolate and is fully capable of oxidizing Pt to Pi for growth on Pt as a sole source. *P. stutzeri* WM3617 is a deletion strain of WM88 in which all Pt oxidation pathways have been deleted, thus rendering it unable to oxidize Pt under any conditions. Other than their different Pt oxidation capabilities, these two strains are isogenic and were used in the soil competition assay. *P. stutzeri* WM88 and WM3617 were given different antibiotic resistance markers through a Tn5 transposon delivery vector. *P. stutzeri* WM88 was given a Cm resistance marker using a previously reported Tn5 transposon delivery vector, pRL55 (57). The resulting Cm resistant strain is referred to as *P. stutzeri* RP1. A Km resistance marker was inserted into *P. stutzeri* WM3617 using a previously reported Tn5 transposon delivery vector, pRL27 (9). The resulting strain from this conjugation is referred to as *P. stutzeri* RP2.

Conjugation and Mutagenesis of Reporter Bacteria *P. stutzeri* RP1 and RP2

Insertion of the antibiotic cassettes into each of the starting strains was carried out as previously described using a donor strain BW20767, which carries transfer functions and the respective conjugal plasmid vectors that carry the antibiotic cassettes within a transposable region (57). In brief, recipient and donor strains were grown to mid-exponential phase in LB broth (WM88 and WM3617), LB broth containing ampicillin (*E. coli* BW20767/pRL55), or LB broth containing kanamycin (*E. coli* BW20767/pRL55) at 37°C. Donor and recipient strains were mixed in a ratio of 1 (50 µl):10 (500 µl),

respectively, and collected on a 0.2-µm Nalgene® filter. The filter containing the cells was incubated overnight on LB agar medium at 37°C overnight to allow for conjugation. After incubation, all cells on the filter were suspended in 1x "M" MOPS buffer, followed by direct plating onto agar solidified GluMOPS media containing the appropriate P source and antibiotic, in order to select for desired Tn5 transposon insertion mutants. WM88 derived Cm resistant exoconjugants were selected for on GluMOPS containing Pt and CM while WM3617 derived exoconjugants were selected for on GluMOPS containing Pi and Km. Selection plates were incubated at 37°C overnight, and the resulting colonies were the Pt oxidizing, *P. stutzeri* RP1 and non-Pt oxidizing RP2.

Phenotype Verification of Tn5 Mutant Reporter Bacteria

Growth curves were generated for *P. stutzeri* wild-type (WM88) and *P. stutzeri* Tn5 insertion mutants RP1 and RP2 to confirm that all critical genes were intact and growth rates were similar between all the strains to be used in the competition assay. *P. stutzeri* strains WM88, RP1, and RP2 were added from overnight LB cultures in triplicate into four different media, LB, GluMOPS + Pi, GluMOPS + Pt, and GluMOPS + No P source. Growth curve assays or each cultures were carried out over 24 hours at room temperature in a 96 well plate with mild agitation in a final volume of 5ul. Optical density measurements were taken every 20 minutes at A450nm using a BioTek Synergy plate reader. Generation times were then calculated for growth analysis.

Soil Preparation for Competition Assay

Soil that has never been treated for any P was gathered from Chico University Farm and extensively mixed for homogeneity via vortex on maximum speed. To confirm that the naturally present organisms in the soil sample to be used for the competition assays were not Cm and/or Km resistant, 0.1 g of soil was mixed with 0.9 mL of GluMOPS media to make a soil suspension, which was serially diluted in sterile to 10^{-4}. The 10^{-2} to 10^{-4} dilutions were plated onto LB + Cm and LB + Km agar plates. This critical step was carried out to ensure that the growth on the Cm or Km LB plates during the competition assay exclusively represented *P. stutzeri* RP1 or RP2, respectively.

After the preliminary assessment of the soil, 900 g of the same soil sample was equally distributed into six different autoclaved jars (150 g per jar), and setup in replicate for three different treatment types, Pi, Pt, or No P. *P. stutzeri* RP1 and RP2 were grown overnight in LB broth, and 1.5 mL of each organism was diluted into 4 mL of sterile water. The soil in each of the jars was immediately inoculated with the entire 7 mL mixture containing both reporter strains, followed by thorough mixing. Concurrently, a viable cell count was immediately performed on the freshly mixed soil samples to obtain the actual cells per gram added into the soil. Refer to Fig. 3 for illustration.

Nutrient Addition to Soil Samples

All necessary nutrients (carbon, nitrogen, etc.) other than P were added in higher concentrations than present in the environment to all six jars at day 0, and the same concentrations would be added in GluMOPS media. The nutrients were added again in excess at day 12 and day 39, additional supplements of Pi and Pt were added to their

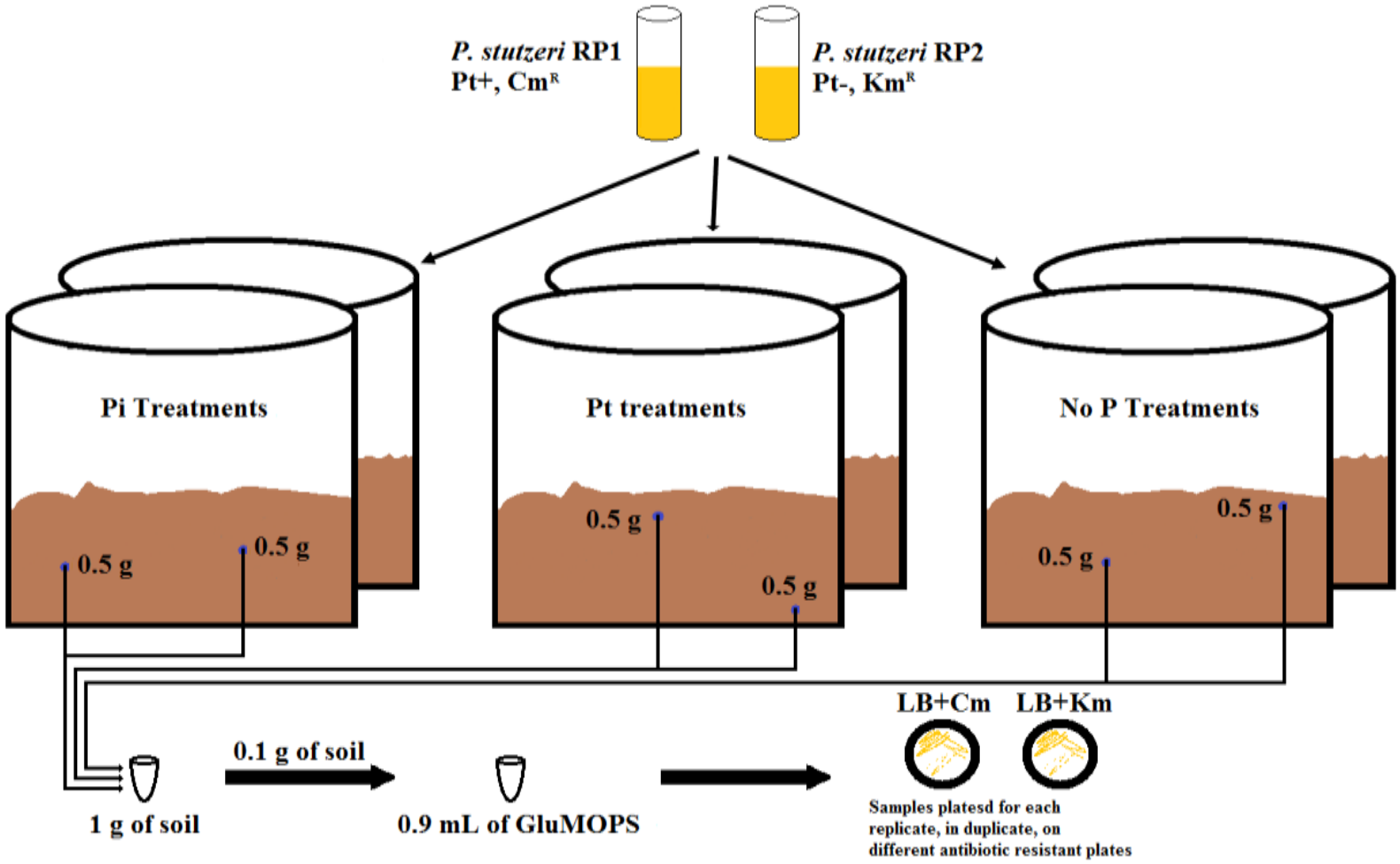

FIG. 3. Description of the soil preparation, soil recovery, and cell counting for reporter bacteria added into soil samples for the soil competition assay

respective treatment jars at a concentration of 0.83 µmol per gram of soil. No P source of any kind was added to the No P treatment jars (substituted with sterile water), but all other nutrients were still added. All jars were mixed thoroughly after nutrient addition.

