This is to certify that the

thesis entitled

The Role of Trace Copper in the Transformation of Carbon Tetrachloride by Pseudomonas Stutzeri KC

presented by

Hae Kyung Kim

has been accepted towards fulfillment
of the requirements for

M.S. degree in Environmental Engineering

Craig S. Criddle
Major professor

Date May 1998

THE ROLE OF TRACE COPPER IN THE TRANSFORMATION OF CARBON TETRACHLORIDE BY *PSEUDOMONAS STUTZERI* KC

By

Hae Kyung Kim

A THESIS

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE

Department of Civil and Environmental Engineering

1998

ABSTRACT

THE ROLE OF TRACE COPPER IN THE TRANSFORMATION OF CARBON TETRACHLORIDE BY *PSEUDOMONAS STUTZERI* KC

By

Hae Kyung Kim

Pseudomonas stutzeri KC secretes a low-molecular-weight factor when grown under iron limiting conditions [4,7]. This factor fortuitously transforms carbon tetrachloride to carbon dioxide, formate and other non-volatile products under denitrifying conditions without the production of chloroform. The factor can be activated for CT transformation by diverse cell types. Maximized and reliable transformation of CT is only achieved when trace exogenous copper is combined with the secreted factor and active cells. This work demonstrates that exogenous copper participates at the reaction level in CT transformation. The reaction requires only catalytic amounts of copper - more than ~ 6.4 μM of copper did not increase the rate of transformation. In fact, increasing level of copper inhibited transformation and cell growth. Cell types sensitive to copper mediated CT transformation at faster rates that did cell types resistant to copper.

ACKNOWLEDGMENTS

I would like to express my appreciation, first of all, to Dr. Craig S. Criddle for giving me opportunity and encouragement to pursue my experiments. His persistent support, guidance, and patience led me where I am today. Special thanks goes to Dr. Mike Dybas for his help on various experiment procedures and advice and to Dr. Robert Hickey for his valuable critical review of this thesis.

This work was supported by the NSF Center for Microbial Ecology at Michigan State University under NSF grant BIR-9120006.

Last but not least, I thank my parents for their love and supports.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

k_d	first order decay coefficient, hr^{-1}
M	total mass of substrate in the system
X	active organism concentration (protein), mg/L
Xo	initial active biomass concentration, mg/L
Clo	initial concentration of substrate in the aqueous phase, mg/L
T	theoretical or true biomass transformation capacity, mg substrate / mg protein
k′	second order rate coefficient, L/mg protein-hr
k	first order rate coefficient, hr^{-1}
t	time, hours
A	$V_L / (V_L + HcV_G)$
Hc	Henry's constant (-)

CHAPTER 1

INTRODUCTION

Previous research on *Pseudomonas stutzeri* KC has established that the degradation of carbon tetrachloride (CT) is a cometabolic process including two key elements. One of these elements is a small (≤ 500 Da) extracellular molecule secreted under iron-limiting conditions. The second is a cell factor(s) that can be supplied by diverse cell types, including *Pseudomonas fluorescens* and *Escherichia coli* [23: Tatara unpublished data]. Production of the cell associated factor is a robust trait of many microorganisms and is not dependent on iron or oxygen levels. By contrast, production of the extracellular molecule is achieved by strain KC only under iron limiting (≤ 1.0 μM) conditions [4,7]. Copper also plays a role in the transformation of CT.

Copper is required for the nutrition of nearly all organisms. It mainly serves as an inorganic component within enzymes [22]. The role of copper for CT degradation by strain KC presents an interesting paradox. At pH 8.2, 6.4 μM copper is toxic for aerobic growth, but this level of copper supports degradation rates that are higher than rates obtained at lower concentrations. Under most circumstances, the free cupric ion appears to be responsible for toxic effects on cell growth [8,19]. In order to maximize the rates of CT degradation for field applications, the level and role of copper should be clearly

understood. Previous research provides some evidence for the direct participation of copper at the reaction level. It includes the data obtained from experiments with *Pseudomonas fluorescens* and inhibited CCl_4 transformation by cyanide, a metalo-center inhibitor [23,24].

One possible role of copper is as a component in the active site of the secreted factor. When cells are grown in the absence of copper or at very low copper concentrations, they tend to lack the potential to degrade CT unless extra copper is added (24: Tatara et al.). Another possible scenario is direct coupling of copper with CT; with reduced copper acting as an election donor for CT reduction.

The range of pH values over which CT degradation occur is broad, but the optimum for CT transformation is approximately 8.5 as measured by Tatara [23]. Copper speciation likely influences the effect of pH. Biotransformation experiments performed with fixed copper concentrations and varied pH levels can conceivably isolate the effects of copper speciation revealing toxic species and changes in biomolecule function at the reaction level.

The primary objective of this research was to investigate the role of copper in the kinetics of CT degradation and to ascertain at what level it participates in the transformation. It may be possible to inject the secreted biomolecule directly into a contaminated site along with added copper rather than injecting KC cells. Because the cells required to regenerate

the secreted factor can be native to the contaminated site, ecological and transport issues raised by the introduction of non-native organisms are avoided.

CHAPTER 2

MATERIALS AND METHODS

Organisms. *Pseudomonas* sp. strain KC (DSM deposit no. 7136, ATCC deposit number 55595), derived originally from aquifer solids from Seal Beach, CA (Criddle et al., 1990), was routinely maintained on nutrient agar plates. The copper sensitive and tolerant strains of *Pseudomonas syringae* Al487 and Al 513R were obtained from University of California, Berkeley (Courtesy, S. Lindow). They were routinely cultured on King's medium B (KB) at 30° C before inoculated to nutrient broth (3). *Pseudomonas fluorescens* (ATCC deposit no. 13525) was obtained from the culture collection of the Microbiology Department at Michigan State University.

Chemicals. All chemicals used were ACS reagent grade (Aldrich or Sigma Chemical Co.). All water used in reagent preparation was deionized 18 Mohm resistance or greater. Carbon tetrachloride (99 % purity) was obtained from Aldrich Chemical Co., Milwaukee, WI.

Media. Synthetic groundwater medium (SGM) contained (per liter deionized water): 0.455 g of $NaSiO_3$ $9H_2O$, 0.16 g of Na_2CO_3, 0.006 g of $NaSO_4$, 0.02 g of KOH, 0.118 g of $MgCl_2$ $\bullet 6H_2O$, 0.0081 g of $CaCl_2$ $\bullet 2$ H_2O, 6.81 g of KH_2PO_4, 0.8 g NaOH, 1.6 g

$NaNO_3$, 1.6 g acetate, and 1 ml of trace element solution. The trace element solution contained (per liter deionized water): 0.021 g $LiCl_2$, 0.08 g $CuSO_4 \bullet 5H_2O$, 0.106 g $ZnSO_4 \bullet 7H_2O$, 0.06 g $TiCl_4$, 0.03 g KBr, 0.03 g KI. 0.629 g $MnCl_2 \bullet 4H_2O$, 0.036 g $SnCl_2 \bullet 2H_2O$, and 0.3 g $FeSO_4 \bullet 7H_2O$. In certain experiments, copper was omitted from the trace element solution. The pH of SGM was adjusted to 8.2 by addition of NaOH pellets followed by titration with 1M NaOH, then autoclaved at 121 °C for 25 min. Nutrient broth and nutrient agar (Difco) plates were prepared according to manufacturer's instructions. Nutrient broth cultures were grown aerobically at 20 °C 150 rpm to stationary phase (12 hours). 1 % (v/v) of this culture was used as the inoculum for experiments to evaluate the effects of trace copper.

Groundwater. Groundwater from a CT-contaminated aquifer in Schoolcraft, MI, was used to prepare an enrichment of indigenous flora. Groundwater samples were obtained manually by withdrawing groundwater with a Teflon bailer from a 2 inch steel well screened at 30 feet below the water table. Groundwater samples were stored in pre-sterilized, sealed Nalgene carboys or in Wheaton bottles equipped with Teflon lined caps at 4 °C.

Analytical methods. Carbon tetrachloride was assayed by gas chromatography (GC) by removing a headspace sample for analysis. A five-point external calibration curve was prepared by diluting a primary standard (8.2 μg of CT per μl of methanol) in SGM medium to achieve concentrations bracketing that of assay samples. Assay and calibration samples were carried out in 28 ml serum tubes (Bellco Glass no. 2048-00150) sealed with

Teflon lined butyl rubber septa (West Co. no. 935326) and aluminum crimp seals. Nitrate, nitrite, and acetate ions were assayed by ion chromatography (Dionex model 2000ISP ion chromatography with suppressed conductivity detection equipped with a Dionex Ionpak AS4-A anion exchange column and utilizing a 1.8 mM bicarbonate/ 1.7 mM carbonate mobile phase at a ml/min). Chromatograms were recorded and data integrated using Turbochrom 3 software (Perkin Elmer Corp.). External standard calibration curves, which bracketed the concentrations of the test samples, were prepared by diluting primary ion standards into deionized water having at least 18 Mohm resistance. Measurements of pH were made with an Orion model 720A pH meter. Optical density measurements were measured at 660 nm using a Shimadzu UV -160 spectrophotometer.

CHAPTER 3

EFFECT OF INITIAL pH AND COPPER CONCENTRATION ON GROWTH

Materials and Methods.

Effects of Copper on Growth. To insure that toxicity did not limit the rate of CT degradation at high copper concentration, studies were performed evaluating the effects of copper on the growth rate of *Pseudomonas fluorescens, Pseudomonas stutzeri KC,* and *Pseudomonas syringae*. Starter cultures were prepared by transferring cells from nutrient agar plates to previously autoclaved nutrient broth using aseptic technique. After 12 hours of growth in nutrient broth, a 1 % (v/v) inoculum was transferred to SGM or Schoolcraft groundwater. Copper was added just prior to inoculation as $CuSO_4 \bullet 5H_2O$. A growth curve for *Pseudomonas fluorescens* in SGM was obtained using 28 ml balch tubes and 250 ml flakes for denitrifying and aerobic growth conditions, respectively. Balch tubes containing 5 ml of SGM were passed through an anaerobic hood interlock three times to remove oxygen from the headspace then sealed with rubber stoppers. Balch tubes and flasks containing SGM were autoclaved for 20 minutes and allowed to equilibrate for at least for 1 day before nutrient broth inoculation. This procedure prevented excess phosphate precipitation after autoclaving. Optical density values at 660 nm (OD_{660}) were obtained from aerobic samples containing 0, 0.32, 3.2, 6.4, 9.6, 12.8 μM copper for approximately every 6 hours for 40 hours. OD_{660} values were also

obtained from denitrifying samples containing 0, 0.32, 3.2, 6.4, 9.6 μM copper approximately every 5 days for 27 days. Initial and final pH was measured for each sample.

Growth of strain KC in SGM and Schoolcraft groundwater was evaluated using 500-ml Wheaton bottles and 250-ml flasks for denitrifying and aerobic growth conditions, respectively. Bottles with loosened caps were passed through an anaerobic hood interlock three times to remove oxygen from the headspace. Aerobic and denitrifying cultures grown in Synthetic Groundwater containing 0 - 12.8 μM copper were sampled approximately every 4 hours for 36 hours. Total protein accumulation was monitored using the modified Lowry protein analysis at 660 nm [14]. Schoolcraft groundwater was amended with acetate (750 mg/L) and nitrate (450 mg/L) for denitrifying growth and with acetate alone for aerobic growth. After pasteurization at 65 °C for 8 hours, phosphate was also added to give a final concentration of 10 mg/L. Samples of culture grown in amended Schoolcraft groundwater were taken approximately every 4 hours for 36 hours, and total protein accumulation was monitored using the modified Lowry protein analysis. Initial and final pH for each sample was measured.

Pseudomonas syringae was grown aerobically in a shake flask with SGM containing copper concentrations that depended upon the strain : a copper sensitive strain Al487 provided by S. Lindow from University of California, Berkeley was routinely cultured on King's medium B (KB) at 30° C before inoculated to nutrient broth [3]. After 12 hours growth in nutrient broth, a 1 % (v/v) inoculum was transferred to SGM and grown at 0,

0.32, 1.28, 3.2, 12.8, 32 μM copper ; the copper tolerant strain Al513R (provided by S. Lindow) was grown at 0, 0.32, 1.28, 12.8, 32, 64μM. Samples were taken approximately every 12 hours for 114 hours. Protein accumulation was monitored by Lowry at 660 nm after all samples reached stationary phase [14]. Initial and final pH were measured.

Effect of pH on Growth. Growth of *Pseudomonas fluorescens* and *Pseudomonas syringae* as a function of initial medium pH was determined by preparing synthetic groundwater at various pH levels. NaOH pellets and 1M NaOH solution were used to adjust solution pH to the desired level. Growth curves were obtained for *Pseudomonas fluorescens* in SGM 28-ml balch tubes and a 250-ml shake flask at pH values of 6.5, 7, 7.5, 8, 8.5. Each sample was prepared in triplicate. Anaerobic samples were rendered anoxic by passage through an anaerobic glove box, where the samples were sealed under an atmosphere of 98% N_2 and 2% H_2. For aerobic cultures, optical density was measured every 6 hours for 42 hours; for denitrifying culture, optical density was measured approximately every 5 days. Initial and final pH were measured for each sample.

Copper sensitive and copper tolerant *Pseudomonas syringae* strains were grown aerobically at initial pH values of 6.5, 7, 7.5, 8, 8.2, 8.5 and monitored for growth by the Lowry protein assay. Samples of culture grown in 250-ml flakes were removed approximately every 12 hours for 114 hours. After all samples reached stationary phase, protein accumulation was monitored by the Lowry assay at 660 nm.

Results and Discussion

Rapid transformation of CCl_4 requires cell factors, notably membranes and NADH, which may be obtained from many different cell types plus a secreted biomolecule produced by *Pseudomonas stutzeri* KC [23]. In the presence of secreted factor, aerobically-grown *Pseudomonas fluorescens* transformed CT at a pH of 8.2 in synthetic groundwater (Tatara, 1996). The maximum growth rate under aerobic conditions (Figures 3.1 and 3.2) was about 10 times greater than the growth rate under denitrifying conditions at pH 7 (Figure 3.3 and 3.4). Increasing levels of added copper had a profound effect on the growth of cells grown under aerobic but not under denitrifying conditions.

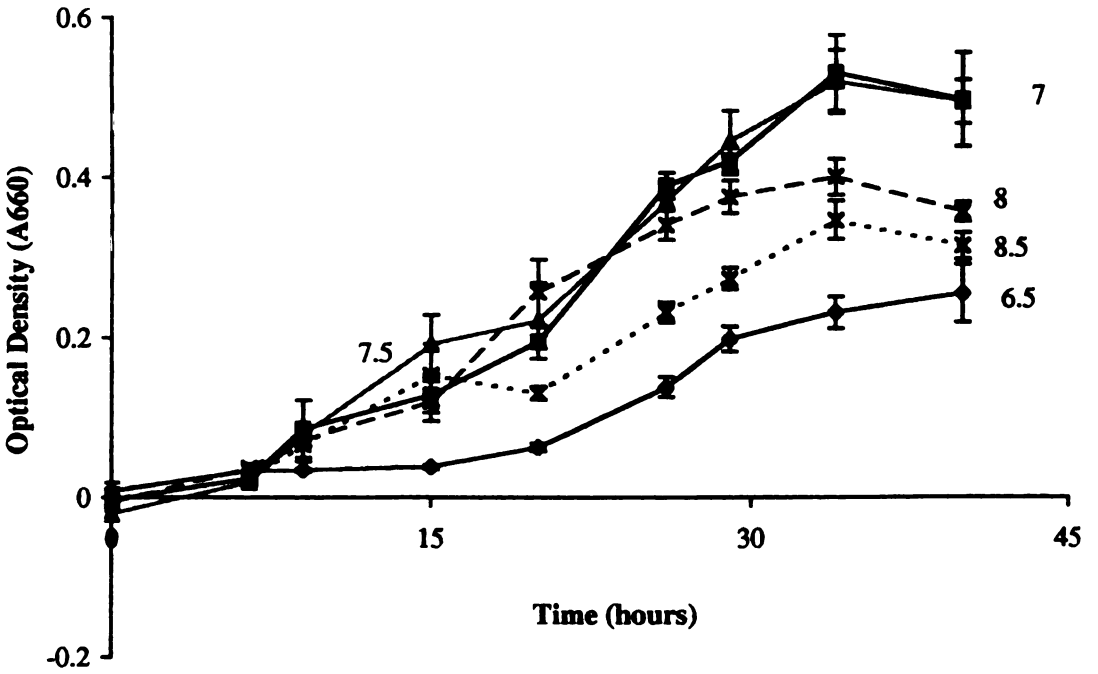

Figure 3.1 Effect of pH on aerobic growth of *Pseudomonas fluorescens* in synthetic groundwater. Labels on plot indicates initial pH.

