INFLUENCE OF SIDEROPHORE PRODUCING BACTERIA AND ORGANIC LIGANDS ON PHASE DISTRIBUTION OF CADMIUM AND ITS UPTAKE BY BRASSICA NAPUS IN THE PRESENCE OF GOETHITE

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

Stephen Anthony Mirabello

August 2006

ABSTRACT

Cadmium is a non essential trace element, which is toxic at very low concentrations. While Cd can occur at high concentrations as a result of the weathering of Cd-rich minerals, most high concentrations are of human origin. *Brassica* have been shown to be hyper-accumulators of cadmium and are therefore good candidates for the application of phytoremediation to remediate soils with Cd contamination. The processes leading to successful phytoremediation are not fully understood, and may be dependant upon the bacterial community present in the rhizosphere. Bacteria in the vicinity of plant roots may degrade plant root exudates that would otherwise act to bind metals and bacteria can produce metal binding ligands such as siderophores. This study was conducted to determine if the presence of siderophore producing bacteria will affect cadmium uptake by *Brassica napus* (canola) in a model system.

An apparatus was designed that included the dominant components of a phytoextraction system, a solid phase (modeled using γ-FeOOH, goethite), a liquid matrix (chemically defined MMS-2 medium in which metal speciation may be calculated), a plant (*B. napus*) and a siderophore producing bacterium (*Burkholderia cepacia*). Physical separation of the plant from the Fe was needed to discern plant uptake of Cd and was accomplished by utilization of dialysis membranes. Components of the system were studied independently before combining for short term plant uptake experiments.

The system was kept at a pH of 6.4 to model a typical soil pH. Studies of Cd adsorption on γ-FeOOH in MMS-2 revealed that adsorption was fast, and that desorption was biphasic and incomplete within the time frame of the experiment. A linear equilibrium adsorption isotherm for Cd on γ-FeOOH was developed with a

sorption constant, K_d, of 2600 mL/g. A linear sorption relationship was also developed for Cd sorption by the walls of the glass reactor vessel, with a volumetric sorption constant, K_g (unitless), which is proportional to the surface area to volume ratio at a given volume.

Bacterial growth was measured at two initial substrate concentrations, 68 and 272 mg/L dextrose added to the MMS-2 media. Stationary phase was achieved in approximately 40 hours after inoculation. Siderophore production by *B. cepacia* was measured by the Chrome Azurol Sulfonate (CAS) technique, and yielded a maximum concentration of 39 μM for the experiments.

Cd speciation, phase distribution, and uptake by *B. napus* were perturbed in the experimental system by the addition of salicylate, citrate, ethylenediamine tetraacetic acid (EDTA), a suspension of *B. cepacia* and its associated siderophore, or the supernatant containing the *B. cepacia* siderophore. Changes in phase distribution of Cd upon addition of ligands with defined Cd binding constants were not consistent with the values predicted by MINEQL+, where CdEDTA>CdCitrate>CdSailcylate. Salicylate desorbed more Cd than citrate, and was chosen along with EDTA for plant uptake experiments. Cell suspensions and siderophore containing supernatant were shown to enhance the adsorption of Cd under the experimental conditions.

Addition of plant roots to a γ-FeOOH suspension resulted in a rapid capture of goethite onto the roots, as a result it was impossible to distinguish Cd in the plant root from Cd sorbed to the FeOOH. A dialysis membrane was utilized to separate roots from the Fe solid phase. The presence of the dialysis membrane did not create a detectable change in adsorption of Cd.

Plant uptake experiments were conducted over a short time scale. An eight hour equilibration step was utilized after perturbations of Cd-γFeOOH adsorption equilibrium (by addition of ligand) and followed by six hours of plant uptake. Both

salicylate and EDTA were effective in increasing the phytotextraction of Cd. Salicylate may have more potential of these two ligands for field application because of the concerns associated with toxicity of and the mobilization of the Cd-EDTA complex. *B. cepacia* and its supernatant with dissolved siderophores did not improve uptake of Cd by canola, but did desorb more Cd.

BIOGRAPHICAL SKETCH

Stephen Mirabello, a lifelong Staten Island resident attended and graduated from Staten Island Technical High School in 2000. Stephen graduated with a Regents diploma with honors as well as induction into the National Honor Society. He then attended SUNY College of Environmental Science and Forestry in Syracuse where he majored in Forest Engineering. While at ESF, in addition to the core curriculum, Stephen completed advanced courses in biology, chemistry and geology. He also enjoyed teaching and was employed as and received course credit for his work as a Teaching Assistant in courses such as Physics and Dendrology. He graduated Magna Cum Laude with a Bachelor of Science in 2004. Stephen then attended Cornell University where he majored in Environmental Engineering in the School of Civil and Environmental Engineering and minored in Biological Engineering. Stephen was a TA for CEE 453 and CEE 351 in addition to serving as a TA trainer for the College of Engineering.

ACKNOWLEDGMENTS

Stephen would like to acknowledge the academic and financial support of the CEE school in this research with special thanks to Dr. Len Lion, Beth Ahner, Ruth Richardson, and Janice Thies for lab supplies, reagents, and experimental advice. Cultures of *B. cepacia* were provided by Dr. Bill Ghiorse, Microbiology. Hydroponic cultivation was made possible through assistance from Tim Vadas, BEE. *B. napus* seeds were supplied by Roger Spanswick, BEE. Assistance with microscopy was provided by Barbara Eaglesham. General experimental support was provided by Anthony Hay, Microbiology, Murray McBride and Kathy Howard, Crop and Soil Science. Experimental support was provided by all members of the Lion, Richardson Ahner, and Thies labs.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Introduction and Literature Review

Cadmium is a non-essential trace element, which is toxic at very low concentrations (EPA Maximum Contaminant Level 5μM). Cadmium can occur at high concentrations because of natural weathering of minerals, but most high concentrations in soils are of human origin (Nriagu 1980). A common source of contamination is from the application of fertilizers, both inorganic and organic. Phosphate fertilizers contain Cd as an impurity, which then accumulates in the soil over time. An organic source of fertilizer is biosolids that are produced from wastewater treatment, but biosolids are also high in Cd, as well as other heavy metals. Other than fertilizers, Cd may be in soil because of deposition from smelting and refining industries (Nriagu 1980). Cadmium is also found in PVC as a stabilizer, in glass and plastic pigments, and also in Nickel Cadmium batteries. These sources have resulted in Cd becoming a primary contaminant at many EPA superfund (29.8%) and Brownfield sites (Maier et al. 2000). Cadmium may become a threat to human health is if it is ingested, either directly from the soil, or from plant matter that has extracted the metal from the soil. It is the purpose of this research to illuminate the influence of the microbial community on the Cd uptake by canola, and to consider how these interactions may apply to soil remediation technologies.

It is possible to remove the Cd from contaminated soil so as to alleviate the threat to human health. One method to accomplish this is phytoremediation, which is the use of green plants to remove contaminants from the soil. In the case of organic contaminants, plants may be able to degrade the contaminants within the plant tissues. In the case of heavy metals, which cannot be degraded (such as Cd), they must be extracted from the soil. Phytoextraction is the term given to this form of plant based bioremediation, where the metal may accumulate in the roots, steams or leaves of the remediation plant.

One such plant, *Brassica napus* (common name, canola) has been studied for its phytoextraction capabilities. Canola was grown for 47 days on soil contaminated with 100 mg Cd/kg and the accumulation of Cd in the roots, leaves and stems was measured (Carrier et al. 2003). It was found that the plants grown long-term had little to no signs of stress (leaf chlorosis, growth retardation). Further, the most Cd was found in the leaves, with a concentration of 250 mg Cd/kg dry mass, which is 2.5 times the concentration in the soil. The Cd was found in the vacuoles of the cells, where it did not appear to interfere with primary cell functions. It is because of this high tolerance for Cd that canola is an excellent candidate for use in phytoextraction and merits further study.

The ability of any plant to remove metals from the soil is dependent upon the speciation of the metal and chemistry of the rhizosphere. Regardless of the soil type, the chemistry of the rhizosphere is strongly influenced by the microbial community that is present. In order to better understand the processes that may control hyper-accumulation of metals we must first understand the plant-microbe-metal interactions that occur in the rhizosphere.

A plant can only extract Cd that is bioavailable, which does not include metal that is sorbed to the soil. Essential metals, such as Zn^{2+}, may also be sorbed to soils, and require chemical intervention from plants and microbes to make them available. Fe in aerobic soils is typically in the form of oxides with low solubility and chemical intervention from plants or bacteria is also thought to be important in accessing this essential metal. Bacteria can enhance the availability of iron through the release of iron chelating compounds called siderophores. A bacterium may produce many types of siderophores, including hydroxomates, catecholates and carboxylic acids. *Burkholderia*, a genera that has been shown to live on the roots of *B. napus (*see appendix), can produce several siderophores, shown in Figure 1 (Klumpp et al. 2005).

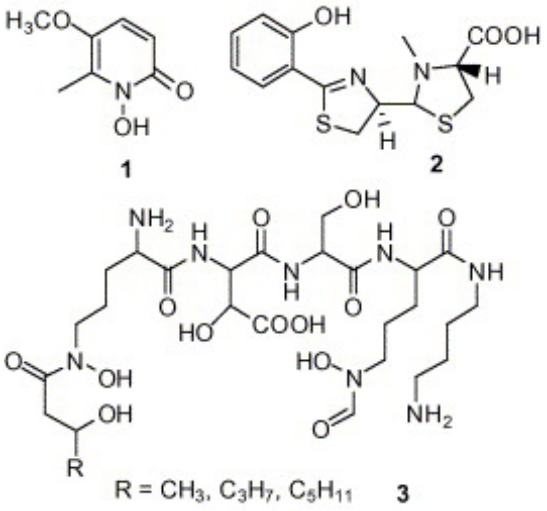

Figure 1: Siderophores produced by *B. cepacia* ATCC #25416 1) cepabactin 2) pyochelin 3) ornibactin

Iron specific chelating compounds are released by graminaceous plants and are called phytosiderophores. In addition, plants can exude organic acids that will form complexes with metals. It has been shown that the presence of siderophores will promote desorption of Pb from an iron oxide (FeOOH) surface (Dubbin and Ander 2003). Further, a related study has shown that siderophores are effective in desorbing Cd and Pb from a kaolonite clay at a moderate pH range (Hepinstall et al. 2005). Other extracellular compounds of bacterial origin, such as extracellular polymers, have also been shown to be effective in the desorption and mobilization of metals, specifically Cd and Cu(II) (Jensen-Spaulding et al. 2004).

In a previous study (Bertrand et al. 2001), the root-associated bacteria of a soil in which *B. napus* was grown were isolated and identified. The study aimed to identify Gram-negative bacteria that grew in the rhizoplane and endorhizosphere (within a plant root) in a Nitrogen depleted soil. The bacteria that were isolated were

used to inoculate plants that were grown hydroponically and the increase of plant dry mass that resulted because of the bacterial presence was recorded. After sequencing, it was found that the genus *Phyllobacterium* resulted in the largest increases in plant dry mass, 52% and 35% in control and N-deficient soil, respectively. In addition it was found that *Pseudomonas* was the most abundant bacterial genus associated with the roots of plants studied (Bertrand et al. 2001). In another study, a siderophore producing strain of *Kluyvera ascorbata* was used inoculate the roots of canola in a nickel uptake experiment, and was shown to decrease the toxic effects of the metal (Burd et al. 2000). These studies helped identify some bacteria that may naturally be associated with a potential metal accumulator and helped emphasize the importance of plant-microbe interactions in plant function.

Methods

The objective of this research is to determine the impact of bacterial and their siderophores on the speciation and bioavailability of Cd in a phytoextraction system. This was accomplished in the following steps: 1) The relationship for Cd adsorption onto a γ-FeOOH solid phase was evaluated and incorporated in an isotherm model. 2) Several strains of siderophore producing bacteria were cultured to obtain siderophores for use, along with commercially available siderophores. 3) The change in adsorption of Cd by γ-FeOOH over time was evaluated subsequent to the addition of the siderophores and cell suspensions. 4) Cd bioavailability (as measured by plant uptake experiments) was determined by exposure of canola plants to suspensions containing Cd, Fe oxides and the addition of organic ligands, bacteria or siderophores. It is anticipated that siderophores will increase the aqueous phase concentration by

promoting the desorption of Cd from Fe oxide and ultimately increase the bioavailability of Cd.

Cd- γ–FeOOH

All flasks used were first acid washed for 24 hours with 0.1 N HNO_3 (Reagent grade) made with distilled water followed by a 24 hour wash in 0.1 N HNO_3 (trace metal grade) made with deionized-distilled water. Flasks were rinsed with deionized distilled water 6 times in between the 24 hour wash steps. After acid washing, flasks were air dried completely, and coated with a silanating solution, by filling the flask 80% full and then swirling the solution for 10 seconds. The silanating solution consisted of 5% dimethyldichlorosilane in CCl_4.

Goethite, γ–FeOOH (Pfizer), was suspended in deionized water and the pH reduced to 3.5 to remove any easily bound surface metals as described previously (Benjamin and Leckie 1979). The acid washed mineral was then used to prepare a 100g/L stock suspension in deionized water that served as the source of γ–FeOOH for all future experiments. Properties of the γ–FeOOH used in this research are listed in Table 1. The Fe oxide suspension was shaken vigorously before each use to ensure consistent suspended solids concentration.

Table 1 Properties of γ-FeOOH

pH point of Zero Charge (PZC)	~7.2
BET surface area , m^2/g	22
Oxide Sites/nm^2	10

Minimal Mineral Salts Media (MMS) was used for all experiments; its composition is listed in Table 2. The media used 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) as pH buffer.

Table 2: MMS-2 Recipe

Compound	Concentration (mg/L)
$CaCl_2 * 2H_20$	30
$MgSO_4$	35
$(NH_4)_2SO_4$	120
KNO_3	15
$NaHCO_3$	0.84
HEPES	5950
Dextrose	68 / 272

A Cd^{2+} stock was prepared by diluting a 1g/L Cd $(NO_3)_2$ (2% HNO_3 solution) stock to 10 mg/L in MMS (2% HNO_3). This diluted stock served as the source of cadmium for experiments, except where otherwise specified.

Cadmium concentrations were measured on a Perkin Elmer Atomic Adsorption Spectrophotometer using a Graphite Furnace (AAS-GF). All standards used were acidified to 2% acid (HNO_3).

Cd adsorption to γ-FeOOH

Dextrose free MMS-2 media (100mL), 2.5 mL of 100g/L FeOOH stock, and 10 μL of 1g/L $Cd(NO_3)_2$ were added to a 125 mL Erlenmeyer flask (Pyrex, model 4980) and the pH was adjusted to 6.4 by addition of 1 N NaOH. After pH adjustment, triplicate flasks were placed on a shaker table (Innova 2000 platform shaker, New Brunswick Scientific) at 150 rpm to ensure mixing. Samples were collected in the following manner: Flasks were removed from shaker table and gently mixed by hand to resuspend any settled oxides. A uniform suspension (1.5 mL) was then pipetted into a 1.5mL centrifuge tube, and was spun at 13500x g (Marathon Micro A, Fisher Scientific) for 2 min. The resulting supernatant (1.4 mL) was pipetted into a 1.5 mL vial, two drops (100μL) of 1 N HNO_3 were added for preservation, and the acidified sample was stored until measurement. This procedure was used for all samples involving FeOOH.

Isotherm – Combined

Dextrose free MMS-2 media (100 mL), 2.5 mL of 100 g/L FeOOH stock, and a Cd mass ranging from 0.5 μg to 50 μg were added to each flask respectively (Benjamin and Leckie 1979). The volume of Cd stock added was not greater than 0.5 mL, to prevent large volume changes (and additional wall sorption). Flasks were adjusted to pH 6.4 with 1 N NaOH, placed on a 150 rpm shaker, and allowed to equilibrate for 24 hours prior to sampling. The amount of Cd in solution was measured as described above, and Cd adsorbed to the surfaces was determined by difference.

Isotherm – Blank

Dextrose free MMS-2 media (100 mL) and a Cd mass ranging from 0.5 μg to 50 μg were added to each flask respectively. Each volume of Cd stock added was not greater than 0.5 ml. Flasks were adjusted to pH 6.4 with 1 N NaOH, placed on a 150 rpm shaker and allowed to equilibrate for 24 hours prior to sampling. Additional sampling was also conducted at 5 days. The amount of Cd in solution was measured, and Cd sorbed to the surface of the glass was determined by difference. The same procedure was also carried out for flasks containing 50 mL of dextrose free MMS-2 media.

pH- Adsorption Edge

Dextrose free MMS-2 media (50 mL), reduced to pH 2 was added to 1.365 ml γFeOOH solution (100 g/L) and 0.575 mL Cd^{2+} (10 mg/L) solution. Each flask was adjusted to a pH value ranging from 3 to 8 by the addition of 1 N NaOH. The flasks were allowed to equilibrate for 24 hours on a 150 rpm shaker table prior to sampling. The amount of Cd in solution was measured, and Cd sorbed to the surfaces was determined by difference.

Desorption Kinetics

Dextrose free MMS-2 (100 mL), 2.5 ml of FeOOH (100 g/L) and 10 μg of Cd^{2+} (10 mg/L) were equilibrated at pH 6.4 for 24 hours, the pH was then reduced to pH 4 by the addition of 1 N HNO_3. The flasks were placed on a 150 rpm shaker table and samples were periodically taken. The same starting concentrations were also equilibrated for 24 hours at pH 4 prior to sampling. The amount of Cd in solution was measured, and Cd sorbed to the surfaces was determined by difference.

Bacterial Growth/Siderophores

Strain and Reconstitution

Burkholdaria cepacia, ATCC # 25416, was chosen as a representative siderophore producing bacteria to utilize in this project. *B. cepacia* is a gram negative, pseudomonad that is commonly found in mixed soil communities. It was received from the American Type Culture Collection (ATCC) as a freeze dried sample that was rehydrated in 10 mL of autoclaved PYG (1g/L peptone, 1 g/L yeast extract, and 3 g/L glucose) medium. An aliquot, 0.1 mL, of the cell suspension was then spread onto MMS-2, and CAS plates (described below) for culturing. The plates were incubated at 37 C until sufficient colonies formed for utilization. Aseptic technique was used, all glassware, solutions, and work areas were sterilized prior to use.

MMS-2/ CAS Plates

One liter of pH 6.4 adjusted MMS-2 (68 mg/L dextrose), 1.5% bacteriological agar was prepared. The solution was microwaved at 1 minute intervals until all of the agar was dissolved and then autoclaved for 1 hour. When the autoclaved solution was cool enough to hold, plates were poured and allowed to gel under a sterile laminar flow hood.

Chrome Azurol Sulfonate (CAS) media containing 60.5 mg CAS was dissolved in 50 mL deionized water, mixed with 10 mL of 1 mM $FeCl_3*6H_20$, 10 mM HCl, and 72.9 mg hexadecyltrimethyammonium bromide (HDTMA) in 40 mL water (Milagres et al. 1999). This solution was then added to solution of 15 g agar, 0.5 g NaCl, 1 g NH_4Cl, 0.3 g KH_2PO_4, 5.96 g HEPES, and 3 g of casamino acids in 750 mL deionized water. The pH was adjusted to 6.4 by the addition of 1 N NaOH and the solution was autoclaved for 45 min. Plates were poured and allowed to cool in a sterile laminar flow hood. The CAS medium is a dark blue color when plates are poured, but will turn orange in the presence of siderophores (Bano and Musarrat 2003).

B. cepacia Growth Curve

A loopful of *B. cepacia* grown on a CAS plate was suspended in 10 mL of autoclaved pH 6.4 adjusted MMS-2 (68 mg/L dextrose) and vortexed for 10 seconds to ensure complete mixing. 50 mL of sterile pH 6.4 adjusted MMS-2 (68 mg/L dextrose) plus 1 mL of cell suspension was poured into a 250 mL side arm Erlenmeyer flask. Triplicate flasks were placed on a table shaker at 150 rpm and room temperature (~25 C) for the duration of the growth curve measurements. The optical density at 600 nm (OD 600) was measured on a Spectronic 20 (Bausch & Lomb) and recorded periodically once every hour to two hours. The same procedure was repeated for MMS-2 (272 mg/L dextrose) with a starting volume of 100 mL.

Siderophore Quantification / EDTA curve

A modified CAS indicator solution was prepared as described previously (Schwyn and Neilands 1987). HDTMA (170 mg), 91 mg CAS, 120 μL of 2% $FeCl_3 * 6H_20$ solution, and 5.95 g HEPES were diluted to 1 L in deionized water. The pH of the solution was adjusted to 5.6 using 1N NaOH and the solution was stored for future use.

Siderophore containing solutions were obtained by centrifuging cell suspensions at 6000x g for 10 min (Beckman, Model J2-21), and removing by pipette the needed volume of supernatant.

Equal volumes of CAS indicator solution and siderophore solution were added to a 4 mL cuvette, capped, and then sealed with parafilm. The cuvettes were then shaken by hand and allowed to react. After two hours the absorbance spectrum for each sample was read as was a blank containing equal volumes of CAS indicator solution and dextrose free MMS-2 (Hewlett Packard 8452A Diode Array Spectrophotometer).

An EDTA standard curve was prepared to mimic a siderophore. CAS indicator, dextrose free pH 6.4 adjusted MMS-2, and 1 mM EDTA were added to cuvettes to produce EDTA concentrations ranging from 0 to 20 μM. The cuvettes were then capped, sealed, shaken, and allowed to react. After two hours, the absorbance spectrum of each sample was read.

Plant Growth

Brassica napus var. Quantum seeds were germinated by placing them on a moist paper in a darkened environment for several days (~4-6). When seedlings were ~ 50 mm in length with both cotyledons exposed they were moved to a 50 mm hydroponic growth cup. The cup was filled with 63.5 mm (1/4") black acrylic beads to provide a support for growth. The cup was suspended into a 40 L opaque container with *Brassica* growth medium, see Table 3.