Soil Recovery and Cell Counting of Reporter Bacteria

Every three days, soil was removed from each sample jar and bacteria were recovered to determine the concentration of each of the reporter bacteria strains. Before every recovery, the soil was thoroughly mixed for homogeneity. Then, two 0.5 g soil samples were extracted from two distinct locations per jar; these are plugs A and B. Plug A and plug B were then immediately mixed together in a microcentrifuge tube. From that 1 g mixture, 0.9 g of soil was stored at -4°C for DNA extractions in the future, and the remaining 0.1 g of soil from the mix was placed into a separate tube containing 0.9 mL of GluMOPS buffer for DNA extractions. Tubes were vortexed at maximum speed for 30 seconds and soil particles were allowed to settle for 5 minutes. 2 mL of the resulting supernatant was removed and stored at -4°C. The remaining supernatant from the extraction mix was diluted in series from 10^{-1} to 10^{-4} in 1x "M" MOPS buffer. 10^{-2} to 10^{-4} dilutions were plated onto LB + Cm (*P. stutzeri* RP1 selection) and LB + Km (*P. stutzeri* RP2 selection) agar plates. All plates were then incubated at 37°C overnight. The following day, colonies of *P. stutzeri* RP1 and RP2 were counted in duplicate from dilution plates containing between 30-300 colonies. Refer to Fig. 3 for illustrations.

DNA Extraction and Polymerase Chain Reaction for Denaturing Gradient Gel Electrophoresis

DNA was extracted from 0.9g of previously frozen (-4°C) soil used in the soil competition assay described above. Mo Bio PowerSoil® DNA Isolation Kit was used to extract the DNA from the soil, and the quality and concentration of the DNA obtained was examined on a 0.7% (wt/vol) TAE agarose gel stained with ethidium bromide (EtBr, 0.5 g/liter). In order to ensure the extracted DNA would yield PCR (Polymerase Chain Reaction) product, the extracted DNA was diluted from 10^0 to 10^{-2}, primarily to allow for the removal and/or dilution of natural PCR inhibitors present in the soil. All diluted DNA samples were amplified at the V3 high variable region of 16S rDNA. The forward primer 341F (5'-CCT ACG GGA GGC AGC AG-3'), contains a GC-rich clamp (5'-CGC CCGCCGCGC CCC GCGCCC GTC CCGCCGCCCCCGCCC-3') (58). 341F-GC allows for viewing bands in DGGE analysis, since the GC clamp does not denature along with the extracted DNA. Reverse primer 534R (5'-ATT ACC GCG GCT GCT GG-3') and 907R (5'-CCG TCA ATT CMT TTGAGT TT-3') were both used in PCR reactions, however each data set only had one of the reverse primer for all samples in the data set (58). All PCR assays utilized GoTaq® Hot Start Green 2x Master Mix (Master Mix). Two rounds of PCR were used to prevent the over or under amplification of any given OTUs, it's the same concept of using PreCR®, dilution of natural inhibitors present in soil, allowing for accurate amplification of DNA present in soil based on their concentrations in the soil. The first round of PCR reactions contained in a 20 µl final volume: 2x Master Mix, 0.5 µM of 341F-GC primer, 0.5 µM of 534R/907R primer, and 3 µl of 10^{-2} diluted DNA. The PCR program was as follows: 94°C for 1 min, 52°C for 30 s,

and 72°C for 1 min, all for 30 cycles. This was followed by 10 min at 72°C and a cooling period to 4°C. The second round of PCR reactions contained in a 45 µl total reaction volume: 2x Master Mix, 0.5 µM of 341F-GC primer, 0.5 µM 534R/907R primer, and 2 µl of round one PCR product. The PCR program was as follows: 94°C for 10 min, 52°C for 30 s, and 72°C for 1 min, all for 10 cycles. This was followed by 10 min at 72°C and a cooling period to 4°C. 5 µl of round two PCR product was analyzed on a 1.5% TAE agarose gel prior to DGGE analysis. This allowed the determination of PCR product concentration and quality needed to normalize the amount of DNA used in the DGGE assays.

All DGGE gels were ran using the Dcode universal detection system (Bio-Rad Laboratories, Hercules, CA). PCR amplified 16 rRNA gene sequences were applied to 7% (wt/vol) acrylamide gel in 0.5x TAE buffer with denaturant gradients ranging between 40-65%. 100% denaturant contained 7M urea and 40% (vol/vol) deionized formamide. All gel well were loaded with a normalized DNA concentration of 250 ng, and electrophoresis was carried out in 1x TAE buffer at 58°C for 15 hours at 50v. Gels were stained with 1x TAE buffer with EtBr (10 mg/ml) for 30 min, and immediately de-stained in 1x TAE for 15 min. Gels were viewed with a KODAK imaging system. Gel bands of interest were cut out and transferred into 50 µl of sterile water and frozen for potential sequencing.

CHAPTER III

RESULTS

Pilot Experiment for Soil Competition Assay

A pilot assay was carried out to ensure that desired bacteria could be recovered from any soil sample efficiently and predictability, which is essential for obtaining reliable data during the soil competition assay. In the pilot experiment, a combination of *P. stutzeri* RP2 (Pt-, Km^R) and *S. marcescens* Tn5-Km^R strains were added to soil samples at a predetermined initial concentration. Bacterial cells were then immediately recovered from the soil and viable cell counts were performed with selection on LB-Km plates. *S. marcescens* Tn5-Km^R was used as an indicator strain in this pilot study for the sole purpose that it can be easily differentiated from *P. stutzeri* RP2 (Pt-, Km^R) on Km resistant plate, due to its bright pink pigment. This allowed a viable cell count to be performed for both added bacteria on the same selection plates, simultaneously, shown in Fig. 4.

P. stutzeri RP2 (Pt-, Km^R) was added at 9.88×10^6 cells/gram of soil, verified through a viable cell count at the time of addition, and an immediate soil sample (day 0) recovered 9.4×10^6 cells/gram of soil. *S. marcescens* was added at 8.5×10^6 cells/gram of soil were recovered upon immediate sampling. The accuracy of bacterial recovery from the soil during the recovery process indicates that when we see changes in the soil competition assay, we can be sure that the population changes are true reflections of the

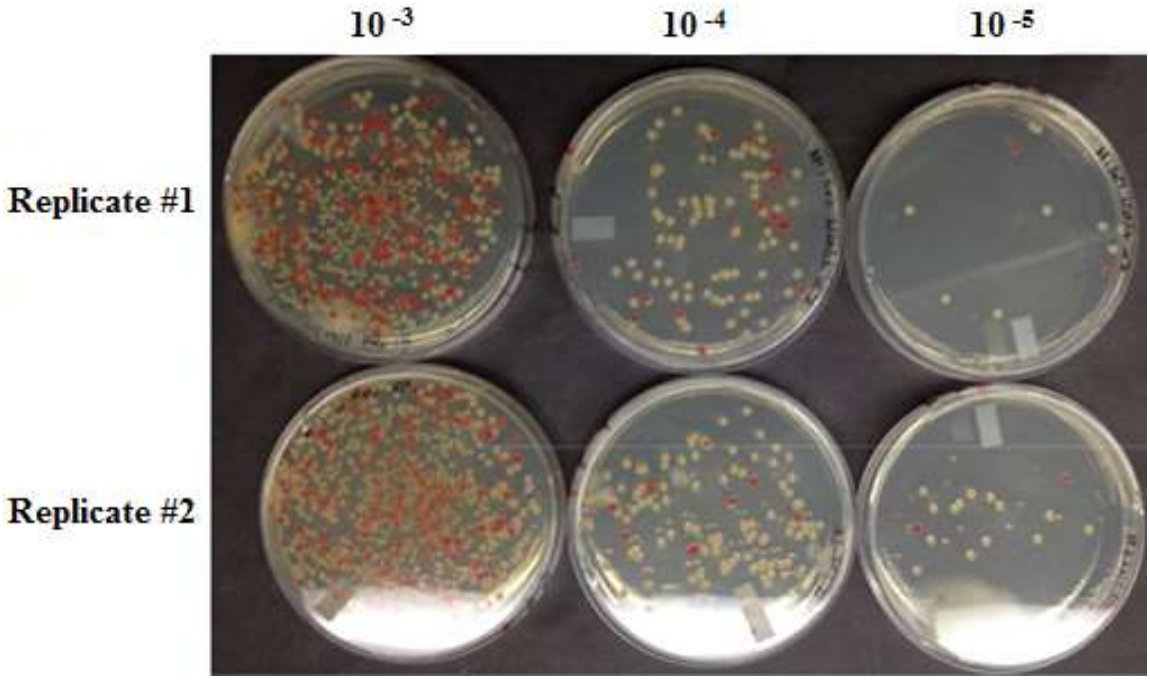

FIG. 4. *P. stutzeri* RP2 (Pt-, Km^R, beige colonies) and *S. marcescens* (Tn5-Km^R, bright pink colonies) recovery in duplicate. Left to right, dilutions are 10^{-3} to 10^{-5}.

changes that the bacterial community is facing, ruling out the possibility that the bacterial recovery is inconsistent.