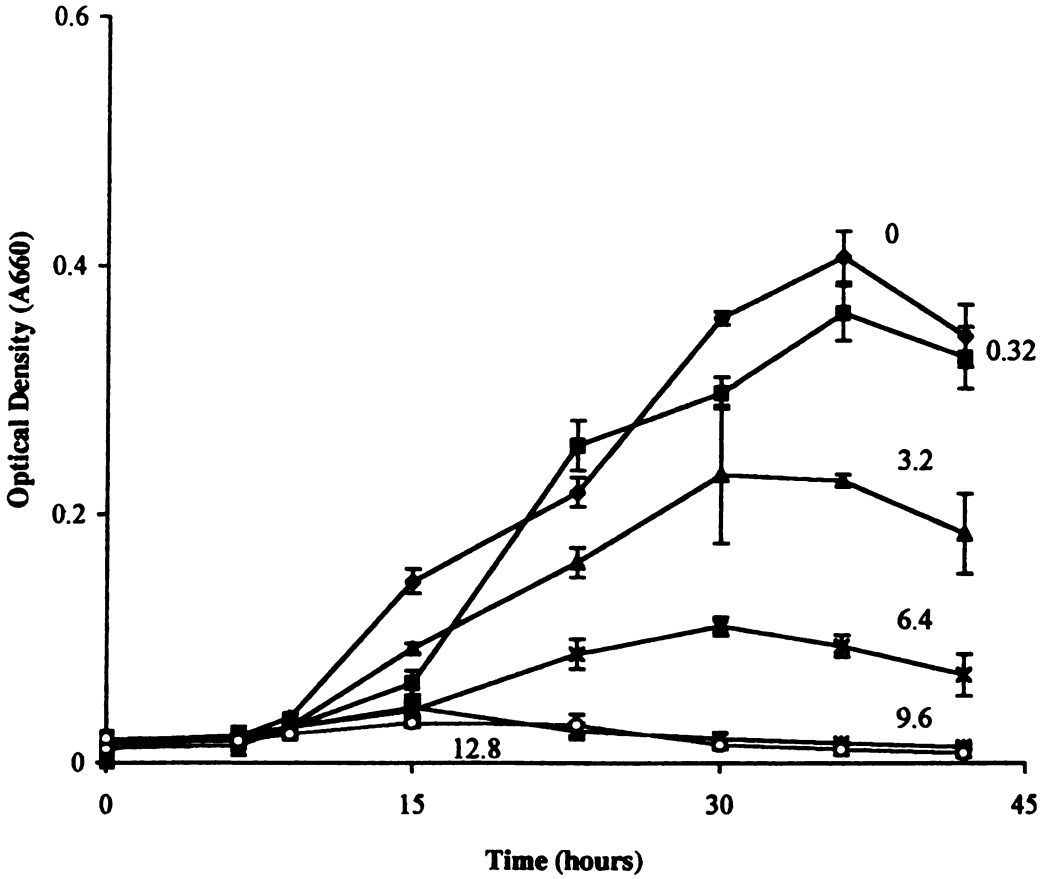

Figure 3.2 Effect of trace copper on aerobic growth of *Pseudomonas fluorescens* in synthetic groundwater. Labels indicate added copper concentration in micromolar units.

A possible explanation for copper toxicity in the aerobic cultures is copper-catalyzed production of toxic forms of oxygen during respiration, such as superoxide or hydroxyl radicals. Superoxide can cause oxidative destruction of lipids and other biochemical components [6,15]. The hydroxyl radical OH• also attacks many of the organic substances present in cells and can cause cell lysis.

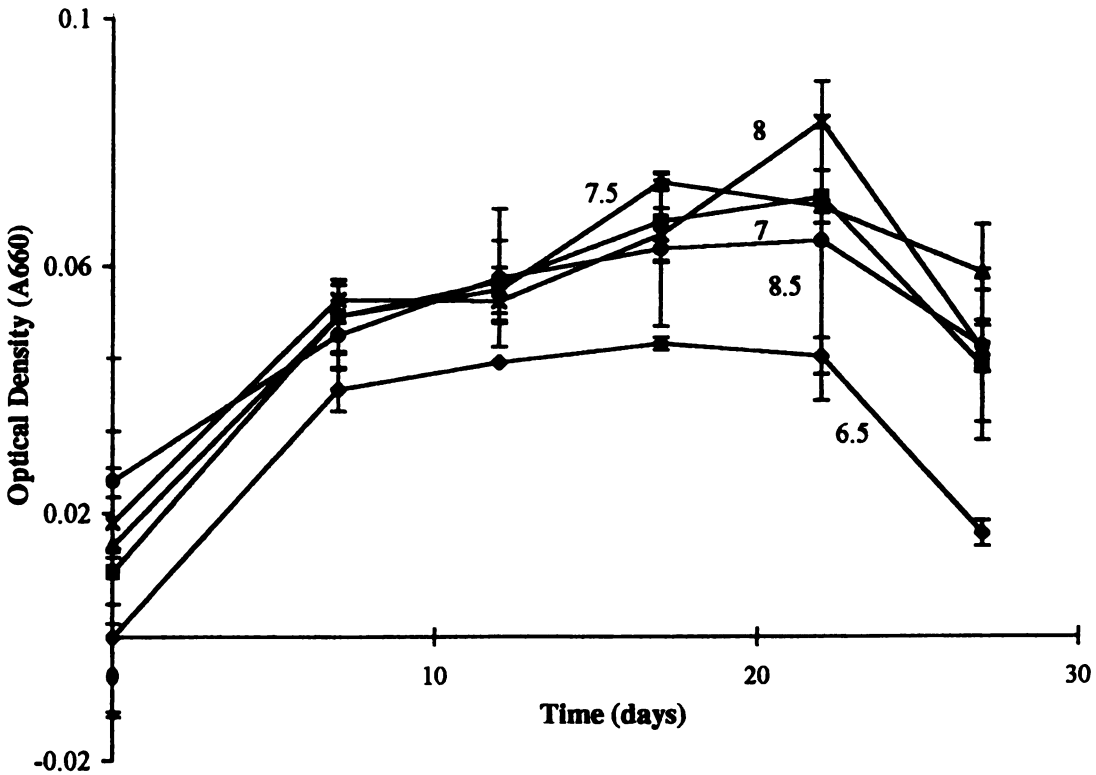

Figure 3.3 Effect of pH on anoxic growth of *Pseudomonas fluorescens* in synthetic groundwater. Labels indicate initial pH.

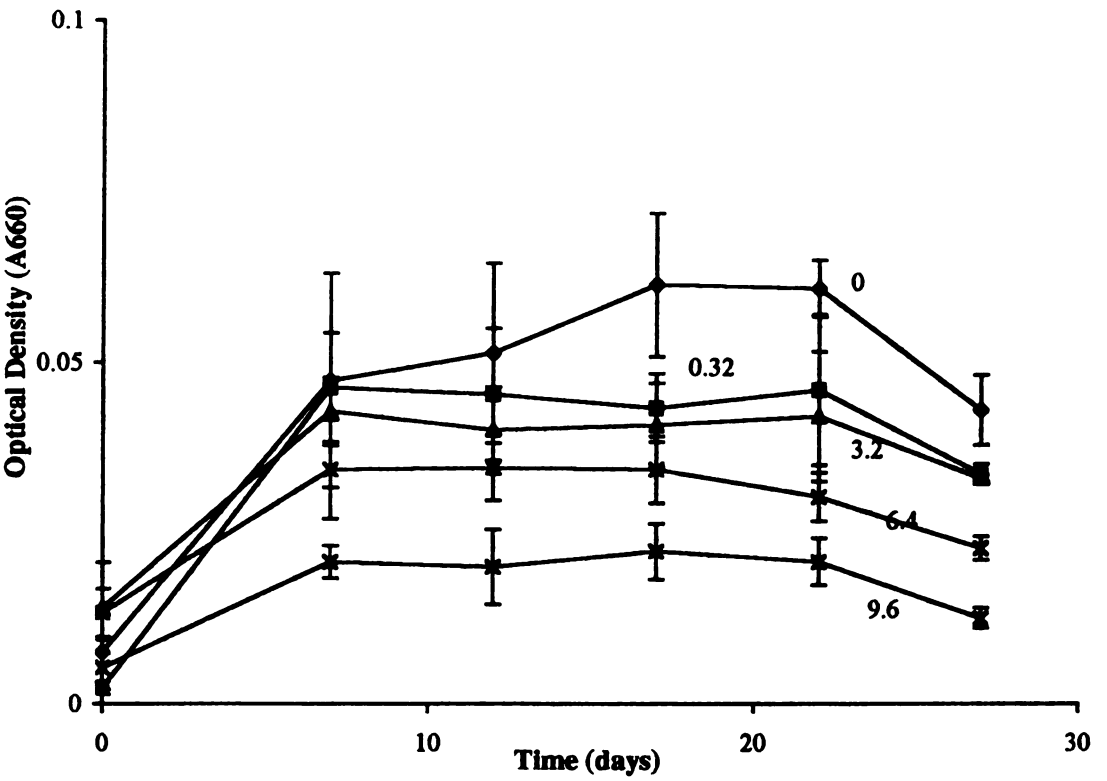

Figure 3.4 Effect of trace copper on anoxic growth of *Pseudomonas fluorescens* in synthetic groundwater. Labels indicate added copper concentration in micromolar units.

Unlike *P. fluorescens,* the growth rate of *Pseudomonas stutzeri* KC under denitrifying conditions was as fast as its growth rate under aerobic conditions (Figure 3.5 and 3.6). Like *P. fluorescens*, however, copper tolerance of strain KC was higher under denitrifying conditions than under aerobic conditions. Low concentrations of copper inhibited growth, but to a lesser extent than observed for *Pseudomonas fluorescens.*

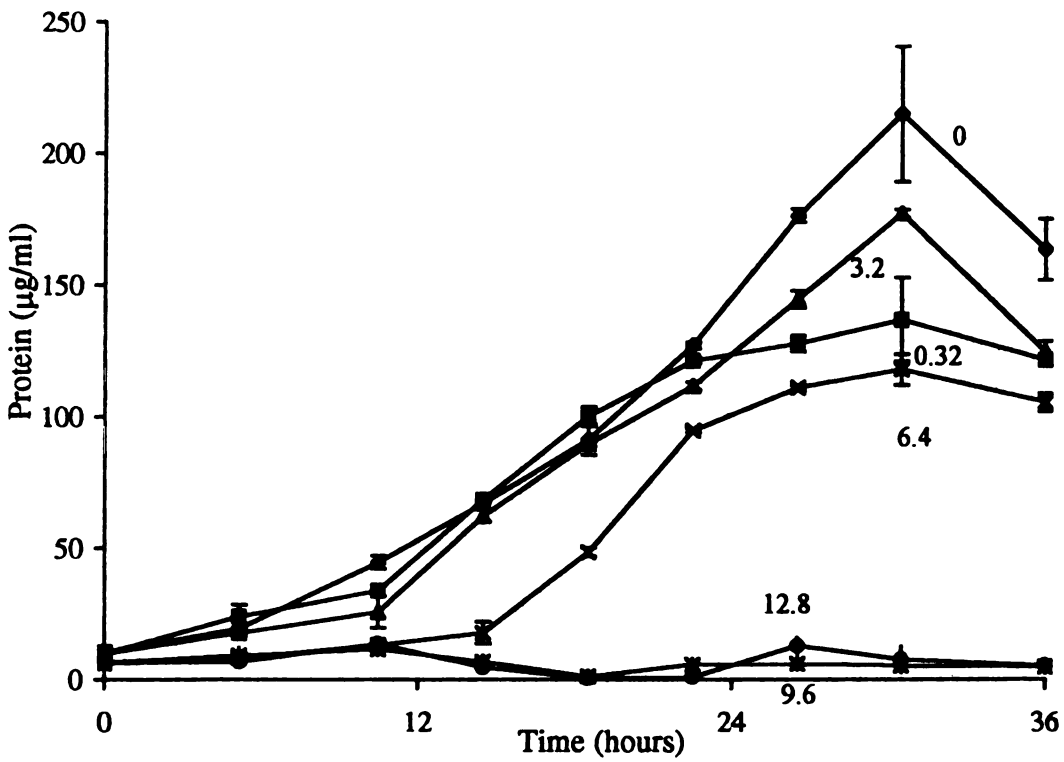

Figure 3.5 Effect of copper on aerobic growth of *Pseudomonas stutzeri* KC in synthetic groundwater. Labels on plots indicate added copper concentration in micromolar units.

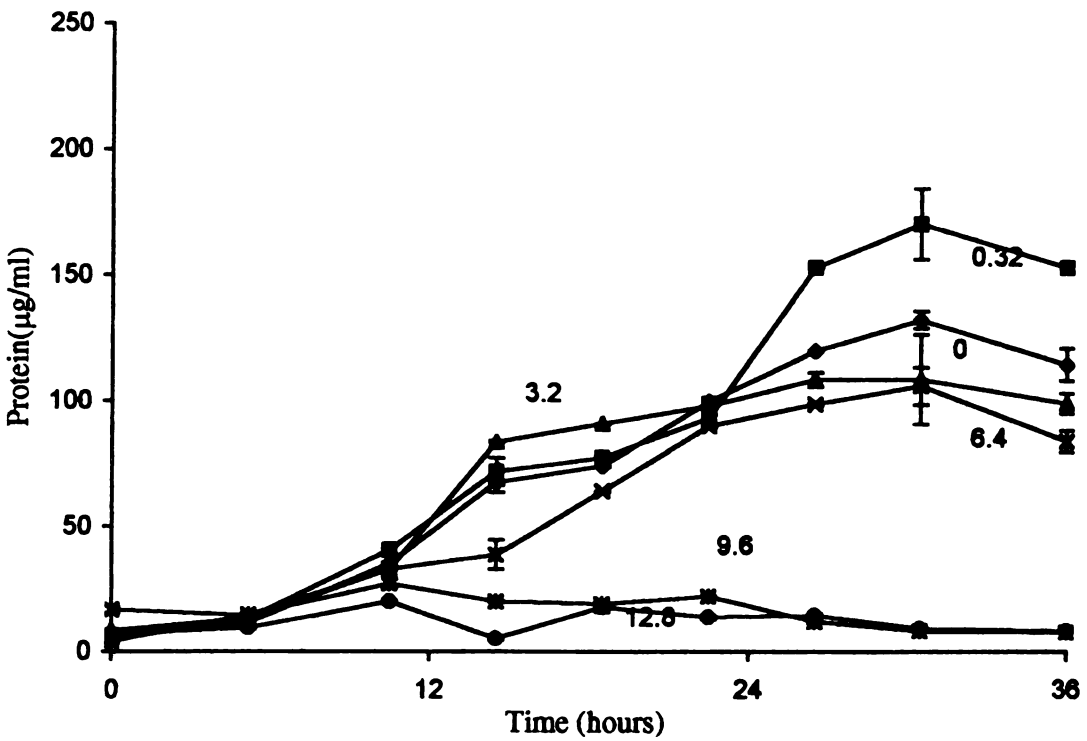

Figure 3.6 Effect of copper on anoxic growth of *Pseudomonas stutzeri* KC in synthetic groundwater. Labels on plots indicate added copper concentration in micromolar units.

In Schoolcraft groundwater, growth of strain KC was more inhibited than in synthetic groundwater, probably because of higher background levels of trace metals in the groundwater (Figure 3.7 and 3.8).

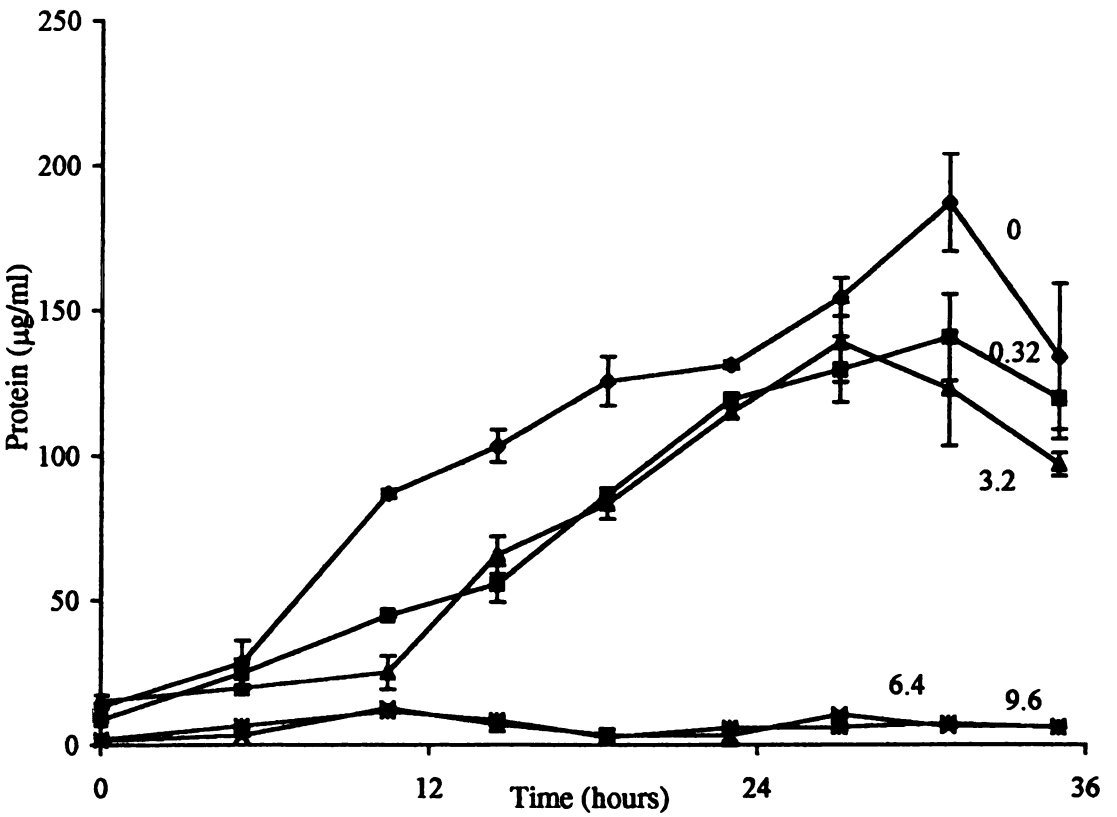

Figure 3.7 Effect of copper on aerobic growth of *Pseudomonas stutzeri* KC in Schoolcraft groundwater. Labels on plot indicate added copper concentration in micromolar units.

