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Factors Influencing the Competitive Advantage of *Pseudomonas* sp. Strain KC for Subsequent Remediation of a Carbon Tetrachloride Impacted Aquifer

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FACTORS INFLUENCING THE COMPETITIVE ADVANTAGE OF *PSEUDOMONAS* SP. STRAIN KC FOR SUBSEQUENT REMEDIATION OF A CARBON TETRACHLORIDE IMPACTED AQUIFER

By

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ABSTRACT

FACTORS INFLUENCING THE COMPETITIVE ADVANTAGE OF *PSEUDOMONAS* SP. STRAIN KC FOR SUBSEQUENT REMEDIATION OF A CARBON TETRACHLORIDE IMPACTED AQUIFER

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To be effective for bioremediation purposes, organisms that are added to a contaminated environment must be able to survive and compete with the indigenous organisms. However, to minimize ecological disturbance, colonization should ideally be constrained. These paradoxical objectives can be satisfied by creating a temporary niche in which the introduced organism can grow, persist, and express the necessary genes. When the niche is no longer maintained, the organism dies out. A simple but powerful niche adjustment is alkalinity addition. Modifying the pH of a neutral or acidic environment to near 8.0 decreases the bioavailability of essential trace metals, such as iron, favoring those organisms that possess efficient trace metal scavenging systems. Alkaline niche adjustment with *Pseudomonas* sp. strain KC, a denitrifying aquifer organism originally isolated under alkaline conditions, was evaluated. Bioaugmentation with strain KC offers both pathway and kinetic advantages for the removal of carbon tetrachloride (CT), provided it can be transported through porous media and can grow and compete with indigenous organisms. Growth of strain KC in batch cultures as measured by μ_{max} and yield was more efficient than that of its competitors in Schoolcraft groundwater. In the stationary phase, strain KC was highly persistent at pH 8.2, but died rapidly when carbon dioxide was added to reduce the pH of the medium. These observations indicate that alkaline niche adjustment can be used to both facilitate and limit colonization of strain KC, and to control expression of its biodegradative activity.

I humbly dedicate this thesis to Jesus Christ, Lord and Savior of my life, without whom I am nothing, and to my parents who raised me in His love.

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It is my desire to show appreciation and give due credit to the people that made the completion of this research and thesis possible.

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b	decay coefficient, day^{-1}
K_S	half-velocity coefficient, mg/L
μ_m	maximum specific growth rate, day^{-1}
S	rate-limiting substrate concentration, mg/L
t, T	time, days
X	concentration of microorganisms (or protein), mg/L
So	the initial nitrate concentration, mg/L
S	time dependent nitrate concentration, mg/L
k_m	the maximum specific rate of substrate utilization, $hour^{-1}$
S_{min}, R*, J	substrate concentration at which growth and decay are equal, mg/L

CHAPTER 1

INTRODUCTION

In-situ Remediation with *Pseudomonas* sp. Strain KC

In-situ bioremediation offers the potential for remediation of many contaminated sites. The two most important options for *in-situ* bioremediation are biostimulation, the addition of growth-limiting factors to increase the concentration of indigenous organisms, and bioaugmentation, the introduction of organisms with unique catabolic capabilities. Both approaches have advantages and disadvantages. For bioaugmentation, transport of the added microorganisms and competition between the added microorganisms and indigenous populations present formidable challenges. A possible means of surmounting the challenge of competition is to create a temporary niche that favors growth and maintenance of the introduced organism. The niche adjustment evaluated in this work was addition of alkalinity to favor growth and maintenance of *Pseudomonas* sp. strain KC.

Carbon tetrachloride (CT) transformation by indigenous denitrifying microorganisms typically leads to the production of chloroform (CF). In a field-scale experiment at the Moffett Naval Air Station, for example, CF production resulted from acetate addition to biostimulate indigenous denitrifying populations (Semprini *et al.*, 1992). *Pseudomonas* sp. strain KC is a denitrifying microorganism that rapidly transforms CT to CO_2, formate, and an unidentified non-volatile product, without the production of CF (Criddle *et al.*, 1990, Lewis *et al.*, 1993, Dybas *et al.*, 1994 a). The requirements for CT transformation by strain KC are: (1) adequate concentrations of nitrate and electron donor, (2) iron-limiting conditions, (3) trace levels of copper, and (4) an incubation temperature of 4-23°C