Furthermore, in order to determine if the soil samples had any residual P compounds that could be used by bacteria as a P source for growth, we monitored the concentration of *P. stutzeri* RP2 (Pt-, Km^R) over 15 days (Fig. 5). The goal was to confirm that the soil was not compromised with heavy P nutrients that would later affect the population growth of oxidizers and/or non-oxidizers of Pt. It was observed that *P. stutzeri* RP2 (Pt-, Km^R) cell concentrations continued to decrease over time in the soil sample where No additional P source was added (Fig. 5). Pt was added on day 6, and the concentration of *P. stutzeri* RP2 (Pt-, Km^R), continued to decrease, as expected (Fig. 5). The Pt addition was

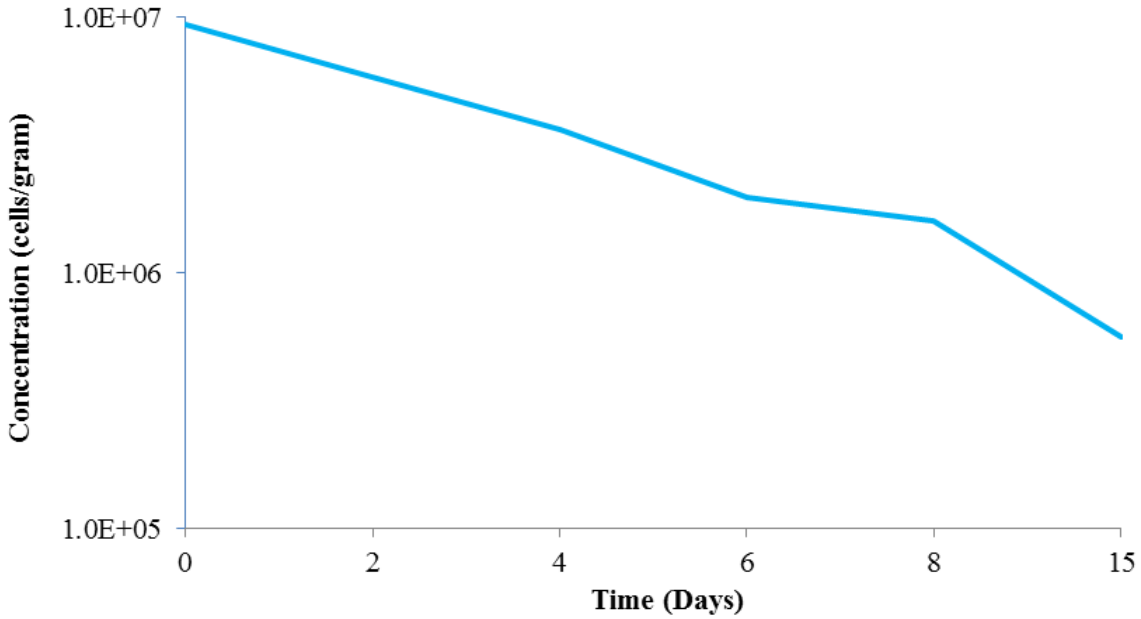

FIG. 5. *P. stutzeri* RP2 (Pt-, KmR) preliminary soil extractions over a 15 day time period. Six extractions were performed at various days. Pt was added after day 6 extraction.

important to add for this non-oxidizer, to ensure that there would not be an unknown effect from the addition Pt that would cause the *P. stutzeri* RP2 (Pt-, Km^R) to grow, under conditions where it should not be growing. This pilot experiment verified that our Pt non-oxidizing strain, *P. stutzeri* RP2 (Pt-, Km^R), does not grow in the presence of Pt in a soil environment, and that our tracing system for the reporter bacteria was reproducible and accurate as far as tracing reporter bacteria population changes.

Phenotypic Verification of Tn5 Mutant Reporter Bacteria

Once the extraction techniques were established and reproducible, the mutant strains *P. stutzeri* RP1 (Pt+, Cm^R) and RP2 (Pt-, Km^R) were compared against the wild-type *P. stutzeri* for confirmation of similar generation times (Fig. 6). This would ensure that the mutagenesis used to construct the antibiotic resistant strains did not disrupt

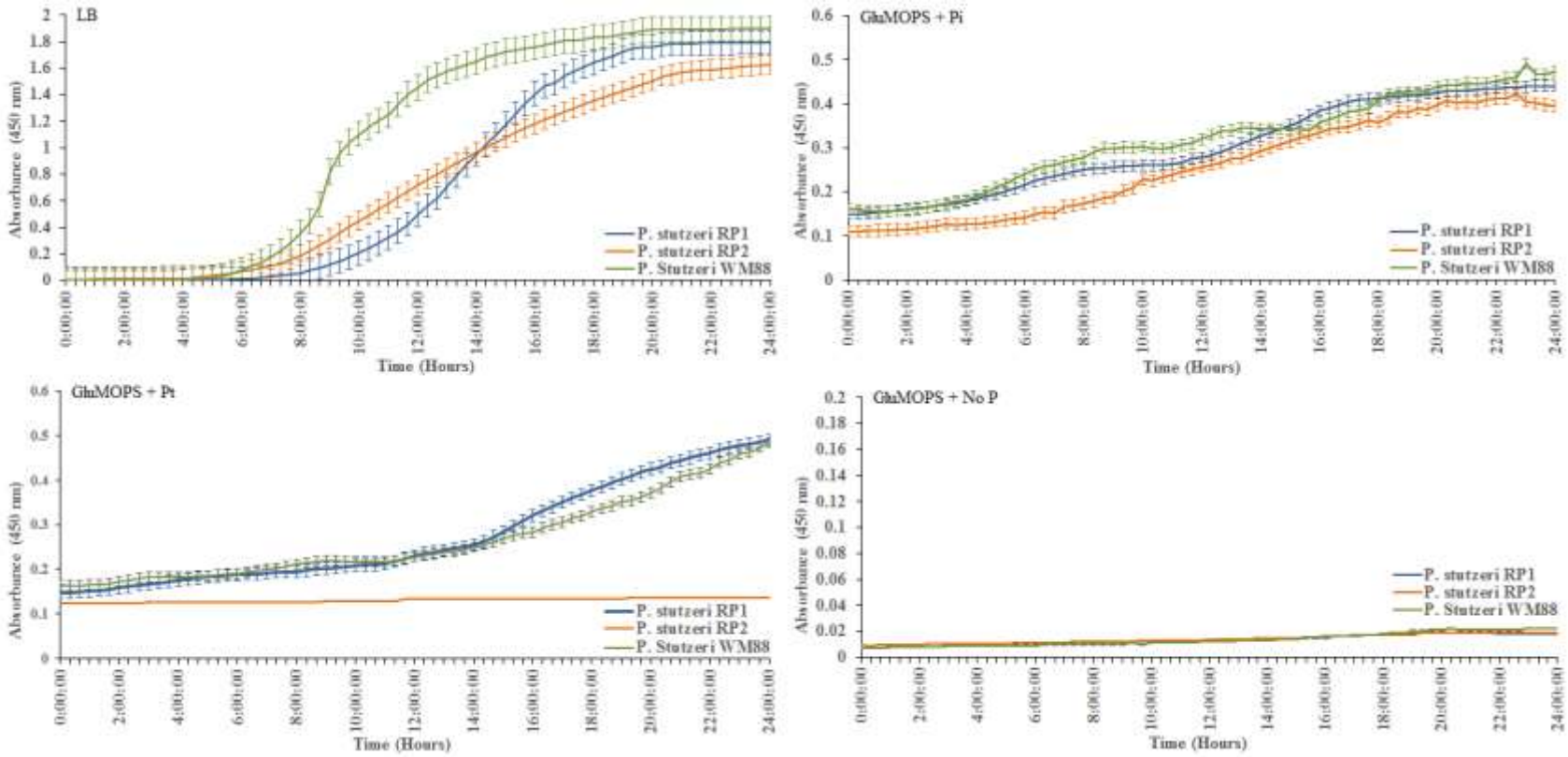

FIG. 6. Growth curves based on 450nm absorbance over 24 hours *P. stutzeri* RP1 (Pt+, Cm^R), RP2 (Pt-, Km^R), and WM88 (wild-type) are shown in all growth curves. Standard error bars are shown. Organism growth shown in LB broth, GluMOPS + Pi media, GluMOPS + Pt media, and GluMOPS + No P media. Y-axis are not standardized in all figures to show distinct growth patterns in different media. GluMOPS + No P media graph was performed on a separate day with different overnight cultures, thus their absorbance value at time 0 is inconsistent with the time 0 on the other graphs.

TABLE 1. Generation times, in minutes, derived from triplicate averages from growth curves for organism wild-type *P. stutzeri* RP1 (Pt+, CmR), and *P. stutzeri* RP2 (Pt-, KmR) in four different types of media with varying nutrient sources

	LB broth	GluMOPS+Pi broth	GluMOPS+Pt broth	GluMOPS+P broth
P. stutzeri WM88	61	590	930	-
P. stutzeri RP1	60	625	890	-
P. stutzeri RP2	57	595	-	-

essential functions for growth which would alter the outcome of the growth competition assay in soil. Generation times for our mutants (Table 1) were calculated based on growth curves performed in triplicate (Fig. 6) for verification that all critical genes were still intact. The strains were observed to have very minimal differences in growth rates, from any one strain to another, in a given type of media. As expected, no strains grew in media without a P source (No P) and *P. stutzeri* RP2 (Pt-, Km^R) did not grow in media which Pt was the only P source provided. The similarities in generation times between wild type *P. stutzeri* WM88 and the Tn5 induced antibiotic resistant reporter strains indicate that *P. stutzeri* RP1 (Pt+, Cm^R) and *P. stutzeri* RP2 (Pt-, Km^R) are not impacted in growth rate under these conditions and are therefore fit to use in a growth competition assay.