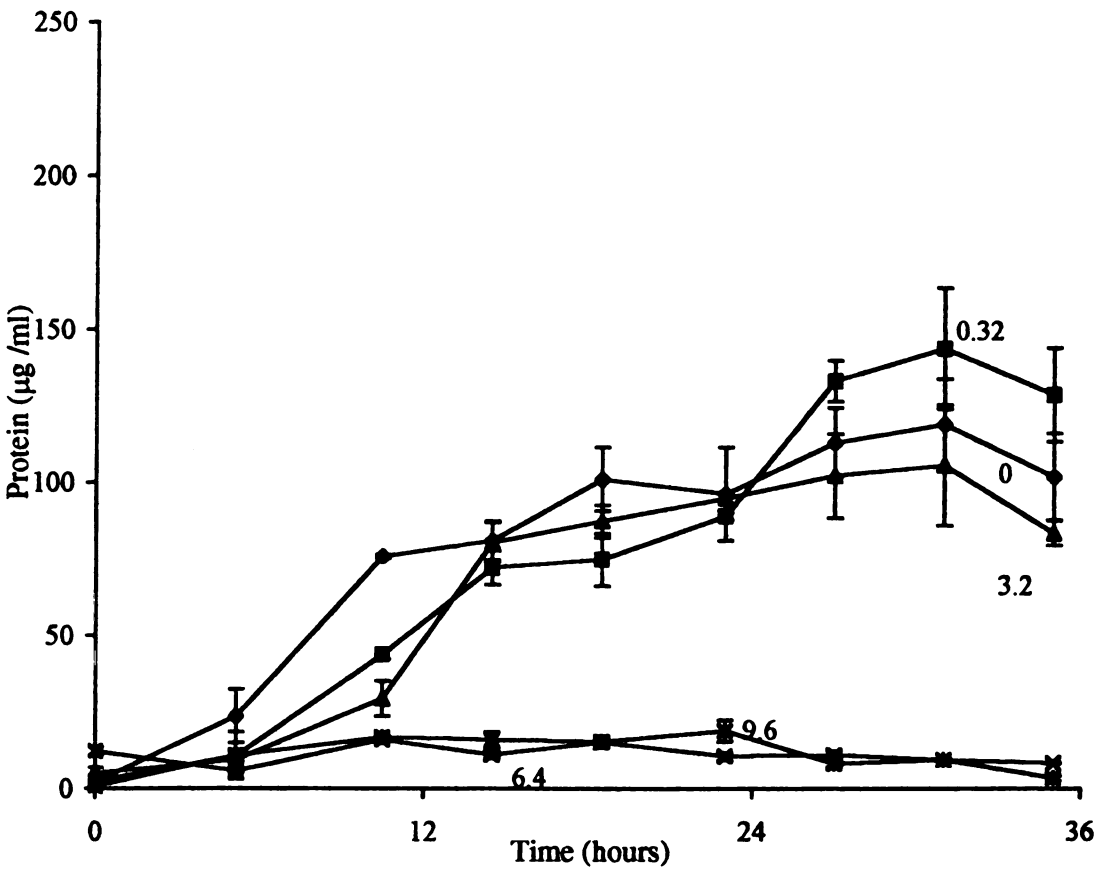

Figure 3.8 Effect of copper on anoxic growth of *Pseudomonas stutzeri* KC in Schoolcraft groundwater. Labels on plot indicate added copper concentration in micromolar units.

Maximum growth rate was obtained at pH 7.5 for both the copper sensitive *Pseudomonas syringae* strain Al487and the copper tolerant strain Al513R. Low levels of copper inhibited growth of strain Al487. For Gram-negative bacteria, copper -resistance is associated with a unique set of proteins : CopA and CopC in the periplasmic space, CopB in the outer membrane. These proteins are induced by copper. Their likely function in copper resistance is sequestration of copper ions in the periplasm, preventing entry of copper ions into the cytoplasm [18].

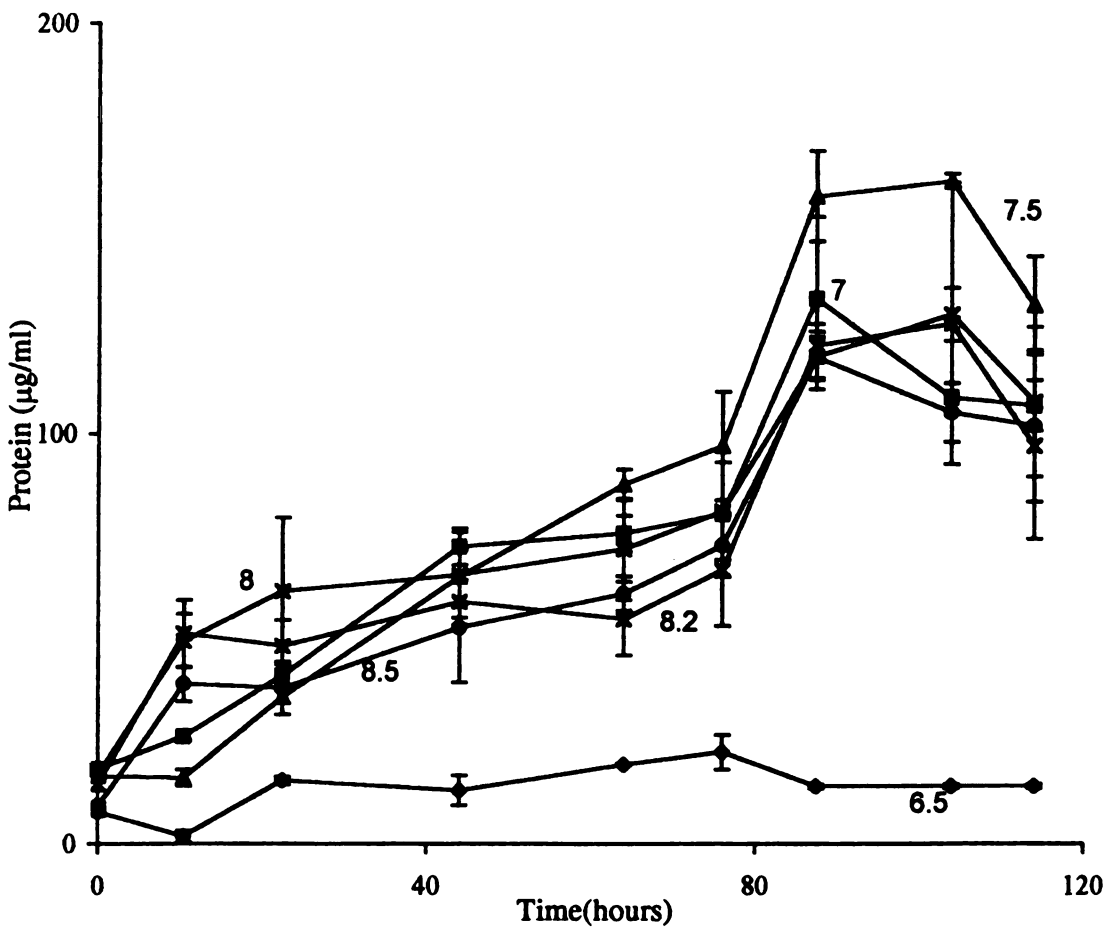

Figure 3.9 Effect of pH on aerobic growth of copper-sensitive *Pseudomonas syringae* in synthetic groundwater. Labels on plot indicate initial pH.

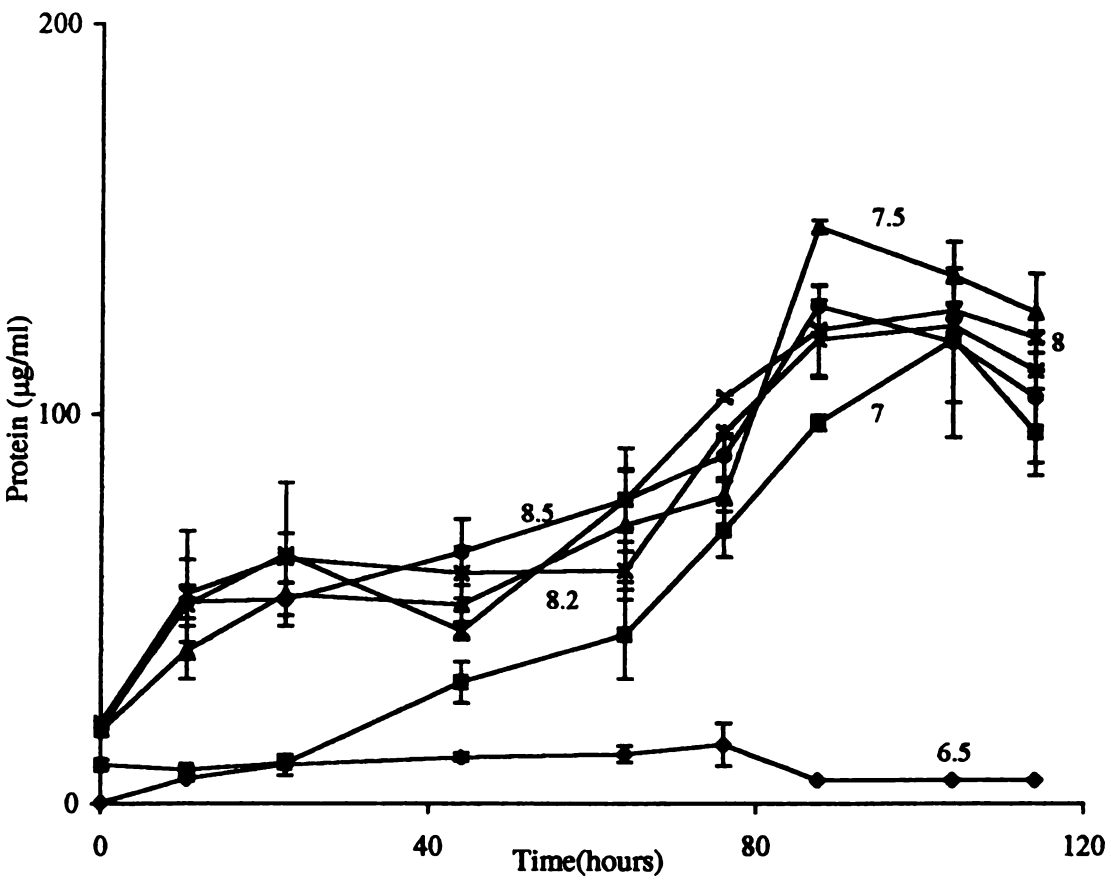

Figure 3.10 Effect of pH on aerobic growth of copper-tolerant *Pseudomonas syringae* in synthetic groundwater. Labels on plot indicate initial pH.

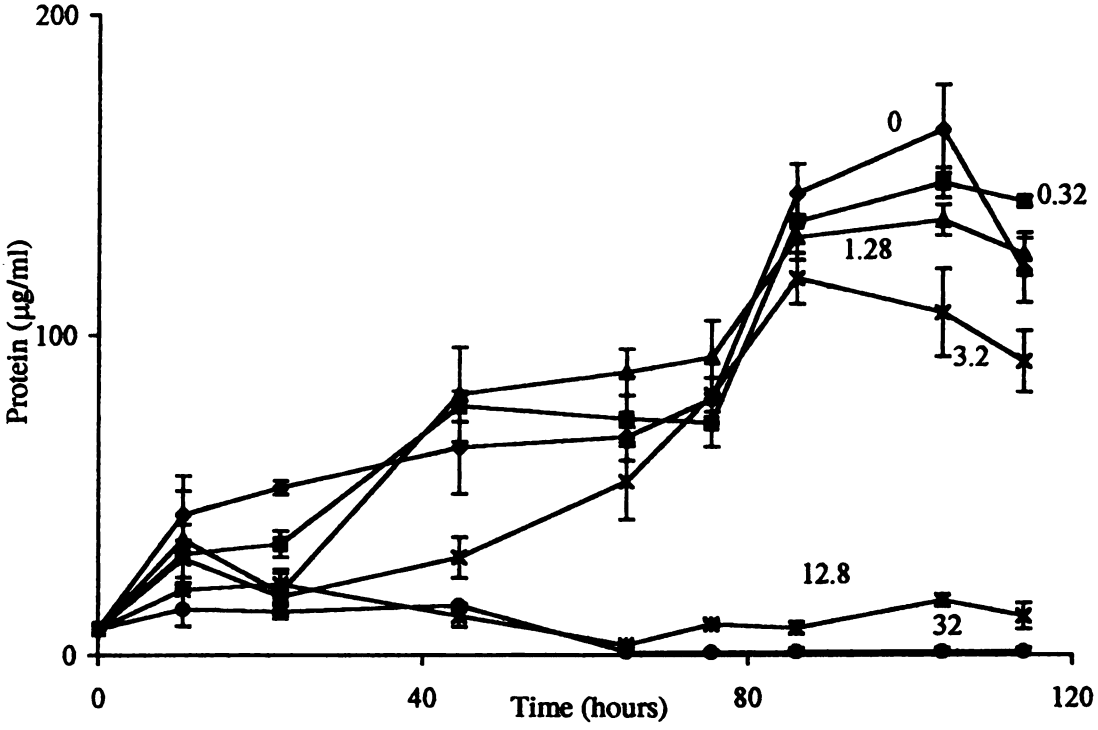

Figure 3.11 Effect of copper on aerobic growth of copper-sensitive *Pseudomonas syringae* in synthetic groundwater. Labels on plot indicate added copper concentrations in micromolar units.

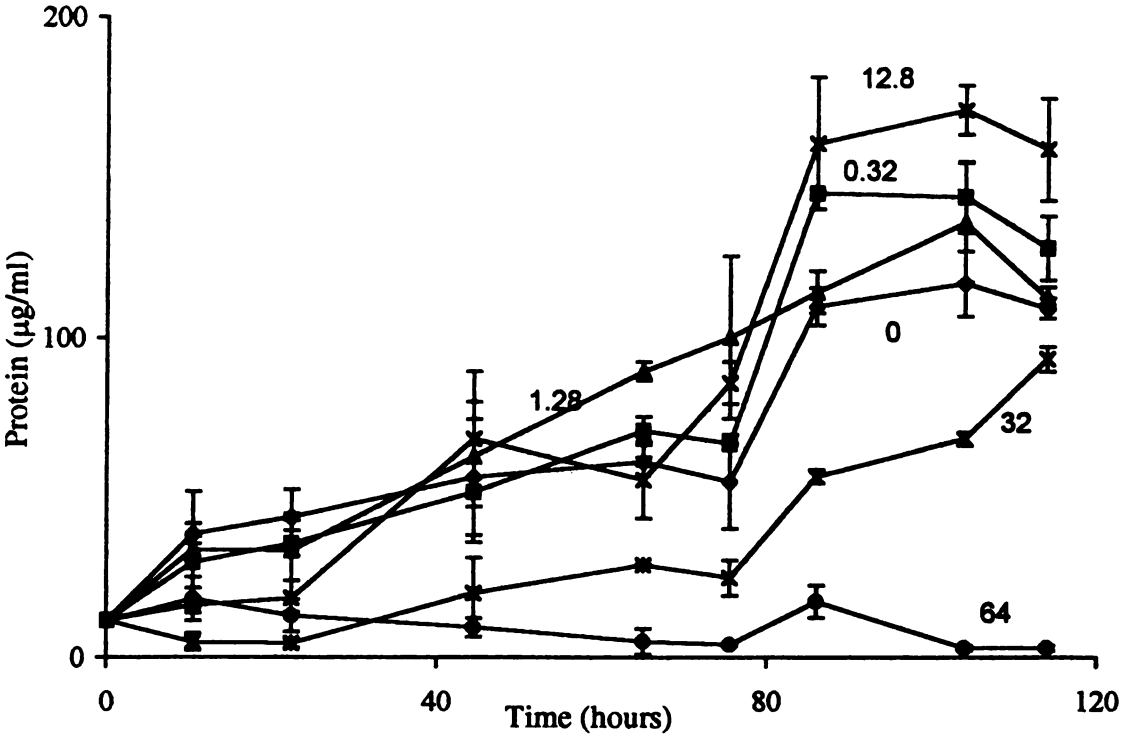

Figure 3.12 Effect of copper on aerobic growth of copper-tolerant *Pseudomonas syringae* in synthetic groundwater. Labels on plot indicate added copper concentrations in micromolar units.

CHAPTER 4

DETERMINATION OF THE EFFECTS OF TRACE COPPER ON THE BIODEGRADATION OF CARBON TETRACHLORIDE

Preparation of partially purified culture supernatant. In order to determine whether the presence of trace copper affects the transformation of carbon tetrachloride at the reaction level, cultures of strain KC grown with and without added trace concentrations of copper were grown for approximately 24 hours in Synthetic Groundwater Medium (SGM), then fractionated by centrifugation and ultrafiltration. Following an initial screening for CCl_4 transformation activity, cells were harvested by centrifugation (15 minutes, 5,000 rpm), and the supernatant filtered through a 0.2 μM filter 3 times. Filtered supernatant was further fractionated by filtration through Amicon 10, 000 molecular cut-off filters in a Coy anaerobic glove box (95% N_2 5% H_2 atmosphere). The resulting filtrate was then lyophilized. CT transformation assays were performed by dissolving the lyophilized fraction in 4.5 ml SGM, combining the resulting solution with 0.5 ml samples of cell washed factor, under a N_2 atmosphere, spiking with CCl_4 (10 μg/ liter), and assaying CCl_4 levels by gas chromatography, as described in Chapter 2.