Table 3 : Hydroponic growth medium for *Brassica napus*

Component	Concentration
KNO_3	1.214 mM
$Ca(NO_3)_2{*}4H_2O$	0.7857 mM
$MgSO_4{*}7H_2O$	2.413 mM
$(NH_4)_2HPO_4$	1.0715 mM
KH_2PO_4	1.0715 mM
Na_2MoO_4	0.1074 μM
KCl	10.71 μM
H_3BO_3	5.337 μM
$ZnSO_4{*}7H_2O$	0.4286 μM
$MnSO_4{*}H_2O$	0.4285 μM
$CuSO_4{*}5H_2O$	0.1073 μM
$Fe(NO_3)_3{*}9H_2O$	6.000 μM
HEDTA	6.000 μM

The hydroponic medium pH was adjusted to 6.3 by the addition of 1N KOH. Plants were grown under an integrated light system that provided 17 Moles of incident photons per day. Water level, pH, and conductivity were checked every two days and restored to starting conditions by the addition of deionized water and/or growth medium. Plants were large enough for uptake experiments after ~3 weeks from the transfer to the hydroponic cups.

Perturbations:

Equilibrated Cd-FeOOH in MMS-2 suspension (50 mL) in which 90% of Cd was adsorbed (Cd_{total} 5.75 μg, 51.3 nmoles, plus $FeOOH_{total}$ 1.365 g yields 30 ppm adsorbed Cd as calculated using the adsorption isotherm) was perturbed by adding 50 mL of pH 6.4 adjusted dextrose free MMS-2 alone or MMS-2 and 1:1 mole ratio (51.3 nmoles) of sodium citrate, sodium salicylate, or Na_4EDTA. The same procedure was also repeated for the addition of 50 mL of stationary phase cell suspension or

supernatant. Triplicate flasks were placed on a shaker at 150 rpm and samples were collected periodically for 5 days. The amount of Cd in solution was measured, and Cd sorbed to the surface was determined by difference.

The above procedure was repeated for 30 ppm Cd-FeOOH, 85% sorbed (Total Cd 4μg, 36 nmoles, $FeOOH_{total}$ 0.0718 g as calculated from the adsorption isotherm). Flasks were also wrapped in foil to prevent UV degradation of added ligand (36 nmoles for 85% sorbed), or siderophore in stationary phase suspension/supernatant. Triplicate flasks were placed on a shaker table in an environmental controlled chamber without UV light. The shaker table operated at 150 rpm and samples were collected periodically for 5 days. The amount of Cd in solution was measured, and Cd sorbed to the surfaces was determined by difference.

Plant Uptake Model System

Apparatus

A set up was designed that separated the different phases of the experiment but allowed for experimental Cd transfer. A 125 ml silanated Erlenmeyer flask served as the container with a dialysis tube draped inside. Images of the apparatus are shown in Figures 2 and 3.

Dialysis Tubing

18 cm strips were cut of 6,000 Molecular weight cut off (MWCO) regenerated cellulose dialysis tubing (Spectra-Por, #132665). One end of each strip was folded in half three times and then closed with a zip tie. The closed strips were then soaked in deionized water for at least one hour prior to use.

Phase Separation

50 mL of equilibrated 30 ppm Cd-FeOOH in MMS-2 suspension at 85% Cd sorbed (Cd_{total} 4 μg, $FeOOH_{total}$ 0.0718 g as calculated by isotherm) was present in the flask before perturbations. Foil wrapped flasks were perturbed by the addition of 50 mL total of dextrose free MMS-2 alone or MMS-2 with 1:1 ligand (36 nmoles) of salicylate or EDTA. 20 mL of the MMS-2 was added directly to the flask, the remaining 30 mL was added in a dialysis tube that was then draped into the flask. The ligand was added 70:30 (flask: tube) to ensure that the concentration of ligand was the same inside and outside the tube.

Figure 2 : Image of reactor vessel and dialysis tubing separate (left) and combined (right)

A similar procedure was conducted for the addition of stationary phase cells or supernatant. 20 mL of supernatant was added to the flask and 30 mL was added inside the dialysis tube. A concentrated cell suspension was created by resuspending the remaining pellet from the supernatant centrifuge tube. The cell suspension was added to the system so that the concentrations of cells inside and outside the dialysis tube were equal.

After equilibration, plants were added to the system by placing them in the 30 mL dialysis tube, which was open at the top of the flask.

Figure 3 : Image of foil wrapped reactors plus *B. napus*

Dialysis Sorption

50 mL of dextrose free MMS-2, 4 μg Cd^{2+}, and 0.0718 g FeOOH were equilibrated overnight (minimum 12 hours). This yielded 85% adsorption of Cd with 30 ppm of Cd on the FeOOH (from isotherm). A perturbation was conducted in the presence and absence of dialysis tubing to determine Cd adsorption to the tubing. The control (no dialysis tube) was perturbed by adding 50 mL of MMS-2 directly into the flask. Flasks with dialysis tubing had 20 mL MMS-2 added to the flask and 30 mL added in a dialysis tube, as described previously. The triplicate flasks were placed on a shaker table at 150 rpm and samples were taken periodically for 24 hours from both outside and inside the dialysis tube at each time point. The aount of Cd in solution was measured, and Cd sorbed to the dialysis tube surface was determined by difference.

Perturbation / Plant Uptake Schedule

Two schedules were followed for the plant uptake experiments. Flasks were set up and perturbed as described in "Phase separation" section. Prior to the introduction of a seedling, each plant was rinsed in deionized water for 10 minutes before placement in dialysis tube. This was done to remove any HEDTA that might still remain on the roots, and would chelate cadmium. Each plant was removed from the growth cup, and any associated roots that were torn from the plant were also rinsed and added to the tube.

Triplicate flasks were allowed to equilibrate for 5 hours after perturbation before the addition of the plant. All perturbations were performed in a UV free chamber to prevent photodegradation. Immediately before plant addition, a sample was taken from inside and outside the dialysis tube, followed by a weighing of the flask. The mass of the flask was used to help track water volumes and transpiration. The plant was then placed into the dialysis tube and the shaker table speed was reduced to 50 rpm. This speed was selected to allow mixing of the water, but to avoid damage to the plant tissue. Plants remained in the tube for 3 hours and then were removed for analysis. After plant removal, the flask was first weighed and then a sample was taken from inside and outside the dialysis tube.

The second uptake experiment followed a modified procedure only differing in the equilibration time and time for plant uptake. Triplicate flasks were allowed to equilibrate for 8 hours prior to the addition of the plant. The plant then remained in the flask for 6 hours before removal for analysis.

Plant Cd Composition/Ashing

Plants were removed from dialysis tubing and were immediately rinsed in deionized water for 10 minutes to remove any dissolved Cd^{2+} transferred with solution on the roots. Following rinsing, roots of each plant were cut from the shoots and the

roots and shoots were placed in crucibles and weighed. The crucibles were covered in aluminum foil and placed in a 100 ºC oven overnight (minimum 12 hours) and were then weighed again. The plant material was then crushed into a powder using a ceramic pestle, the crucible was covered with foil and placed in a 600 ºC muffle furnace for 6 hours (Perkin_Elmer 1994). The resulting ash was then dissolved in 1 mL of 1 N HNO_3, diluted to 10 mL with deionized water in a 20 mL culture tube (VWR, KIMAX 51, borosilicate glass) and stored until analysis.

Microbial Communities (DAPI).

Cell counts were performed to estimate both the concentration of *B. cepacia* cells added in the cell suspension and also the amount of microbes present on the roots before and after addition of the plant.

B. cepacia

One mL of a 1:10 dilution of *B. cepacia* suspension culture in DI water was added to a 1.5 mL centrifuge tube along with 2 drops (20 μL) of a 0.2 mg/ml DAPI solution. The tube was wrapped in foil and placed on a shaker Table at 55 rpm (Lab Line Orbit Shaker) for 10 minutes. A 500 μL of the sample was then combined with 25 mL sterilized DI water and concentrated on a 25 mm, 0.2 μm pore size membrane filter using vacuum filtration. The filter was then moved to a glass slide and 1 drop of Citiflour was added prior to placement of a cover slip. Slides were stored in the dark until microscopic viewing.

B. napus root communities

A sample of root was added to 10 mL of sterilized DI water in a 15 mL culture tube. The sample was then vortexed on high for three 30 second pulses to promote removal of tenacious microbes, but not high enough to destroy the roots. 1 mL of

resulting suspension was stained with DAPI as described previously. The plant root was then removed from the suspension and weighed.

Cell Counts

Slides were analyzed under UV light using a Nikon Eclipse E400 scope, and 100x lens. 10 fields (900 μm^2 each) were randomly chosen and used for counts. A negative control (same procedure, but with DI water in place of cell suspension) was used for all experiments.

Results

In all graphs, error bars indicate the S.D. of triplicates

Cd- γ–FeOOH

Adsorption

The Cd – γ–FeOOH suspension came into equilibrium within 3 minutes (see Figure 4). The dissolved Cd concentration remained relatively unchanged for the next 6 hours, after which samples were not taken. This result is consistent with previous studies using γ–FeOOH, where an ultimate equilibration time of 24 hours was used (Benjamin and Leckie 1981b).

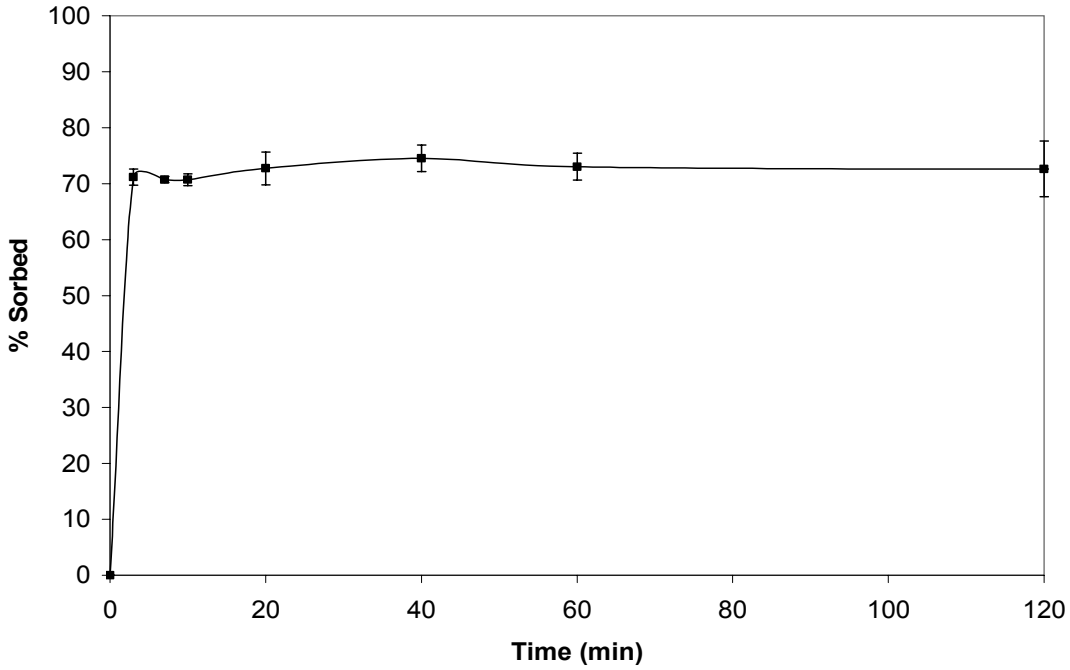

Figure 4: Plot of percent Cadmium sorbed onto γ–FeOOH as a function of time at pH 6.4

Isotherms

Both the blank and γ-FeOOH flasks produced linear isotherms when mass sorbed (μg) was plotted as a function of Cd concentration (μg/L). The mass sorbed onto the surfaces, either the glass wall or goethite, was calculated by difference. The results are plotted in Figure 5, below. As can be seen, the addition of FeOOH produced a steeper curve, which indicates a larger amount of Cd adsorption.

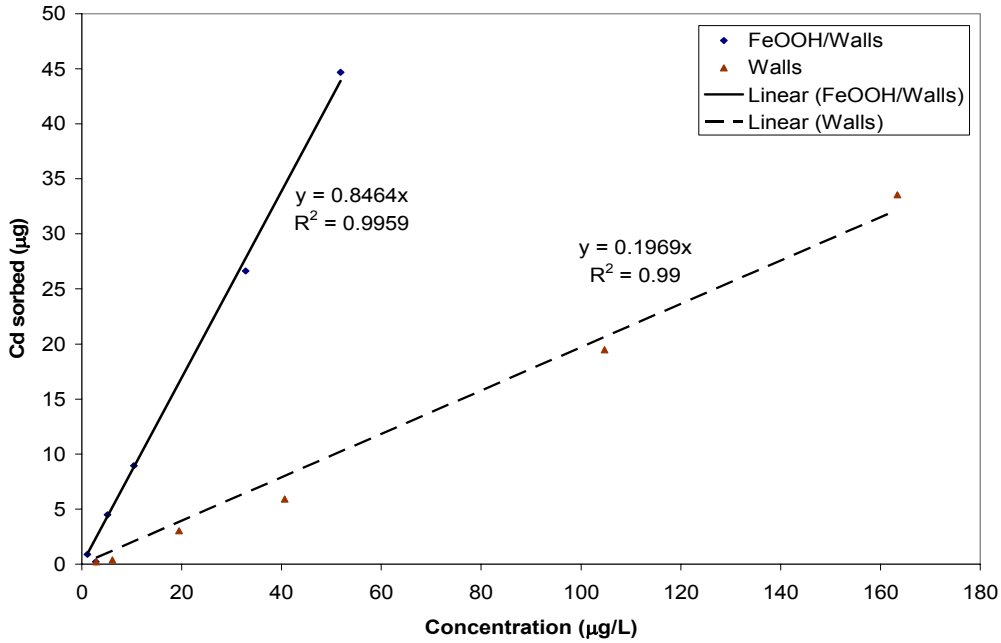

Figure 5 : Plot of Sorbed Cd (µg) as a function of soluble Cd concentration (µg/L) for γ FeOOH and blank flasks at pH 6.4

The linearity of the Cd adsorption isotherms on the glass reactor surface and in the reactor containing γFeOOH simplified the formulation of an isotherm for the Fe surface. A linear isotherm could be used for Cd on γ-FeOOH in MMS-2 at pH 6.4 and could be calculated using the two graphs. Additional adsorption (above the wall loss with zero Fe) was assumed to be caused by the addition of FeOOH, and the corresponding amount sorbed was calculated by the difference between the two curves. The following relationship was used to model adsorption.

$$S = K_d * C$$

Where S is Cd sorbed onto FeOOH (µg/g)

C is concentration of Cd in solution (µg/L)

and K_d is a constant relating the S and C (L/g)

The isotherm is applicable from 0 to 60 μg/L. A plot of the isotherm over the applicable concentrations is shown in Figure 6.

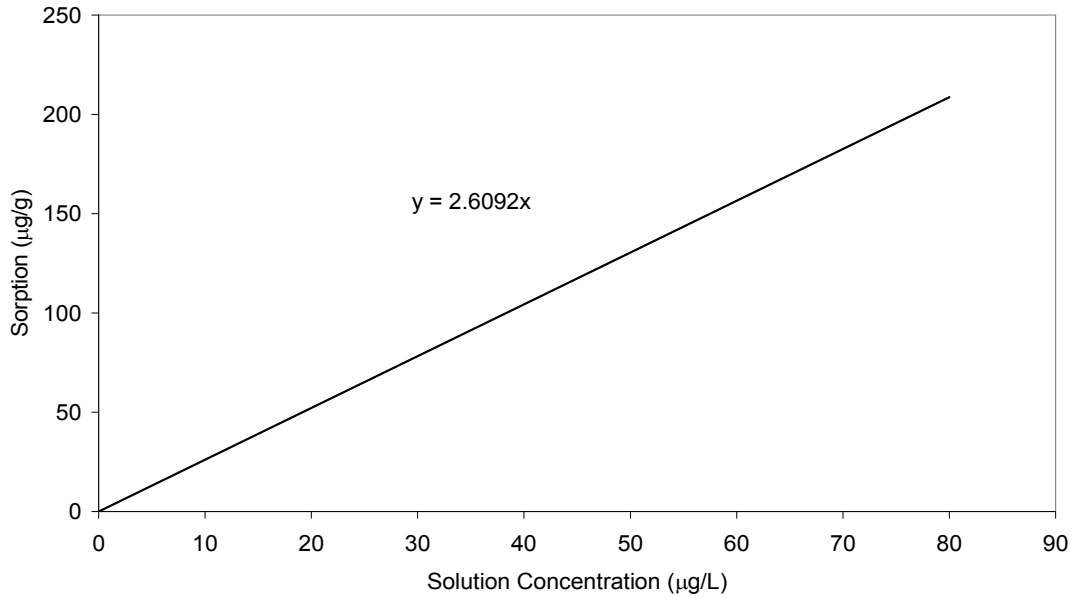

Figure 6 : Isotherm for Cd on γ-FeOOH in MMS-2 at pH 6.4

The steepness of the blank adsorption isotherm, as seen in Figure 5, indicates that wall loss is not negligible in this system. When calculating a mass balance for Cd, a relationship for wall sorption needed to be established to make accurate predictions. Wall sorption is related to the amount glass surface contacting the solution at each volume, therefore a blank isotherm for an experimental design volume of 50 mL was conducted in addition to the blank isotherm at 100 mL. The blank isotherm for 50 mL is shown in Figure 7 below.

Wall sorption had a variable temporal behavior, with a higher sorption initially, which decreased to a steady value by 5 days after addition of Cd (Figure 5). This variable behavior was not seen in flasks containing FeOOH, as the concentration of Cd measured at 1 day was identical to values measured at 5 days after addition of Cd.

As can be seen in Figures 5 & 7 the blank reactor isotherm slope for the 50 mL flasks (0.1155) is much less than the 100 mL flasks (0.1969), as would be expected because there is less surface area in contact with the solution.

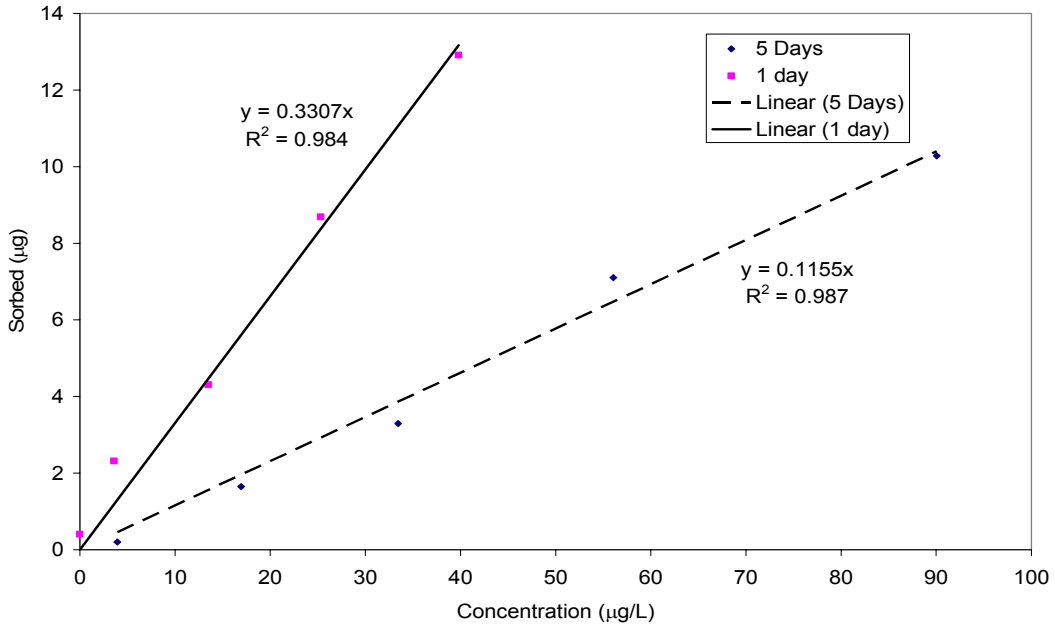

Figure 7: Plot of Cd sorbed (µg) onto walls as a function of Cd concentration (µg/L) at a design volume of 50 mL at 1 and 5 days

In order to simplify later calculations, a volumetric relationship for wall adsorption of Cd was determined. The previous data was analyzed to account for the volume present in the 50 mL and 100 mL volume flasks. The volume present in each flask was not exactly 50 or 100 mL because of the varying amount of Cd solution and the corresponding base added to reach pH 6.4. The resulting curves are shown in Figure 8, and show that at 50 mL there is a higher adsorption per unit volume than at 100 mL.