Soil Competition Assay

To gain a better understanding of how Pt might alter bacterial communities in the soil, a soil competition assay was designed to determine how exogenous Pt addition to a soil environment might alter the concentrations of Pt oxidizing bacteria relative to Pt non-oxidizing bacteria. In this assay, both reporter strains were added into a series of soil samples that were exposed to different treatments (Pi, Pt, and No P) over a 45 day period.

Both strains were mixed into the same soil samples, and all soil samples were analyzed for reporter bacteria concentration over time. For all treatment types, the initial inoculation of each strain, represented by day 0 (Fig. 6), was followed by an immediate addition of essential non-P nutrients (nitrogen, carbon, etc.) This was performed to ensure that adequate nutrients (with the exception of P) were available for growth, and to deplete and residual P sources that the bacteria could use in the soil samples. P treatments were added on day 12 and 39, along with the same additional nutrients added on day 0. Figure 7 represents *P. stutzeri* RP1 (Pt+, Cm^R) and RP2 (Pt-, Km^R) concentration changes over time in soil samples to which Pi was added at the induced time points. There was an initial decrease of cell concentration from day 0 to day 9, for both *P. stutzeri* RP1 (Pt+, Cm^R) and RP2 (Pt-, Km^R), as expected, due to lack of a P source. Pi treatment on day 12 (Fig. 7) resulted in an increase in cell concentrations for both strains, which plateau at day 30, subsequently increasing after Pi was added again on day 39. This does not seems to be apparent for *P. stutzeri* RP2 (Pt-, Km^R), an explanation for this will be discussed later.

In the Pt treated soil samples (Fig. 8), Pt treatments were added on day 12 and 39. An initial decrease of cell concentration from day 0 to day 9, in both *P. stutzeri* RP1 (Pt+, Cm^R) and RP2 (Pt-, Km^R), was observed as expected. At day 12, Pt was added and cell concentrations of *P. stutzeri* RP1 (Pt+, Cm^R) increased significantly, while concentrations of *P. stutzeri* RP2 (Pt-, Km^R) remained low. At day 39, Pt treatment was added again and both strains increased in cell concentration for 12 days.

In the soil samples to which no P source was added (Fig. 9), again, the initial inoculation concentration of each strain was measured at day 0, followed by an

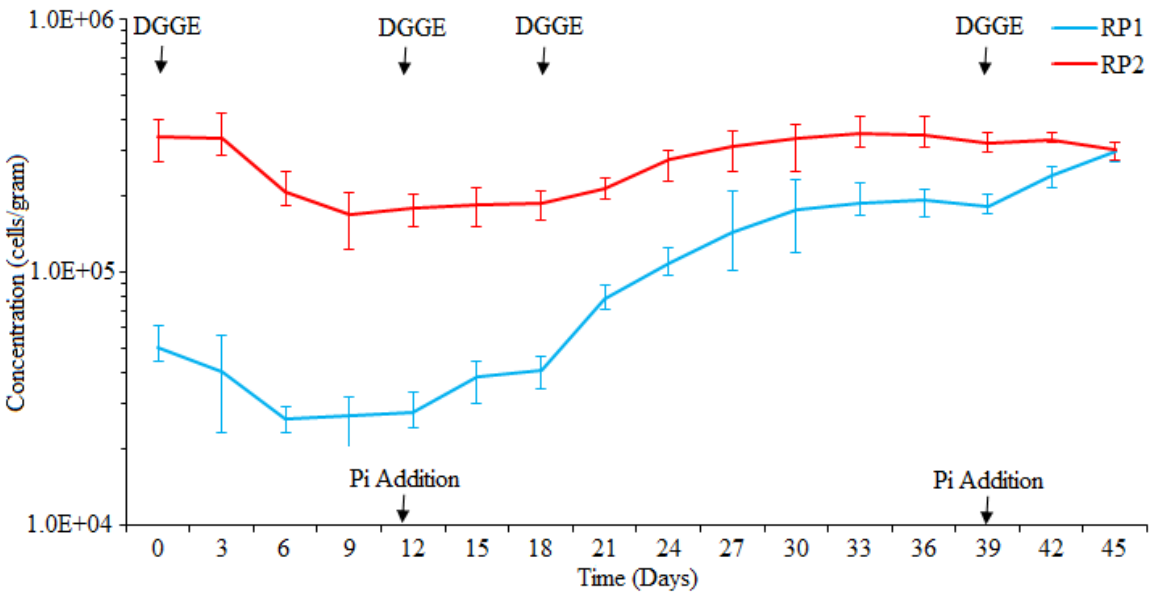

Fig. 7. The effects of Pi addition on reporter bacteria *P. stutzeri* RP1 (Pt+, CmR) and RP2 (Pt-, KmR) cell concentrations over time in soil. Pi was added at day 12 and 39. All essential non-P nutrients were added on day 0, 12, and 39. Cell concentrations are based on an average from viable cell count assays of soil extractions in duplicate soil samples with the same treatments. Range bars are shown. DGGE arrows represent soil samples used in the DGGE assay.

immediate inoculation of non-P nutrients (nitrogen, carbon, etc.). No P treatments were (Pt-, KmR), as expected. At day 12 (Fig. 9), only C, N, and S sources were added on day 12 and 39. There is an initial decrease of cell concentration from day 0 to day 9, in both *P. stutzeri* RP1 (Pt+, CmR) and RP2 added and cell concentrations of *P. stutzeri* RP1 (Pt+, CmR) and RP2 (Pt-, KmR) continued to gradually decrease, indicating the absence of a P source in the soil samples. At day 39, again, a No P treatment was added.

Denaturing Gradient Gel Electrophoresis

To determine how Pt addition might be altering the overall bacterial community composition in these soil samples, DGGE was utilized to examine bacterial

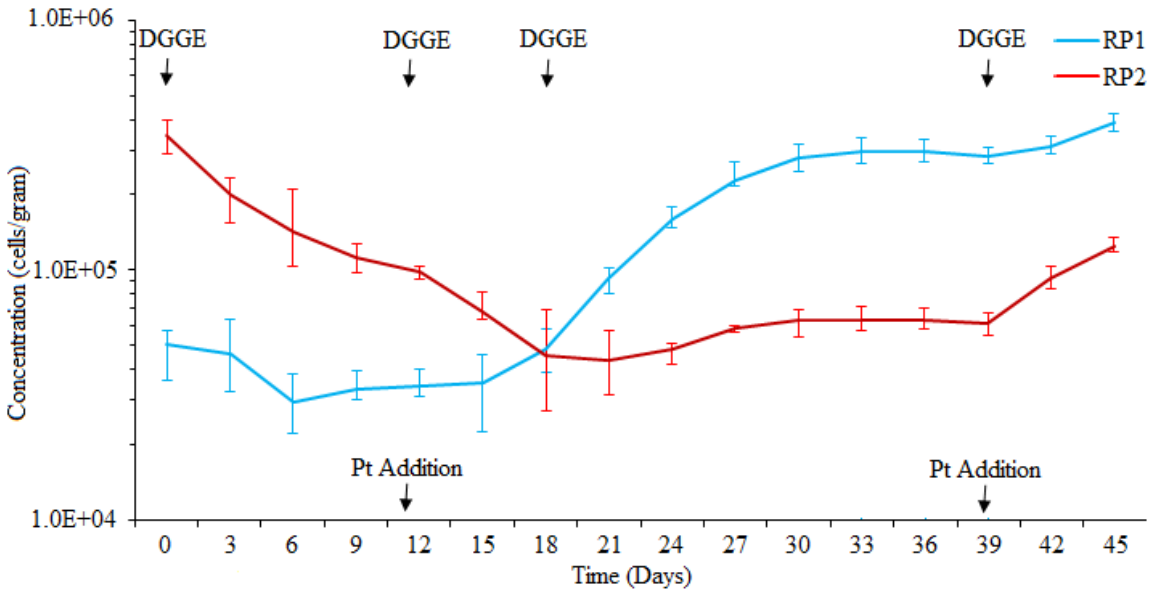

FIG 8. The effects of Pt addition on reporter bacteria *P. stutzeri* RP1 (Pt+, CmR) and RP2 (Pt-, KmR) cell concentrations over time in soil. Pt was added at day 12 and 39. All essential non-P nutrients were added on day 0, 12, and 39. Cell concentrations are based on an average from viable cell count assays of soil extractions in duplicate soil samples with the same treatments. Range bars are shown. DGGE arrows represent soil samples used in the DGGE assay.

population changes at time points before and after the addition of Pi (positive control), Pt, and No P (negative control). To visualize potential changes in the microbial population over time in each of the treated soil samples described above, two different primer sets were used to amplify the 16S rRNA genes from the soil samples for DGGE analysis. One set of the primers (314F-GC and 907R) was only used once, as it did not produce any viable results, but it was included to show the approach we took to gather our results (Fig. 10). The second set of primers (341F-GC and 534R) was the second set that was used to gather data, since this primer set allowed for greater and more distinctive separation of OTUs on the DGGE gels than the 341F-GC and 907R counterpart. Thus, 341F-GC and 534R primer set data on DGGE gels will be the main source for the conclusions that are