Cell factors. *Pseudomonas fluorescens* cells grown in the presence and absence of trace copper SGM were harvested at OD 660 values of approximately 0.192 and 0.187

respectively. Cell and suspended fractions were separated by centrifugation (10 min, 3000 rpm). Cell pellets were resuspended to one tenth their original volume in fresh medium of the identical composition used for growth. To evaluate the effect of copper, cell and supernatant combinations of *Pseudomonas stutzeri* strain KC and *Pseudomonas fluorescens* were mixed. Copper was added to selected samples as $CuSO_4 \bullet 5H_2O$ from 80mg/L or 800mg/L stock solutions to give the desired final concentration. Each sample was prepared in triplicate, rendered anoxic by passage through an anaerobic glove box, sealed under an atmosphere of 98% N_2 and 2% H_2, spiked with 10 µl of a 20 mg/L aqueous stock solution of CT, placed on a shaker, and assayed for CT degradation by GC analysis of the headspace.

To prepare enrichments of indigenous microflora from Schoolcraft groundwater, two 500-ml samples of Schoolcraft groundwater were placed into sterile 1000-ml flask and amended with 500 mg/L acetate and 430 mg/L nitrate. One flask received 0.32 µM copper. After 5 days of shaking, both groundwater samples received an additional spike of 500 mg/L acetate. Stimulated indigenous flora were harvested at optical densities of 0.234 and 0.279 for cultures grown in the absence and presence of trace copper, respectively, and centrifuged to separate cells from supernatant. Cell supernatant combinations of Schoolcraft indigenous flora and *Pseudomonas stutzeri* KC were prepared in the same way as described previously for *Pseudomonas fluorescens.*

Modeling. Tatara (1995) demonstrated that disappreance of CCl_4 and appearance of products could be quantified with a simple model in which transformation activity is assumed to decay with time:

$$\frac{-dMc_T}{dt} = k'XCaq \cdot e - kdt = k\frac{Vaq}{(Vaq + HcVg)}Mc_T \cdot e - kdt \qquad (1)$$

where M_{CCl4} =total mass of CCl_4 in the system = Caq(Vaq+HcVg), k= first order rate coefficient (min^{-1}), k_d = first order decay coefficient (hr^{-1}). Equation (1) can be integrated to obtain M as a function of time:

$$M = Mo\exp[\frac{k}{k_d}\frac{Vaq}{(Vaq + HcVaq)}(\exp(-k_d t) - 1)] \qquad (2)$$

The endogenous decay term k_d includes loss of activity caused by cell death and by depletion of reducing power required for monooxygenase activity. Another simplified case is obtained when product toxicity is the dominant factor causing loss of transformation activity.

$$M = Mo\frac{F\exp(-k'AFt)}{Xo - \frac{Clo}{T}\exp(-k'AFt)} \qquad (3)$$

Where Clo is initial concentration of substrate in the aqueous phase (mg/L), F = Xo – Clo /T.

Disappearance of the target compounds was modeled using both equations (2) and (3). Kinetic parameters were estimated by nonlinear regression using Statistica (MathSoft, Gaithersburg, MD).

Results and Discussion

Kinetics of CCl_4 transformation. The dependence of CCl_4 transformation rates on concentration of copper and disappearance of CCl_4 activity was evaluated by plotting the logarithm of mass of CCl_4 versus time. A simple model invoking first-order degradation with respect to CCl_4 concentration failed to describe the transformation (Figure 4.1). However, the endogenous decay model of equation 2 did describe the data, suggesting gradual decay on depletion of a key reactant. In the endogenous decay model, loss of transformation activity is controlled by time, and is not affected by the amount of nongrowth substrate transformed. This contrasts with the transformation capacity model of equation 3 that also successfully described the data. In this case, the decay of transformation activity is directly proportional to the amount of nongrowth substrate transformed.

Table 4.1 Comparison of kinetic coefficients for transformation of CT by *P. stutzeri* KC using biomolecule secreted from SGM with 0.32 μM of copper and *P. fluorescens* grown with copper. Best fit for the parameters of equation 3 (X_o =187.08 mg/L and Henry's constant = 1).

Equation	Added copper (μM)	k′(L/mg-hr)	k(hr)	k_d	T(μg/mg)	Correlation coefficient r^2
First-order	12.8	0.016	2.99	NA*	NA	0.881
2	12.8	0.046	8.18	1.255	NA	0.989
3	12.8	0.039	6.9	NA	0.19	0.991

NA* indicates that data is not available.

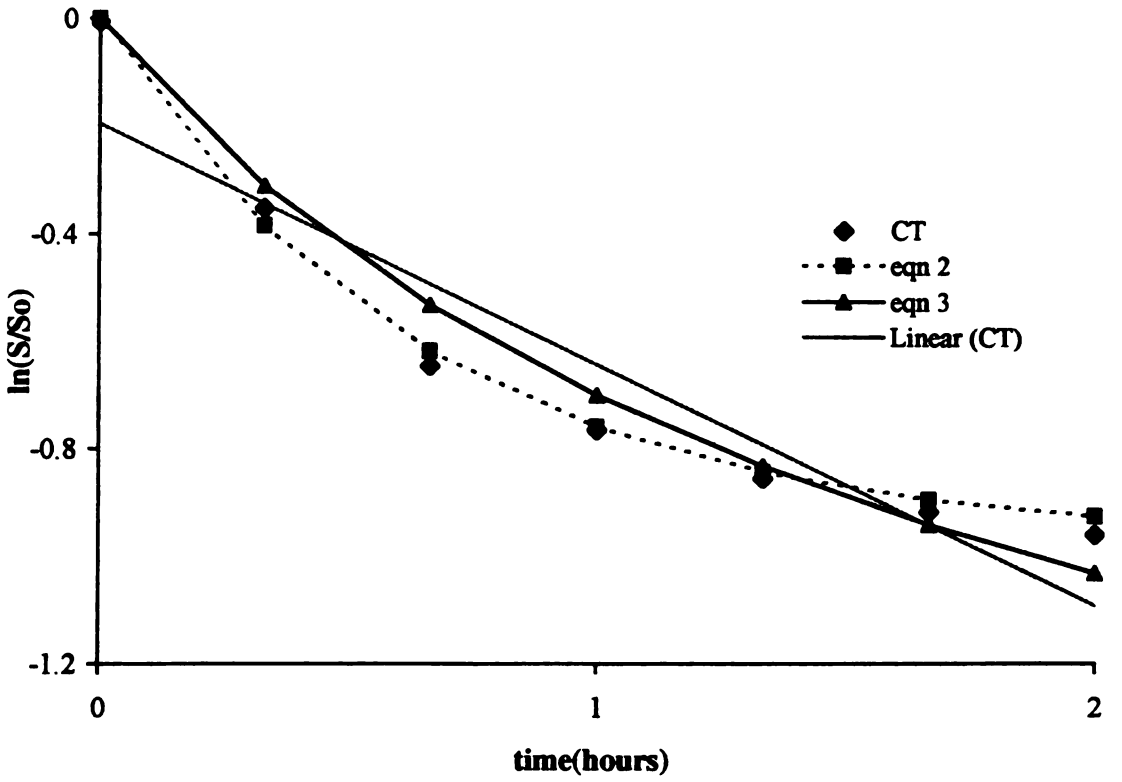

Figure 4.1 Transformation of CT by biomolecule secreted from SGM with 0.32 μM of copper and *Pseudomonas fluorescens* grown from SGM without copper. Fitting parameters are summarized in Table 4.1.

Effects of Copper. Examination of CT degrading activity with selected combinations of cell and freeze-dried fractions of *P. stutzeri* KC revealed a distinct effect due to the presence of copper. Tatara (1995) reported previously that rapid transformation of CCl_4 was only obtained with samples containing added copper and omission of only 1μM copper was sufficient to prevent CCl_4 transformation. The present work confirmed this finding. As shown in Figure 4.2, the effect of added copper on CT degradation was most striking in samples of freeze-dried fractions grown without copper. Samples from freeze-dried fractions grown without copper did not degrade CT to any appreciable extent, but when exogenous copper was added, rapid and appreciable degradation of CT was observed. Exogenous copper added to samples grown in the presence of copper also increased the rate of CT degradation, but not to the same extent as samples grown without copper.

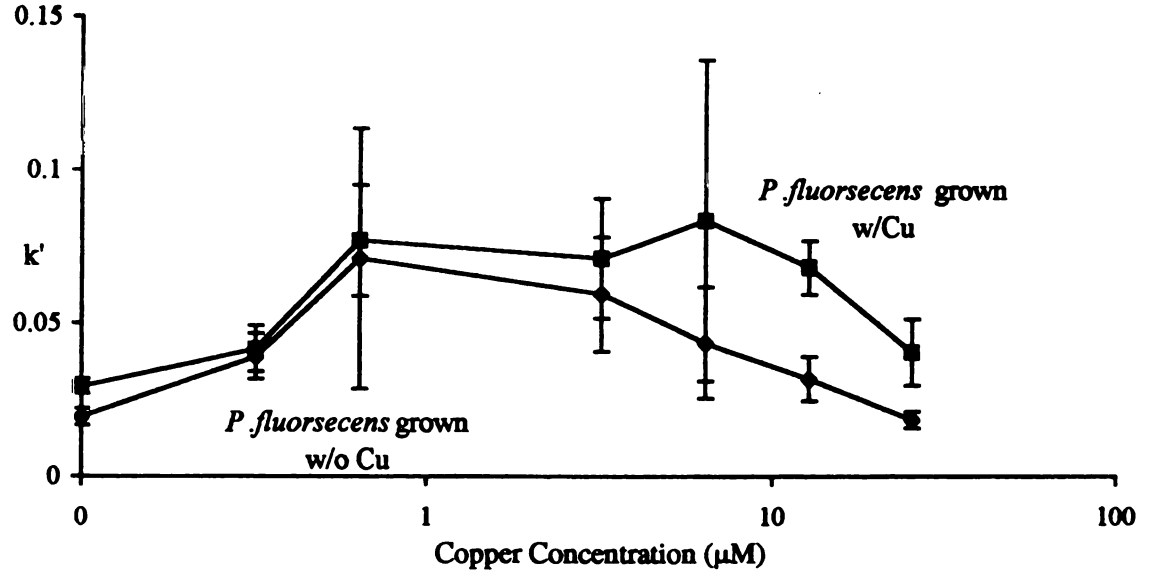

Figure 4.2 Second order coefficients for CT degradation for *P. fluorescens* grown with and without copper using biomolecule from a KC culture grown without copper (Biomolecule was harvested from 2 different cultures: biomolecule from the first culture for experiments with 0, 0.32, 3.2 μM Cu^{2+}; biomolecule from the second culture was used for experiments with 0.64, 6.4 and 25.6 μM Cu^{2+}).

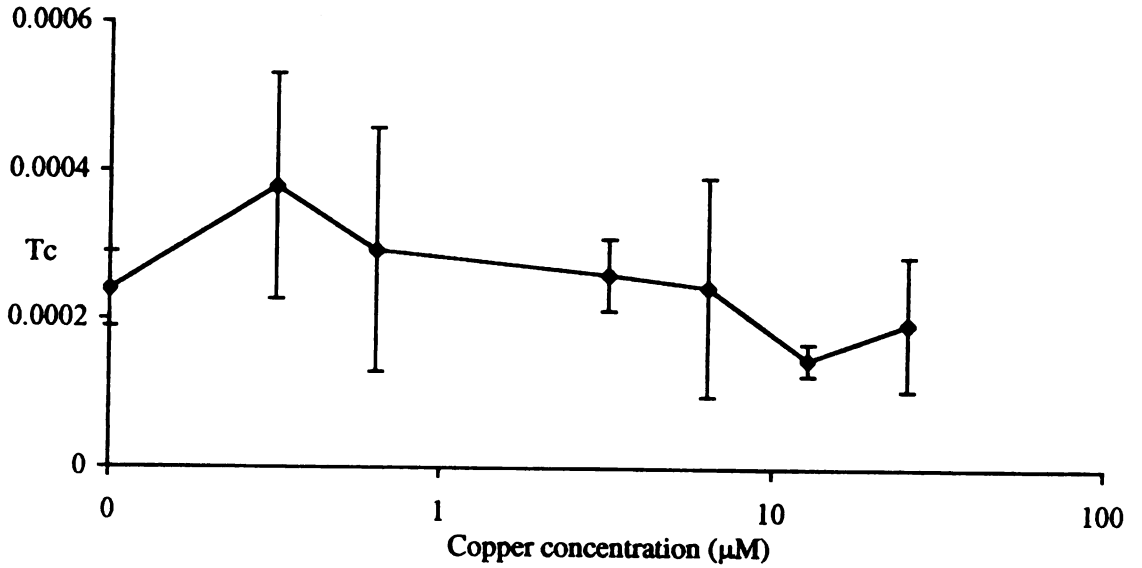

Figure 4.3 CT transformation capacity for *P. fluorescens* grown without copper and using secreted biomolecule from a KC culture grown without copper (Biomolecule was harvested from 2 different cultures: the first culture for experiments with 0, 0.32, 3.2 μM Cu^{2+}; biomolecule from the second culture was used for experiments with 0.64, 6.4 and 25.6 μM Cu^{2+}).

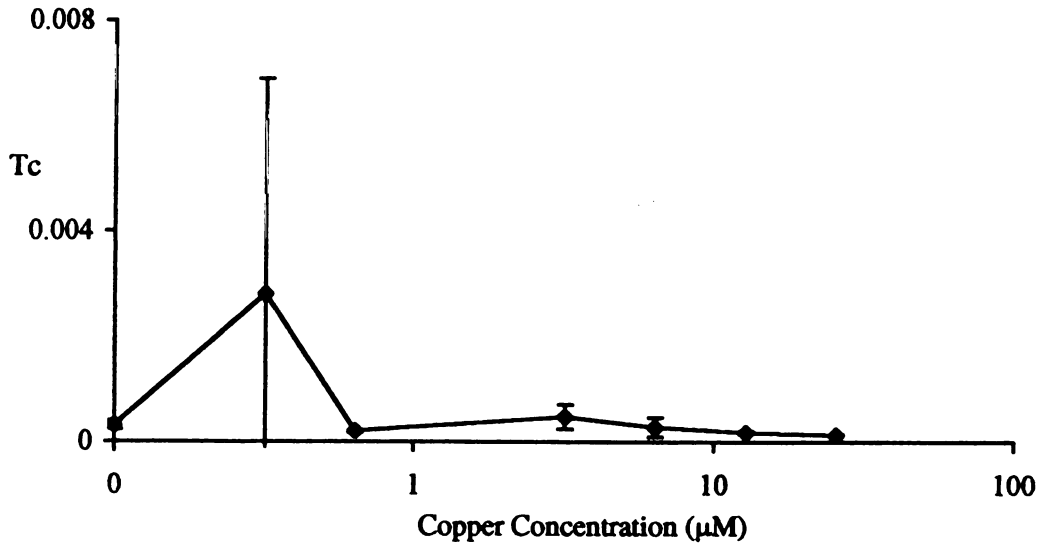

Figure 4.4 CT transformation capacity for *P. fluorescens* grown with copper and using secreted biomolecule from a KC culture grown without copper (Biomolecule was harvested from 2 different cultures: the first culture for experiments with 0, 0.32, 3.2 μM Cu^{2+}; biomolecule from the second culture was used for experiments with 0.64, 6.4 and 25.6 μM Cu^{2+}).

Copper toxicity. Copper addition just prior to initiation of the CT degradation assay markedly increased the extent of CT degradation, and this was true for cells grown in the presence or absence of copper. At low copper concentrations, CT transformation activity increased linearly as exogenous copper concentration was increased. Beyond a certain level, however, additional copper provided no benefit. Copper concentrations over the range of 6.4 μM did not increase degradation activity significantly. A reduced rate of activity was observed at 25.6 μM.

The rate of CT degradation and the effects of copper concentration depended upon the method of biomolecule production. Freeze-dried fractions grown in SGM without copper exhibited greater sensitivity to exogenous copper concentrations and higher tolerance to high copper concentrations. On the other hand, freeze-dried fractions grown in SGM with copper did not show the same sensitivity to copper concentrations. Transformation activity remained stable at copper concentrations of 0.64 μM to 12.8 μM, but rapidly declined at higher levels.

When microorganisms encounter excessive copper concentrations, detoxification is mediated by various mechanisms include energy dependent efflux, intracellular sequestration, and extracellular complexation [10]. The presence of copper in the medium can induce proteins that bind the copper and thereby detoxify it. Competition between such proteins and the secreted factor may explain the higher activity levels of biomolecule in synthetic groundwater in the absence of copper.