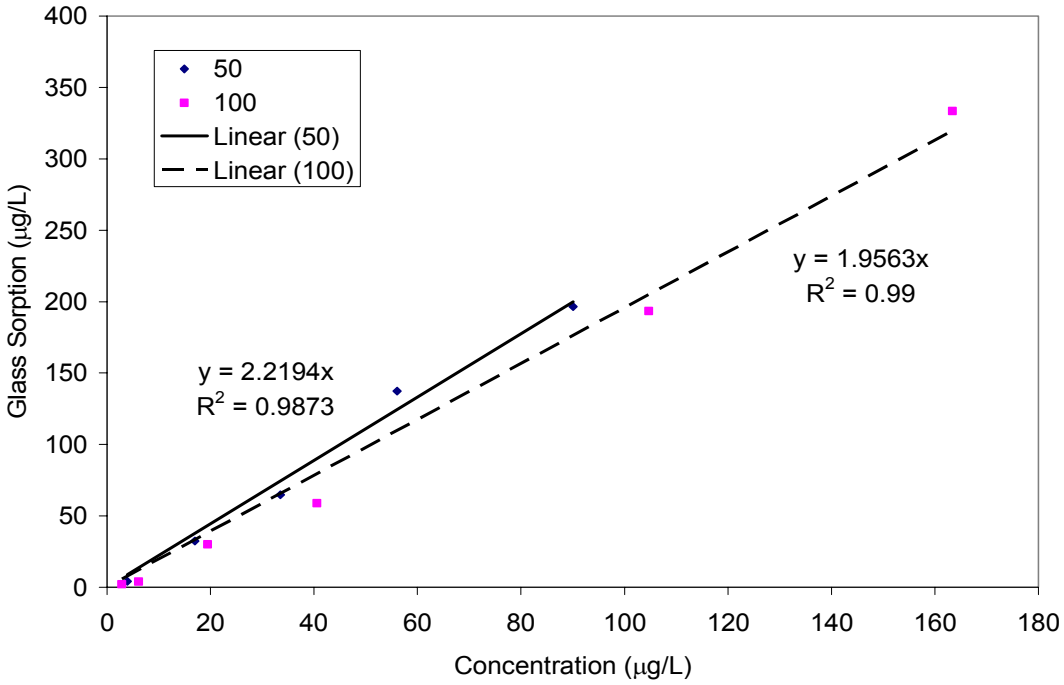

Figure 8 : Volumetric sorption of Cd onto flask walls at design volumes 50 and 100 mL respectively

The volumetric sorption amounts shown are proportional to the area to volume ratio, A/V, for a flask filled to those specified volumes. The area (cm^2) and volume (ml) were calculated using an inverted cone approximation and measured dimensions from the 125 mL Erlenmeyer flask. This data was used to create a relationship for wall sorption which is described below

$$Cd_g = K_g * C * V$$

Where Cd_g is the cadmium sorbed to the flask wall (µg)

C is the same as described before

V is the volume of the flask (L)

K_g is a constant that relates amount in solution to amount sorbed

K_g can be approximated for any volume from 50 mL to 100 mL using the relationship below

$$K_g = a_g * \frac{A_x}{V_x}$$

Where a_g is a constant, 1.74 (cm), determined by a two point calibration, and $\frac{A_x}{V_x}$ is the area to volume ratio at volume, x (cm^{-1}).

The above relationships were used to formulate a mass balance for Cd in the flasks which is presented below. The cadmium in the flasks can either be found in solution, on the walls, or on the goethite.

$$Cd_T = C*V + K_d*C*Cs*V + K_g*C*V$$

Where $C, V, K_d,$ and K_g are as described previously

Cs is the concentration of FeOOH in the flask (g/L)

And Cd_T is the total amount of Cd in the flask.

The above equation can be manipulated to yield a relationship for fraction sorbed (fs), which is independent of Cd concentration.

$$fs = 1 - \frac{C*V}{Cd_T} = 1 - \frac{1}{1 + K_d*Cs + K_g}$$

Performing a simple calculation for a blank 50 mL flask (Cs = 0, Kg = 2.22), will yield that 69% of the Cd in the flask is sorbed to the wall. The wall is actually the dominant surface to which cadmium is sorbed in the experimental system, not the FeOOH.

pH Adsorption Edge

Experiments to determine the pH dependence of Cd adsorption were conducted three times, and the data summarized in Figure 9. The data show an overall increase in adsorption as the pH increases, which has been shown previously (Benjamin and Leckie 1979).

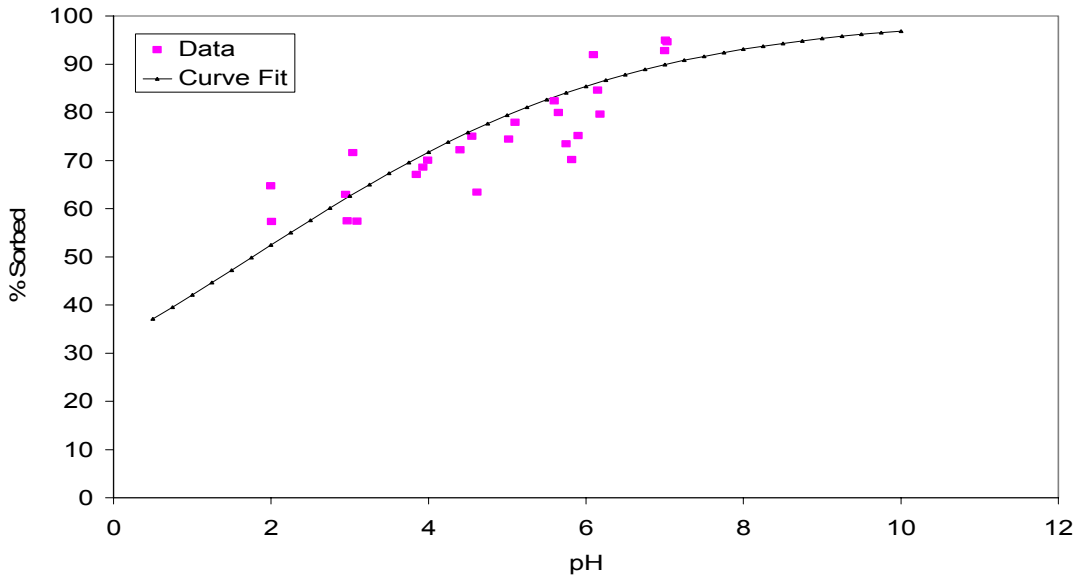

Figure 9 : Adsorption Edge for Cd of g-FeOOH at pH 6.4. Cd_T 5.75 µg, $FeOOH_T$ 0.1365 g and design volume of 50 mL.

The low slope of the data indicates that the system is not very sensitive to pH changes. A general relationship used to model metal sorption as a function of pH, which relies upon exchange of hydrogen for metal ions has been described previously (Benjamin and Leckie 1981a). The assumed reaction is:

$$SOH_x + M \leftrightarrow xH^+ + SOM$$

The equilibrium constant for the reaction, P, is therefore given by:

$$P = \frac{(H^+)^x * SOM}{SOH_x * M}$$

Where SOH_x is the surface with bound hydrogen ions (g/L)

M is the free metal ion in solution (µg/L)

H^+ is the free hydrogen ion concentration (Molar),

SOM is the metal that is bound to the surface (µg/l)

The above equation can be manipulated to solve for x and P. As shown below

$$\log \frac{SOM}{M * SOH_x} = x * pH + \log P$$

To translate this equation to the known variables, *Cs* replaced *SOH* (under the assumption that few surface sites were occupied by adsorbed metal), *SOM* was calculated by difference from *M*, which was a measured quantity. A plot of the resulting data is shown in Figure 10, with a corresponding value of 0.13 for *P* and 0.18 for *x*. This means that every mole of metal ions adsorbed are exchanged for only 0.18 moles of hydrogen, instead of a maximum of two. The variables x and P were used to create a fitting curve, as shown in Figure 9. A previous studie found an x value of 1.8 for Cd adsorption onto amorphous iron oxides at pH 7 (Benjamin and Leckie 1981b).

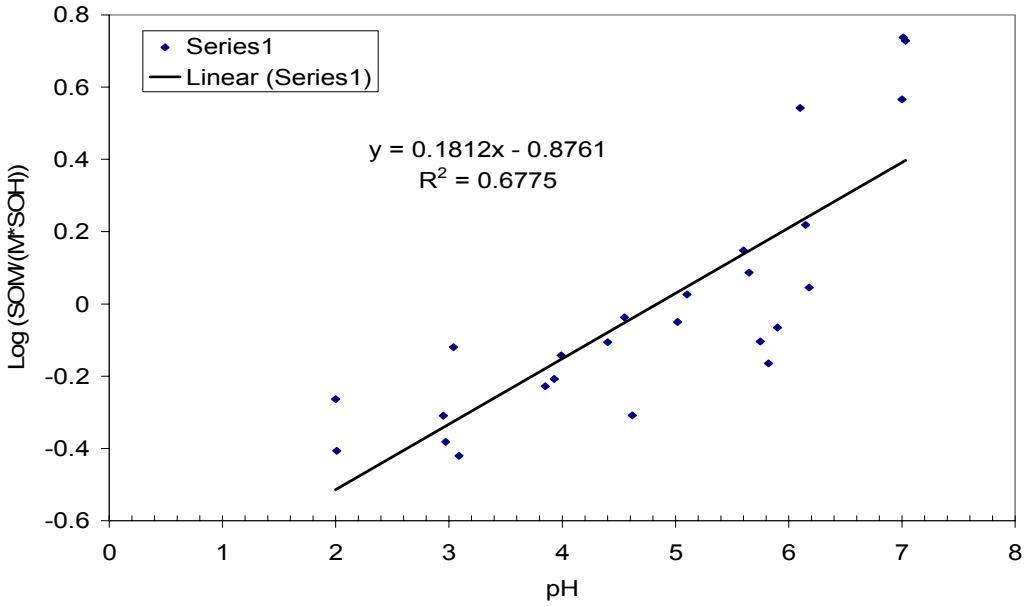

Figure 10 : Transformation of Adsorption Edge data to find x and P

Desorption

The desorption of Cd from the γ-FeOOH system was biphasic as seen in Figure 11. Upon a drop of pH 6.4 to 4 there was an initial rapid drop in adsorbed Cd followed by a much slower decrease over the next few hours. The forward adsorption

reaction at pH 4 yielded an equilibrium of approximately 40% adsorbed. If desorption was complete it would approach this value but the lowest value attained in desorption was only 58%. These results show that only partial desorption of Cd can be expected to occur over several hours.

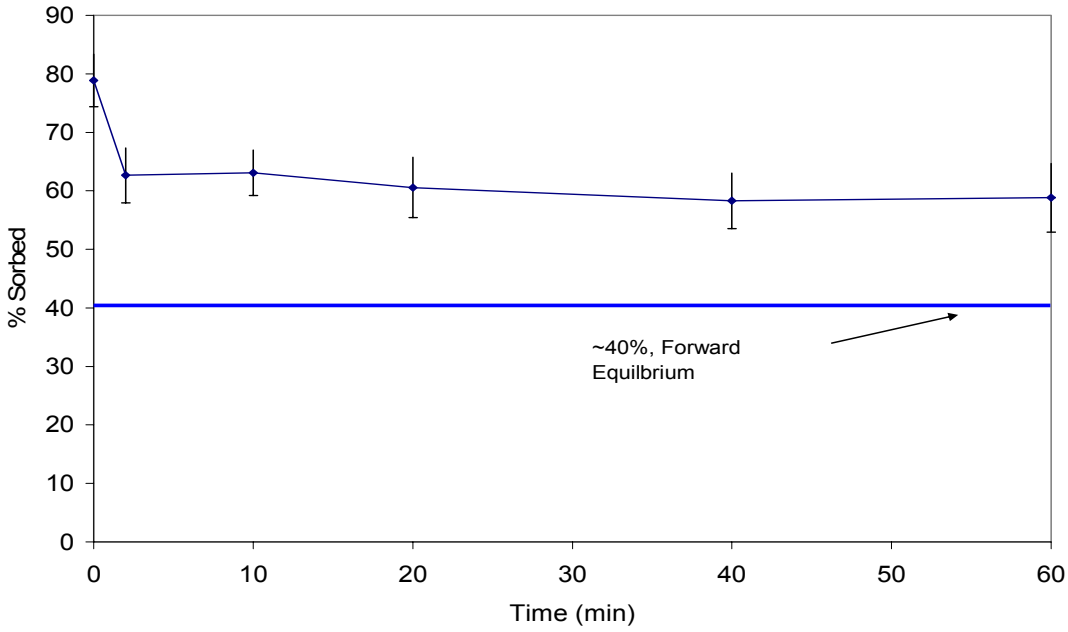

Figure 11 : Desorption of Cd from γ-FeOOH caused by a drop of pH

Bacterial Growth

MMS-2/CAS Plates

B. cepacia grew on both MMS-2 and CAS plates. Growth was much more rapid on CAS plates, so they were chosen as the media to grow cultures on in preparation for measurement of the growth curves. Initial CAS plates streaked with *B. cepacia* did not have any orange spots to indicate the presence of siderophores, but

spots did appear with later streakings. This change was probably a result of the selection of *B. cepacia* cells that needed to produce siderophores to acquire ferric iron from the media.

Growth Curve

The growth of *B. cepacia* in suspension with MMS-2 is shown in Figure 12. The Spectronic 20 was calibrated so that initial reading was greater than zero, to show any initial loss of cells. When a measurement was taken, it was compared against a flask filled with the same volume of DI water as a control. This was needed to account for the fluctuations in lamp output. The growth curve indicates that the culture was in lag phase until hour 10 after inoculation, followed by a growth phase until hour 33. The culture experienced a long stationary growth phase at OD 600 ~ 0.16 which lasted until hour 100, at which point the cell cultures were harvested and stored for later use. The cell growth medium (MMS-2) did not contain any added iron, so in order to obtain this necessary macronutrient the cells acquired needed Fe from trace contamination in the media reagents. The Fe deficient media likely promoted siderophore production by *B. cepacia*.

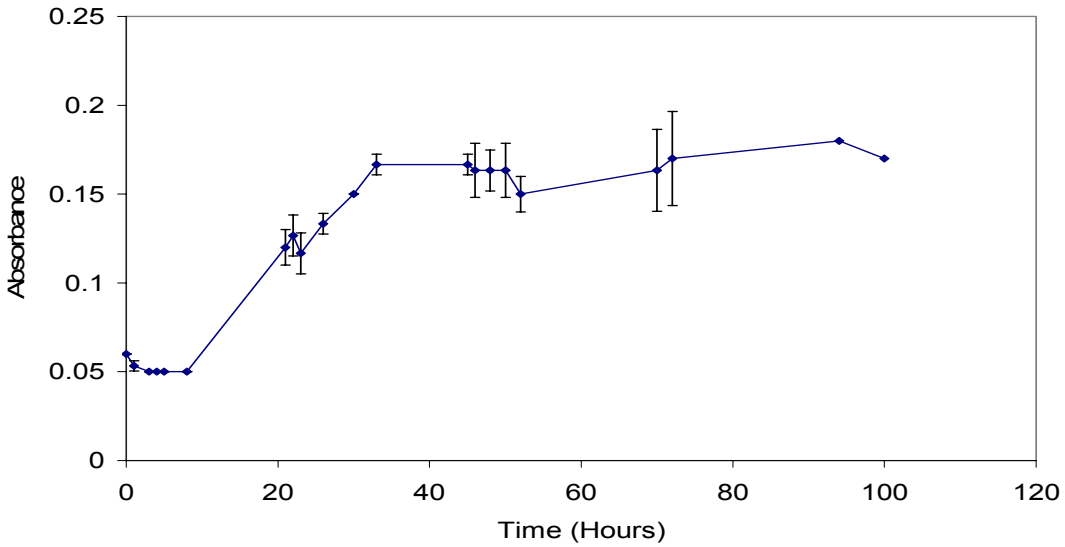

Figure 12 : Growth curve for *B. cepacia* on 68 mg dextrose/L MMS-2 pH adjusted to 6.4

A second growth curve was conducted at a dextrose concentration of 272 mg/L, 4 times the original concentration. This was done to produce more cells, and also more siderophores. The volume of the culture was increased to 100 mL in order to produce the same cell suspension in fewer flasks, but still allowing sufficient headspace for oxygen transfer. The resulting growth curve (Figure 13) was produced with a lag phase lasting until hour 7, followed by growth until hour 40.

The growth rate, μ, can be estimated by using exponential growth equations: (Madigan et al. 2003). Hours 9 and 37 marked the beginning and end of growth phase respectively, and the corresponding ODs were used to approximate growth rate. OD was converted to Klett units and approximately 2.4 generations had passed during the 28 hours of growth phase (~12 hour doubling time). The growth rate is approximately 0.06/hr and was approximated by the following equation:

$$\mu = \frac{\ln 2}{t_d}$$

Where μ is the growth rate, and t_d is the doubling time

The stationary growth phase at OD 600 ~0.32 lasted until the end of experiment at hour 54. Overall, the increase in dextrose concentration produced approximately double the amount of cells and reached stationary phase in the same amount of time. Final cell suspensions were either stored for future use or used immediately in siderophore quantification or perturbation experiments.

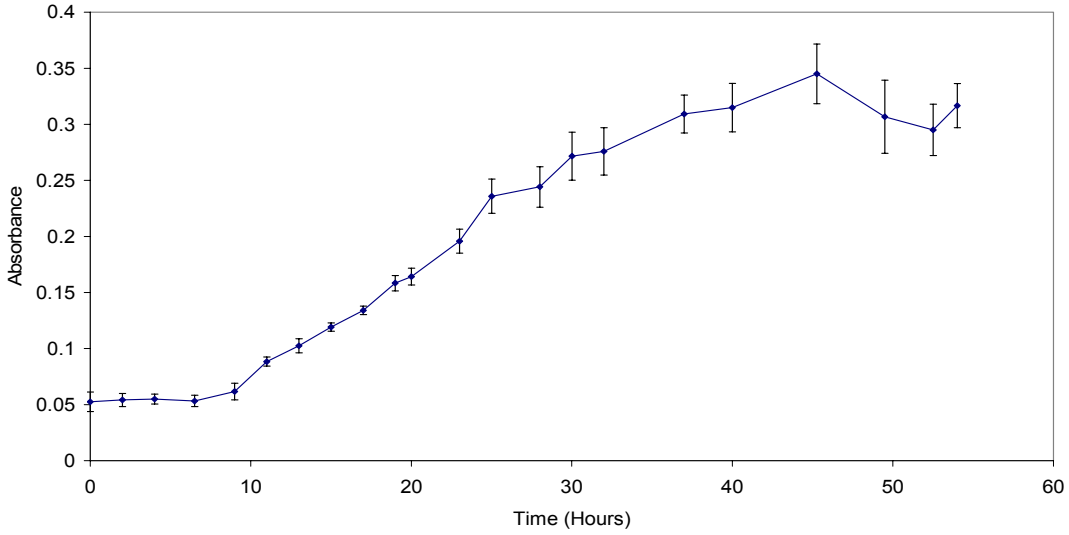

Figure 13: Growth curve for *B. cepacia* on 272 mg dextrose/L MMS-2 pH adjusted to 6.4

Siderophore Quantification/ EDTA standard curve

The CAS complex, which is comprised of a CAS molecule, ferric ion and a HDTMA ion, is responsible for giving the CAS media a blue color. This complex has been previously characterized and has a maximum absorbance at 630 nm (Schwyn and Neilands 1987). When a strong ligand/chelator is added, it will remove iron from the

complex and a color change will result. With increasing ligand concentration the solution will change from blue to green to orange. Any chelator that binds Fe^{3+} stronger than CAS, such as EDTA, is acceptable for calibration purposes. Absorbance spectra for the CAS/EDTA standards are shown in Figure 14. The calibration curve, shown in Figure 15, was calculated by taking the ratio of absorbance of a standard to blank cuvette (uninnoculated MMS-2 medium added to CAS indicator solution) at 630nm.

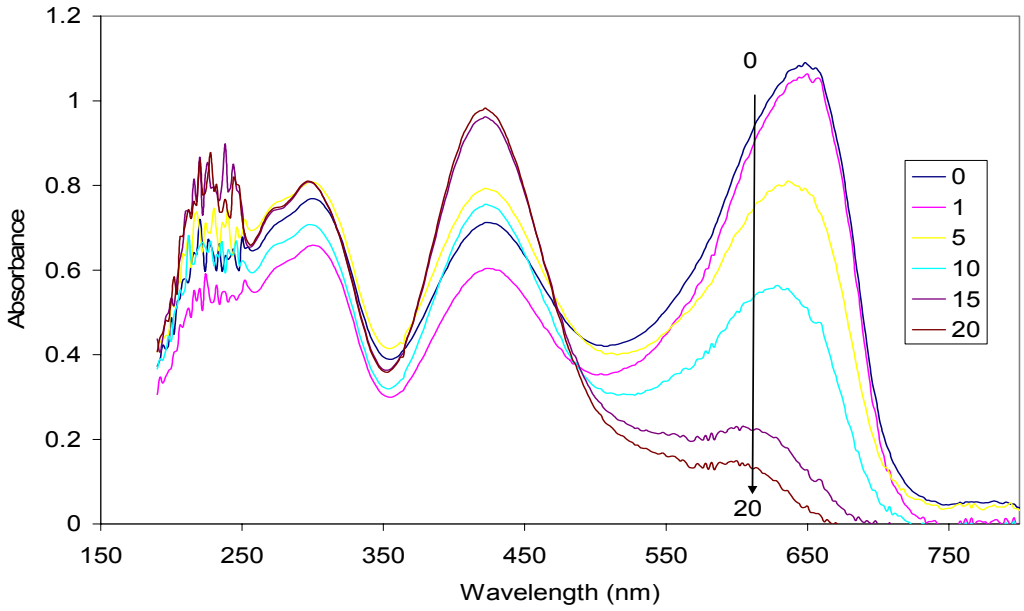

Figure 14 : UV spectra for EDTA in using CAS media

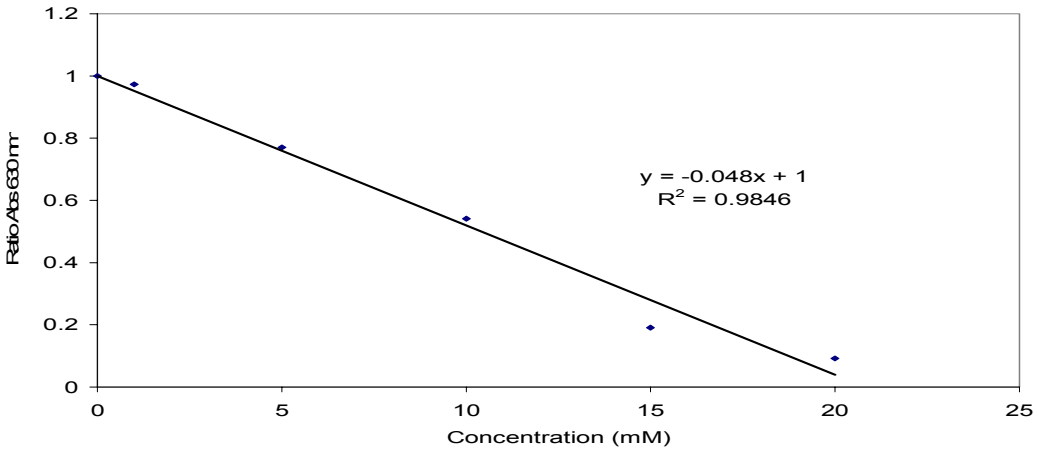

Figure 15 : EDTA – Siderophore calibration curve

The CAS solution has 15 μM of Fe, and can not be used accurately for siderophore concentrations much greater than this, with 20 μM chelator being the upper limit. The EDTA standard curve found in this work was virtually identical to those described for a wide variety of siderophores and was assumed to be accurate for quantifying siderophores produced by *B. cepacia* (Schwyn and Neilands 1987).