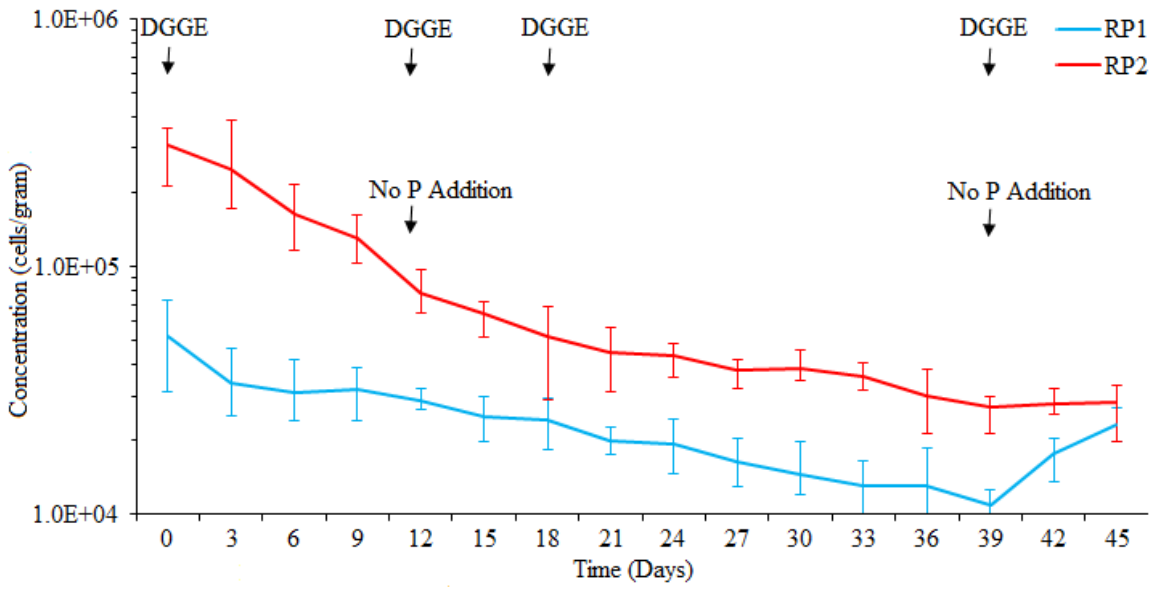

FIG. 9. The effects of No P addition on reporter bacteria *P. stutzeri* RP1 (Pt+, CmR) and RP2 (Pt-, KmR) cell concentrations over time in soil. No P was added at day 12 and 39. All essential non-P nutrients were added on day 0, 12, and 39. Cell concentrations are based on an average from viable cell count assays of soil extractions in duplicate soil samples with the same treatments. Range bars are shown. DGGE arrows represent soil samples used in the DGGE assay.

drawn since greater differentiation is more likely to occur, and it is more likely that an individual organism will be present in a single band, rather than multiple organisms in a single band. Table 2 summarizes all OTUs of interest, identified as having differences among the soil samples or time points, across the different gels.

Each DGGE assay was performed with four different time points from all three different soil samples (Pi, Pt, and No P). DGGE gels were originally performed for each individual soil samples, meaning the PCR products from any given replicate was not mixed with the other replicate that shared its treatment type, this can be seen in Fig. 11. Furthermore, this is the only gel where replicates were shown separately, since we mixed PCR products of the replicates after we noticed that there was too much variability even

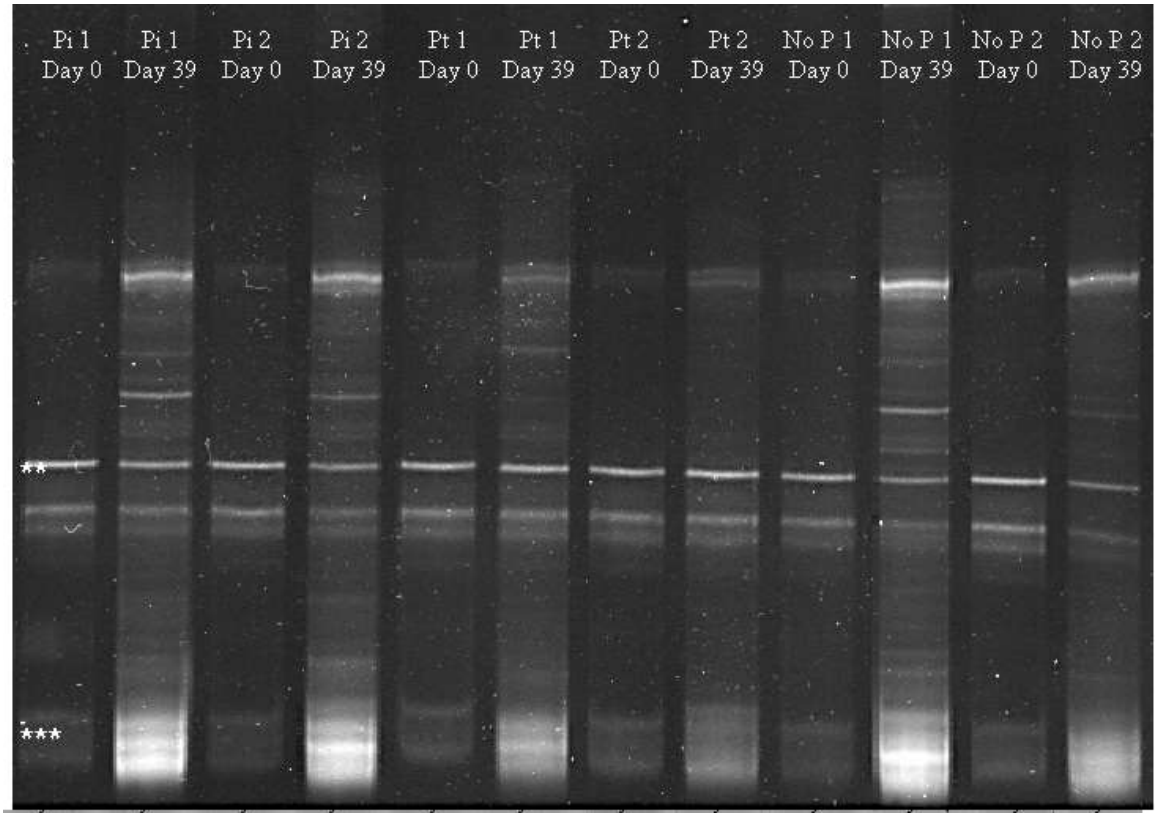

Fig. 10. DGGE patterns of 16S rDNA PCR product on a 40-60% denaturing gradient, with a negative image of EtBr-stained pattern. Primers 341-GC and 907R were used. Band sizes are 566 nucleotides with an attached 39 nucleotide GC clamp. Both treatment soil samples #1 and #2 were used for PCR and DGGE. No results were able to be gathered from this gel.

between replicates. Thus we felt that mixing the replicate samples of the each individual treatment type would not result in biased data, due to the original variability, and that it would allow for a more simple, yet comprehensive analysis. Figure 12 shows DGGE gels that had replicate PCR products mixed.

The time points chosen for analysis were determined based on specific factors predicted to influence the bacterial community in the soil samples. Day 0 was selected to represent the baseline population that existed in the soil samples prior to any treatments. Day 12 was chosen to represent bacterial population changes after 12 days of P

TABLE 2. Relevant bands from Figs. 11, 12, and 13 grouped together as unique organisms, based on their consistencies in band separation patterns, band intensity patterns, and Pt oxidation abilities throughout the aforementioned figures. Their population changes are shown in relation to Pt's presence in soil

Band organism	Respective figures	Population change	Pi Oxidation abilities
Band 1	Fig. 11, 12	Decrease	Cannot oxidize Pt or survive until Pi recycling occurs
Band 2	Fig. 11, 12	Decreases	Cannot oxidize Pt or survive until Pi recycling occurs
Band 3	Fig. 11	Increase	Can oxidize Pt or survive until Pi recycling occurs
Band 4	Fig. 11	Decrease	Cannot oxidize Pt or survive until Pi recycling occurs
Band 5	Fig. 11	Increase	Can oxidize Pt or survive until Pi recycling occurs
Band 6	Fig. 11, 12	Increase	Can oxidize Pt or survive until Pi recycling occurs
Band 7	Fig. 12	Increase	Can oxidize Pt or survive until Pi recycling occurs
Band 8	Fig. 12	Decrease	Cannot oxidize Pt or survive until Pi recycling occurs

starvation. Day 18 was selected to allow analysis of population changes 6 days after different P treatments were added. Lastly, day 39 was selected to identify any long term changes in bacterial populations in the soil samples, in response to the added P sources.