(transformation is inhibited above 25°C; growth is inhibited above 30°C). Iron-limited conditions can be achieved by adjusting the pH to near 8.0 (Tatara *et al.*, 1993) or by adding iron chelators (Lewis *et al.*, 1993). Copper is required for CT transformation (Tatara *et al.*, 1993), but inhibits growth at neutral pH (Criddle *et al.*, 1990). The transformation is cometabolic, and it is linked to mechanisms for trace metal scavenging (Criddle *et al.*, 1990, Tatara *et al.*, 1993). No similar activity has yet been detected in other isolates or in indigenous enrichment communities (Criddle *et al.*, 1990, Lewis *et al.*, 1993). Thus, use of strain KC for bioaugmentation may offer certain pathway and kinetic advantages, provided it can compete with indigenous organisms and can express the genes needed for CT degradation in environments that contain appreciable levels of iron.

The value of pH adjustment in creating a niche favorable for growth of strain KC is associated with changes in speciation of iron and copper at high pH. Iron solubility (as ferric ions) is lowest in the pH 8.0-8.2 range (Stumm *et al.*, 1981). Addition of base results in the precipitation of ferric salts from solution. Consequently, base addition favors strain KC and other organisms capable of scavenging iron in low iron environments. However, upon adjustment of pH to near neutral conditions, say, for example, after mixing with carbonated waters, then trace copper becomes inhibitory to growth. These considerations suggest that pH control may be used to both enable and confine colonization of strain KC.

In this thesis, the effectiveness of niche adjustment and the potential for successful bioaugmentation with *Pseudomonas* sp. strain KC is evaluated in groundwater samples. Evidence is provided demonstrating that alkaline niche adjustment favors growth of strain KC giving it a competitive advantage over the indigenous flora.

Kinetic characterization of both *Pseudomonas* KC and the native flora found in the Schoolcraft aquifer under alkaline conditions is crucial for determining the outcome of experiments in which *Pseudomonas* KC is in direct competition with the aquifer consortium. Kinetic parameters obtained will be used in modeling efforts for field scale studies. With this data it will be possible to predict the approximate yield of biomass expected from certain substrate concentrations facilitating estimation of the degree of carbon tetrachloride degradation.

Theory of Competition

There has been much debate in literature over the topic of predicting the qualitative outcomes of mixed-growth competition using kinetic parameters obtained on organisms grown alone (Hansen *et al.*, 1980, Tilman, 1981). When microbial strains compete for the same limiting nutrient in continuous culture, resource-based competition theory predicts that only one strain will survive and all others will die out (Hansen *et al.*, 1980). Experimental results have shown that two species were observed to coexist only if either (1) each was limited by a different resource and met the theoretical criteria for coexistence or (2) the species were limited by the same resource and did not differ significantly in their resource requirements (Tilman, 1981). Taylor and Williams (1975) concluded that to sustain a mixed population of a number of species in a chemostat-type continuous-flow system it is necessary that there are at least as many growth-limiting substrates as there are different species.

The experiments reported herein attempt to create single-nutrient competition for nitrate. The superior competitor as predicted by resource-based competition theory will be the organism with the lowest resource requirement, as measured by R* (Tilman, 1981). This

resource requirement is also termed subsistence or "break-even" concentration of the limiting resource, defined by the J parameter in other literature (Hansen *et al.*, 1980). Predictions using the J parameter have been confirmed in the case of auxotrophic bacterial strains competing for limiting tryptophan (Hansen *et al.*, 1980). Rittman and McCarty (1980) have also referred to this resource requirement parameter labeling it S_{min}. Although all three parameters mentioned above are named differently, each are defined as the substrate concentration at which growth and decay are equal (Hansen *et al.*, 1980, Tilman, 1981, Rittman *et al.*, 1980). The derivation of this parameter (hereafter referred to as S_{min}) begins with Monod's (1942) formulation of microbial growth which was later modified by van Uden (1967) to consider organism decay as well:

$$\mu = \mu m \frac{S}{(S+Ks)} - b$$

also, $\mu = (dX/dt)/X$ = specific growth rate, day^{-1}

where:

b = decay coefficient, day^{-1}

K_S = half-velocity coefficient, mg/L

μ_m = maximum specific growth rate, day^{-1}

S = rate-limiting substrate concentration, mg/L

t = time, days

X = concentration of microorganisms, mg/L

This crucial parameter (S_{min}) is found from the differential form of the equation for μ by letting $dX/dt = 0$ (Rittman *et al.*, 1980):

$$Smin = Ks \frac{b}{\mu m - b}$$

When the substrate concentration is equal to or below S_{min}, there is no net growth of microorganisms (Criddle *et al.*, 1991).