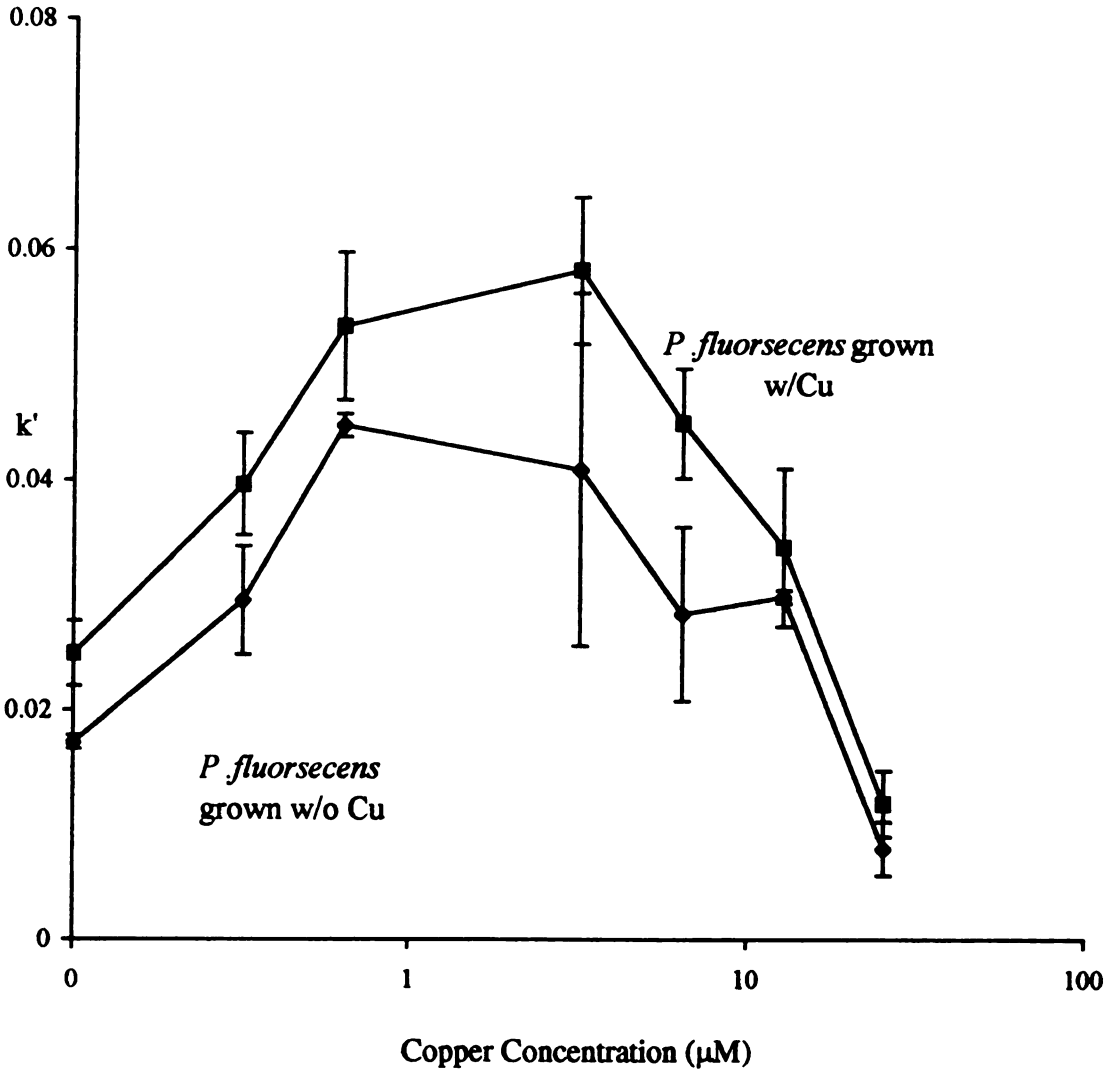

Figure 4.5 Second order coefficients for CT degradation for *P. fluorescens* grown with and without copper using biomolecule from a KC culture grown with copper (Biomolecule was harvested from 2 different cultures: biomolecule from the first culture for experiments with 0, 0.32, 3.2 μM Cu^{2+}; biomolecule from the second culture was used for experiments with 0.64, 6.4 and 25.6 μM Cu^{2+}).

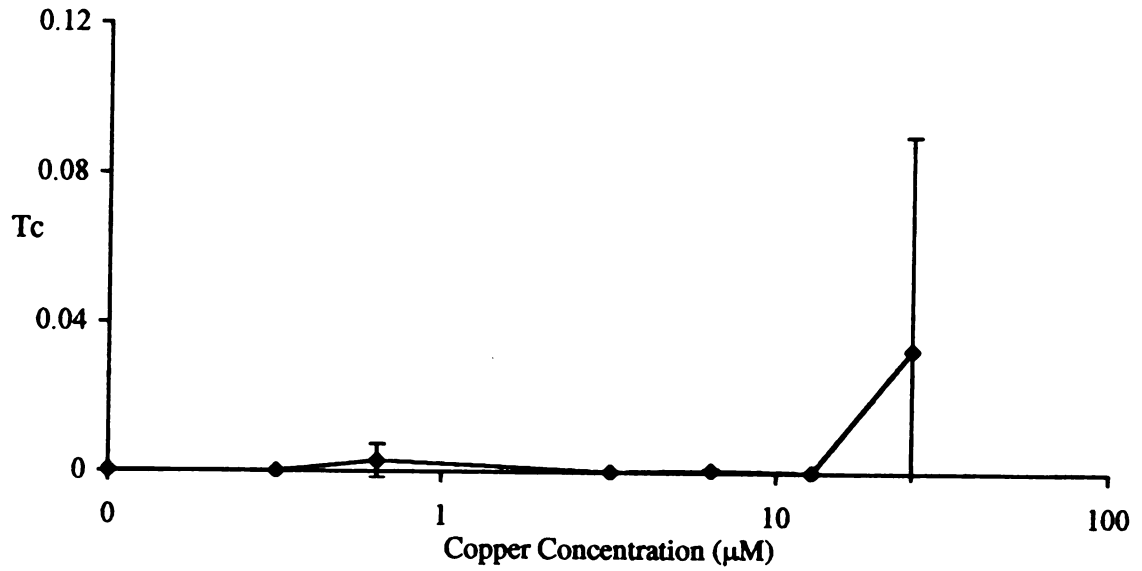

Figure 4.6 CT transformation capacity for *P. fluorescens* grown without copper and using secreted biomolecule from a KC culture grown with copper (Biomolecule was harvested from 2 different cultures: the first culture for experiments with 0, 0.32, 3.2 μM Cu^{2+}; biomolecule from the second culture was used for experiments with 0.64, 6.4 and 25.6 μM Cu^{2+}).

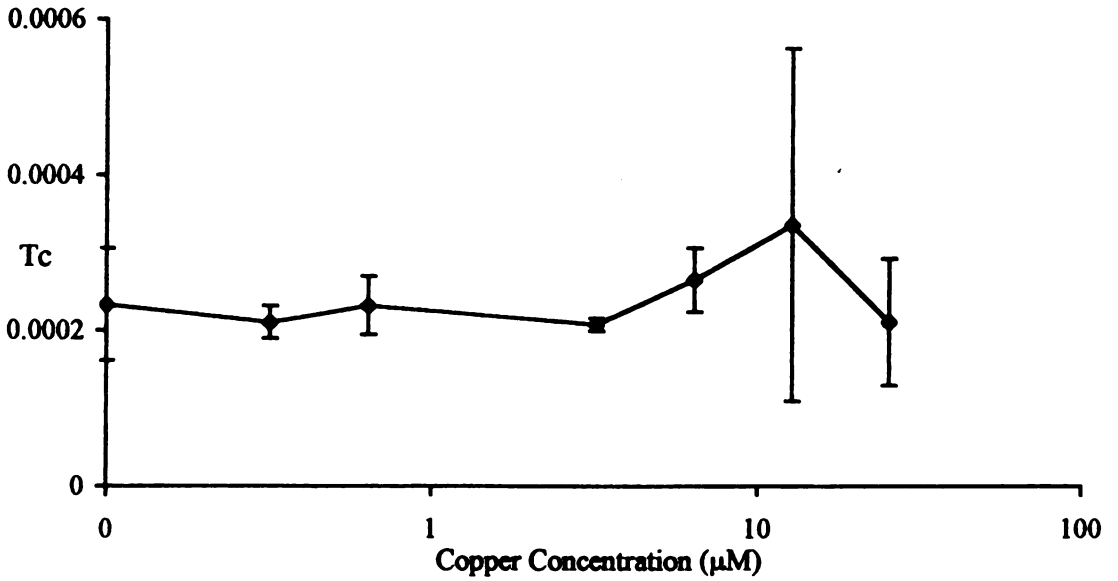

Figure 4.7 CT transformation capacity for *P. fluorescens* grown with copper and using secreted biomolecule from a KC culture grown with copper (Biomolecule was harvested from 2 different cultures: the first culture for experiments with 0, 0.32, 3.2 μM Cu^{2+}; biomolecule from the second culture was used for experiments with 0.64, 6.4 and 25.6 μM Cu^{2+}).

Previous research has established that rapid transformation of CCl_4 requires both cell factors and a secreted factor present in supernatant of *Pseudomonas stutzeri* KC. This study establishes that the role of copper is at the reaction level. Rapid and reliable CCl_4 transformation was obtained when cells fractions were reconstituted with a freeze-dried supernatant fraction from strain KC, confirming the need for both extracellular and intracellular factors. Micromolar levels of copper dramatically enhanced CCl_4 transformation, suggesting that copper participates directly in the reaction, perhaps in a catalytic role.

The results of this work support the conclusion that transformation of CCl_4 by strain KC proceeds by a complex mechanism. The transformation appears to involve three factors: a cell fraction, a supernatant fraction, and copper. Evidence for this hypothesis include the following observations: (1) the freeze-dried supernatant did not transform CT unless cell factors were provided; (2) the freeze-dried fraction harvested from culture grown in SGM without copper did not exhibit high transformation rates, even though cell factors were provided; (3) and the rate of transformation and persistence of activity increased as the added copper increased to an upper limit, depending on the method used to prepare freeze-dried fractions. Tatara (1996) demonstrated that a cytoplasmic component is not required for transformation, and that NADH does not reduce the secreted factor directly. A possible role for copper is in the reduction of the secreted factor possibly in conjunction with some component of the cell membrane. Alternatively, copper may be an essential component of the secreted factor. The secreted factor may function as a mediator of electron transfer between the cell and the CCl_4 molecule. Of significance is the fact the

rate of transformation of secreted factor does not increase linearly as the concentration of copper is increased. Above a certain concentration, additional copper does not increase the transformation rate. At high copper concentrations, decreased transformation rates were observed. At low levels, copper may provide electrons for CCl_4 reduction, but at high levels, reduction or regeneration of the secreted biomolecule may be shut down by copper toxicity.

Stimulated indigenous flora. Regeneration of activity by organisms indigenous to the Schoolcraft aquifer was examined at various copper concentrations. As shown in Figure 4.8, indigenous flora successfully regenerated the activity of a freeze-dried fraction containing the secreted biomolecule. This finding supports previous reports (Tatara; 1995) and indicates that the ecological and transport issues raised by the introduction of KC non-native organism might be avoided by stimulating indigenous flora and adding the secreted factor (plus trace copper). The activity observed with the stimulated flora was greater than that observed with *Pseudomonas fluorescens*.

For samples containing biomolecule harvested from culture grown with copper, the rate of CT degradation activity suddenly increased at an exogenous copper concentration of 3.2 μM and remained high thereafter. On the other hand, freeze-dried fractions from cultures grown in the absence of copper SGM had lower rates of transformation for added copper level exceeding 6.4 μM.

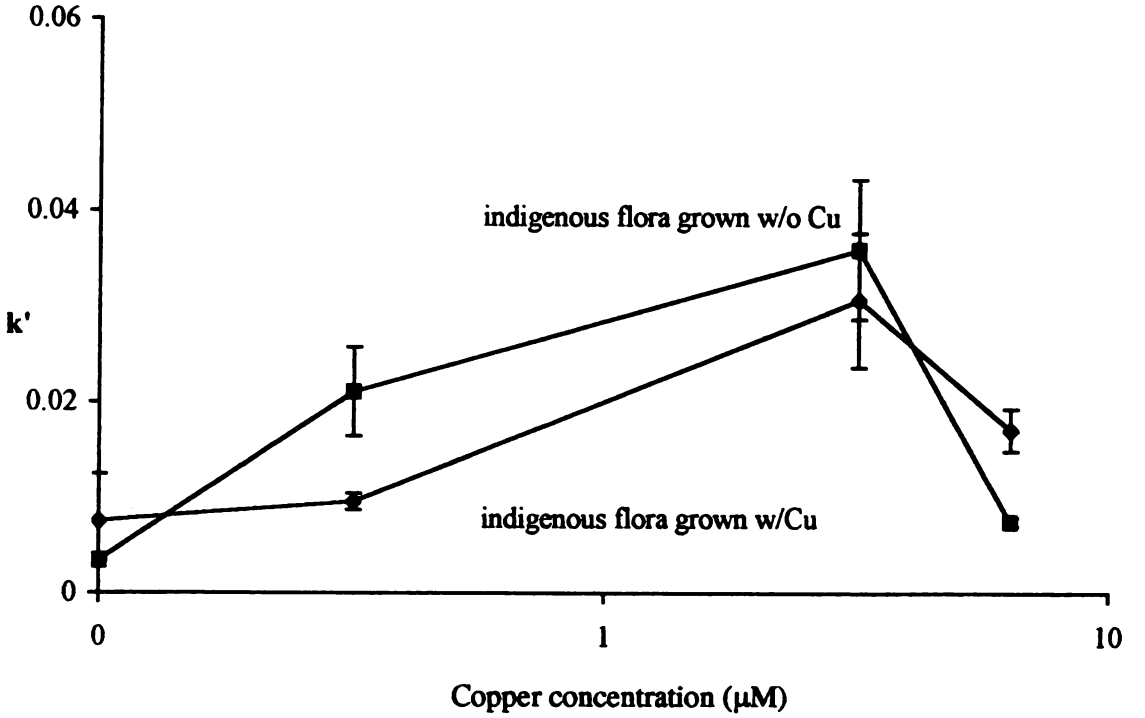

Figure 4.8 Second order rate coefficients for CT degradation for indigenous flora grown with and without copper using biomolecule from a KC culture grown without copper.

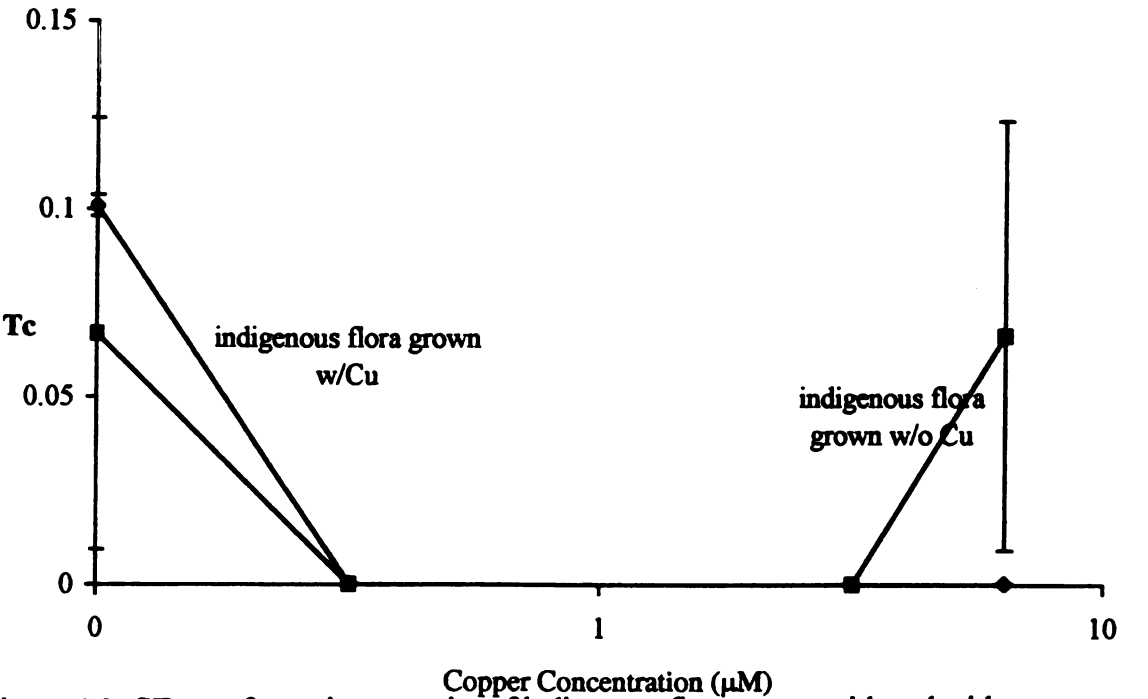

Figure 4.9 CT transformation capacity of indigenous flora grown with and without copper using biomolecule from a KC culture grown without copper.

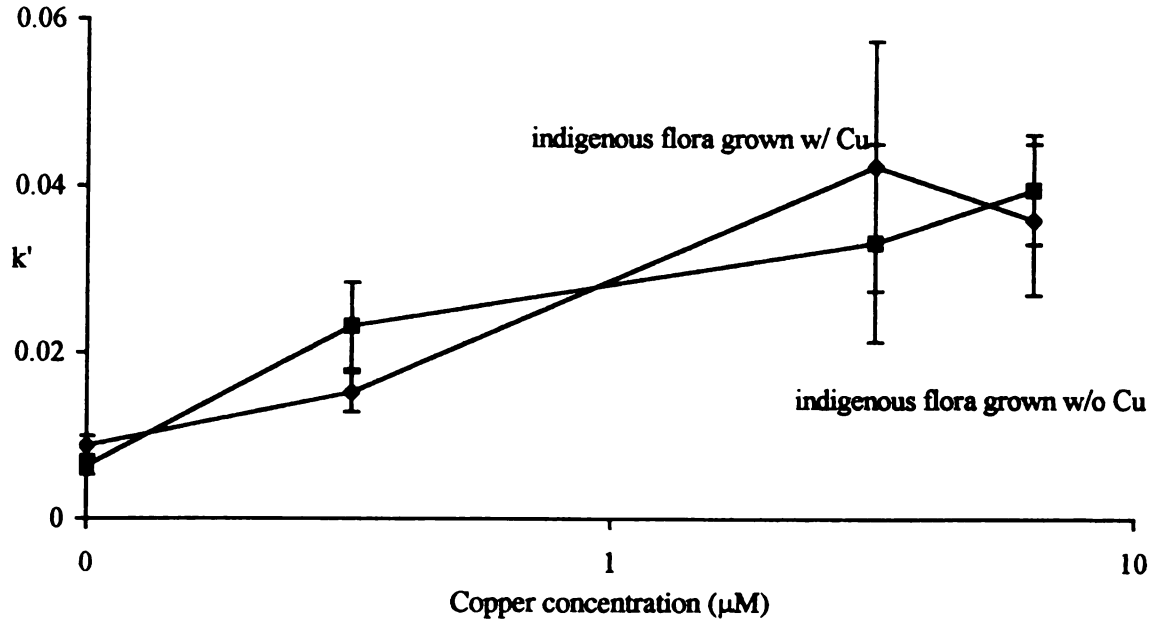

Figure 4.10 Second order rate coefficients of CT degradation for indigenous flora grown with and without copper and biomolecule from a KC culture grown with copper.