The attention for the DGGE analysis will be in regards to the increase and decrease in band intensity within the Pt soil samples over time, and their subsequent comparisons to the positive (Pi) and negative (No P) controls. These observed changes in band intensities can indicate whether a specific organism, believed to be represented by one band, is a Pt oxidizer or non-oxidizer. We believe that each individual band

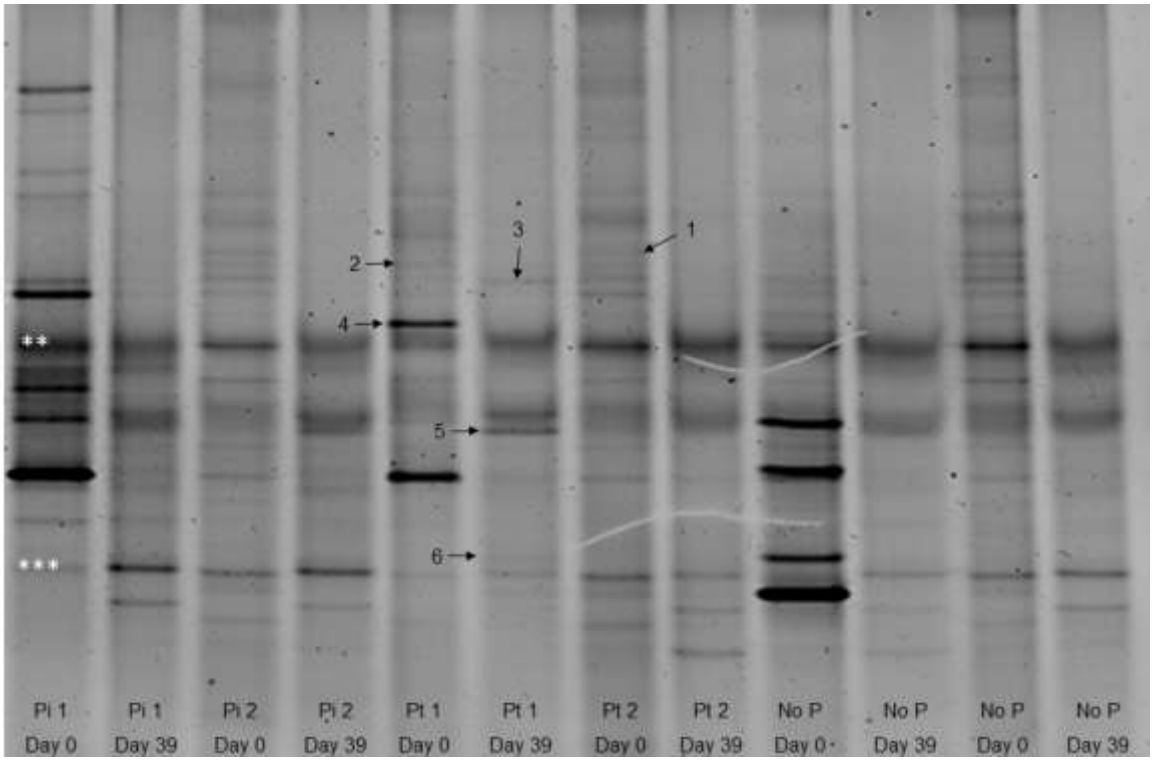

Fig. 11. DGGE patterns of 16S rDNA PCR product 40-65% denaturing gradient, with a negative image of EtBr-stained pattern. Primers 341F-GC and 534R were used. Band sizes are 193 nucleotides with a GC clamp (39 nucleotides). Both replicates for all soil treatments were used for PCR, mixed, then ran on DGGE. Soil bacterial community diversity is shown at days 0 and 39. The symbols ** and *** were added to help orientate when comparing bands across different gels.

represents a single organism. Thus observed changes in band intensities can indicate if a band is increasing or decreasing in population. Table 2 condenses the results from the DGGE gels (Figs. 11 and 12) by grouping different band patterns of interest from across the three different gel figures. The bands are grouped together according to the high probability that they represent the same organism across all gels, as mentioned in Table 2. These results were formulated based their similarities in band separation, band intensity, and probability of Pt oxidation abilities across all three gels. For the remaining part of this paper, any given band should be considered an individual organism. For example,

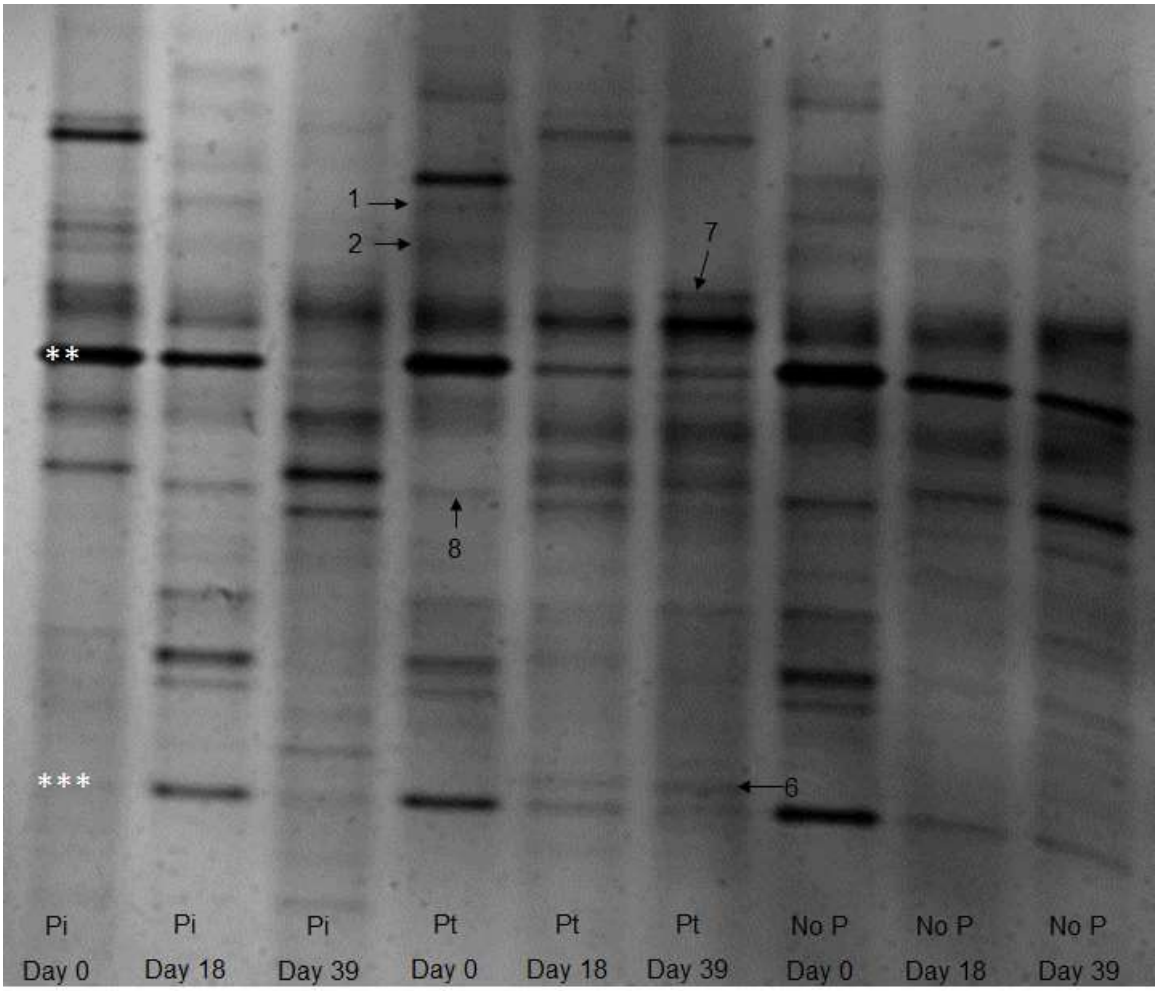

Fig. 12. DGGE patterns of 16S rDNA PCR product 44-61% denaturing gradient, with a negative image of EtBr-stained pattern. Primers 341F-GC and 534R were used. Band sizes are 193 nucleotides with a GC clamp (39 nucleotides). Both replicates for all soil treatments were used for PCR, mixed, then ran on DGGE. Soil bacterial community diversity is shown at days 0, 18, and 39. The symbols ** and *** were added to help orientate when comparing bands across different gels.

band 1 can be seen in figures 11 and 12. Furthermore, Table 2 also summarizes the observed population (physiological) changes over time for each band in response to Pt addition into the soil. The changes in population numbers are directly interpreted via the changes in band intensity.

Each band indicated in Table 2 is believed to have undergone a change in population size over the time period examined in the Pt treated soil samples, as seen by band intensity fluctuations (Figs. 11 and 12). Decreasing band intensities over time were observed for four bands, bands 1, 2, 4, and 8. Band 1 had strong intensity at day 0 (Figs. 11 and 12) but their band intensities decrease significantly at day 18 (Fig. 12), followed by a lack of band presence at day 39 (Figs. 11 and 12). Band 2 has a strong presence at day 0 (Figs. 11 and 12), however, at day 18 and 39 its presence completely disappears indicating that band 2 is no longer detectable in the soil sample. Band 4 (Fig. 11), showed an extremely strong presence on day 0, but by day 39, its population size was non-detectable. Band 8 has a peculiar pattern in regards to its population change, on day 0 (Fig. 12) strong band intensity is present, however the band disappears at day 39 (Fig. 12). All bands (organisms) 1, 2, 4, and 8 were unable to maintain their original population sizes in the Pt treated soil samples, albeit to differing degrees. Nevertheless all four bands were unable to be detected through PCR at day 39.