It is readily obvious that such a parameter is a powerful tool for predicting outcomes of single-nutrient competition between organisms when kinetic parameters have been obtained for the organisms grown alone. The following predictions can be made for competition between two types of organisms: (i) if two strains have equal μ_m's and b's, the strain with the lower K_S wins; (ii) if two strains have identical K_S's and b's, the strain with the higher μ_m wins; and (iii) if two strains have different K_S's and μ_m's, but in spite of this still have identical S_{min}'s, then the species or strains will coexist indefinitely (Hansen *et al.*, 1980).

In this thesis, kinetic parameters were determined for *Pseudomonas* sp. strain KC and the indigenous microorganisms in Schoolcraft groundwater. Using these kinetic parameter values and resource-based competition theory, it is possible to predict the winner of direct competition between *Pseudomonas* sp. strain KC and the indigenous microorganisms in Schoolcraft groundwater.

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.

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.

Media. Medium D contained per liter of deionized water: 2.0 g of KH_2PO_4, 3.5 g of K_2HPO_4, 1.0 g of $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 3.0 g of sodium acetate, 2.0 g of sodium nitrate, 1 ml of 0.15 M $Ca(NO_3)_2$, and 1 ml of trace nutrient stock TN2. Stock solution TN2 contained per liter of deionized water: 1.36 g of $FeSO_4 \cdot 7H_2O$, 0.24 g of $Na_2MoO_4 \cdot 2H_2O$, 0.25 g of $CuSO_4 \cdot 5H_2O$, 0.58 g of $ZnSO_4 \cdot 7H_2O$, 0.29 g of $Co(NO_3)_2 \cdot 6H_2O$, 0.11 g of $NiSO_4 \cdot 6H_2O$, 35 mg of Na_2SeO_3, 62 mg of H_3BO_3, 0.12 g of NH_4VO_3, 1.01 g of $MnSO_4 \cdot H_2O$, and 1 ml of H_2SO_4 (concentrated). Typically, medium D is adjusted to pH 8.2 using KOH pellets followed by 3 M KOH stock solution. Medium D was prepared and dispensed in 28 mL serum tubes, 120 ml serum bottles, or 500 ml Wheaton bottles. Nutrient broth and nutrient agar (Difco) plates were prepared according to manufacturer's instructions. Cultures were grown at 20°C with 150 rpm shaking under aerobic or denitrifying (N_2 head space) conditions.

Groundwater. Groundwater from a CT-contaminated aquifer in Schoolcraft, MI, was used in all batch. Groundwater samples were obtained manually by withdrawing groundwater from a 2" steel well screened at 30 feet below the water table with a Teflon®

bailer. Groundwater samples were stored in pre-sterilized sealed Nalgene® carboys or in Wheaton bottles equipped with Teflon® lined caps at 4°C.

Analytical methods. Nitrate, nitrite, and acetate ions were assayed by ion chromatography (Dionex model 2000i/SP ion chromatograph 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 1 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. Protein was determined by the modified Lowry method, with bovine serum albumin as the standard (Markwell *et al.*, 1981). Optical density was measured at 660 nm using a Shimadzu UV-160 spectrophotometer.

CHAPTER 3

EFFECTS OF INITIAL pH MEDIUM ON GROWTH OF STRAIN KC AND SCHOOLCRAFT AQUIFER ORGANISMS

Materials and Methods

Growth Curve and kinetic parameters of strain KC in medium D. Strain KC starter cultures were prepared by transferring cells from nutrient agar plates to previously autoclaved 28 ml test tubes containing nutrient broth using aseptic technique. After 24 hours, previously autoclaved test tubes containing medium D were inoculated with a 1% inoculum of the nutrient broth grown culture. One hundred milliliter aliquots of medium D at pH 8.2 under anaerobic conditions (nitrogen head space) were inoculated with a 48 hour 1% medium D inoculum. Growth was followed using serial dilutions and nutrient agar plate counts. Each colony forming unit was scored after 6 days of incubation at 20° C. The geometric mean and the standard deviation for the geometric mean was used to statistically analyze the data. Total protein accumulation was monitored using the modified Lowry protein assay (Markwell *et al.*, 1981).