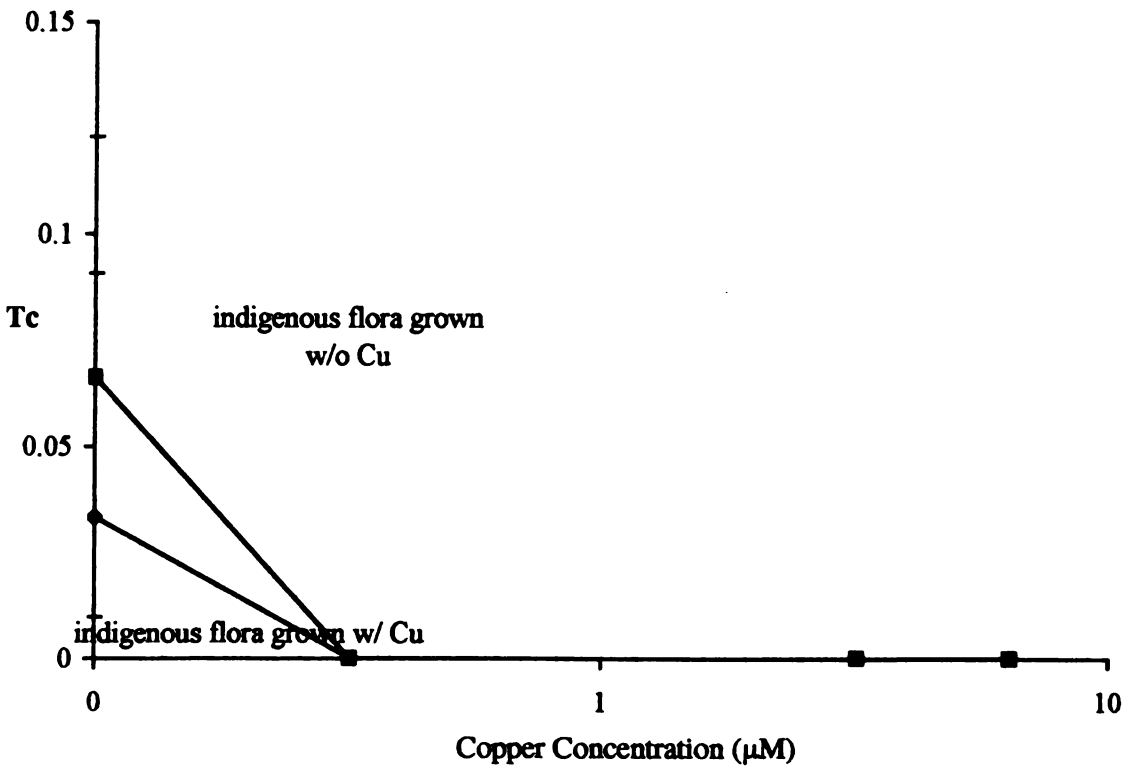

Figure 4.11 CT transformation capacity of indigenous flora grown with and without copper and using biomolecule from a KC culture grown with copper.

Compared to pure cultures like *Pseudomonas fluorescens*, the mixed culture of Schoolcraft aquifer flora had a relatively high standard deviation, perhaps reflecting variability in the populations present.

CHAPTER 5

ISOLATION OF COPPER EFFECT ON BIOMOLECULE

Materials and Methods. To better isolate the effects of copper on the secreted biomolecule produced by *P. stutzeri* KC, copper tolerant and sensitive *Pseudomonas syringae* strains were obtained from the University of California, Berkeley (Courtesy, S. Lindow). The copper sensitive strain Al487 and the copper tolerant strains Al513R were originally isolated from almond trees and bean plants respectively. They were routinely cultured on King's medium B (KB) at room temperature before inoculation to nutrient broth (3). *Pseudomonas syringae* strains were grown in the absence of trace copper in synthetic groundwater for 32 hours and harvested at OD 660 values of approximately 0.2. Cell and suspended fractions were separated by centrifugation (10min, 3000rpm). Cell pellets were resuspended to one tenth their original volume in fresh medium of the identical composition used for growth. CT transformation assays were performed by adding 0.5 ml of *Pseudomonas syringae* culture to 4.5 ml of lyophilized supernatant containing secreted biomolecule produced by *Pseudomonas stutzeri* strain KC, as described in Chapter 4. Copper was added to select samples as $CuSO_4 \bullet 5H_2O$ using a 80mg/L solution or 800mg/L to achieve final concentrations of 0, 0.32, 3.2, 32, 64 μM. Each sample was prepared in triplicate, rendered anoxic by passage through an anaerobic glove box, sealed under an atmosphere of 98% N_2 and 2% H_2, spiked with 10 μl of a 20

mg/L aqueous stock solution of CT, placed on a shaker, then monitored for CT degradation by injection of headspace samples on a gas chromatograph.

Modeling. Assuming product toxicity is the dominant factor causing loss of transformation activity, the disappreance of the target compounds was modeled with equation (3) in Chapter 4, where Xo is the initial *Pseudomonas syringae* (mg/L). The observed yield on acetate for both cultures was calculated using dry weight measurements obtained by filtering a known volume through a pre-weighted 0.2 μm pore filter membrane (Gelman Sciences). Membranes were dried 6 hours at 104 °C and weighed. Ion chromatography was used to measure the acetate consumption. The observed yield was calculated by dividing the increased in dry weight biomass concentration by the total acetate consumed over the identical time period. Actual substrate data at specific time points were used, but dry weight measurements were interpolated from a plot of dry weight versus time. This was necessary because substrate and dry weight data time points were taken at different times. Raw data and supporting calculations are provided in Appendix B.

Results and Discussion

As shown in Figure 5.1, copper sensitive and copper tolerant strains exhibited different patterns of transformation at varied copper concentrations. The rate was low when no copper was added to sensitive cells and suddenly increased as concentration of copper increased. The rate did not increase significantly beyond 3 μM and finally decreased.

Tolerant cell did not show as large an increase in rates, with little decrease in the rate of transformation at higher copper levels. In fact, the rate of CT transformation was highest at the highest copper concentration (Figure 5.1). This difference was even more apparent for copper sensitive cells combined with secreted factor from strain KC cells grown with copper (Figure 5.2). In this case, the rate of transformation decreased drastically for 32 μM copper. The CT degradation rate of copper tolerant cells decreased as the added copper concentration increased.

Examination of CT degrading activity with selected combinations of cell and freeze-dried fractions of *P. stutzeri* KC indicated that the presence of copper in the growth medium of strain KC influenced the transformation pattern obtained. When copper was not present, the rate of degradation and sensitivity toward trace amount of copper addition was higher than when the secreted factor was produced in the presence of copper. This suggests that copper has additional indirect effects on the transformation, beyond its direct effects at the reaction level. When microorganisms encounter excessive copper, detoxification is mediated by various biochemical mechanisms including energy dependent efflux, intracellular sequestration, and extracellular complexation [9]. The presence of copper can induce the production of proteins that detoxify copper by chelating it. These induced proteins may compete with secreted factor. This may explain higher activity for biomolecule produced in synthetic groundwater in the absence of copper.

The rationale for use of copper sensitive and copper tolerant cells was that copper tolerant cells would transform CT more rapidly at high copper concentrations. This was not

observed. Instead, rapid transformation was associated with limited transformation capacity. This may indicate that toxic intermediates are produced causing cell damage or death during CT degradation. Alternatively, copper sensitive cells may have higher rate of degradation because copper tolerant cells produce chelating proteins that compete with the secreted factor for copper.

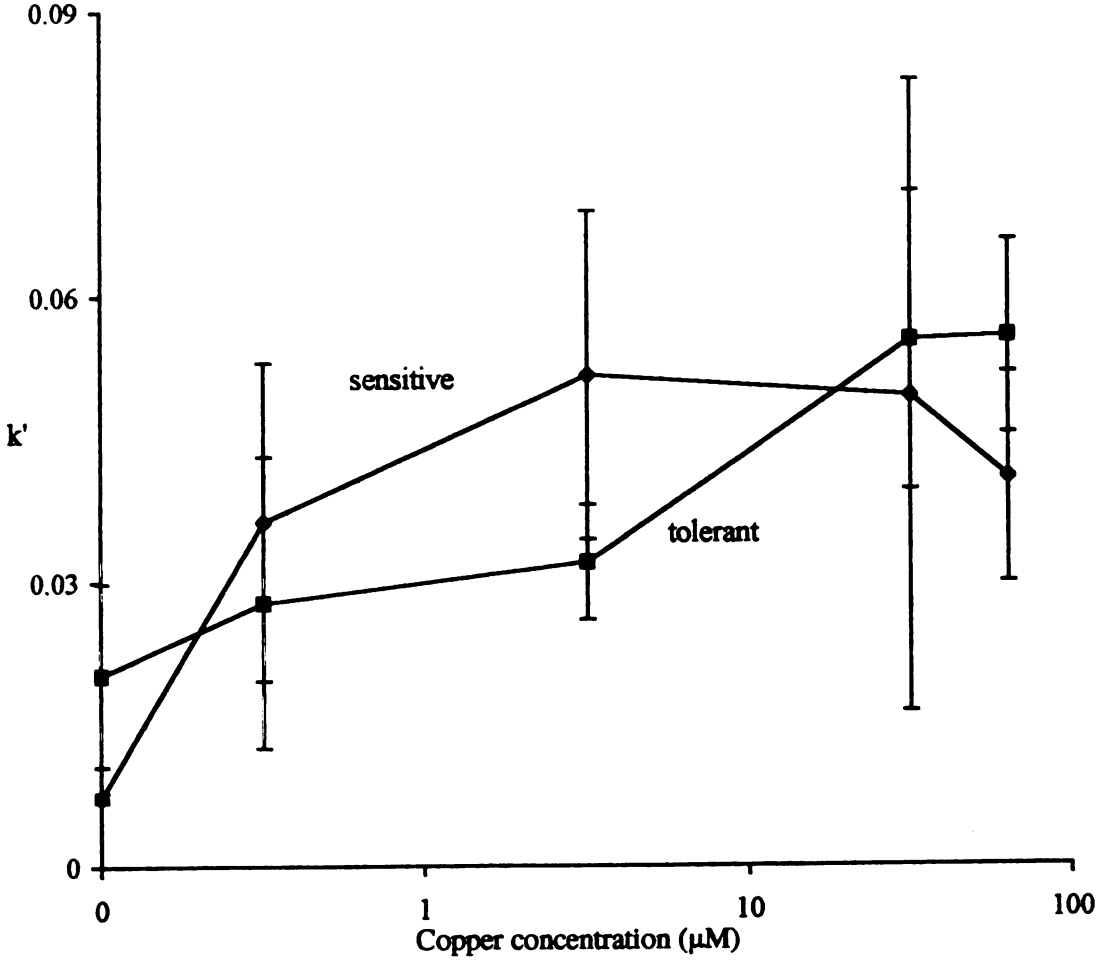

Figure 5.1 Pseudo-second order rate coefficients for copper-sensitive and copper-tolerant *P. syringae* strain grown without copper using secreted biomolecule from a KC culture grown in the absence of copper.

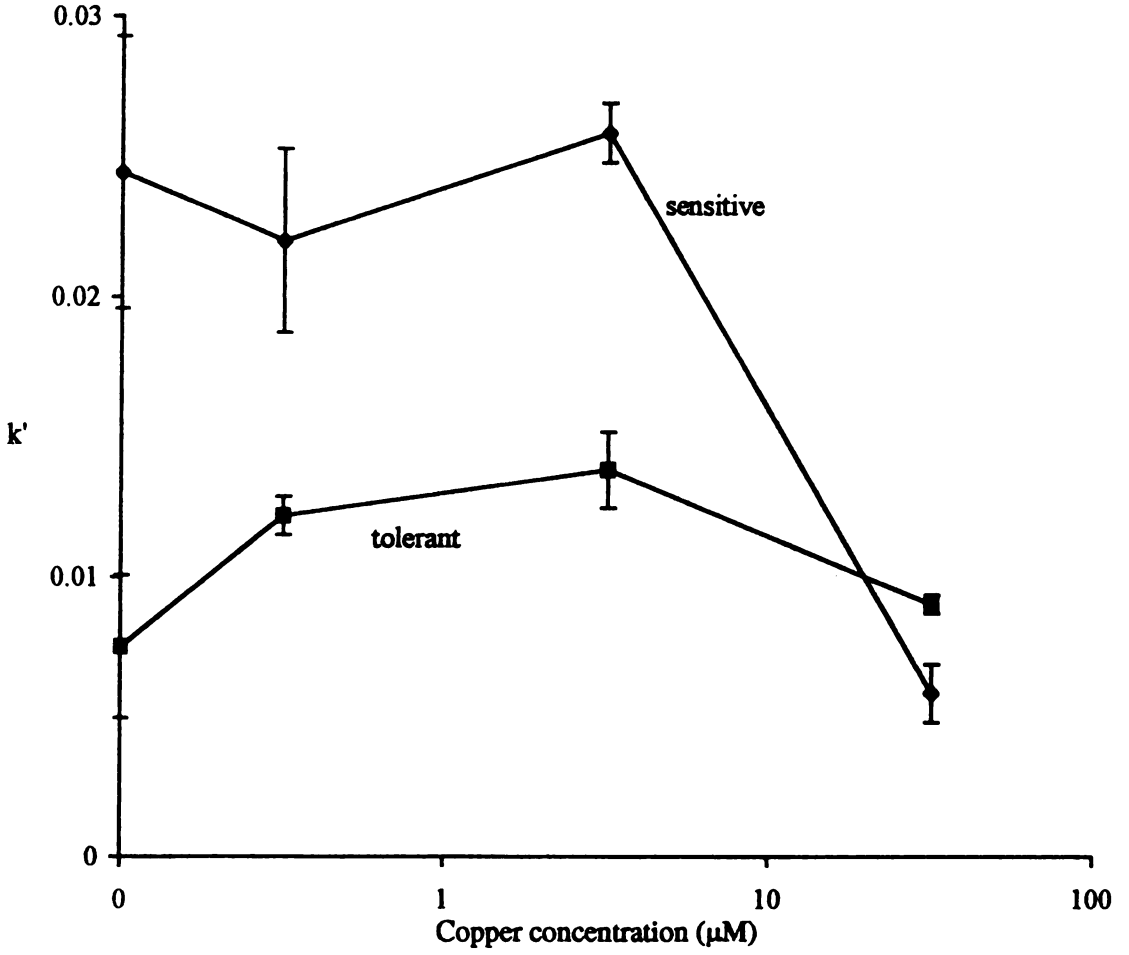

Figure 5.2 Pseudo-second order rate coefficients for copper-sensitive and copper-tolerant *P. syringae* strains grown without copper using secreted biomolecule from a KC culture grown in the presence of copper.

CHAPTER 6

ENGINEERING APPLICATION

The introduction of novel organisms into a new environment is termed bioaugmentation. Many important scientific issues must be considered in evaluating the potential ecological consequences of the planned introduction of organisms into the environment. These include survival and reproduction of the introduced organisms, interactions with other organisms, and the effects of the introduced organisms on ecosystem function. When considering the environmental application of microorganisms, ecological issues are critical.

This study presents a generally favorable outlook for use of the secreted factor produced by *P. stutzeri* KC in field applications. Use of the secreted factor with biostimulated indigenous flora avoids problems associated with the ecology and transport of non-native organisms. Although mixed cultures of indigenous flora from Schoolcraft aquifer had a high capacity for CT transformation, the pattern of transformation depended on the biomolecule production environment. The role of mixed cultures of aquifer flora on CT degradation should be investigated more thoroughly.

Additional work is needed to identify the specific factor(s) that initiate decay of regeneration of the secreted factor and reduction of copper (II) to copper (I) in order to

provide electrons for CT transformation. Instead of adding cupric copper, CT transformation may be evaluated with direct cuprous addition.

This thesis has addressed the catalytic role of copper on biochemical components and processes responsible for cometabolism of CCl_4 degradation. It has raised several questions: What is the secreted factor made of? Why is it produced? What are the factors preventing it from being produced while growing in synthetic groundwater, and is there any way to minimize inhibitory factors preventing its activity? How can we prevent decay of activity during filtration? Once we obtain these fundamental answers about generation and optimization of purification and storage conditions, many engineering applications of these secreted factors are likely to become apparent.

Another question of interest is: What are the differences between the effects of copper obtained in the lab and its effects in field application? Addition of trace copper to groundwater to stimulate CT degradation leads to a paradox: higher copper addition may be needed to achieve high rates of CT degradation, but high copper concentrations may also kill KC or indigenous flora. Therefore, close monitoring and research before application is recommended in order to enhance CT degradation.