Organisms that are of particular interest in the DGGE assay are those that increased in population size in response to Pt treatment. For example, there are minimal to no indications that band 3 is even present in the soil samples on day 0 (Fig. 11), however on day 39 (Fig. 11), band intensity had increased in the gel, indicating that bacterial population density had increased over the 39 day period. Furthermore, band 5 (Fig. 11), does not have a band that can be observed at day 0, but it slowly emerges as a faint band on day 39. Additionally, Band 6 is not visible on day 0 (Figs. 11 and 12), but appears to increase in intensity on day 18 (Fig. 12) and even more so on day 39 (Figs. 11 and 12). This organism is of particular interest because the increase in band intensity

overtime is only seen in the Pt treated soil samples. Lastly, band 7 has the same type of pattern as band 3, it is minimally present at day 0 (Fig. 12) and barely discernible. However, at day 18 (Fig. 12), we see there is a slight increase in the intensity, and at day 39 (Fig. 12), band 7's presence in the soil is highly apparent, pointing to a strong overall growth over the 39 days. In Fig.12, the increase in population density of band 7 seems to be Pt-dependent. In summary, bands 3, 5, 6, and 7 generally seemed to increase their population densities over the 39 day time course in the Pt treated soil samples.

CHAPTER IV

DISCUSSION

Soil Competition Assay

The primary goal of the soil competition assay was to analyze the effects of different P compounds (Pi, Pt, No P) on Pt oxidizers and non-oxidizers. Since current farming and forestry practices utilize Pt fertilizers and phosphine products, it was essential to study the effects of exogenous Pt on bacterial populations present in the soil. To our knowledge, no studies that examine the effects of these practices on soil microbial communities that have been done. Considering the well-established ability of many soil bacteria to oxidize Pt to Pi, it seems likely that such practices could result in dramatically altering soil microbial communities as well as the P balance of the environment. Such alterations could have significant consequences on the growth of macroorgansims in these environments as well as Pi runoff levels, ultimately affecting an entire ecosystem.

One vital question we asked was, does adding Pt to a soil environment have significant impact on Pt oxidizing and non-oxidizing bacterial populations? By following two isogenic organisms, *P. stutzeri* RP1 (Pt+, Cm^R) and *P. stutzeri* RP2 (Pt-, Km^R) we were able to determine that Pt addition in soils drastically favored the growth of the Pt oxidizing strain relative to the non-oxidizing strains. Our results suggest that Pt addition to soils could also lead to higher densities of other Pt oxidizing bacteria, which would out compete the non-oxidizers in Pt treated soil samples, as seen in our experiment. Similar

experiments in regards to ammonia-oxidizing bacteria have been conducted (59-61). These investigators concluded that ammonia-oxidizing bacterial populations increased when ammonia was introduced into their soil environment. As with ammonia addition, Pt addition to soil would likely result in a large scale change in bacterial community composition.

A critical point to note when observing figures 7-9 is that the cell densities for both *P. stutzeri* RP1 (Pt+, Cm^R) and RP2 (Pt-, Km^R) likely increased higher than 10^6 cells/gram (as shown on y-axis), in some instances. However, the reason for the lack of this observation is based upon an extraction limit. In all of the pre-trial experiments with the same soil sample used in the experiment, we noticed that the extraction concentrations at any given time had an absolute recoverable limit of 10^6 cells/gram even if 10^{10} cells/gram were inoculated. This is why figures 7-9 do not show extracted population densities larger than 10^6 cells/gram, when higher cell concentrations might be expected. Each individual soil sample had consistent extraction limits when compared to other soil samples used in the assay, across all time points. Thus, the data observed was truly due to population changes of *P. stutzeri* RP1 (Pt+, Cm^R) and RP2 (Pt-, Km^R), and not due to fluctuations in extraction limits from one time point to another. Similar extraction limit issues were observed in an experiment analyzing heavy metal resistances in microbes. These investigators extracted 771 isolates from soil samples with an extraction limit of 10^4 cells/gram (62). We concluded that our observed consistencies among our own extraction limit assays and the results from the previous study assured that the lack of observable growth beyond 10^6 cells/gram was due to an extraction limit, not due to an actual lack in growth.

The Pi treatment (positive control) soil samples represents the growth patterns of our reporter organisms in an environment with significant free-Pi (Fig. 7). Given that generation times between *P. stutzeri* RP1 (Pt+, Cm^R) and RP2 (Pt-, Km^R) are nearly identical, and that the only varying factor between these two reporter organisms is the *ptx*D gene knockout in *P. stutzeri* RP2 (Pt-, Km^R), we were able to accurately analyze real-time cell concentration changes over time in regards to both organisms. As expected, we noticed cell death over the first few days when free-Pi was limited or non-existent in the soil, followed by a rapid increase in cell population after Pi addition at day 12. At day 39, *P. stutzeri* RP1 (Pt+, Cm^R) does not seem to increase in overall cell density, which is attributed to the extraction limits discussed earlier. *P. stutzeri* RP2 (Pt-, Km^R) cell density on the other hand was low at day 0, thus this strain displayed a more accentuated overall growth pattern throughout the time points. We would expect a rapid overall growth of all organisms present in this soil sample treatment, since Pi is the primary P source and all essential nutrients were also added in what we predicted to be in excess concentrations.

The Pt treatment (Fig. 8) resulted in an initial decline in cell concentrations for both reporter organism populations, as expected and is similar to our Pi treatment sample observations. Pt addition on day 12 did not have an effect on *P. stutzeri* RP1 (Pt+, Cm^R) until day 15, after which cell concentrations began to increase. The lag growth with Pt as a P source was also observed in our generation time assay. Since Pt is a non-preferential P source, a lag time was expected after treatment on day 12 to allow for the transcription and translation of -the *ptx* operon, which is required for Pt oxidation. *P. stutzeri* RP2 (Pt-, Km^R) continued to decline in cell density until day 21, after which the cell density increases slowly until day 39. However, when the Pt treatment is added again on day 39,

we see a rapid increase in cell concentration for both *P. stutzeri* RP1 (Pt+, Cm^R) and RP2 (Pt-, Km^R). We believe this event likely stems from three consecutive processes. 1) The oxidation of Pt to Pi by *P. stutzeri* RP1 (Pt+, Cm^R) and other Pt oxidizers in the soil sample, followed by 2) A successive recycling and release of excess converted Pi by *P. stutzeri* RP1 (Pt+, Cm^R) and the other Pt oxidizers into the soil, and 3) A subsequent consumption of the released Pi by *P. stutzeri* RP2 (Pt-, Km^R) and the other non-oxidizers present in the soil sample. These results suggest that cross-feeding of Pi may occur between *P. stutzeri* RP1 (Pt+, Cm^R) and RP2 (Pt-, Km^R). If *P. stutzeri* RP2 (Pt-, Km^R), and potentially other non-oxidizing bacteria, can survive without Pi in a harsh Pt environment, they seem to eventually benefit from *P. stutzeri* RP1 (Pt+, Cm^R), and potentially other oxidizing bacteria, during times when Pt is the primary P source. This relationship is highly plausible due to analogous patterns observed between bacteria in other biogeochemical cycles such as carbon and nitrogen.

Finally, the No P treatment (Fig. 9, negative control) shows a constant decrease in bacterial count over time. This is essentially a negative control since no P sources of any kind were introduced into the No P soil sample. The results obtained were as expected, and verified that no other unaccounted P sources were available for use in the soil samples used.

The three different soil sample treatments (Pi, Pt, and No P) have shown results that reflect hypothesized outcomes for the experiment. Specifically, the changes observed in *P. stutzeri* RP1 (Pt+, Cm^R) and RP2 (Pt-, Km^R) in response to Pt are similar to changes observed in microbes in response to reduced carbon and nitrogen compounds (63). These oxidizers of essential elements regulate key nutrients for all microbial

populations present in their respective environments. The implications of the interactions between *P. stutzeri* RP1 (Pt+, Cm^R) and RP2 (Pt-, Km^R) are monumental in regards to gaining knowledge about P cycling in the environment. Not only have we examined the effects of different P compounds on these two bacterial populations, but we can logically state that these interactions are likely to occur for the other bacterial species present in the soil, with similar growth patterns in response to the respective treatments of Pi, Pt, and No P.

Denaturing Gradient Gel Electrophoresis

The soil competition assay provides an insight on the specific interactions between our Pt oxidizing and non-oxidizing reporter bacteria. Based on logic and our soil competition results, an organism that is capable of oxidizing Pt has a clear advantage for growth during conditions where Pt is the sole or major P source, while organisms that are not capable of oxidizing Pt are disadvantaged. Currently, studies concerning reduced P compounds are limited to examining the natural presence of reduced P compounds in the environment and identification of pathways used by bacteria to utilize reduced P compounds are primary P sources (1-17, 22, 23, 25-30, 32, 34, 35, 39-43, 56, 57, 64). However, investigations which examine the environmental consequences of reduced P compounds influx on microbial populations are nearly non-existent.