Bacterial cultures were grown as described above and were harvested at the beginning of stationary phase and the concentration of siderophores in solution was quantified. An example of a CAS absorbance spectra in media containing the supernatant from the *B. cepacia* suspension is shown in Figure 16, where the lower curve corresponds to the presence of the supernatant. As seen the addition of supernatant yielded a lower absorbance at 630 nm, indicating the presence of a chelator, such as a siderophore. The resulting ratio at 630 nm (0.27) when used with the EDTA calibration curve indicates the amount of siderophore in the cuvette. This concentration is half of the supernatant concentration since the supernatant is added 1:1 to the CAS indicator solution. The siderophore that is most likely produced by *B. cepacia* is cepabactin (Meyer et al. 1989), although *B. cepacia* can also produce other

siderophores as well, such as pyochelin and ornibactin as shown in Figure 1 (Klumpp et al. 2005). The amounts of siderophore produced by the *B. cepacia* suspensions are listed in Table 4, in addition to the number of times the culture has been restreaked since revitalization of the freeze dried culture. It should be noted that on 3/12/06 the cultures switched from being grown in 50 mL at 68 mg/L dextrose to 100 mL at 272 mg/L dextrose. In general, the amount of siderophores produced by *B. cepacia* increase with increased dextrose and also with successive restreakings.

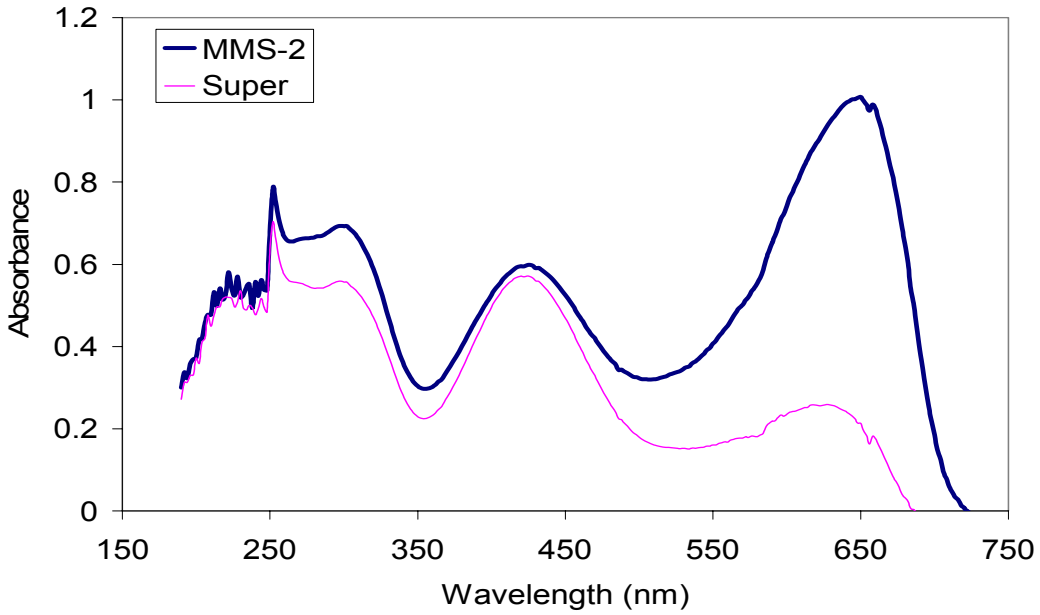

Figure 16 : Example of a *B. cepacia* siderophore CAS spectrograph

Table 4 : Siderophore concentration for supernatant at different restreakings

Restreak #	Date	Ratio at 630 nm	Siderophore Concentration (μM)
1	12/5/2005	1.000	0.000
2	12/11/2005	0.995	0.191
3	2/6/2006	0.832	7.019
4	3/12/2006	0.539	19.215
5	4/23/2006	0.277	30.108
6	5/22/2006	0.271	30.382
7	6/8/2006	0.076	38.492

* assuming 1:1 siderophore:Fe complex

Plant Growth

Seedlings were grown for ~3 weeks under conditions described previously, but did not exhibit uniform growth rates throughout this time. During the first week growth was very slow and replacement of water and media was every other day. On average 0.5 L of deionized water or growth medium was needed every two days to return the water level to 40 L and the conductivity to ~1000 μS. Emergence of true leaves typically occurred at the beginning of the second week, and was accompanied by an increase in growth medium utilization. After three weeks of growth, the seedlings were taking up about 2 L/day of growth medium and were large enough to cover the surface area of the tub (~0.4 m^2). Some plants grown at the end of May and the beginning of June had buds and flowers that bloomed. For consistency, plants that did not have buds, or a large central stem from which the flowers would bloom were chosen for uptake experiments. Different uptake results might be obtained if more mature plants were used in experiments.

Perturbations

All of the perturbations of FeOOH equilibrated with Cd displayed an initial rapid drop in Cd adsorption, followed by different behavior depending upon the treatment. The first perturbation, which compared MMS-2, cell suspension and supernatant (siderophore concentration 7.02 μM), is shown in Figure 17. After the increase in Cd concentration (a decrease in % adsorbed), readsoprtion occurred (presumably to newly wetted reactor walls) this was then followed by a slow desorption. At the end of four days after perturbation(Figure 18), the release of Cd reached the level predicted by the Cd mass balance equation for all treatments.

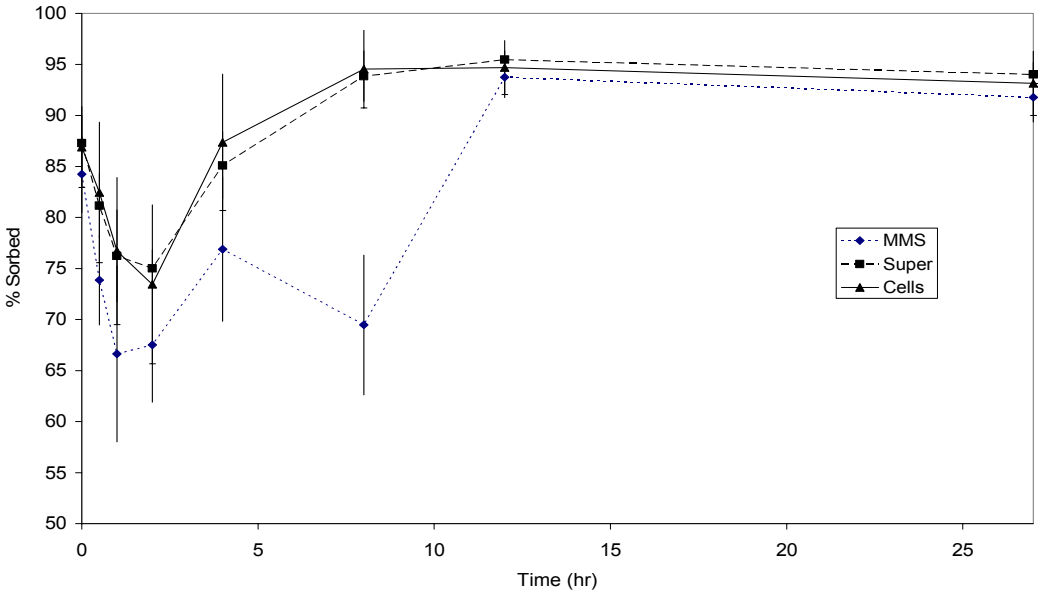

Figure 17 : First perturbation of addition of supernatant and cells. Siderophore concentration: 7.02 μM for first 27 hours

A simulation for the addition of ligands was run using MINEQL+. In addition to the MMS-2 species, Fe^{3+} was included in the same amount present in the added FeOOH. The pH was set to 6.4 and an open system model was used (CO_2 exchange with atmosphere). The results of the simulation showed that Fe speciation was

governed by precipitation of hematite (Fe_2O_3) and the calculated Cd speciation is listed in Table 5. In the absence of wall or FeOOH adsorption all of the Cd in the MMS-2 would be mainly in the forms of Cd^{2+}, $CdCl^{+}$, and $CdSO_4$ AQ. The addition of the ligands resulted in the formation of complexes with Cd in the given order CdEDTA > CdCitrate >> CdSalicylate. The model did not account for metal adsorption to iron oxides. According to this model EDTA would be most effective at desorbing Cd^{2+} from any surface as a result of the chelation of Cd^{2+} ions upon the addition of the ligand.

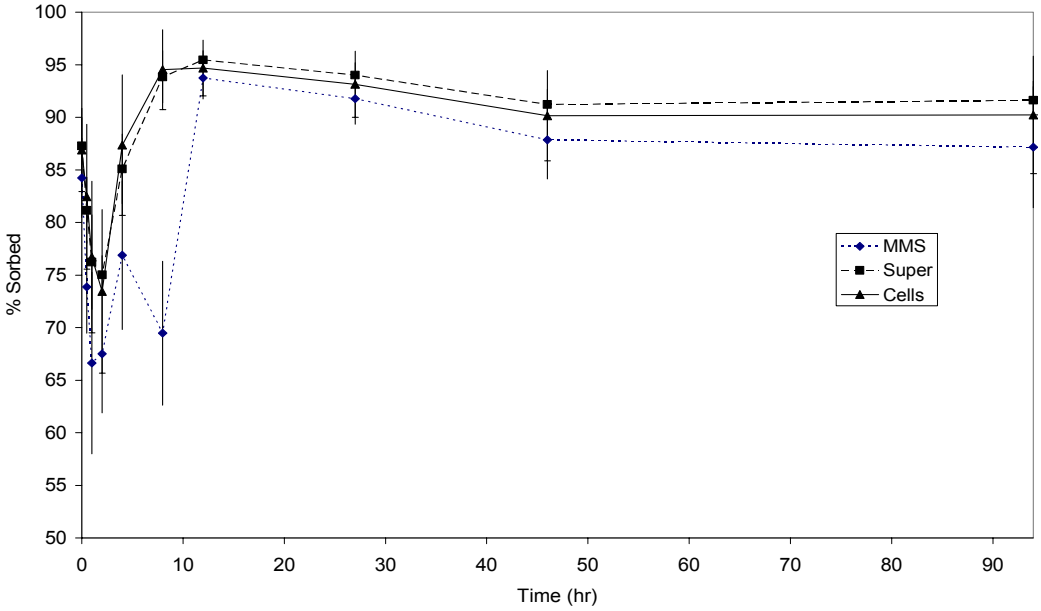

Figure 18: First perturbation of addition of supernatant and cells. Siderophore concentration: 7.02 μM, results for 94 hours

Citrate and salicylate were chosen for experimental use because they are both known exudates for Pseudomonads. Citrate is excreted to solubilize iron in times of Fe limitation, while salicylate is a precursor in the synthesis of catecholate siderophores (Crosa et al. 2004) . The first experimental results from perturbation of Cd adosprtion onto FeOOH by the addition of ligands are shown in Figure 19. The

plot shows an initial drop in Cd adsorption followed by a rapid increase for citrate and salicylate, and a slower increase for EDTA starting at four hours. At t = 24 hours, citrate and salicylate adsorption levels were the same as predicted by the mass balance equation applicable to Cd phase distribution in the absence of ligand. Sorption levels in the presence of EDTA were lower than predicted by the Cd isotherm and are consistent with the MINEQL+ prediction. Regrettably, no MMS-2 control was included for this first experiment as a reference.

Table 5 : Speciation of Cd calculated by Mineql+ in MMS-2 + added ligand at pH 6.4

Cd Species	MMS-2	Citrate	Salicylate	EDTA
Cd^{2+}	75.35%	75.07%	75.35%	21.18%
$Cd(OH)_3^-$	0.00%	0.00%	0.00%	0.00%
$Cd(OH)_4^{2-}$	0.00%	0.00%	0.00%	0.00%
$Cd(OH)^+$	0.02%	0.02%	0.02%	0.00%
$Cd(OH)_2$ Aq	0.00%	0.00%	0.00%	0.00%
Cd_2OH^{3+}	0.00%	0.00%	0.00%	0.00%
CdOHCl Aq	0.00%	0.00%	0.00%	0.00%
$CdHCO_3^+$	0.10%	0.10%	0.10%	0.03%
CdH_2Citrate	0.00%	0.00%	0.00%	0.00%
CdHCitrate	0.00%	0.00%	0.00%	0.00%
CdHEDTA	0.00%	0.00%	0.00%	0.00%
$CdCl^+$	3.00%	2.97%	3.00%	0.84%
$CdCl_3^-$	0.00%	0.00%	0.00%	0.00%
$CdCl_2$ Aq	0.01%	0.01%	0.01%	0.00%
$CdCO_3$ Aq	0.03%	0.03%	0.03%	0.01%
$Cd(CO_3)_3^{4-}$	0.00%	0.00%	0.00%	0.00%
$CdNO_3^-$	0.03%	0.03%	0.03%	0.01%
$CdPO_4$	0.00%	0.00%	0.00%	0.00%
$Cd(SO_4)_2^{2-}$	0.23%	0.23%	0.23%	0.06%
$CdSO_4$ Aq	21.20%	21.12%	21.20%	5.97%
$CdCitrate_2$	0.00%	0.00%	0.00%	0.00%
CdCitrate	0.00%	**0.46%**	0.00%	0.00%
CdEDTA	0.00%	0.00%	0.00%	**71.99%**
CdSalicylate	0.00%	0.00%	0.00%	0.00%

The supernatant perturbation was repeated, this time examining aged versus fresh supernatant. The concentrations of siderophores in the aged and fresh supernatants were 19 μM and 30.1 μM, respectively. A plot of the results can be seen in Figure 20. The desorption of Cd was initially much slower than previous perturbations, with similar kinetic behavior for both siderophore and cell additions. The aged supernatant desorbed the least Cd of all the perturbations followed by the supernatant and cells which behaved similarly. The MMS-2 desorbed the most Cd of all the perturbations, the final concentration was within the predicted value from the mass balance equation. All of the perturbations reached a minimum value in Cd adsorption at approximately 8 hours after the initial perturbation.

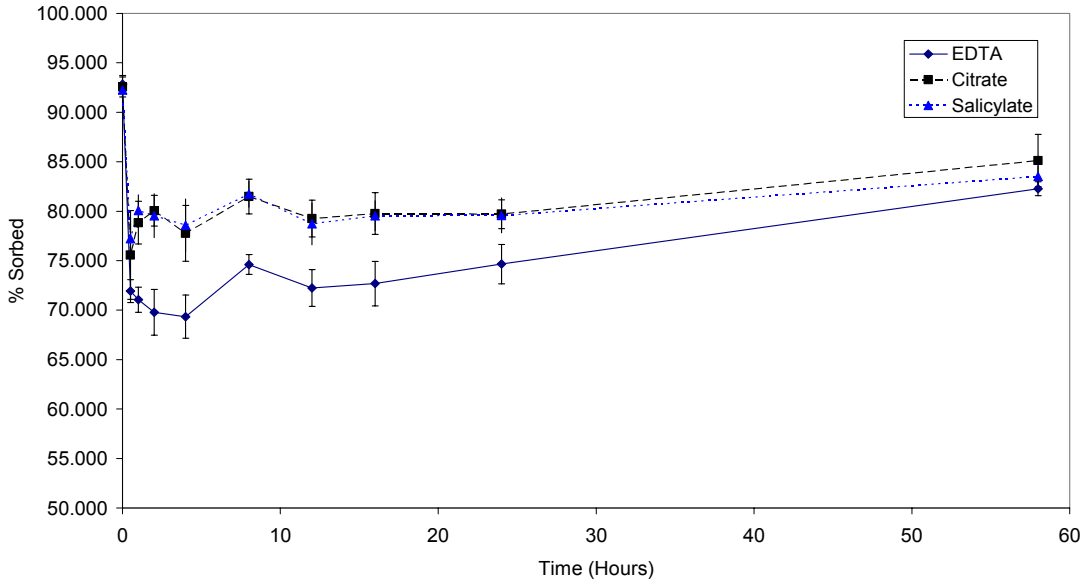

Figure 19 : First perturbation by addition ligand. Ligand, cadmium total, 51.3 nmoles

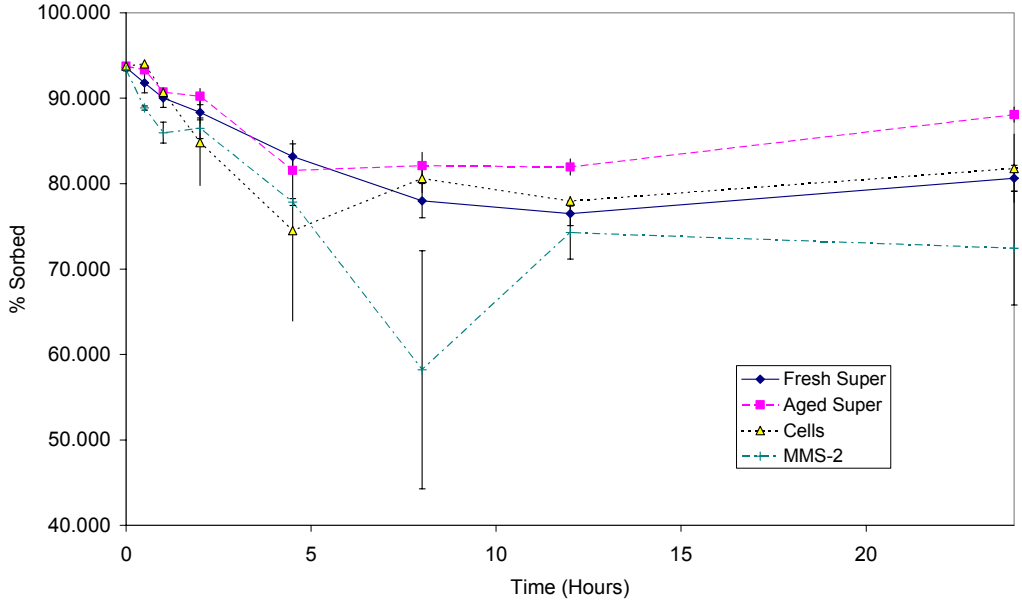

Figure 20 : Second perturbation by addition of supernatant (fresh and aged) and cells. Siderophore concentration Fresh : 30.1 µM, Aged 19.2 µM

The final perturbation performed was a replicate of the ligand mediated desorption. This repetition included an MMS-2 control for reference, and the results are shown in Fig 21. In general the flasks started off below predicted percent adsorption, with only the citrate flasks being near the predicted 85% value at the start. All flasks, except the one ones containing added citrate showed an initial desorption of Cd followed by a rapid resorption. After 8 hours, the MMS-2 control had more Cd adsorbed and returned to near predicted levels by the end of the experiment. The percent Cd adsorbed in flasks containing citrate remained high and eventually sorbed the same amount as the MMS-2 control as seen in Figure 22. More Cd was initially desorbed by EDTA and salicylate than the MMS control and the percent Cd adsorbed in these treatments remained lower than the control for the first 24 hours. After five hours

both the salicylate and EDTA perturbations began to readsorb Cd and eventually reattained the starting levels of Cd sorption after 12 hours as seen in Fig 21

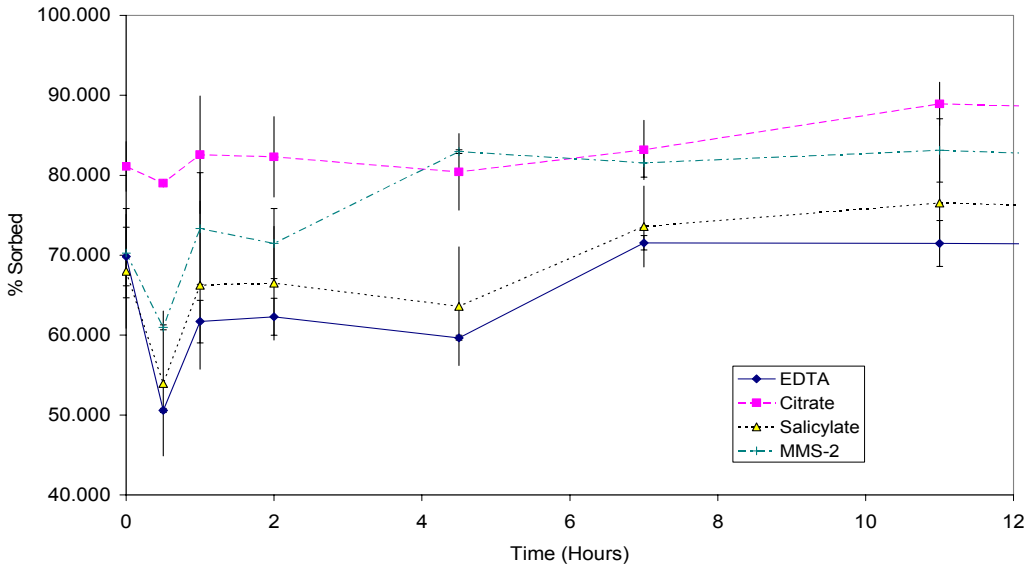

Figure 21 : Second perturbation by addition of ligand. Ligand, cadmium total, 36 nmoles, up to 12 hours

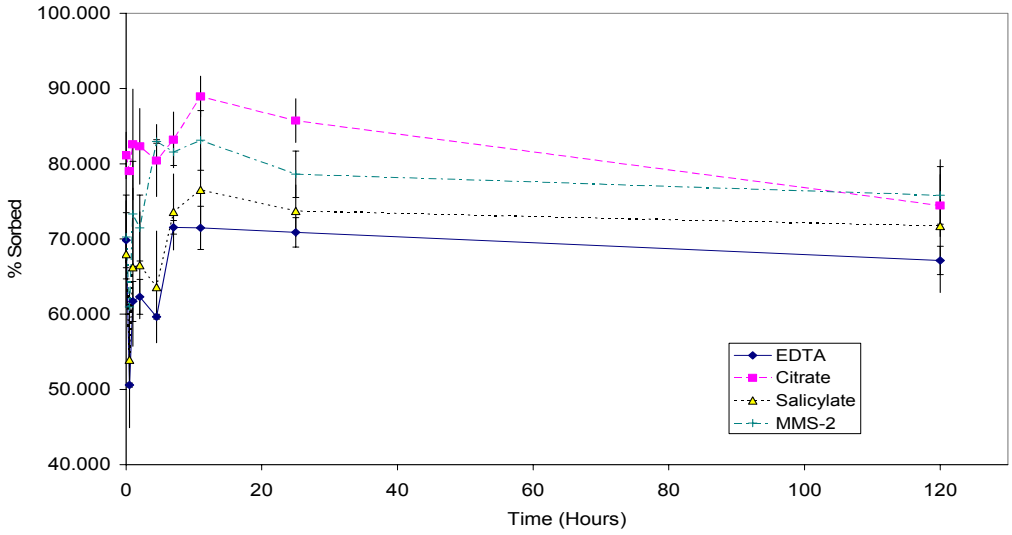

Figure 22 : Second perturbation by addition of ligand. Ligand, cadmium total, 36 nmoles, up to 5 days

Plant Uptake Model

Dialysis Sorption

Dialysis tubing was introduced into the experimental reactor system for plant uptake experiments. Sorption of Cd to the dialysis tubing was evaluated in the same manner in which the previous perturbations were conducted. The results are shown in Figure 23. The plots for the Cd concentrations frequently overlapped and were also noisy. At the end of the experiment it was not possible to discern the amount of Cd sorbed onto the dialysis bag. If there was any sorption onto the bag it was lost in the inherent error of the experiment.