A factor that severely limited this research was consistent production of the secreted factor. A small quantity of biomoelcule was produced for each study. Since activity of the biomolecule is not consistent from culture-to-culture, it is difficult to conclude that differences in CT transformation were due to various cell components. Also the increase

in pH caused by addition of freeze dried supernatant may lead to some unknown effect on CT transformation.

CHAPTER 7

CONCLUSIONS

1. Trace amounts of copper are needed to enhance the rates of transformation of CT.

2. Copper affects the CT transformation at the reaction level.

3. Above a certain concentration, copper exerts toxic or inhibitory effects that reduce transformation, even in copper-tolerant cells.

4. Growth of cells in media without copper resulted in the production of secreted factor with higher transformation activity.

5. When the secreted factor was produced by cells grown in synthetic groundwater with 0.32 μM of copper:

- Copper-tolerant *P. syringae* had lower CT transformation rates compared to the copper-sensitive *P. syringae*; except at high copper addition (32 μM).
- CT transformation activity was observed at low copper levels (range of 0 μM to 6.4 μM).
- Copper-tolerant *P. syringae* had a high CT transformation capacity, suggesting reduced toxicity.

6. When the secreted factor was produced by cells grown in synthetic groundwater without copper:

- Higher rates of CT transformation were observed for both copper-tolerant and copper-sensitive cells.
- The highest transformation capacity observed during these experiments was obtained at 0.32 μM copper for the copper-tolerant cells and when no copper was present for the copper-sensitive cells.
- The rates of CT transformation for copper-tolerant *P. syringae* did not increase in proportion to increment of copper concentrations; a decrease in rates was observed at the highest copper concentration tested (32 μM).
- Rates of CT transformation for copper-sensitive *P. syringae* decreased as the amount of exogenous copper increased.

FUTURE WORK RECOMMENDATIONS

1. Investigate possible role and applications of copper for other cometabolic processes.

2. Increase the degradation time for copper-tolerant and copper-sensitive *P. syringae.*

3. Determine how medium components affect secreted factor production and identify methods for maximizing its activity.

4. Enumeration of growth curve according to time interval to improve accuracy.

5. Evaluate CT degradation of indigenous flora grown under denitrifying conditions.

LIST OF REFERENCES

1. Cha, J.S., and D.A. Cooksey. 1991. Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. *Microbiology* **88**:8915-8919.

2. Chang, W., and C.S. Criddle.1995. Biotransformation of HCFC-22, HCFC-142b, HCFC-123, and HFC-134a by methanotrophic mixed culture MM1. *Biodegradation* **6**: 1-9.

3. Criddle, C.S. 1993. Kinetics of Cometabolism. Biotechnology and Bioengineering, 41:1048-1056.

4. Criddle, C.S., J.T.DeWitt, D.Grbric Galic, and P.L. McCarty. 1990. Transformation of carbon tetrachloride by *Pseudomonas* sp. strain KC under denitrification conditions. *Appl. Environ, Microbiol.* 56(11):3240-6.

5. Criddle, C.S, 1989. Reductive Halogenation in Microbial and Electrolytic Model Systems, Stanford University.

6. Diguiseppi, J and I.Fridovich. 1983. Oxy Radicals and their scavenger systems. Vol.I: Molecular Aspects. Elsevier Science Publishing Co., Inc.

7. Dybas, M.J., G.M.Tatara, and C.S. Criddle. 1995. Localization and characterization of the carbon tetrachloride transformation activity of *Pseudomonas* sp. strain KC *Appl. Environ. Microbiol.* 61(2):758-762

8. Gadd, G.M. and A.J.Griffiths 1978. Microorganisms and Heavy Metal Toxicity Microbial Ecology, 4:303-317

9. Gordon, A.S. et al 1992. Growth, Copper-Tolerant Cells, and Extracellular Protein Production in Copper-Stressed Chemostat Cultures of *Vibrio alginolyticus. Appl. Environ. Microbiol.*, 59(1):60-66

10. Harwood-Sears, V. and A.S. Gordon. 1990. Copper-Induced Production of Copper-Binding Supernatant Proteins by Marine Bacterium *Vibrio alginolyticus Appl. Environ. Microbiol.* 56(5):1327-1332.

11. Huges, M.N.,and R.K. Poole. 1991. Metal speciation and microbial growth-the hard (and soft) facts. *J.* of *General Microbiol.* **137**, 725-734

12. Knoll, W.H. 1996. Factors Influencing the Competitive Advantage of *Pseudomonas sp.* strain KC for Subsequent Remediation of A Carbon Tetrachloride Impacted Aquifer., Michigan State University.

13. Lewis, T.A. and R.L.Crawford. 1993. Physiological factors affecting carbon tetrachloride dehalogenation by the denitrifying bacterium *Pseudomonas sp.* strain KC. *Appl. Environ. Microbiol.* 59:1635-1641

14. Markwell, M.A., S.M. Haas, N.E. Tolbert, and L.L. Bieber. 1981. Protein Determination in Membrane Lipoprotein Samples: Manual and Automated Procedures. Methods Enzymol., 72: 296-301.

15. McCord, J.M. 1979. Superoxide dismutase and oxygen toxicity, 109-123. Elsevier Holland, Inc.

16. Menkissoglu, O. and S.E Lindow,.1991. Chemical Forms of Copper on Leaves in Relation to the Bactericidal Activity of Cupric hydroxide Deposits on Plants. Phytopathology, 81: 1263-1270

17. Menkissoglu, O. and S.E. Lindow, 1991. Relationship of Free Ionic Copper and Toxicity to Bacteria in Solutions of Organic Compounds. Phytopathology, 81:1258-1263

18. Mills, S.D.et al 1993. A Two-Component Regulatory System Required for Copper-Inducible Expression of the Copper Resistance Operon of *Pseudomonas syringae. J of Bacteriology,* **175** (6) 1656-1664.

19. Petersen, R. 1982. Influence of Copper and Zinc on the Growth of a Freshwater Alga, *Scenedesmus quadricauda*: The significance of Chemical speciation. Environ. Sci. Technol. 16 (8): 443-447.

20. Schecher, W.D. 1994. Mineql+: A Chemical Equilibrium Program for Personal Computers P&G Co. Hallowell, Maine.

21. Sneathen, mark 1994. Theoretical and Experimental Competitiveness of *Pseudomonas stutzeri* strain KC, Michigan State University

22. Stanier, R.Y. et al 1986. 5th ed. The Microbial World by Prentice Hall, NJ 07632

23. Tartara, gregory michael 1996. Physiology of Carbon Tetrachloride Transformation by *Pseudomonas stutzeri* strain KC, Michigan State University

24. Tartara, G.M., M.J., Dybas, and C.S. Criddle, 1993. Effects of medium and trace metals on kinetics of carbon tetrachloride transformation by *Pseudomonas* sp. strain KC. Appl. Environ. Microbiol. 59:2126-2131.

Appendix A

EFFECT OF INITIAL PH AND COPPER CONCENTRATION ON GROWTH FOR *PSEUDOMONAS FLUORESCENS*, *PSEUDOMONAS* KC, AND *PSEUDOMONAS SYRINGAE*

Table A-1 Effect of initial medium pH on optical density during aerobic growth of *Pseudomonas fluorescens* in synthetic groundwater.[1]

Initial Culture pH	Final Culture pH[1]	Final Optical Density @ 40hrs.(A660)
6.483	6.470 ± 0.0012	0.255 ± 0.036
6.971	8.448 ± 0.0495	0.497 ± 0.058
7.468	9.035 ± 0.0828	0.494 ± 0.027
8.054	9.07 ± 0.0265	0.357 ± 0.012
8.392	8.907± 0.0181	0.314 ± 0.016

Table A-2 Effect of copper concentration on optical density during aerobic growth of *Pseudomonas fluorescens* in synthetic groundwater.[2]
(initial pH 8.172)

Copper concentration (μM)	Final Culture pH[2]	Final Optical Density @ 42 hrs. (A660)
0	9.070± 0.002	0.357 ± 0.034
0.32	8.948 ± 0.0195	0.337 ± 0.001
3.2	8.743 ± 0.0322	0.185 ± 0.032
6.4	8.712 ± 0.0635	0.071 ± 0.017
9.6	8.363 ± 0.0513	0.013 ± 0.002
12.8	8.227 ± 0.0981	0.008 ± 0.004

1. Approximately after 40 hrs.
2. Approximately after 42 hrs.

Table A-3 Effect of initial medium pH on optical density for denitrifying growth of *Pseudomonas fluorescens* in synthetic groundwater.[3]

Initial Culture pH	Final Culture pH[3]	Final Optical Density @27days.(A660)
6.483	6.33 ± 0.0013	0.004 ± 0.002
6.971	7.37 ± 0.0032	0.031 ± 0.012
7.468	8.07 ± 0.0635	0.036 ± 0.008
8.054	8.35 ± 0.0298	0.046 ± 0.004
8.392	8.46 ± 0.0281	0.047 ± 0.012

Table A-4 Effect of copper concentration of optical density on denitrifying growth of *Pseudomonas fluorescens* in synthetic groundwater.[3]
(initial pH 8.15)

Copper concentration (μM)	Final Culture pH[3]	Final Optical Density @27 days.(A660)
0	8.57 ± 0.0024	0.013 ± 0.005
0.32	8.42 ± 0.0325	0.009 ± 0.002
3.2	8.43 ± 0.0315	0.008 ± 0.001
6.4	8.21 ± 0.0365	0.007 ± 0.002
9.6	8.17 ± 0.0213	0.003 ± 0.002

3. Approximately after 27 days.

Table A-5 Effect of copper concentration on protein production for aerobic and denitrifying growth of *Pseudomonas* KC in synthetic groundwater.[4]

(initial pH 8.185)

Copper concentration (μM)	Final Culture pH[4]		Protein Concentration @32 hrs. (μg /ml)	
	aerobic	denitrifying	aerobic	denitrifying
0	8.89 ± 0.0127	8.83 ± 0.0151	163.55 ± 11.55	114.36 ± 6.42
0.32	9.09 ± 0.023	8.97 ± 0.035	121.46 ± 1.31	153.03 ± 1.31
3.2	8.86 ± 0.021	9.01 ± 0.009	124.6 ± 4.03	99.03 ± 4.03
6.4	8.32 ± 0.013	9.18 ± 0.015	105.46 ± 3.43	83.89 ± 4.31
9.6	8.42 ± 0.018	8.459 ± 0.0013	4.79 ± 0.16	7.79 ± 0.16
12.8	8.27 ± 0.012	8.28 ± 0.008	5.36 ± 0.72	8.36 ± 0.72

Table A-6 Effect of copper concentration on protein production for aerobic and denitrifying growth of *Pseudomonas* KC in Schoolcraft groundwater.[4]

Copper concentration (μM)	Final Culture pH[4]		Protein Concentration@32 hrs. (μg /ml)	
	aerobic	denitrifying	aerobic	denitrifying
0	8.93 ± 0.013	8.83 ± 0.012	134.27 ± 25.21	101.97 ± 15.21
0.32	8.91 ± 0.03	9.07 ± 0.031	119.79 ± 14.09	128.69 ± 14.09
3.2	8.52 ± 0.003	8.68 ± 0.008	97.03 ± 4.03	83.69 ± 4.03
6.4	8.32 ± 0.01	8.43 ± 0.017	6.36 ± 0.72	8.46 ± 0.72
9.6	8.36 ± 0.003	8.35 ± 0.007	5.79 ± 0.16	3.4 ± 0.16

4. Approximately after 32 hrs.

Table A-7 Effect of copper concentration on protein production for copper-sensitive and copper-tolerant *Pseudomonas syringae* in synthetic groundwater.[5]
(initial pH 8.109)

Copper concentration (μM)	Final Culture pH		Protein Concentration@114 hrs. (μg /ml)	
	Sensitive	Tolerant	Sensitive	Tolerant
0	9.71 ± 0.014	9.5 ± 0.028	120.14 ± 9.96	109.26 ± 3.09
0.32	9.67 ± 0.001	8.35 ± 0.007	141.79 ± 1.64	128.04 ± 10
1.28	9.66 ± 0.001	9.21 ± 0.014	125.3 ± 6.59	112.33 ± 3.47
3.2	9.56 ± 0.028	*NA	91.79 ± 9.6	NA
12.8	8.13 ± 0.003	9.645 ± 0.035	12.14 ± 4.12	158.82 ± 16.07
32	8.11 ± 0.013	9.115 ± 0.078	1.07 ± 1.05	93.39 ± 3.96
64	NA	8.13 ± 0.028	NA	3.03 ± 0.79

*NA denotes data that was not available.

Table A-8 Effect of initial medium pH on protein production for copper-sensitive and copper-tolerant *Pseudomonas syringae* in synthetic groundwater.[5]

Initial Culture pH	Final Culture pH		Protein Concentration @114 hrs. (μg /ml)	
	Sensitive	Tolerant	Sensitive	Tolerant
6.426	6.415 ± 0.007	6.435 ± 0.021	14.1 ± 0.52	6.07 ± 0.06
6.932	9.01 ± 0.085	8.88 ± 0.24	106.92 ± 6.09	95.47 ± 11.07
7.493	9.46 ± 0.03	9.44 ± 0.028	131.31 ± 11.96	125.98 ± 10.17
7.958	9.575 ± 0.035	9.52 ± 0.028	107.8 ± 18.31	119.68 ± 1.34
8.109	9.51 ± 0.014	9.545 ± 0.035	97.1 ± 22.72	111.25 ± 0.21
8.350	9.61 ± 0.014	9.555 ± 0.007	101.92 ± 18.54	104.42 ± 16.76

5. Approximately after 114 hrs.

Appendix B

ORIGINAL DATA AND CALCULATIONS FOR BIOMASS USED ON MODELING

Table B-1 Original data used for determination of biomass for *Pseudomonas fluorescens* in SGM with and without copper for modeling.

(Samples in triplicate)

Pseudomonas fluorescens								
Time	Average Weight[1]		Stdev Weight		Average OD[2]		Stdev OD	
	W/o Cu	W/Cu	W/o Cu	W/Cu	W/o Cu	W/Cu	W/o Cu	W/ Cu
0	0.0001	0.0001	0	0	0	0.001	0	1.7E-5
8	0.0007	0.0007	5.77E-5	1.03E-11	0.0196	0.022	0.0052	0.003
16	0.0009	0.001	5.77E-5	5.77E-5	0.1032	0.092	0.01	0.01
24	0.0032	0.0025	0.00015	0.00015	0.33	0.242	0.01	0.019
36	0.0042	0.0034	0.00025	0.0001	0.387	0.381	0.012	0.012

OD versus biomass concentration for *P. fluorescens* in SGM w/o copper: Biomass = 0.0099* OD + 0.0002, $r^2 = 0.9797$

OD versus biomass concentration for *P. fluorescens* in SGM w/copper: Biomass = 0.0084* OD + 0.0003, $r^2 = 0.9805$

Table B-2 Original data used for determination of biomass for *Pseudomonas syringae* in SGM with and without copper.

(Samples in triplicate)

P.syringae Copper Sensitive								
Time	Average Weight[1]		Stdev Weight		Average OD[2]		Stdev OD	
	W/o Cu	W/Cu	W/o Cu	W/Cu	W/o Cu	W/Cu	W/o Cu	W/ Cu
0	0.0001	0.0001	0	0	0	0.001	0	1.7E-5
24	0.00075	0.00105	7.07E-5	7.07E-5	0.0177	0.02	0.0038	0.0023
36	0.0019	0.0023	0.00014	0.00014	0.101	0.155	0.0053	0.034
58	0.0041	0.0032	0	0.00014	0.424	0.358	0.024	0.042

P.syringae Copper Tolerant								
Time	Average Weight		Stdev Weight		Average OD		Stdev OD	
	W/o Cu	W/Cu	W/o Cu	W/Cu	W/o Cu	W/Cu	W/o Cu	W/ Cu
0	0.0001	0.0001	0	0	0	0.001	0	1.7E-5
24	0.00065	0.00085	0.00035	7.07E-5	0.0177	0.02	0.0037	0.0023
36	0.0022	0.0017	0	0.00014	0.103	0.124	0.013	0.023
58	0.0039	0.004	0.00014	0.00028	0.403	0.471	0.0025	0.0393

1. Units in g/10ml.
2. Optical density measured in A660.

OD versus biomass concentration for copper-sensitive *P. syringae* in SGM w/o copper: Biomass = 0.0079* OD + 0.0008, $r^2 = 0.9746$

OD versus biomass concentration for copper-sensitive *P. syringae* in SGM w/copper: Biomass = 0.0058* OD + 0.0012, $r^2 = 0.9695$

OD versus biomass concentration for copper-tolerant *P. syringae* in SGM w/o copper: Biomass = 0.0072* OD + 0.0011, $r^2 = 0.9462$

OD versus biomass concentration for copper-tolerant *P. syringae* in SGM w/copper: Biomass = 0.007* OD + 0.0007, $r^2 = 0.9971$

Table B-3 Data and calculations used for determining the observed yield of *Pseudomonas fluorescens, Pseudomonas stutzeri* KC, and *Pseudomonas Syringae* grown in SGM without copper using largest dry weight value.