DGGE is a powerful tool that is commonly used in researching microbial population changes over time in relation to environmental composition changes such as temperature, nutrients, depth, etc. (52-55, 65, 66). In this study, DGGE was used to gain

insight into the changes that may occur in soil bacterial populations as a result of Pt introduction.

However, there are some limitations to DGGE analysis, including three specific challenges we encountered: 1) A possibility of disproportionate DNA amplification of the bacterial community, in comparison to the actual proportions of the bacteria present in our soil samples; 2) Observing band intensity changes and misinterpreting them as actual DNA concentration (bacterial population) changes in the soil. This difference in the initial DNA concentrations that are loaded from one DGGE gel lane to another would result in skewed observations; and 3) The possibility that a single band on any of the gels could actually represent more than one single organism, due to the sheer volume and diversity of the DNA that was amplified from our soil samples. In order to counteract these inherent limitations, we engaged in three respective pre-requisite steps that would assist in achieving results that were as accurate as technologically possible. 1) We diluted the extracted DNA down to 10^{-2} in order to diminish natural PCR inhibitors present in the soil. This was followed by two rounds of PCR in order to decrease the random likeliness of over or under amplification of OTUs, in contrast from just one round of PCR. 2) We ran the second round PCR product on gel electrophoresis to determine DNA concentrations for all soil sample time points used in any given DGGE gel. This permitted normalized DNA concentrations to be loaded into each DGGE lane across all soil sample time points, allowing us to avoid DNA concentration bias when viewing band intensity changes. 3) We selected primers 341-GC and 534R, targeting the V3 high variable region of 16S rDNA, which would produce relatively small OTUs (193 bp). Smaller OTUs are more conductive to greater separation

of bacterial OTUs on the DGGE gels, especially when there are only minor difference in the V3 region. This strategy would be more likely to produce two different visible bands on the DGGE gels for two separate organisms. In addition, we also determined the most narrow gradient range (15-17%) that could be used without the clustering of multiple bands on the top and bottom of the gel. A narrow gradient is valuable in creating further separation between bands that are highly similar at the V3 region, leading to a higher probability of an observed band being a single organism.

Data was analyzed attempting to identify considerable decreases or increases in band intensity over time, assumed to correspond to population decreases or increases of the particular organism represented by that band.

Our first area of analysis was focused on decreasing band intensities, or population densities, in response to Pt addition. The current presumption is that Pt is beneficial to crop production. The results of these practices lead to higher Pt levels in crop soils, and due to the toxicity of Pt to many organisms, it leads to the eventual harming of some organisms in the plant-microbe community. In a specific study involving lettuce crops, it was found that lettuce growth was reduced to 80% or less of its growth potential with even the lowest Pt concentration (0.2 mM) additions into the soil (67). Furthermore, the authors state that root growth and thus nutrient uptake is shunted as well due to Pt additions. It is widely known that bacteria have vast influences in plant growth through the colonization of nodules, roots, stems, and foliage (68-70). It is crucial to consider the potential connection between our observed decreasing bacterial populations and the reported effects on crop plants. This in fact is a significant relationship that is being overlooked in current farming techniques. For example, the

organisms decreasing in band intensity could potentially hold critical roles in the environment such as carbon fixation, nitrogen cycling, sulfur cycling, cofactor production, etc. Such critical roles are highly likely to affect multiple aspects of bacterial-bacterial and plant-bacterial interactions.

Specifically, we have focused on four organisms (bands 1, 2, 4, and 8) that showed strong presence during day 0 in the Pt soil samples and were non-detectable by day 39 (Fig. 11 and 12). It is likely that Pt was either toxic to these species, or they were simply outcompeted in growth by Pt-oxidizing bacteria due to their ability to obtain a useable P source. These organisms may represent crucial bacteria for crop production. Granted, there can be issues in DGGE techniques and/or PCR amplification bias that result in perceived population decreases, but with most of these organisms the decrease in band intensity was grateful over the different time points until they were detectable on day 39. The gradual decrease in density gives us confidence that our observations are based on population decrease, rather than PCR amplification errors throughout the time points.

In contrast, our second group of organisms and arguably the more noteworthy analysis, lies in the increasing band intensities that were observed over the 39 day time period (Figs. 11 and 12). These increases in band intensity (band 3, 5, 6, and 7) represent organisms that grown in population density during conditions in which Pt was the sole P source. There are three possibilities in growth characteristics that would allow for these observed increases in band intensity. 1) Bacteria in the soil were capable of oxidizing Pt to Pi via the *ptx* pathway or a similar pathway, 2) Horizontal transfer of the *ptx* operon occurred between naturally present Proteobacteria in the soil, as previously reported (9).

This would result in population growth of initial non Pt-oxidizing Proteobacteria, and 3) Organisms were able to withstand Pt toxicity, and survive through Pi starvation periods. Due to their resilience, we hypothesize that these organisms had access to Pi once the innate Pt oxidizers converted Pt to Pi, and released some Pi into the environment once their own P metabolic needs were met. This theory is further solidified by our soil competition assay observations, during which *P. stutzeri* RP2 (Pt-, Km^R) was in stationary phase from day 18-24, followed by minor growth at day 27 with the first Pt treatment on day 12, and a major growth at day 39 with the second Pt treatment on day 39 (Fig. 7). The most probable explanation for this observed phenomenon is that *P. stutzeri* RP1 (Pt+, Cm^R) and other Pt oxidizers are recycling Pi into the soil community for other organisms to utilize.

When analyzing our data for the growing organisms (bands 3, 5, 6, and 7), we can visually see one of the three growth characteristics, described above, in all six bands. While figures 11 and 12 are not very distinct gels, there were some limitations in the interpretations. Band 3 seems to be a survivor of Pt toxicity and P starvation or Pt oxidizer (Fig. 11), as its band intensity is strong at day 39 when compared to day 0, signifying overall growth, but it does not tell us through which mechanism (Pt oxidation, horizontal transfer, or survival) the growth occurred. Band 5 (Fig. 11) was only observed at day 0 and day 39, portraying a clear growth in population that could have been through either innate Pt oxidation, horizontal transfer of the *ptx* operon, or through survival. Additionally, band 6 (Figs. 11 and 12) displayed gradual growth over the time course of the experiment. We believe its increase in growth is a result of its innate ability to oxidize Pt. Additionally, band 7 (Fig. 12), has a growth pattern that is rather straight-forward, it

portrays characteristics of a Pt oxidizer, due to its consistent growth over time from day 0 to day 39. Organisms mimicking *P. stutzeri* RP1's Pt oxidation abilities could possibly include bands 5 and 6, and organisms mimicking *P. stutzeri* RP2's non-Pt oxidation abilities could possible include bands 3, 5 and 7. We believe this possibility exists because the growth patterns for these bands in the DGGE gels are consistent with the growth patterns of our reporter bacteria in the soil competition assay. Sequencing would be a start in confirming these assumptions.

In conclusion, the observed growth of these organisms in a Pt environment, where Pt was the sole P source, leads to broader implications of bacterial P redox interactions occurring in the environment. Either through survival, innate Pt oxidation, or horizontal transfer of *ptx*, these organisms were growing and even thriving in a Pt environment. Much like the organisms that decreased in population, the organisms that increased in population can also potentially hold critical roles in the environment such as carbon fixation, nitrogen oxidation, sulfur reduction, cofactor production, etc. The combinations of microbial biogeochemical activities that are harmed and/or benefited by Pt additions are potentially limitless when studying such natural environments containing so many variables.

Future Research

The lack of knowledge is so immense that aggressive research is required to solidify the results of this study. However, we have established that significant interactions are occurring in the environment when Pt is added into soil, and we have confirmed that Pt toxicity can be detrimental or beneficial to organisms in an

environment. The key is comprehending which organisms have crucial roles in agricultural soils containing momentous amounts of Pt-fertilizers, and more broadly, what roles do these organisms have in other environments where Pt and other reduced P compounds are available in excessive amounts? Further research following this study would include sequencing and determining the genus and species represented by bands that were either increasing or decreasing in population size. Additionally, it would be ideal to determine essential nutrient redox processes carried out by these organisms to evaluate Pt addition effects on other biogeochemical cycles. This should be followed by establishing minimum inhibitory concentration of Pt for all organisms of interest in this study.

Pi is a non-renewable source, and the scientific community is still unclear about the biotic and abiotic oxidation of reduced P compounds. Stone and White (14) discovered numerous bacterial species that are capable oxidizing reduced P compounds in the environment, but the exact effects of reduced P oxidation in soil has yet to be determined. As Thao (67) and his team discovered, Pt addition into soil had significant repercussions to crop growth, probably through the effects Pt has on rhizobacterial populations, which inadvertently affected their lettuce population. Biogeochemical P cycling is likely overlooked due to a lack of understanding of this phenomenon. Our data provides some insight into this complex system, and this topic requires additional investigations to help understand P availability, P cycling, P conservation efforts, and to improve P based agricultural methods. This study has provided ample evidence to justify further exploration into bacterial-bacterial and plant-bacterial interactions in

environments where reduced P compounds are prevalent, as well as further investigation into P cycling outcomes based off these interactions.

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