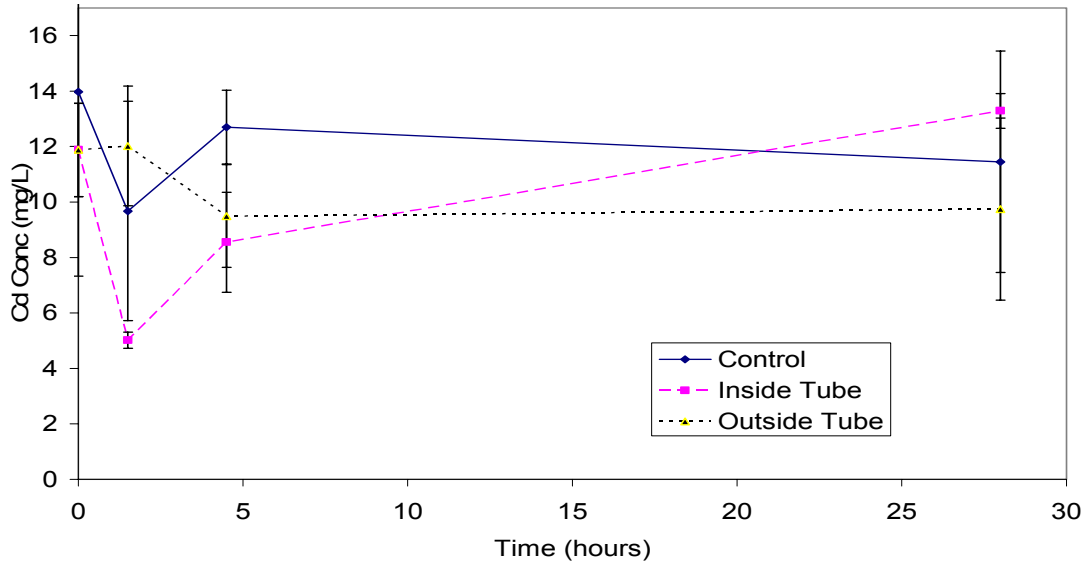

Figure 23 : Calculated sorption of Cd onto dialysis tubing. Control indicates same conditions, but absence of dialysis membrane

Plant Uptake Model

Concentrations of Cd inside and outside the dialysis tube were measured three times during plant uptake experiments, before perturbation, before plant addition, and

after plant removal. Equilibration times of five and eight hours after perturbation were chosen because they represent the times and when soluble Cd was highest in the prior perturbation experiments. Cd concentrations were corrected for volume changes and summed to obtain a total Cd in solution. Cd concentration values were used to evaluate whether the system was in equilibrium before addition of the plant. Soluble Cd (μg) was used to track the loss of Cd from the system. A plot of Cd concentration for an initial evaluation of plant uptake is shown in Figure 24. The term "Flask" denotes the concentration of Cd outside the dialysis tube and "Tube" is the concentration inside the dialysis tube. As seen, the concentrations of Cd in the supernatant and cell perturbations were approximately equal in the tube and flask at five hours, while in the MMS-2 control had a lower Cd concentration in the tube versus the flask. At the end of the experiment the cell and supernatant flasks had a much lower concentration of Cd inside the dialysis tube. When the overall mass of Cd in solution as a function of time is plotted (see Figure 25), a different result can be seen. Overall the Cell and Supernatant flasks (siderophore concentration, 30.4μM) had statistically more ($\alpha = 0.1$) soluble Cd prior to the introduction of the plant. After the addition of the plant the Cell flasks lost the most soluble Cd (Δ Cd was greater between 5 and 8 hours), followed by Supernatant, and then the MMS-2 control. Plant tissue analysis for initial plant uptake experiments was compromised and could not be used to yield reliable data.

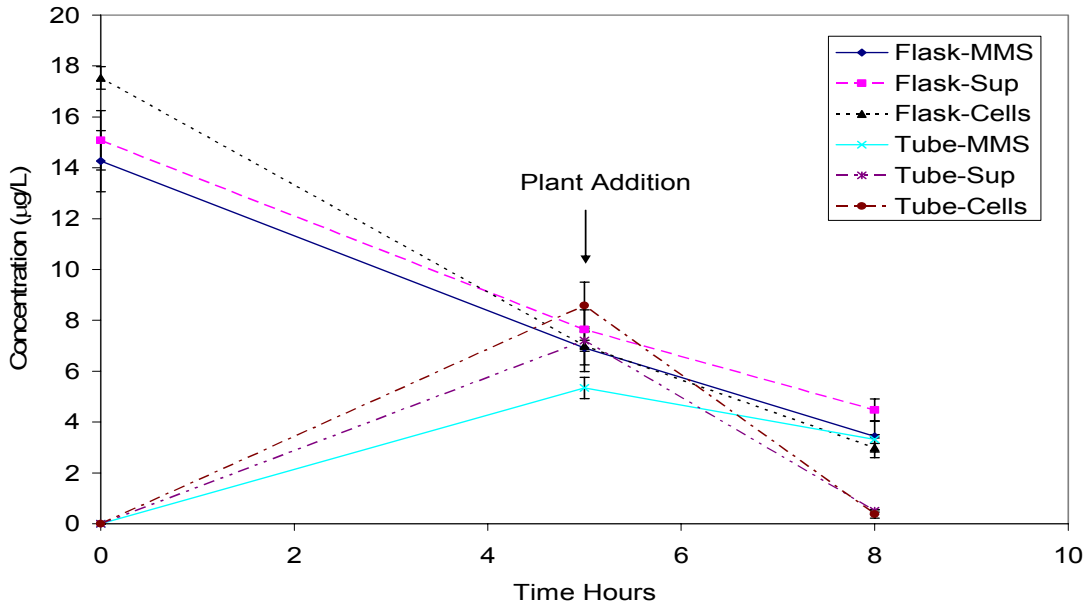

Figure 24 : Concentrations of Cd inside and outside dialysis tube as a function of time for an initial plant uptake experiment

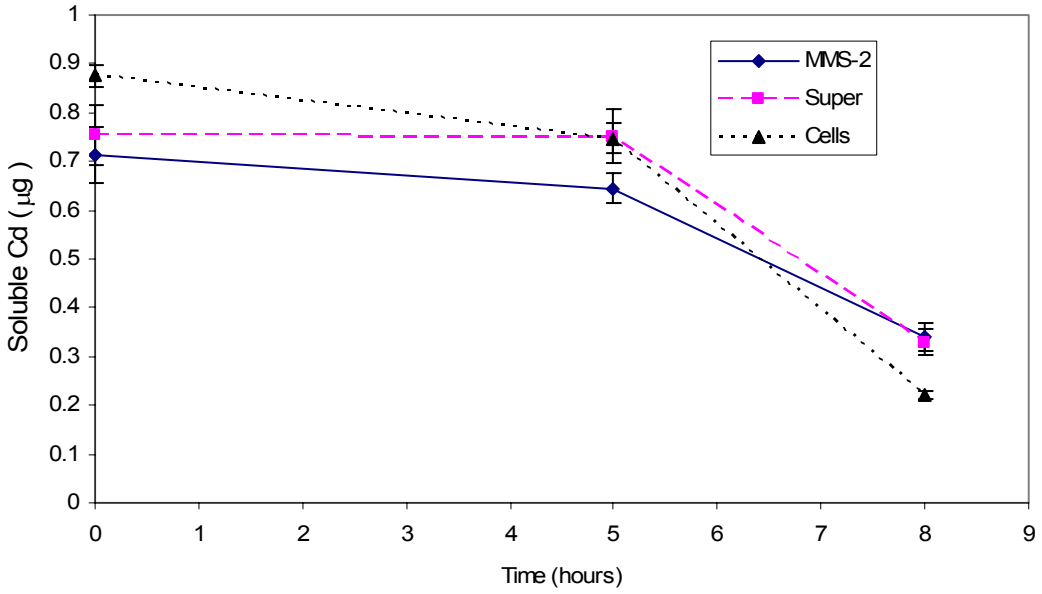

Figure 25 : Soluble Cd (µg) present in flasks as a function of time in an initial plant uptake experiment using cells and supernatant

The difference in the flask weight before and after addition of the plant confirmed evapotranspirational processes. The volume of water lost because of evapotranspiration was deducted from the total for the mass balance. An evaporation experiment (no plant addition) was conducted in parallel, and confirmed that the change in weight of the flask was primarily due to plant mediated processes

When ashed, plant material yielded predominantly white ash, although some yielded grey ash, and some shoot ash had red particles indicating the presence of iron. Some shoot ash also had the smell of reduced sulfur, and some of the shoot ash fizzed when added to the acid dilution. The plant matter had typical water contents; roots typically contained 97%, and shoots were typically 88%.

Plant uptake experiment results with ligands are plotted in the Figures 26-29. EDTA and salicylate were chosen because they produced the most desorption in the perturbation experiments. Equilibration time prior to the addition of plants was extended to 8 hours of perturbation to allow more time for the dissolved concentration of Cd to come to equilibrium. However, the only flask in which the tube and flask concentrations were equal was the EDTA flask; the MMS-2 control and salicylate flasks had statistically higher concentrations than their associated tubes (Fig 26). After the addition of the plant, none of the flasks had the same concentration inside and outside the dialysis tube. The EDTA tube exhibited a greater reduction in concentration.

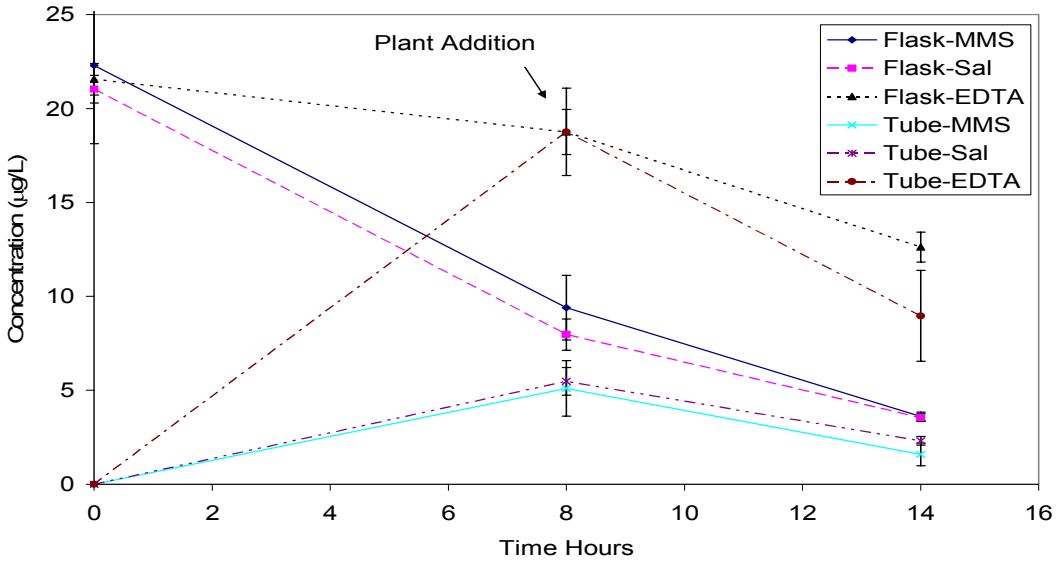

Figure 26 : Concentrations of Cd inside and outside dialysis tube as a function of time for ligand plant uptake experiment

A plot of soluble Cd (Figure 27) yielded similar results. EDTA desorbed more Cd than salicylate, which was not statistically different from the MMS-2 control. EDTA on average did lose more soluble Cd after plant addition, as is indicated by the steeper slope in between hour 8 and 14.

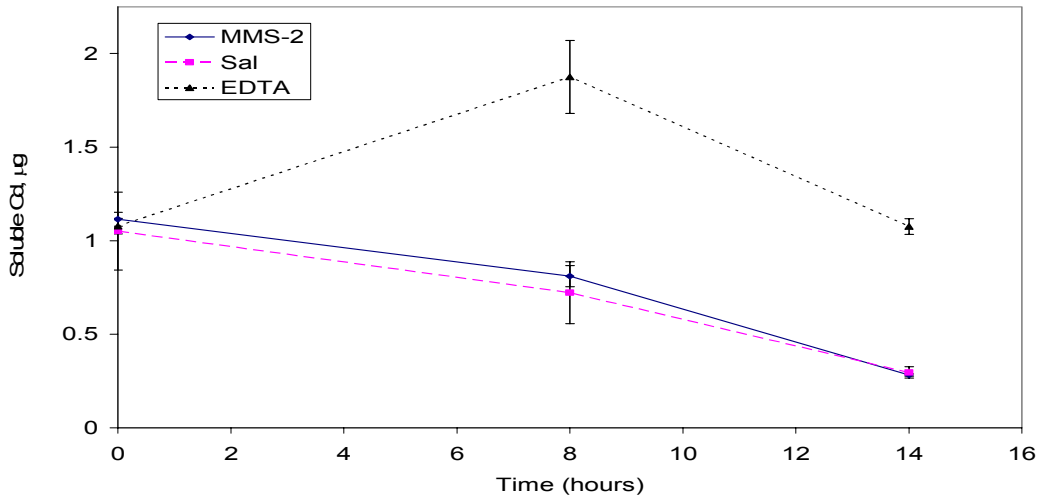

Figure 27 : Soluble Cd(µg) present as a function of time for ligand plant uptake experiments

The composition of the final plant material is plotted in Figure 28. "Base" indicates plant material that was ashed immediately upon removal from hydroponic medium. The amount of Cd that was recovered from the plant material is the difference between the Cd content of a given plant and the baseline value. All of the flasks had statistically higher root Cd content than the baseline, indicating the Cd was taken up by the plant roots. Further, each flask was statistically different from one another. The salicylate flask took up more Cd in the roots than the MMS-2 control, even though there was not a significant difference in the soluble Cd. Overall, the EDTA roots took up the most Cd, and were statistically different from the salicylate. The shoot Cd content was much lower and did not mirror the root content. Among the shoots, the MMS-2 control and EDTA shots are statistically higher in Cd content than the baseline, but not from any other treatment, as seen in Figure 29.

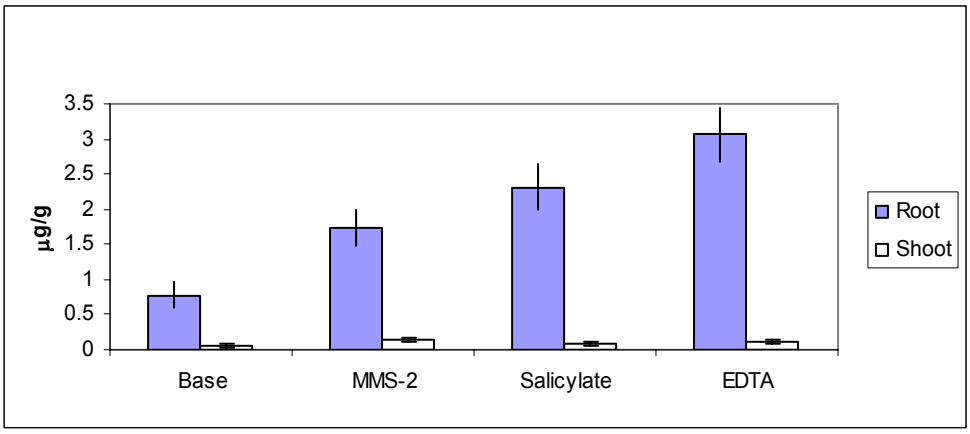

Figure 28 : Cd Content of *B. napus* root/shoots for ligand uptake experiments

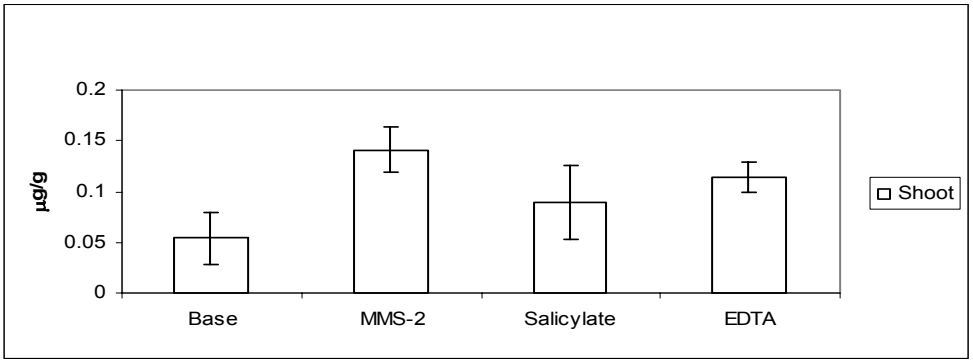

Figure 29 : Cd Content of *B. napus* shoots for ligand uptake experiments

The percent Cd recovered attributed to plant uptake is listed in Table 6, below. The tabulated values were calculated by dividing the Cd plant uptake (Total content – baseline content, Figure 28) by the Cd lost from solution (Figure 27). The salicylate had the highest percent recovery of Cd in the plant tissue, though it was not statistically different from the EDTA treatment. Both additions of the ligand produced a greater bioavailability and percent recovery than the MMS-2 control.

Table 6 : Percent Cd Recovery for Ligand plant uptake experiments

	MMS	Salicylate	EDTA
Average	13	46	29
S.D.	2.1	16	7.2

The results for the plant uptake experiments using cells and supernatant (siderophore concentration 38.5 µM) are shown in Figures 30-33, below. The results for the Cd concentration (Figure 30) and total soluble Cd (Figure 31) are similar to the results observed in the initial supernatant plant uptake experiment. The flasks approached equilibrium at 8 hours after perturbation, but this time the concentration inside the dialysis bags was greater than outside. The Cd concentrations inside the dialysis bag for the cell and supernatant flasks decreased more rapidly than any of the

other treatments in the presence of the plant. The soluble Cd was again higher for the cells and supernatant flasks at the end of 8 hour perturbation. After addition of the plant, both the supernatant and cell soluble Cd decreased to the same amount as the MMS-2 control.