(Samples in triplicate)

Pseudomonas fluorescens				
Initial pH	Acetate consumed (mM)	Dry Weight (mg/L)	Observed Yield (mg cells/mg acetate)	Final pH@ 40 hrs
7	6.78	480	0.407	8.96
8.2	6.73	460	0.404	9.084
Pseudomonas KC				
Initial pH	Acetate consumed (mM)	Dry Weight (mg/L)	Observed Yield (mg cells/mg acetate)	Final pH@ 40 hrs
7	6.89	530	0.413	8.86
8.2	6.65	500	0.399	9.278
Copper sensitive *Pseudomonas Syringae*				
Initial pH	Acetate consumed (mM)	Dry Weight (mg/L)	Observed Yield (mg cells/mg acetate)	Final pH@ 5 days
7	6.58	490	0.395	8.756
8.2	6.47	470	0.388	9.125
Copper tolerant *Pseudomonas Syringae*				
Initial pH	Acetate consumed (mM)	Dry Weight (mg/L)	Observed Yield (mg cells/mg acetate)	Final pH@ 5 days
7	5.68	440	0.341	8.723
8.2	5.99	450	0.360	9.108

Appendix C

EFFECT OF COPPER ON SECOND ORDER RATE COEFFICIENT AND TRANSFORMATION CAPACITY BY SECRETED FACTOR PRODUCED FROM *PSEUDOMONAS* KC

Table C-1 Second order rate coefficient and transformation capacity by secreted factor produced in synthetic groundwater without copper.

Biomolecule[1]	Growth condition	Cu added (μM)	K'(L / mg-h)	Tc (μg CT/mg)	r^2
-Cu[2]	+ Cu[3]	0	0.03 ± 0.002	0.32 ± 8E-02	0.983
		0.32	0.042 ± 0.0075	2.8 ± 4.1	0.983
		0.64	0.077 ± 0.0181	0.2 ± 2E-02	0.987
		3.2	0.071 ± 0.0195	0.47 ± 0.2	0.95
		6.4	0.083 ± 0.0524	0.3 ± 0.2	0.983
		12.8	0.068 ± 0.0087	0.2 ± 1E-02	0.99
		25.6	0.04 ± 0.0109	0.1 ± 7E-03	0.87
	- Cu[4]	0	0.02 ± 0.003	0.24 ± 5E-02	0.99
		0.32	0.039 ±0.0075	0.38 ±0.2	0.98
		0.64	0.071 ± 0.0424	0.32 ± 0.2	0.99
		3.2	0.059 ± 0.0186	0.26 ±5E-02	0.99
		6.4	0.044 ± 0.018	2.8 ± 0.1	0.977
		12.8	0.032 ± 0.0072	0.47 ± 2E-02	0.977
		25.6	0.018 ± 0.0027	0.24 ± 5E-02	0.90

Table C-2 Second order rate coefficient and transformation capacity by secreted factor produced in synthetic groundwater with copper.

Biomolecule[1]	Growth condition	Cu added (μM)	K'(L / mg*h)	Tc (μg CT/mg)	r^2
+Cu[5]	+ Cu[3]	0	0.025 ± 0.0028	0.23 ± 7E-02	0.977
		0.32	0.04 ± 0.0044	2.1 ± 2E-02	0.987
		0.64	0.053 ± 0.006	0.23 ± 4E-02	0.987
		3.2	0.058 ± 0.0063	0.21 ± 8E-03	0.983
		6.4	0.045 ± 0.005	0.27 ± 4E-02	0.99
		12.8	0.034 ± 0.007	0.34 ± 0.2	0.99
		25.6	0.012 ± 0.003	0.21 ± 8E-02	0.907
	- Cu[4]	0	0.017 ± 0.0006	0.29 ± 3E-02	0.99
		0.32	0.03 ±0.0047	0.19 ±5E-02	0.98
		0.64	0.045 ± 0.001	2.89 ± 4.5	0.99
		3.2	0.041 ± 0.0153	0.23 ±7E-02	0.99
		6.4	0.028 ± 0.008	0.63± 0.4	0.99
		12.8	0.03 ± 0.001	0.16 ± 7E-03	0.977
		25.6	0.008 ± 0.002	33.2 ± 57.3	0.95

1. Biomolecule harvested in 2 different cultures : a first set for experiments with 0, 0.32, 3.2 μM Cu^{2+} ; a second set for experiments with 0.64, 6.4 and 25.6 μM Cu^{2+}
2. Secreted factor produced in synthetic groundwater without copper.
3. *P. fluorescens* grown in 0.32 μM synthetic groundwater.
4. *P. fluorescens* grown in synthetic groundwater in absence of copper.
5. Secreted factor produced in 0.32 μM synthetic groundwater.

Table C-3 Second order rate coefficient and transformation capacity by secreted factor produced in synthetic groundwater with copper and stimulated indigenous flora grown with and without copper in Schoolcraft groundwater.

Biomolecule	Growth condition	Cu added (μM)	K'(L / mg*h)	Tc (μg CT/mg)	r^2
-Cu[6]	+ Cu[7]	0	0.01 ± 0.005	101 ± 2.8	0.79
		0.32	0.01 ± 0.001	3.2E-01 ± 0.2	0.81
		3.2	0.03 ± 0.007	1.3E-01 ± 6E-03	0.97
		6.4	0.02 ± 0.002	3E-01 ± 0.2	0.88
	- Cu[8]	0	3E-03 ± 1E-04	67 ± 57.4	0.72
		0.32	0.02 ±0.005	1.1E-01 ± 2E-03	0.96
		3.2	0.04 ± 0.007	1.3E-01±7E-03	0.98
		6.4	0.01 ± 4E-04	60 ± 57.2	0.93
+Cu[9]	+ Cu[7]	0	0.01 ± 0.001	33 ± 57.4	0.85
		0.32	0.02 ± 0.002	0.2± 7E-02	0.93
		3.2	0.04 ± 0.015	0.3 ± 0.1	0.97
		6.4	0.04 ± 0.009	0.2 ± 0.1	0.96
	- Cu[8]	0	0.01 ± 0.001	67 ± 56.6	0.90
		0.32	0.02 ±0.005	0.2 ± 0.1	0.98
		3.2	0.03 ± 0.012	0.3 ± 0.1	0.98
		6.4	0.04 ± 0.007	0.1 ± 2E-02	0.98

6. Secreted factor produced in synthetic groundwater without copper.
7. Indigenous flora grown in 0.32 μM synthetic groundwater.
8. Indigenous flora grown in synthetic groundwater in absence of copper.
9. Secreted factor produced in 0.32 μM synthetic groundwater.

Appendix D

EFFECT OF COPPER ON FIRST ORDER RATE COEFFICIENT AND DECAY COEFFICIENT BY SECRETED FACTOR PRODUCED FROM *PSEUDOMONAS* KC

Table D-1 First order rate coefficient and decay coefficient by secreted factor produced in synthetic groundwater without copper.

Biomolecule[1]	Growth condition	Cu added (μM)	K(min^{-1})	K_d (min^{-1})	r^2
-Cu[2]	+ Cu[3]	0	0.098 ± 0.014	0.0098 ± 0.0032	0.987
		0.32	0.2 ± 0.0359	0.022 ± 0.00078	0.98
		0.64	0.2 ± 0.035	0.019 ± 0.0046	0.983
		3.2	0.246 ± 0.0466	0.017 ± 0.008	0.983
		6.4	0.31 ± 0.068	0.037 ± 0.011	0.99
		12.8	0.26 ± 0.029	0.034 ± 0.0063	0.983
		25.6	0.23 ± 0.01	0.055 ± 0.0079	0.99
	- Cu[4]	0	0.08 ± 0.0097	0.011 ± 0.0022	0.983
		0.32	0.124 ± 0.014	0.0066 ± 0.00115	0.99
		0.64	0.241 ± 0.0641	0.022 ± 0.0073	0.99
		3.2	0.186 ± 0.0135	0.0135 ± 8.7E-04	0.99
		6.4	0.16 ± 0.038	0.0199 ± 0.0073	0.96
		12.8	0.173 ± 0.013	0.035 ± 0.00396	0.99
		25.6	0.16 ± 0.029	0.04 ± 0.011	0.987

Table D-2 First order rate coefficient and decay rate by secreted factor produced in synthetic groundwater with copper.

Biomolecule[1]	Growth condition	Cu added (μM)	K(min^{-1})	K_d (min^{-1})	r^2
$+Cu^5$	$+ Cu^3$	0	0.085 ± 0.012	0.012 ± 0.0044	0.97
		0.32	0.121 ± 0.014	0.014 ± 0.005	0.98
		0.64	0.157 ± 0.0069	0.015 ± 0.00032	0.987
		3.2	0.17 ± 0.042	0.017 ± 0.0095	0.987
		6.4	0.152 ± 0.015	0.015 ± 0.0027	0.99
		12.8	0.158 ± 0.0124	0.023 ± 0.0022	0.99
		25.6	0.044 ± 0.0045	0.0098 ± 0.0008	0.947
	$- Cu^4$	0	0.072 ± 0.015	0.0096 ± 0.0054	0.987
		0.32	0.092 ± 0.017	0.0096 ± 0.0055	0.957
		0.64	0.137 ± 0.0006	0.011 ± 0.0006	0.99
		3.2	0.17 ± 0.025	0.022 ± 0.007	0.977
		6.4	0.123 ± 0.011	0.011± 0.004	0.99
		12.8	0.13 ± 0.005	0.023 ± 0.002	0.99
		25.6	0.03 ± 0.0047	0.008 ± 0.00073	0.97

1. Biomolecule harvested in 2 different cultures : a first set for experiments with 0, 0.32, 3.2 μM Cu^{2+} ; a second set for experiments with 0.64, 6.4 and 25.6 μM Cu^{2+}
2. Secreted factor produced in synthetic groundwater without copper.
3. *P. fluorescens* grown in 0.32 μM synthetic groundwater.
4. *P. fluorescens* grown in synthetic groundwater in absence of copper.
5. Secreted factor produced in 0.32 μM synthetic groundwater.

Table D-3 First order rate coefficient and decay coefficient by secreted factor produced in synthetic groundwater with copper and stimulated indigenous flora grown with and without copper in Schoolcraft groundwater.

Biomolecule	Growth condition	Cu added (μM)	K(min^{-1})	K_d (min^{-1})	r^2
-Cu[6]	+ Cu[7]	0	0.061 ± 0.03	0.0246 ± 0.018	0.887
		0.32	0.135 ± 0.055	0.0375 ± 0.0194	0.91
		3.2	0.31 ± 0.119	0.037 ± 0.0159	0.93
		6.4	0.162 ± 0.035	0.031 ± 0.007	0.87
	- Cu[8]	0	0.025 ± 0.01	0.024 ± 0.0058	0.87
		0.32	0.154 ± 0.052	0.037 ± 0.016	0.98
		3.2	0.31 ± 0.063	0.036 ± 0.0067	0.97
		6.4	0.089 ± 0.045	0.021 ± 0.0169	0.96
+Cu[9]	+ Cu[7]	0	0.21 ± 0.078	0.039 ± 0.011	0.92
		0.32	0.099 ± 0.038	0.008± 0.0087	0.94
		3.2	0.35 ± 0.009	0.03 ± 0.00052	0.983
		6.4	0.35 ± 0.153	0.03 ± 0.0146	0.987
	- Cu[8]	0	0.148 ± 0.085	0.0144 ± 0.002	0.96
		0.32	0.153 ± 0.0176	0.029 ± 0.00052	0.977
		3.2	0.295 ± 0.144	0.022 ± 0.0115	0.987
		6.4	0.3 ± 0.061	0.023 ± 0.0026	0.99

6. Secreted factor produced in synthetic groundwater without copper.
7. Indigenous flora grown in 0.32 μM synthetic groundwater.
8. Indigenous flora grown in synthetic groundwater in absence of copper.
9. Secreted factor produced in 0.32 μM synthetic groundwater.

Appendix E

EFFECT OF COPPER ON SECOND ORDER RATE COEFFICIENT AND TRANSFORMATION CAPACITY BY SECRETED FACTOR PRODUCED FROM *PSEUDOMONAS* KC

Table E-1 Second order rate coefficient and transformation capacity by secreted factor produced in synthetic groundwater in absence of copper.

Biomolecule	Cell factor	Cu added (μM)	K'(L / mg*h)	Tc (μg CT/mg)	r^2
-Cu[1]	Sensitive[2]	0	0.007 ± 0.0005	6.5 ± 56	0.96
		0.32	0.036 ± 0.0167	0.3 ± 0.2	0.97
		3.2	0.052 ± 0.0173	0.18 ± 1E-02	0.99
		32	0.049 ± 0.033	0.17 ± 2E-02	0.99
		64	0.041 ± 0.011	0.11 ± 8E-02	0.98
	Tolerant[3]	0	0.02 ± 0.0096	0.12 ± 3E-02	0.79
		0.32	0.028 ± 0.0153	0.35 ± 0.1	0.98
		3.2	0.032 ± 0.006	0.29 ± 0.2	0.96
		32	0.055 ± 0.016	0.2 ± 2E-02	0.99
		64	0.056 ± 0.01	0.16 ± 1E-02	0.97

Table E-2 Second order rate coefficient and transformation capacity by secreted factor produced in synthetic groundwater in presence of copper.

Biomolecule	Cell factor	Cu added (μM)	K'(L / mg*h)	Tc (μg CT/mg)	r^2
+Cu[4]	Sensitive[2]	0	0.024 ± 0.0048	0.29 ± 0.2	0.98
		0.32	0.022 ± 0.0033	3.3 ± 57.5	0.99
		3.2	0.026 ± 0.0011	0.4 ± 6E-02	0.98
		32	0.006 ± 0.001	99.4 ± 0.5	0.94
	Tolerant[3]	0	0.008 ± 0.0025	2435000 ± 421800	0.95
		0.32	0.012 ± 0.0007	1859000 ± 322000	0.99
		3.2	0.014 ± 0.0014	63 ± 55	0.97
		32	0.009 ± 0.0003	2497000 ± 217900	0.97

1. Secreted factor produced in synthetic groundwater without copper.
2. Copper-sensitive *P. syringae* grown in synthetic groundwater without copper.
3. Copper-tolerant *P. syringae* grown in synthetic groundwater without copper.
4. Secreted factor produced in synthetic groundwater containing 0.32 μM copper.

Appendix F

EFFECT OF COPPER ON FIRST ORDER RATE AND DECAY COEFFICIENT BY SECRETED FACTOR PRODUCED FROM *PSEUDOMONAS* KC

Table F-1 First order rate coefficient and decay coefficient by secreted factor produced in synthetic groundwater in absence of copper.

Biomolecule	Cell factor	Cu added (μM)	K(min^{-1})	K_d (min^{-1})	r^2
-Cu[1]	Sensitive[2]	0	0.035 ± 0.0017	0.0051 ± 0.00176	0.86
		0.32	0.21 ± 0.073	0.031 ± 0.0089	0.983
		3.2	0.258 ± 0.1	0.032 ± 0.0145	0.99
		32	0.165 ± 0.056	0.023 ± 0.0086	0.99
		64	0.165 ± 0.0206	0.04 ± 0.0041	0.88
	Tolerant[3]	0	0.28 ± 0.138	0.096 ± 0.023	0.917
		0.32	0.154 ± 0.114	0.0195 ± 0.021	0.99
		3.2	0.33 ± 0.131	0.043 ± 0.014	0.99
		32	0.244 ± 0.067	0.035 ± 0.0075	0.983
		64	0.329 ± 0.12	0.049 ± 0.0169	0.877

Table F-2 Second order rate coefficient and transformation capacity by secreted factor produced in synthetic groundwater in presence of copper.

Biomolecule	Cell factor	Cu added (μM)	K(min^{-1})	K_d (min^{-1})	r^2
+Cu[4]	Sensitive[2]	0	0.133 ± 0.0165	0.017 ± 0.0042	0.987
		0.32	0.1 ± 0.012	0.0072 ± 0.0024	0.99
		3.2	0.137 ± 0.026	0.012 ± 0.0046	0.98
		32	0.034 ± 0.01	0.0084 ± 0.0056	0.96
	Tolerant[3]	0	0.031 ± 0.01	0.0005 ± 0.00057	0.957
		0.32	0.055 ± 0.0019	0.0035 ± 0.0032	0.987
		3.2	0.066 ± 0.012	0.0061 ± 0.0046	0.967
		32	0.042 ± 0.0097	0.0049 ± 0.00845	0.97

1. Secreted factor produced in synthetic groundwater without copper.
2. Copper-sensitive *P. syringae* grown in synthetic groundwater without copper.
3. Copper-tolerant *P. syringae* grown in synthetic groundwater without copper.
4. Secreted factor produced in synthetic groundwater containing 0.32 μM copper.

Appendix G

PRELIMINARY STUDY

- **Effect of Initial medium pH on CT transformation**

The range of pH values over which CT degradation occurs is broad, but the optimum for CT transformation is approximately 8.5 by Tatara [23]. An assay was conducted using a fixed copper concentration and varied pH to examine the effect of speciation of copper and change of biomolecule function at the reaction level. This pH range was first tested using the growth of *Pseudomonas fluorescens* to make sure there was no negative effect. Copper concentrations of 0.64, 6.4, 12.8, 25.6 μg in form of $CuSO_4 \bullet 5H_2O$ were added to SGM prior to increasing the pH to 6.3 and 7.3 in order to achieve speciation of copper. A better understanding of copper speciation in the CT transformation and toxicity could be obtained by experiments like those performed in this work, but the care must be taken to account for the increase in pH caused by addition of freeze-dried supernatant.

- **Competition theory**

One of the possible scenario for secreted factor produced by *P.stutzeri* KC could be to compete with other cells for nutrients. In order to verify this, biomolecule was added to starting exponential period of *P. fluorescens* growing in SGM. Plates count data showed that there was no significant difference on cell number on *P. fluorescens* in the absence or presence of biomolecule. However, addition of freeze-dried supernatant delayed production of the greenish-yellow pigments (siderophores) normally produced by *P. fluorescens* in the stationary phase.