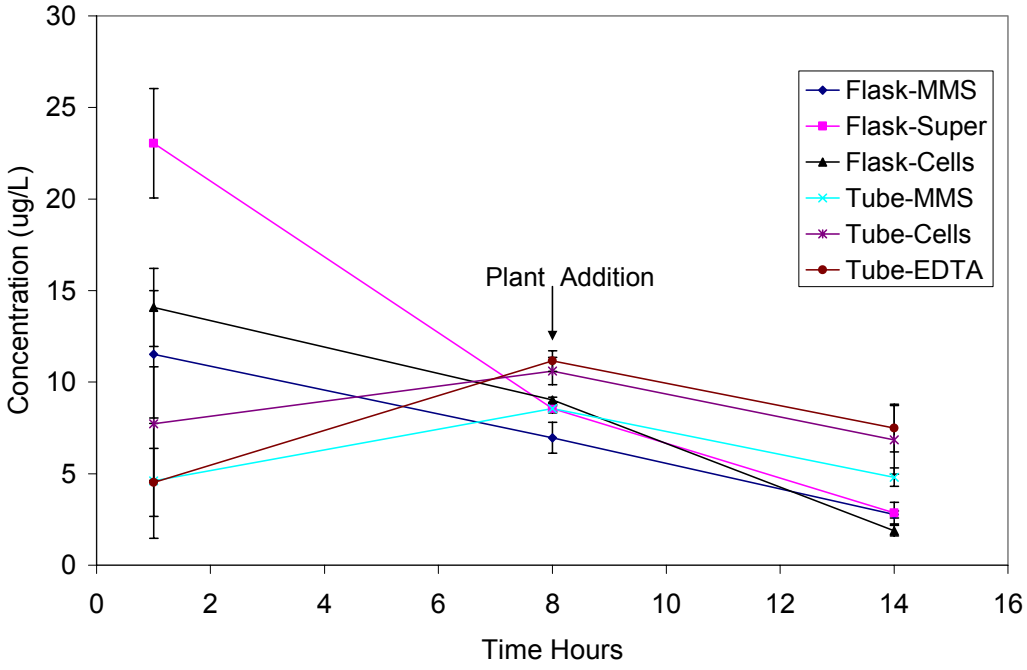

Figure 30 : Concentrations of Cd inside and outside dialysis tube as a function of time for cells/supernatant plant uptake experiment

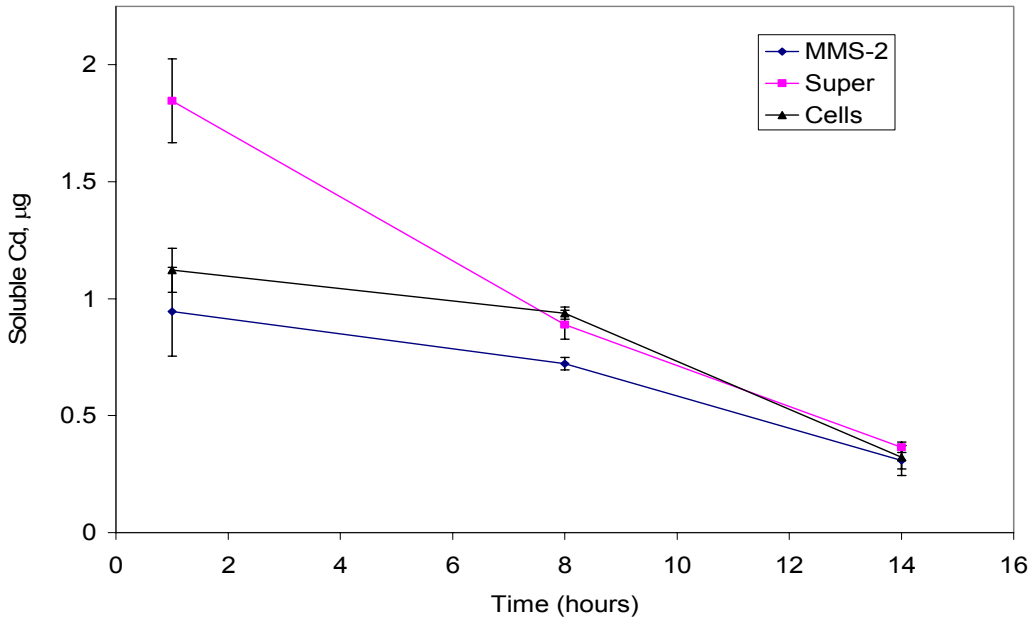

Figure 31 : Soluble Cd (µg) present as a function of time for cells/supernatant plant uptake experiments

Results of the plant uptake are shown in Figure 32. All the flask plants had more Cd than the baseline, which indicates uptake of Cd into the roots. Overall the supernatant and cell exposed plants had a lower mean Cd level than the MMS-2 control, although none of the treatments were statistically different from one another. The Cd composition of the shoots (Figure 33), followed the same pattern, and no clear distinctions could be made. Table 7 lists the percent recovery for the three treatments and does not indicate any clear statistical difference among treatments.

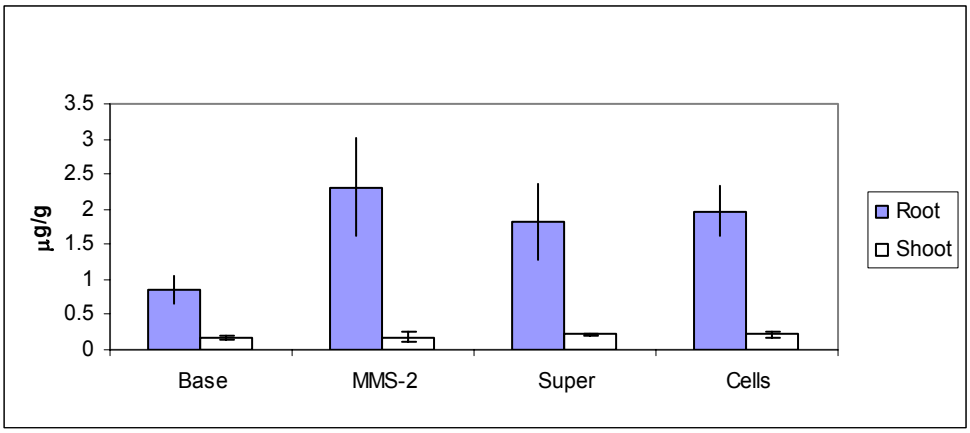

Figure 32 : Cd Content of *B. napus* root/shoots for cells/supernatant uptake experiments

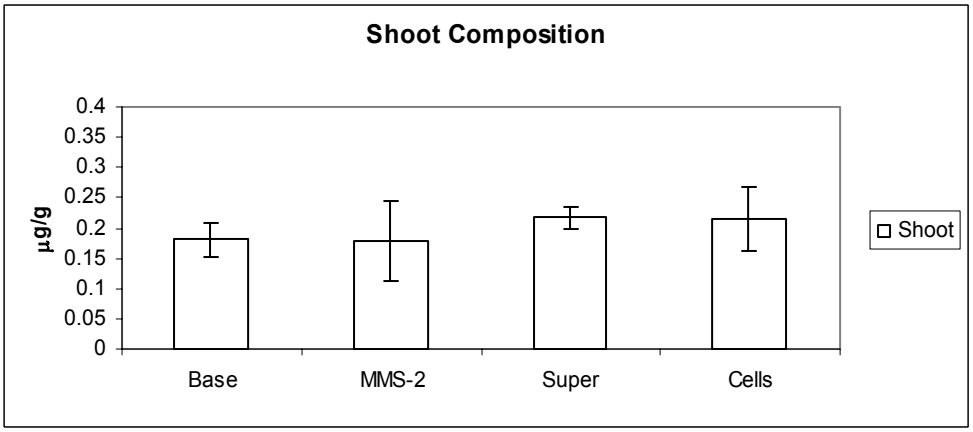

Figure 33 : Cd Content of *B. napus* shoots for cells/supernatant uptake experiments

Table 7 : Percent Cd Recovery for Cell/Supernatant plant uptake experiments

	MMS	Supernatant	Cells
Average	32	24	23
S.D.	14	8.0	0.88

Cell Counts/DAPI

DAPI is a non specific dye that will bind to DNA, as a result the UV images shown represent all microbes living on the plant roots, not just bacteria. DAPI, when present in low concentrations of DNA will fluoresce as blue, but at higher DNA concentrations it may fluoresce as green or yellow. Although a green fluorescence may indicate the presence of polyphosphate granules as well. As a result, the diverse population of microbes living on the plant roots yielded a very colorful image when viewed under UV light. Two images of this diverse population are shown in Figure 34. The microbes living on the roots were of many phenotypes including coccolid, elliptical and chain varieties. The microbes lived planktonically (in suspension) and were also present in flocs, as can been seen on the left image.

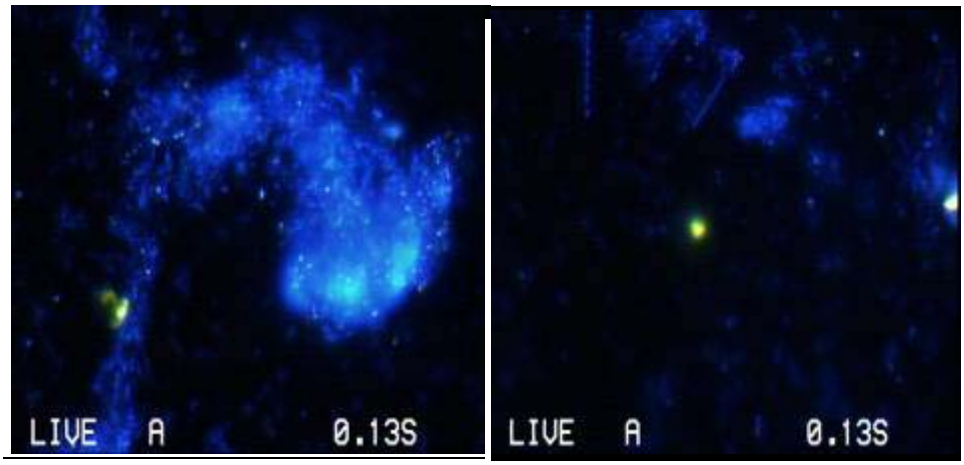

Figure 34 : DAPI UV images of microbes living on *B. napus* roots

After exposure to Cd there was a large reduction the apparent number of microbes living on the plant roots. This can be seen in the left image of Figure 35, while the right image shows a dilute pure culture of *B. cepacia.* The results of the cell counts are listed in Table 8 below. The Baseline value indicates the number of microbes present immediately upon removal from the hydroponic medium, while the

Cd/Cell count indicates the amount of microbes present on the roots after a plant uptake experiment using *B. cepacia* cells. The counts indicate that there are statistically less microbes present on the roots after exposure to Cd and a *B. cepacia* suspension. The OD 600 of the added *B. cepacia* suspension was 0.29.

The baseline value was used to determine the amount of root associated microbes that would be present in plant uptake experiments. Using the plant roots added to each flask, and the volume of the dialysis tube, 30 mL, there was an average of 4E+09 microbes/L present. This value is an order of magnitude less than the concentration of *B. cepacia* cells added. This result confirms an initial assumption that the added cell suspension would have a greater amount of cells than the indigenous microbial community.

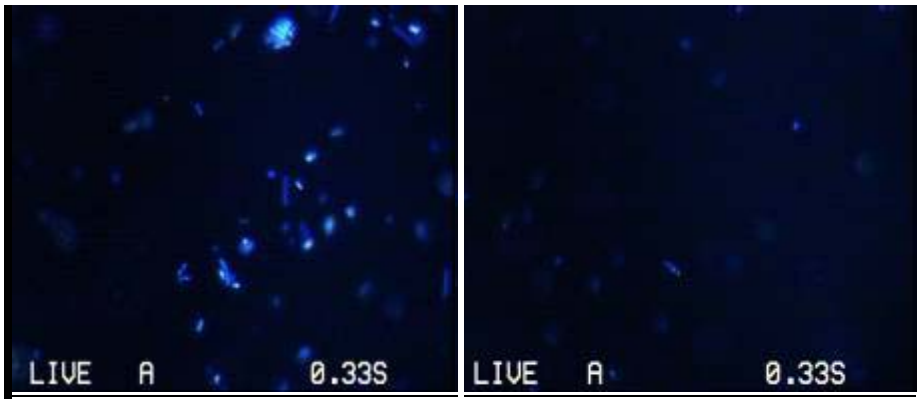

Figure 35 : DAPI UV image of microbes surviving on *B. napus* roots after Cd exposure (left) and dilute *B. cepacia* cultures (right)

Table 8 : Cell Counts from DAPI stained slides

	Cells/L	Cells/g wet root	S.D.
Control	0.00E+00	-	-
B. cepacia	4.81E+10	-	-
Baseline	-	2.97E+07	3.01E+06
Cd &Cell	-	1.23E+07	6.26E+06

Discussion

There were some problems encountered when conducting the experiments that required modification of the original design. Initially there was some difficulty in creating an isotherm of Cd on γ-FeOOH, caused by the choice in media. It was found, that even with the nutritionally restrictive MMS-2 media (68 mg dextrose /L), microbes readily grew and contaminated the flasks. Since dextrose was not a factor in metal speciation it was easily removed, and the isotherms could be determined without worry of microbial contamination.

Initially the experimental flasks used were not silanated, which resulted in the production of isotherms that were identical for the presence and absence of goethite. This result suggested that Cd binding goethite could not be distinguished from Cd loss to the flask walls. To determine the γ-FeOOH isotherm, flasks were silanated, and the concentration of goethite was increased to 2.5 g/L. Even after silanation, the wall sorption proved to be important to the phase distribution of Cd in the flask. According to the mass balance equation developed on page 24, when there is no FeOOH in the flask, 69% of the Cd will be sorbed at a volume of 50 mL. The high Cd adsorption by the silanated glass was the primary reason for resorption of Cd in the perturbation experiments. Initially in the perturbation experiments, only the glass surface in contact with the 50 mL volume was exposed. Upon the addition of 50 mL more media, more glass surface was wetted and acted like a sponge for the soluble Cd that was desorbed from the FeOOH. The possibility of an initial high Cd sorption by glass that desorbs to a steady state value may explain the long term results from the first perturbation experiment, Figures 17 and 18. All treatments had a rapid desorption of Cd came primarily from the FeOOH phase, followed by a sorption onto the newly exposed glass wall. Evidently Cd desorbed from the glass wall (after hour 12) over the next few days, which is reflected in the decreasing percent sorbed.

The slow desorption of Cd from the glass may also help explain the biphasic nature of the desorption of Cd in response to a pH reduction, in Figure 8. The initial drop in pH brings the percent adsorbed down to ~65%, the predicted value of Cd sorption in the absence of goethite, which is then followed by a slow desorption. It is possible that nearly all of the Cd desorbed initially was from the goethite, and the only remaining sorbed Cd is on the glass, which desorbs slowly. A blank desorption experiment under the same conditions was not carried out, but could serve to confirm any dynamics of wall desorption. At pH 6.4, the majority of the cadmium is sorbed onto the wall and is shown to not be very sensitive to pH change (see Figure 9). Wall adsorption dominates Cd phase distribution at the Cd concentrations used, and its low sensitivity to pH can explain the low sensitivity to pH change evidenced in the adsorption edge.

The temporal response of the "blank" conditions could not be readily explained by any known mechanism. It is possible that there was organic or microbial contamination of the deionized water that initially bonded Cd and was adsorbed. Over 5 days the organic compounds could degrade (from UV light or cell death) and release Cd back into solution. If it occurred, this may be fixed by UV irradiating the water prior to use in adsorption experiments(to destroy organics).

An initial explanation postulated for the readsorption of Cd in the ligand perturbation experiment (Figure 19) was the decay of ligand caused by photodegradation. The flasks were subsequently wrapped in foil and kept away from UV light sources (such as sunlight). This precaution did not prevent resorption of Cd, as can be seen in Figure 21. Therefore, the decrease in soluble cadmium was attributed to adsorption on the glass wall. The presence of a ligand provided a driving force to delay, but not prevent adsorption of Cd onto the glass wall.

At higher experimental Cd concentrations wall sorption may follow a non linear isotherm, and may be less of an issue. A Langmuir isotherm is an isotherm that reduces to a linear isotherm at lower concentration, and reaches a maximum value at higher concentration. The equation for a Langmuir isotherm is

$$\Gamma = \frac{\Gamma_{max} * K * C}{1 + K * C}$$

Where Γ is sorption (μg/g)

Γ_{max} is the maximum sorption (μg/g)

K is the inverse of half maximum sorption concentration (L/μg)

C is the concentration of the sorbate (μg/L)

An expanded isotherm for glass sorption should be developed in order to determine if glass sorption approach a maximum at higher Cd concentrations. The isotherm for Cd on γ-FeOOH was also modeled as linear but has been studied previously and has been described by Langmuir isotherm (Neubauer and Furrer 1999), and also by a Freundlich isotherm (Benjamin and Leckie 1979). A Freundlich isotherm is modeled by the following equation:

$$\Gamma = k * C^{\frac{1}{n}}$$

Where Γ is sorption (μg/g)

k is an experimental constant (L/g)

n is an experimental constant, typically >1 (unit less)

C is the concentration of the sorbate (μg/L)

The Freundlich isotherm predicts that a sorbent can never reach a maximum surface concentration, further there is no region of linearity (except when n=1). In the previous study the lowest pH where a Fruendlich isotherm was used was 7.3, well above the pH of 6.4 used here. Further, the prior study used a much wider range of Cd

concentrations, with a maximum of 3500 μg/L , 3 orders of magnitude above the maximum used here. Over a small range of low concentrations a Freundlich or Langmuir isotherm could be approximated by a linear isotherm. The previous study also showed that the γ-FeOOH sorption achieved in this thesis was far from saturation, and the iron surface could accommodate higher concentrations of adsorbed Cd in future experiments. This property is important and suggests that Cd desorbed from the reactor wall could be readily and rapidly sorbed onto the available sites on γ-FeOOH. Although Cd was likely being redistributed between Fe and the glass during experiments the steady state concentration remained approximately constant.

Some issues arose with respect to siderophore production and quantification. *B. cepacia* can produce three different types of siderophores (as shown in Figure 1). The exact type of siderophore produced in this study is not known. It could not be identified as a hydroxomate or catocholate using any described techniques. The ferric complex formed when combining the supernatant with a $FeCl_3$ solution or CAS assay precipitated within minutes of mixing. This observation is consistent with an initial description of cepabactin, where it was identified as an antibiotic that formed an insoluble ferric complex (Itoh et al. 1979). Formation of an insoluble complex may allow the Fe to adhere to the cell surface, where it can then be utilized, but is somewhat counter intuitive since siderophores are thought to be produced to solubilize Fe and thereby enhance its bioavailability

Previous studies have shown that Desferroxamine B (DFOB), a commercially available hydroxomate siderophore, will increase sorption of Cd to goethite at concentrations used in this study (Neubauer and Furrer 1999). Conversely, the same study also showed that at higher DFOB concentrations (>10μM) Cd sorption is decreased. Previous studies of siderophore-heavy metal interactions have been

dominated by DFOB and have yielded some binding constants including the ones for Cd, listed in Table 9 below.

Table 9 : Binding constant for siderophore DFOB and Zn, Cu, and Cd

	Zn^{2+}	Cu^{2+}	Cd^{2+}
$Log([MLH]/[M^{2+}][LH^{2-}])$	9.55	13.54	7.88
$Log([MLH_2^{+}]/[M^{2+}][LH_2^{-}])$	7.88	12.94	5.58
$Log([MLH_3^{2+}]/[M^{2+}][LH_3])$	4.29	7.66	3.32

The constants predict that charged Cd-DFOB complexes comprise at least 5% of the total dissolved cadmium from pH 5.5 to 8.5 at a 10:1 DFOB to metal ratio and I =0.1 (Neubauer et al. 2002). The charged species were shown to adsorb to goethite, while uncharged complexes (which dominate at pH>8) were negatively adsorbed.

Cepabactin is a hydroxomate siderophore that forms a hexadentate complex like DFOB, but it is cyclic rather than linear in nature. Cepabactin itself is a bidentate siderophore, and must form a 3:1 complex to complete the hexdentate structure. The charge of a Cd-cepabactin complex could explain increased Cd sorption in the presence of cepabactin at pH 6.4, but the binding constants for Cd-cepabactin are not yet known.

The speciation of Cd may be further complicated by the presence of pyochelin. Pyochelin is a catecholate siderophore that also has two sulfide groups. Pyochelin has been proposed to form a 1:1:1 complex with cepabactin and ferric iron, although the stability of the 1:1:1 complex is much lower than the 3:1 cepabactin complex with Fe (Klumpp et al. 2005). To better understand the reaction that controlled Cd speciation in the experiments reported in this thesis the types of siderophore produced by the experimental strain of *B .cepacia* need to be identified. Further, these siderophores

should be collected and used to establish binding constants for heavy metals, such as Cd.

The EDTA calibration for siderophore activity proved useful for this application. One drawback was the low maximum siderophore detection limit (20μM) that was nearly exceeded during these experiments. Future use of the CAS technique should use an indicator solution that has at least twice the amount of Fe^{3+} (30μM) to allow for the accurate quantification higher siderophore concentrations. This drawback may also be remedied by the dilution of the siderophore sample prior to addition of the CAS solution.

Initial plant uptake experiments showed that there was a very high attachment of γ-FeOOH onto plant roots. Attempts were made to ash the roots along with the adherent goethite, and then dissolve the result in an acid solution. However, the FeOOH would not fully dissolve in the acid solution. A dilution of 10^3 still yielded a solution with undissolved FeOOH. It was concluded that it would be impossible to determine the difference between Cd in the plant tissue and Cd sorbed on the FeOOH if the roots were in direct contact with the Fe suspension. A regenerated cellulose dialysis membrane was then introduced into the experimental system to separate the roots from the Fe solid phase. The membrane proved successful in not allowing FeOOH to attach to the roots, and further, the membrane did not adsorb any detectable amount of Cd.

While the dialysis tubing did not adsorb Cd, it did have an influence on Cd speciation, particularly in the presence of the *B. cepacia* cell suspension and supernatant. The concentrations of siderophores in the first plant uptake experiment and the final perturbation experiment, 30.1 μM and 30.4 μM respectively, were essentially identical. However different amounts of Cd desorption occurred in these experiments. It is possible, although unlikely, that the type of siderophores produced

varied between the two experiments. If this occurred it could help explain different amounts of Cd desorbed. It is also possible that the cell supernatant had a synergistic reaction with compounds that were released from the dialysis tubing. The tubing contains glycerol in addition to cellulose, and requires rinsing before use to remove the glycerol. *B. cepacia* is not known to produce any cellulases that could degrade the cellulose membrane, but can readily degrade glycerol. The cell supernatant is not well defined, and may contain compounds such as extracellular proteins and enzymes in addition to the detected siderophores. The tubing was soaked for 1 hour prior to use instead of the recommended 3 to 4. Any glycerol that remained after rinsing may have been degraded by *B. cepacia* extracellular enzymes to smaller chain carboxylic acids or ketones, which could act as Cd-binding ligands.

The results from the ligand plant uptake experiment are not completely unexpected. EDTA has been used previously in a hydroponic medium to induce the uptake of heavy metals. A prior study found that Pb-EDTA can be taken up into the shoot tissues of *Brassica* at concentrations much higher than that of the hydroponic media, although uptake was limited due to the phytotoxic effects of very high EDTA concentrations (Vassil et al. 1998). Cd- EDTA complexes have been shown to be readily taken up by *Brassica spp*., which preferentially translocates the complex to the shoots rather than the roots (Grcman et al. 2001). In the absence of EDTA, Cd^{2+} is generally found preferentially in the roots over the shoots in *B. napus* (Nouairi et al. 2006). As seen in Figures 26-29, addition of EDTA elevated the Cd concentration in solution which translated into an increased concentration in both the root and shoot plant tissue. It is possible that the elevated shoot concentration in the presence of EDTA is because of the preferential transport of Cd-EDTA, although the MMS-2 control also showed elevated shoot concentrations. The time allowed for uptake, 8 hours, is likely too short to see any real differences among shoot concentrations.

EDTA has been shown to enhance the phytoextraction of heavy metals, although it is toxic to the soil biota and increases Cd leaching (Nortemann 1999). EDTA metal complex can be biodegraded by specialized bacteria, which, in turn, may prevent the heavy metals from leaching. Heavy metal EDTA complexes, including Cu-,Co-,Ni-,and Cd-EDTA have been shown to be biodegradable to these specialized communities. Secondary treatment has been shown to remove metal EDTA complexes, at cell retention times (θ_c) in excess of 15 days (Nortemann 1999). Recently a strain of *B. cepacia* has been isolated that can subsist solely on Fe(III)-EDTA, and sustain growth at concentrations as high as 1.5 g Fe-EDTA /L (Chen et al. 2005). In the research reported in this thesis *B. napus* was grown in an unsterile hydroponic medium that contained 6 μM EDTA. The only other carbon source available to microbes in the hydroponic medium would have been root exudates. The hydroponic set up is designed to minimize the growth of photosynthetic microbes. The hydroponic media was not fully changed nor sterilized during the growth of seedlings over 80 days, providing ample time for selection of EDTA degrading bacteria.

In the absence of EDTA-degrading microbes, soil treatment with EDTA presents concerns with respect to EDTA of toxicity to plants and enhanced leaching of metals, which has led to the desire for alternatives to synthetic chelating agents for phtyoextraction of heavy metals. Low molecular weight organic acids (LMWOA), such as citrate and gallate, have been applied to contaminated soil and accomplished an increase of Cd concentration in Indian mustard (*Brassica juncea*) (Do Nascimento et al. 2006). This result was attributed to the decreased toxicity of the complexes, not only to the plant, but to the soil biota as well. Salicylate is a LMWOA, and has been shown to increase the uptake of Cd as seen in Figure 28, without greatly increasing the metal concentration (see Figures 22 and 26). Salicylate is more biodegradable than

EDTA and would pose less of a threat with respect to metal mobilization. As a result, salicylate could therefore be a good candidate as a soil amendment for phytoremediation of heavy metals.

Bacterial siderophores have not been shown to be utilized for metal acquisition by plant species (Crosa et al. 2004). In a previous study the presence of phytosiderophores was shown to limit the amount of Cd that could be extracted by *Zea mays,* and therefore served a protective role(Hill et al. 2002). The siderophores used in this study did not prove to be an effective mean to increase Cd accumulation in *B. napus*.

In the uptake experiments conducted here a maximum of 46% of the Cd was recovered in the plant tissue (Table 6), with typical recovery more around 25%. Some of the missing Cd could possibly be removed by desorbing into the DI water rinse after plant removal from the flask. This rinsing was designed to remove any loosely bound Cd from the root surfaces. The Cd may have also simply have resorbed back onto the γ-FeOOH or silanated glass walls. Another possibility is the uptake or sorption of Cd to bacterial cells that were present on the roots and added *B. cepacia* . This hypothesis is supported by Figures 25 and 31, which show slightly more rapid of removal of soluble Cd in the presence of *B. cepacia* cell suspensions.

DAPI cell counts confirm that there was a greater number of cells/L present in the *B. cepacia* suspension relative to the amount of microbes dislodged from living on the plant roots. The cell count may indicate a decrease in root associated microbes present after exposure to Cd, a result that would be consistent with previous studies (Karaca et al. 2002). Cd contamination has been shown to decrease the microbial diversity of bacteria living on *B. napus* roots (see Appendix), and may be supported by the DAPI counts.

Summary and Conclusions

A novel experimental system was constructed that could be of use in the study of phytoremediation. The system was tested using Cd as a model contaminant, γ-FeOOH as the "soil"/solid phase, MMS-2 as the soil solution, and *Brassica napus* (canola) as a representative plant.

The system was perturbed by the addition of salicylate, EDTA, a suspension of siderophore producing bacteria (*Burkholdaria cepacia),* or the supernatant of bacterial cultures that contained siderophores. The experimental set up employed a level of Cd contamination (30 ppm), which may be more representative of a level found in agricultural soils as opposed to industrial contaminated soils. Both salicylate and EDTA were found to be effective in increasing the phytotextraction of Cd. Salicylate may have more potential of the two amendments to field application because of its decreased toxicity to plants and a reduced risk in the leaching of the Cd complex (because salicylate is readily degraded by bacteria). Salicylate is a common microbial and botanical exudate, as well as a siderophore precursor, and is likely to be present in any natural system.

The siderophore-producing bacteria and supernatant did not improve uptake of Cd into canola, but did solublize more Cd in the presence of dialysis tubing. Metal-siderophore interactions are not very well documented, except for those of DFOB, but are clearly important in the speciation of metals. The interaction of Cd and siderophores produced by *B. cepacia* warrants future investigation that may help predict the cycling and fate of heavy metals in contaminated soils.

Recommendations for future research

Several modifications need to be made to improve the performance of the phytoremediation system described in this thesis. High sorption to the reactor walls dominated the phase distribution in these experiments, but may not be an issue at higher Cd concentrations. An equilibrium isotherm should be developed for Cd on glass walls at higher concentrations to evaluate this hypothesis. It is possible that the wall adsorption may still be dominant at high concentrations and alternatives are needed to promote distribution of Cd to FeOOH/solution. A flask with a smaller A/V ratio, would theoretically adsorb less Cd. Further, a different reactor material, that adsorbs less Cd, may also be employed to promote distribution of Cd to the FeOOH/solution components. Polypropylene was used in earlier experiments and adsorbed the same amount of Cd as silanated glass, and may not be a good candidate material. Polycarbonate is a material used in previous studies because of the low adsorption capacity (Benjamin and Leckie 1981a). Teflon is another material that was not explored in this thesis, but is also used when adsorption needs to be minimized. To reduce error associated with different vessels, it may also be useful to explore the use of a vessel that can accommodate many plants, such as a large tub. The system will have to be designed to incorporate dialysis tubing that must separate the plant roots from the FeOOH.

Cd-siderophore interactions were unclear in these experiments. Siderophores were added as a part of an inherently complex bacterial supernatant. The supernatant was not defined with respect to the types of siderophores produced. Future experiments could use defined siderophores added from a pure stock solution. These siderophores should be defined, with respect to the type of Cd complexes they may form. *B. cepacia* or other siderophore producing bacteria should be used to harvest any of the needed siderophores.

System pH may also need to be adjusted to conclusively evaluate the effect of siderophores on plant uptake. Cd-siderophore complexation and the corresponding system adsorption should be studied over a pH range typical of Cd contaminated soil. The results of these studies may dictate the optimum pH for plant uptake experiments.

The experimental system may also be modified to greater discern effect of environmental change as observed by plant uptake. The simplest way to do this would be to allow the plants to have a longer uptake step. This may be accomplished by operating the reactor in a semi-continuous mode, or continuous in the form of a chemostat.

Appendix

Using TRFLP to Determine the Influence of Cadmium on Bacterial Root Communities of *Brassica napus*

Abstract

Cadmium is a non essential trace element, which is toxic at very low concentrations. While Cd can occur at high concentrations as a result of the weathering of Cd-rich minerals, most high concentrations are of human origin. *Brassica napus* (canola) has been shown to be a hyper-accumulator of cadmium and is therefore a good candidate for the application of phytoremediation to remediate soils with Cd contamination. The processes leading to successful phytoremediation are not fully understood, and may be dependant upon the bacterial community present in the rhizosphere. Bacterial communities are known to change in response to environmental stress, including the presence of toxic trace metals. However such changes have not been documented in the case of the adherent populations associated with the roots of plants used for phytoremediation of toxic metals. Bacteria in the vicinity of plant roots may degrade plant root exudates that would otherwise act to bind metals and bacteria, and can produce their own metal binding ligands such as siderophores.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is an established technique for examining the members of bacterial communities and was used to document the expectation that rhizobial bacterial populations of plants used for phytoremendiation will depend, in part, on the presence of toxic metals in the contaminated soils. Specifically, the influence of Cd on the production of siderophore producing bacteria was explored.

B. napus was grown hydroponically in a soil suspension with the presence or absence of a cadmium (100 μg/L). After three weeks the plants were harvested, DNA was extracted, and the roots of plants exposed to Cd were spread on Chrome Azurol S (CAS) plates to detect siderophore producing bacteria. Three siderophore producing isolates were selected and their DNA was extracted. The total DNA was amplified by PCR and digested separately by two restriction enzymes, Hha1 and Msp1. Results of the fragment sizing were analyzed by MATMODEL using an AMMI analysis. Results from both restriction enzymes indicated that, as expected, the presence of cadmium changes the bacterial community structure to one with reduced diversity. However, this difference in community structure was not associated with the presence or absence of siderophore producing bacteria. The bacterial community present at the point of sampling was shown to be strongly influenced by the initial hydroponics community, but was also influenced by the bacterial community associated with the suspended soil. The siderophore producing bacterial isolates were found to be in approximately equal abundance in trials with or without Cd, with one isolate occurring only rarely. A siderophore producing isolate was tentatively identified as belonging to the genus *Burkholdaria.*

Introduction

Cadmium is a non essential trace element, which is toxic at very low concentrations (EPA Maximum Contaminant Level in drinking water = 5μg/L). While Cd can occur at high concentrations as a result of the weathering of Cd-rich mineral phases, most elelvated concentrations result from human activities (Maier et al. 2000). A common source of Cd contamination in soils is fertilizers, both synthetic and organic. Phosphate fertilizers contain Cd as an impurity, which accumulates in the soil over time. Biosolids produced from wastewater treatment that are applied to

soil for use as fertilizer are also high in Cd, as well as other heavy metals. Other than fertilizers, soil Cd may result as a consequence of atmospheric deposition from smelting and refining industries. Cd is also found in PVC as a stabilizer, glass and plastic pigments, and also in Nickel Cadmium batteries. As a result of these varied sources, Cd has become a primary contaminant at many EPA superfund (29.8%) and Brownfield sites (Maier et al. 2000). Cd may become a threat to human health is if it is ingested, either directly from the soil, or from plant matter that has extracted the metal from the soil (Maier et al. 2000).

Since heavy metals, such as Cd, can not be biodegraded, they must be extracted from soils to alleviate the threat to human health. One method to accomplish metal removal is phytoextraction, where the objective is to accumulate metal in the roots, stems or leaves of the remediation plant.

Brassica spp. (which includes Indian mustard, kale and canola) have been studied for their phytoextraction capabilities and some have been identified as a hyperaccumulator of Cd (Nouairi et al. 2006). In one study, researchers grew canola from seeds on soil contaminated with 100 mg Cd /kg (Carrier et al. 2003). The plants were grown for 47 days and the accumulation of Cd in the roots, leaves and stems was measured. It was found that the plants grown long-term had little to no signs of stress (leaf chlorosis, growth retardation). Further, the highest Cd concentration (250 mg Cd/kg dry mass) was found in the leaves, a level is 2.5 times the concentration in the soil. The Cd was found in the vacuoles of the cells, where it would not be expected to interfere with primary cell functions. It is because of this high tolerance for Cd that canola is an excellent candidate for use in phytoextraction and merits further study.

Bertrand et al. (2001) isolated and identified the root-associated bacteria of a soil in which *B. napus* was grown. The study aimed to identify Gram negative bacteria that grew in the rhizoplane (along the root) and endorhizosphere (inside the

root) in an N depleted soil. The bacteria that were isolated were used to inoculate plants that were grown hydroponically and the increase of dry mass that resulted in the presence of bacteria was recorded. After sequencing, it was found that the genus *Phyllobacterium* resulted in 35 to 52% increases in plant dry mass. In addition it was found that *Pseudomonas* was the most abundant genus found in association with the plants studied (Bertrand et al. 2001).

The ability of any plant to remove metals from the soil is dependant upon the speciation of Cd and chemistry of the rhizosphere. Regardless of the soil type, the chemistry of the rhizosphere is strongly influenced by the microbial community that is present. The reasons for why a plant may be a hyper accumulator may include plant-microbe interactions.

A plant can only extract as much Cd as is available. Available Cd does not include metal that remains adsorbed to the soil. Essential metals, such as iron (Fe^{3+}), may be unavailable in soils (in the case of Fe this is because of the low solubility of Fe oxides), and require chemical intervention from plants and microbes to make then available. Bacteria can enhance iron availability through the release of iron-chelating compounds called siderophores, and plants accomplish the same result with phytosiderophores. In addition, plants can exude organic acids that will form complexes with metals. It has been shown that the presence of siderophores will increase the solubility of Pb(II) (Dubbin and Ander 2003). It is reasonable to postulate that Cd, another strongly adsorbed cation, may also have elevated concentrations in the presence of siderophores. Other bacterial compounds, such as extracellular polymers, have also been shown to be effective in the desorption and mobilization of metals, specifically Cd and Cu(II) (Jensen-Spaulding et al. 2004). The coupled plant-microbe-soil interactions that occur in the rhizosphere must be

evaluated if we wish to understand the mechanisms leading to metal accumulation in plants.

A goal of the research reported here is to illuminate the influence of Cd on the microbial community in the roots of canola, and to assess how interactions between Cd and the root-associated bacterial population may influence plant-based soil remediation technologies. Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis was used to examine the composition of the bacterial community on the roots of canola in the presence and absence of Cd. A second research objective was to evaluate the influence of Cd on siderophore production in rhizobial communities using a Chrome Azurol Sulfide (CAS) plating technique. Siderophore producing bacteria were isolated and the Cd-dependency of their presence in the mixed community was assessed using TRFLP. It is hypothesized that the siderophore producing bacteria will be more dominant in the +Cd community than in the Cd free community, if the siderophores play a protective role for the microbes.

Materials and Methods

Hydroponics- Seedling Development

Seeds of *Brassica napus* var. quantum were soaked for 24 hours in distilled water and then transferred to a moist paper towel for germination. After cotyledons emerged, the seeds were transferred to 2" plastic net cups filled with plastic pony beads for support. Each cup was ¾ filled with a growth media containing 1.214 mM KNO_3, 0.7857 mM $Ca(NO_3)_2 \cdot 4H_2O$, 2.143 mM $(NH_4)H_2PO_4$, 2.413 mM $MgSO_4 \cdot 7H_2O$, 10.7 μM KCl, 5.34 μM H_3BrO_3, 0.4285 μM $MnSO_4$, 0.4286μM $ZnSO_4$, 0.107 μM $CuSO_4$, 0.1μM MoO_3, 4 μM $Fe(NO_3) \cdot 3.9H_2O$, and 4 μM HEDTA. The seedlings were grown under fluorescent light in a laminar flow hood at 27 ºC. After 3 weeks of growth the seedlings were removed and placed into flasks containing a soil suspension (described

below). The seedlings were not grown under sterile conditions. To account for the influence of bacteria initially living on the roots, 5 seedlings were transferred to soil-free 125 mL silanted Erlenmeyer flasks filled with the hydroponic medium described above.

Hydroponics – Plant Microbe

All flasks were acid-washed for 24 hours with 0.1 N HNO_3 (Reagent grade) in distilled water and then, washed for 24 hours in 0.1 N HNO_3 (trace metal grade) in deionized water. The flasks were also treated with a silanating solution of 5% dichlorodimethylsilane in carbontetrachloride (CCl_4) following the acid wash to minimize adsorption of cadmium onto the flask walls. Five 125 ml Erlenmeyer flasks were used for each treatment, +Cd, –Cd, and the soil-free control. The soil used for this study was a Collumer soil obtained from the Caldwell field site of Cornell Univeristy. The soil properties (mg/kg) are: P(3), K(71), Mg (48), Ca(788), Al(25), Fe(4), Mn(11.5), Zn(0.5), Nitrate-N (4). The soil had a percent organic matter content of 19000 mg/kg, a pH of 6, and a moisture content of 21.5% (by mass). Each flask (except the controls) had approximately 2.5 g (dry weight) of soil added and mixed with 125 ml of the plant growth medium previously described, but without HEDTA. The +Cd medium had a concentration of 100 μg/L added as $Cd(NO_3)_2$. The medium was adjusted to pH 6 by the addition 0.1M NaOH. Seedlings were placed in the flasks so that the roots were submerged in the media, but some headspace for air exchange remained. Each seedling was held in place with two strips of parafilm at the mouth of the flask. The flasks containing the soil were swirled by hand to create a suspension of soil particles and associated bacteria. Flasks were then placed back in the growth chamber and not mixed during this experiment. The experiment was monitored daily for 3 weeks to ensure proper growth conditions. The summary of the treatments used

is shown in table 10. “Co” is the control flask, “N” is the Cd free flasks, and “Cd” are the flaks with added Cd.

Table 10 Conditions of each treatment used, “Co”, “N”, and “Cd”

Treatment	Hydroponic Growth Medium	Hydroponic Growth Medium (-)EDTA	Hydroponic Growth Medium (-)EDTA, Cd 100□g/L	Caldwell Field Soil
Co	●			
N		●		●
Cd			●	●

Plants were grown under constant light and a constant temperature of 20 ºC. At the end of 3 weeks the plants were removed and the roots were removed with a sterile razor blade. The wet weight of each root was measured. The whole root of the control (Co) and –Cd plants was used for DNA extraction, using a Bio101 Soil extraction kit. The +Cd roots were cut, with half used for a dilution series to determine plate counts of adherent bacteria and to test for siderophore production, and the other half for DNA extraction as described above. The DNA of two soil samples was directly extracted to obtain a profile of the added soil-associated bacteria. The DNA of the plant leaves was also extracted and yielded nuclear DNA and chloroplast DNA, the latter of which can be replicated by bacterial primers.

Siderophore Isolation

The dilution series prepared from the +Cd root tissue sample was prepared using a phosphate buffer adjusted to pH 7 (.25M Na_2HPO_4 and .25M KH_2PO_4). Dilutions ranged from 100 mg of root mass in 100 ml water (10^{-3} dilution), to 10^{-7}. This series was performed in order to obtain an estimate of the number of siderophore

producing bacteria growing on the roots. The dilution series was plated onto Chrome Azurol S (CAS) media containing 60.5 mg CAS dissolved in 90 ml deionized water, mixed with 10 ml of 1 mM $FeCl_3 \cdot 6H_20$, and 10 mM HCl (Milagres et al. 1999). CAS plates were prepared using the root incubation media composition described above with the exception of glutamic acid, biotin, and the substitution of 25 μM HEPES for PIPES buffer (Schwyn and Neilands 1987). One problem encountered with the CAS plating technique was with the preparation of the media. The use of the full compliment of phosphates in the M9 medium resulted in a brown instead of a blue colored solution. The phosphate concentration was reduced to 0.3 g/L, a low enough concentration that would not bind to the iron, and allow the color change. The plates were spread with 0.1 ml of the dilution series. In addition two roots from the dilution series were placed directly onto the plates. The CAS indicator medium has a dark blue color when pouring, but will turn orange upon incubation in locales where there is siderophore production (Bano and Musarrat 2003). After a week of incubation the plates were counted and siderophore producing isolates (associated with orange spots) were restreaked on a fresh plate made with CAS media. After an additional week of incubation the DNA of the isolates was extracted using a Bio101 pure culture extraction kit. The DNA was then quantified using EtBr fluorescence and a BIORAD fluorimeter (Thies 2005). The extracted DNA was amplified by PCR as described below.

PCR

PCR protocol described by (Liu et al. 1997) was followed. Fluorescently labeled bacterial primers, 27f FAM and 1492r (18 pmol), 5 μl extracted community DNA, dNTPs, $MgCl_2$, 10X PCR buffer, BSA, and *Taq* polymerase were added to a microcentrifuge tube. A three minute initial step was used at 94°C followed by 27 cycles of denaturation (30 s at 94°C), annealing (45 s at 56°C), extension (2 min at

72°C), ending with a final extension step of 72°C for 3 min. PCR products were verified by electrophoresis of aliquots (2 μL) in 1.5% agarose in 1x TBE buffer with 5μl EtBr per 100ml solution. A 1 kb ladder was used to determine DNA fragment size. The gel was imaged under UV illumination to indicate binding of EtBr to the DNA and determine if enough DNA was replicated to use for analysis. TRFLP restriction enzymes Hha I, Msp I, were applied independently to cut the labeled DNA. The DNA was digested for 4 hours on a thermal cycler set at 37 ºC, followed by 15 min at 70 ºC to deactivate the enzyme (Thies 2005). The digests were purified using a Performa Dye Terminator Removal (DTR) 96 well plate, and then evaporated using a Speed-Vac. Each sample was then reconstituted with a 10 μl of a denaturant composed of 15 ml formamide in 985ml DI water. Lastly, 15 μl of the size standard, LIZ-500 was added to each well. The resulting mixture of restriction fragments (TRFs) were run on an automated capillary sequencer (ABI-3130), where TRF lengths were determined by comparison with internal standards (Genescan500-Liz). These analyses were performed at the Cornell Bioresource Center. Fragments less than 40 base pairs in length were assumed to be background interference.

Data Analysis

The results of the fragment sizing were analyzed using MATMODEL software using an Additive Main effect and Multiplicative Interaction (AMMI) analysis. Operational Taxonomic Units (OTU) with length between 50 and 600 were used for this analysis. All OTU were rounded to the nearest integer. If there were duplicates (two peaks at the same OUT when rounded), the one with the lower associated area was deleted. Profile data lists peaks at corresponding fragment lengths and the areas of the peak (corresponds to the predominance of the fragment). Any OTU occurring in all of the trials was deleted. Also, any OTU that occurred only once or twice was also deleted. Data was transformed on the basis of presence (1) / absence (0) across the whole range

of OTUs. The resulting data set was analyzed using an Interactive Principal Component Analysis (IPCA).

Results

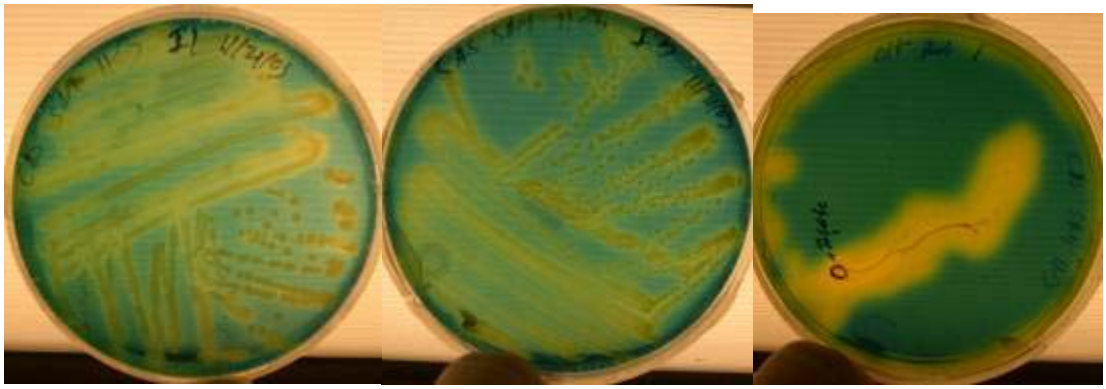

Figure 36 : CAS plates showing Isolate 1, 3, and a root. Plates turn from blue to orange/yellow in the presence of siderophores

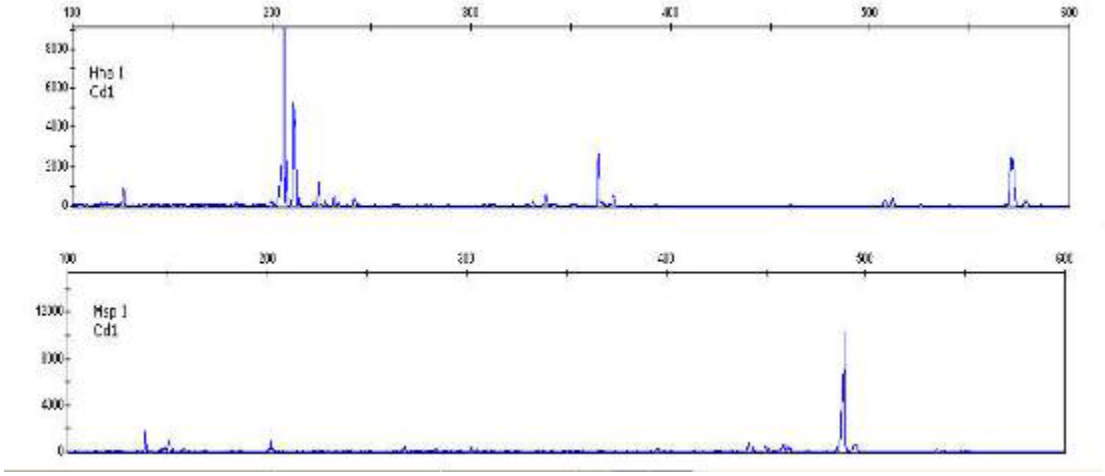

Figure 37 : Electropherograms for Cd1 using Hha I, above, and Msp I, below

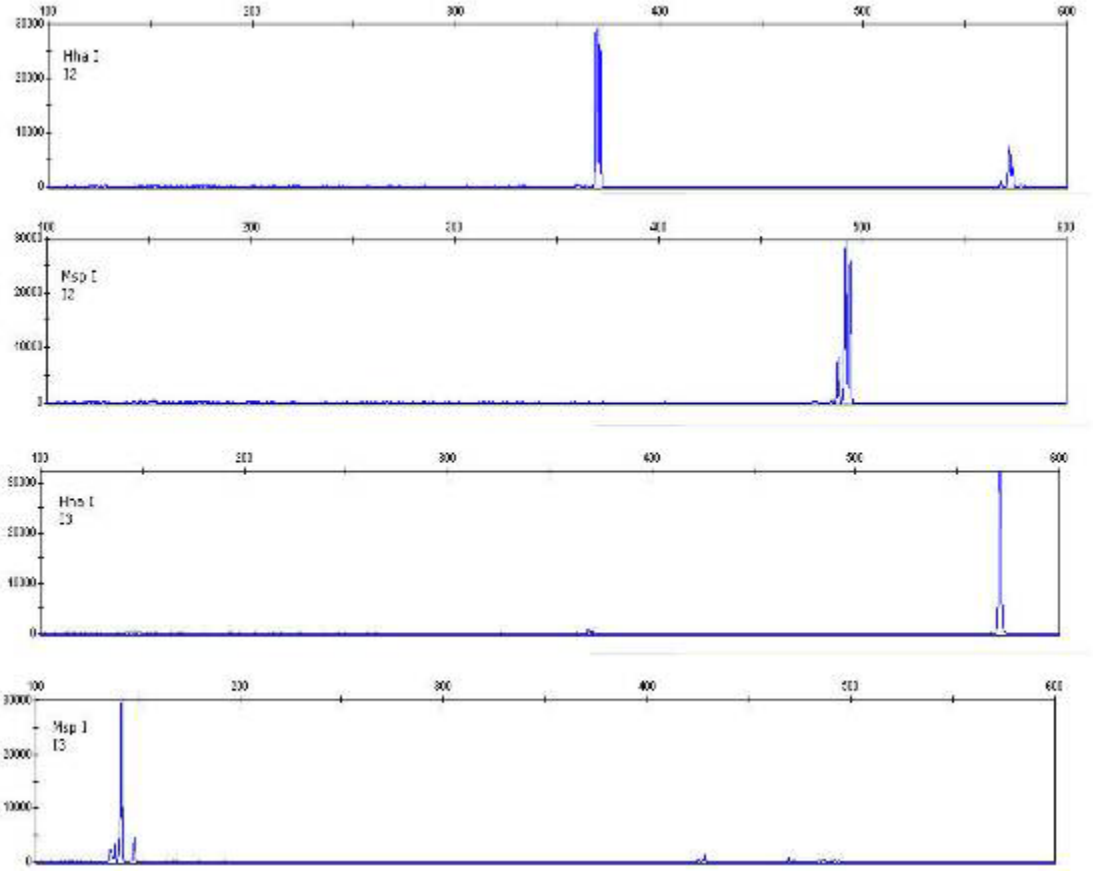

Figure 38: Electropherograms for Isolate 2 (top) using Hha I, above, and Msp I, below, and Isolate 3 (bottom) using Hha I, above, and Msp I, below

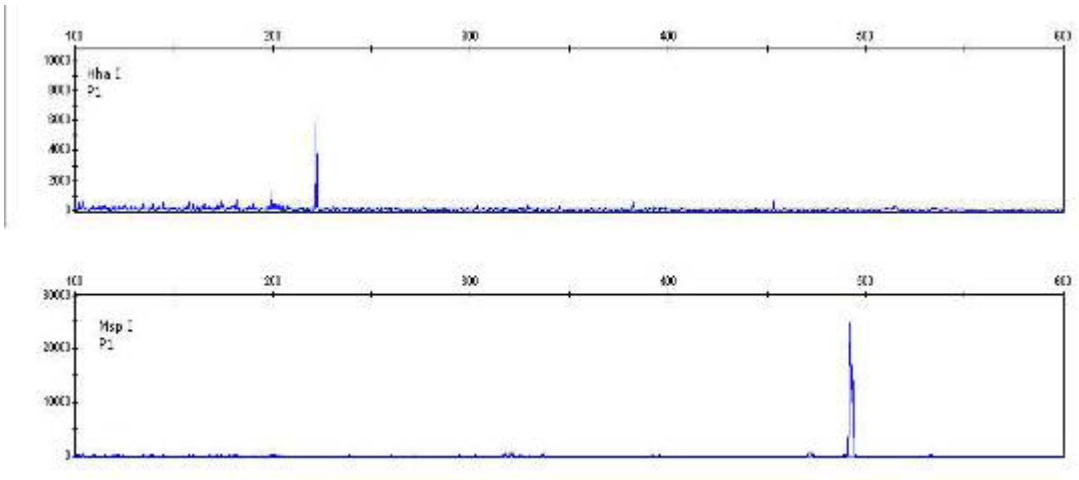

Figure 39 : Electropherograms for Plant matter using Hha I, above, and Msp I, below

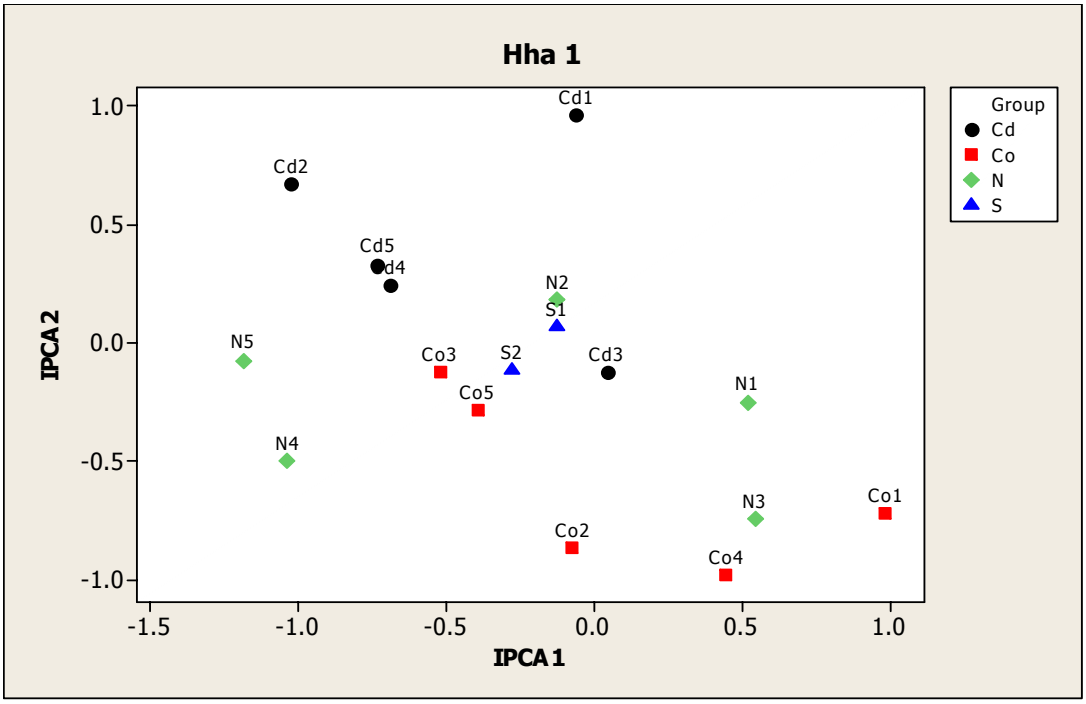

Figure 40 : AMMI analysis of fragments of digestion using Hha1

Axes are Integrative Principal Component Analysis (IPCA)

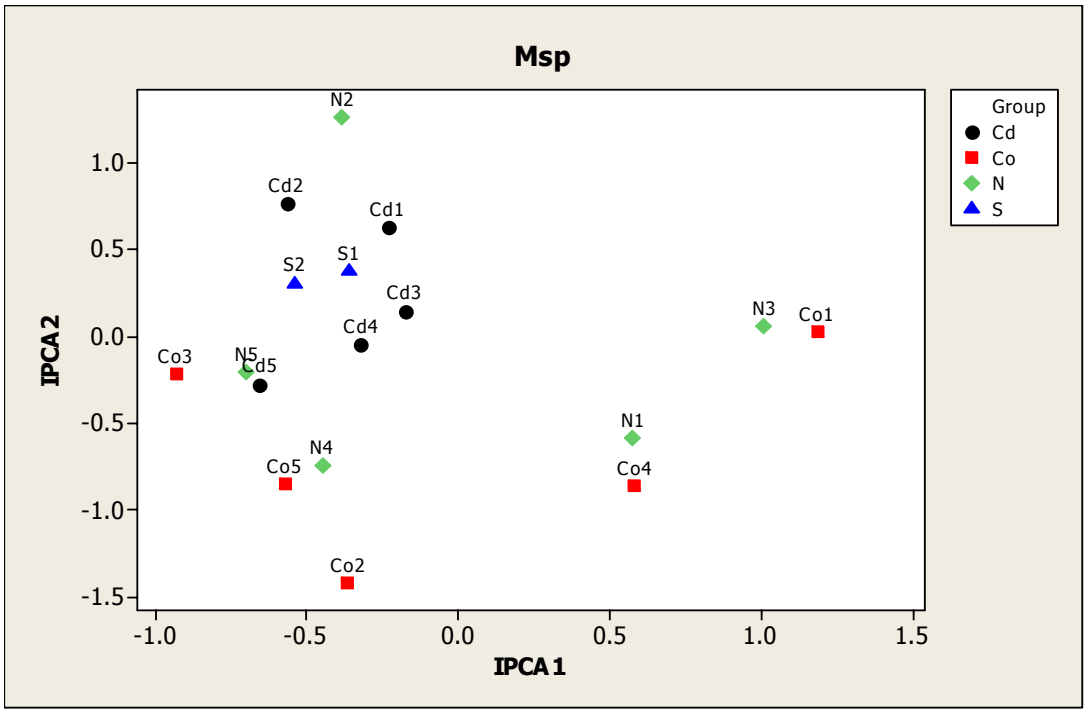

Figure 41 : AMMI analysis of fragments of digestion using Msp1. Axes are Integrative Principal Component Analysis (IPCA)

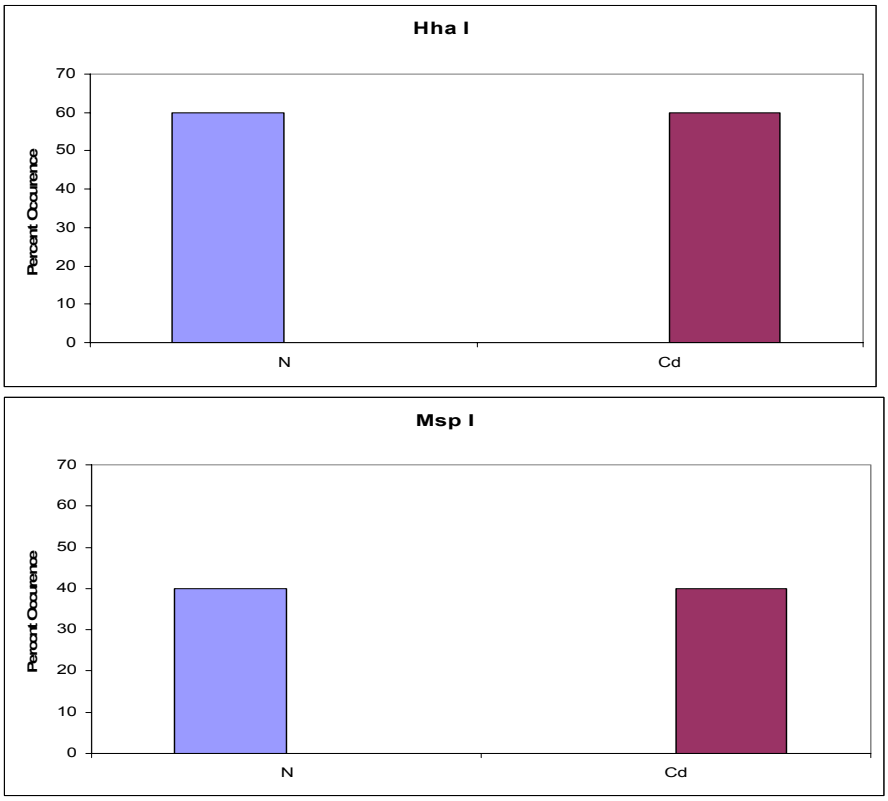

Figure 42 : Percent of Occurrence of Isolate 3 peak in N and Cd trials using Hha I (above) and Msp1 (below)

The CAS plating technique was successful, as can seen in Figure 36. The plates changed from blue to green to orange in the presence of siderophores. A total of 3 bacterial isolates were found that produced siderophores.

The electropherogram shown in Figure 38 indicates the different profiles from the siderophore-producing isolates produced by the different restriction enzymes. The use of multiple enzymes permitted the acquisition of more information for analysis. Siderophore-producing isolate 2 had multiple T-RF peaks regardless of the restriction enzyme that was used. The peaks for isolate 2 were at 369 & 371 for Hha I and 492 & 494 for Msp I. One peak for siderophore-producing isolate 3 was outside of the size

standard, 571 for Hha 1, but inside for Msp I, 142. Larger T-RF's were also observed for other samples, as can been seen in Figure 37, where the large T-RF peaks for Hha I are greater than 500 bp. Any TRF that is greater than 500 is an extrapoltaion based on the upper limit of 500 bp for the size standard. T-RFLP is a powerful tool that can be used to find a "fingerprint" of a microbial community. This can be used to differentiate one community from another, in addition the peaks can be used to identify the individual species that are present.

Figure 39 shows the electropherograms for the DNA extracted from the leaf tissue which was generated to show the chloroplast peak. Previous studies have shown that the T-RF peak for the chloroplast should be at 771 for Hha1, which is above the standard used here (Graff and Conrad 2005). The identification of the chloroplast peak is useful for analysis of the data. Even though it might appear that a certain type of plant associated bacteria is very abundant, the peak may correspond to the inherent chloroplasts. The removal of this peak from analysis may help highlight the important microbes. Even though the known chloroplast dominant peak (771) is above the standard, two dominant peaks were found, at 222 for Hha I and at 492 & 493 for MspI (Figure 36), which may correspond to other plastid or mitochondrial DNA.

Table 11 shows the distribution of OTUs, showing similar results for the quantity of OTUs and the average number across the treatment. The data produced different values for the standard deviation and range depending on the restriction enzyme used. After OTUs were removed using the criteria described in the methods, the total number analyzed in AMMI was reduced to 139 for Hha I, and 153 for Msp I.

The IPCA produced from the reduced data set are plotted in Figures 40 and 41. The peaks for isolate 3 were cross compared and shown to be found in identical proportions (60% for Hha 1, and 40% for Msp 1) for the +Cd/-Cd treatments, as

shown in Figure 42. Isolates 1 and 2 have multiple T-RF peaks that were not as clearly identifiable in the samples as those for isolate 3. While similar sized peaks were observed, the paired peaks were not always found together, so they were not used for comparison.

Isolates were identified using Ribsosomal Database Project (RDP) version 8.1. TRFs, REs, and primers sequences (27f) were input and the and yielded possible genera for the isolates. Isolate 2 had peaks that were common among bacteria, including *E.coli, Salmonella spp. , and Klebsiella spp.* Since the peaks were shared by many types of bacteria, it is not clear to which group isolate 2 belongs. Isolate 3 had peaks at 142 (Msp1) and 571 (Hha1), which were most closely identified with peaks at 141 (Msp) and 568 (Hha1), a strain of *Burkholderia spp*. The reader is cautioned that this is not an exact match, since the measured peaks above 500 were only estimated. However, the match of both RE peaks makes it likely that Isolate 3 has been correctly identified.

Discussion

The CAS method will identify the presence of a siderophore, but not the specific type. This technique, as with all plating methods will exclude the majority of the bacterial population which can not be cultured. It is likely that not all siderophore producing bacteria were able to grow on the medium. The CAS technique alone does not allow the specific type of siderophore to be identified. The type of siderophore is important in understanding the binding of non iron metals, such as Cd and additional analysis is planned. Isolate 3, which produced the most siderophores, shows promise for other applications, such as siderophore collection and may be used for future phytoremediation research.

TRFLP is a powerful tool to examine community structure. This technique will allow differences in the rhizosphere community to be observed, but absent complementary analyses such as sequencing of 16 S ribosomal DNA, TRFLP can not be used to determine the specific species composition of the community. When TRFLP of a community is combined with TRFLP performed on siderophore isolates it can be used to identify the proportion of the community composed of these organisms. Since, two different pieces of DNA may cut at the same length RF by a restriction enzyme, the use of multiple restriction enzymes provides a better indication of species diversity in a community. The number of OTUs obtained with each restriction enzyme is shown in Table 11.

Table 11 : Summarized statistical data for the trials, "Cd", "Co", "N", and background soil, "S."

Hha	O T U	Replicates	Average	Range	SD
Cd	257	5	51	52	21.6
Co	367	5	73	144	57.3
N	487	5	97	157	63.6
S	67	2	34	37	26.2
Msp	O T U	Replicates	Average	Range	SD
Cd	264	5	53	134	54.8
Co	374	5	75	102	42.5
N	491	5	98	98	35.9
S	75	2	38	9	6.4

The two REs yielded IPCA diagrams with similar results, with the Cd and N (-Cd) communities showing differences, with the N community being more similar to the Co communities. The N and Cd communities show deviation from the Control

hydroponic community towards the soil community (S) (see Figure 41). The results from the Msp I may be considered more applicable, because of the decreased presence of OTUs greater than 500 in size relative to Hha I. The analysis was done up to 600 to include the Hha I peak for Isolate 3, which was at 571. The IPCA plots, Figures 40 and 41, showed a tighter clustering of the Cd replicates, with values closer to the soil profile. This would indicate that Cd selected for a less diverse metal resisitant population, which can be also inferred from the lower number of OTUs present in the +Cd treatments.

The difference in community structure at 3 weeks of growth was not because of the presence or absence of the siderophore-producing isolates. As shown in Figure 42, Cd had no effect on the occurrence of the peak associated with Isolate 3. Conclusive results could not be obtained from the other isolates to support their role in changing community structure. This may have been because of the short time allowed for growth, which may have been inadequate for the isolates to establish their role in the community. It would be useful to continue plant growth for an extended period in future research to observe the long term effects.

TRFLP was successfully used to show a change in the root associated microbial community structure because of the presence of cadmium. Although this change in structure is not necessarily because of the presence of the siderophore producing isolates, it is influenced by the introduced soil microbial community. It is not yet known whether the modified community would improve the uptake of cadmium in a phytoremediation setting.

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