The maximum specific growth rate μ_m was obtained for log growth phase cells using plate counts and the relationship: $\mu_m = [\ln(X_f/X_i)] / (t_f-t_i)$, where X_f and X_i represent the geometric mean of the final and initial plate counts, respectively, and t_f and t_i are the final and initial time, respectively. The decay rate was obtained in similar fashion using the relationship: $b = - [\ln(X_f/X_i)] / (t_f-t_i)$. Yield on nitrate was determined by dividing the maximum protein concentration by the initial concentration of nitrate. This estimate is based

on the assumption that all the nitrate was converted to gaseous end products. The maximum specific rate of nitrate utilization km was estimated by dividing the yield on nitrate by the maximum specific growth rate (μ_m/YN). The fraction of electrons diverted for energy f_e and the fraction of electrons used for synthesis f_s were calculated using balanced theoretical stoichiometric relationships between nitrate and cells (Criddle *et al.*, 1991). It was assumed that the dry weight of each cell was equal to exactly twice the measured protein. Protein per strain KC cell is shown in Table 2 and dry weight per strain KC cell can be found in Table 4. These values lend credibility to the above assumption. The ratio of acetate to nitrate was based on balanced stoichiometric relationships using the estimated f_e and f_s values. Yield on acetate was an estimate based on the mathematical relationship: $Ym = (f_s)max$ (h/g), where Ym represents the maximum yield, $(f_s)max$ is the maximum fraction of electrons diverted to synthesis, g is the electron equivalent mass of the electron donor (acetate), and h represents the electron equivalent mass of biomass (4.04 when nitrate is the nitrogen source) (Criddle *et al.*, 1991). See Appendix A for raw data and calculations supporting the above determinations.

Utilization of nitrite as terminal electron acceptor by strain KC. Medium D was prepared as indicated in Chapter 2 of this thesis with the exception of the sodium nitrate addition. Concentrated nitrite and nitrate stocks were used to obtain the desired concentration of each. Strain KC inocula were prepared as above then centrifuged, washed, and resuspended in medium D containing no nitrate. Protein was analyzed using the modified Lowry protein assay.

Effects of pH on growth. The dependence of growth of *Pseudomonas* sp. strain KC and the Schoolcraft aquifer native microbial flora as a function of initial medium pH was determined by preparing medium D at various initial pH levels (modified by addition of nitric acid or NaOH). Ten milliliter aliquots of medium D at pH 7-10 were inoculated

with an aerobic 24 hour 1% inoculum of medium D grown strain KC or aquifer microbial flora and sealed under anaerobic conditions. Growth was assayed for protein levels using the modified Lowry protein assay after 92 hours of growth. The aquifer microbial flora originated from an inoculum of Schoolcraft groundwater into nutrient broth which then served as an inoculum source for the medium D starter culture. Aerobic samples of Schoolcraft flora at pH 8.2 were prepared as above with the exception of being sealed under anaerobic conditions.

Results and Discussion

Effect of alkaline niche adjustment on growth and persistence of indigenous aquifer organisms and strain KC. The growth curve of *Pseudomonas* sp. strain KC in Medium D with an initial pH of 8.2 is illustrated in Figure 1. Total accumulated protein, shown in Figure 2, did not hydrolyze as rapidly as cell number decreased.

Because protein did not hydrolyze before the fourth day, protein measurements taken up to 4 days accurately represent maximum accumulated protein. This result allowed for adequate accumulated protein comparisons of strain KC and Schoolcraft organisms even after strain KC had passed through the decay phase. As shown in Table 1, initial medium pH had a significant effect on the concentration of protein produced in medium D. The buffer capacity of the system will also effect this result but to a lesser degree. The effects of medium pH on growth of strain KC and Schoolcraft aquifer native microbial flora are shown for comparison.

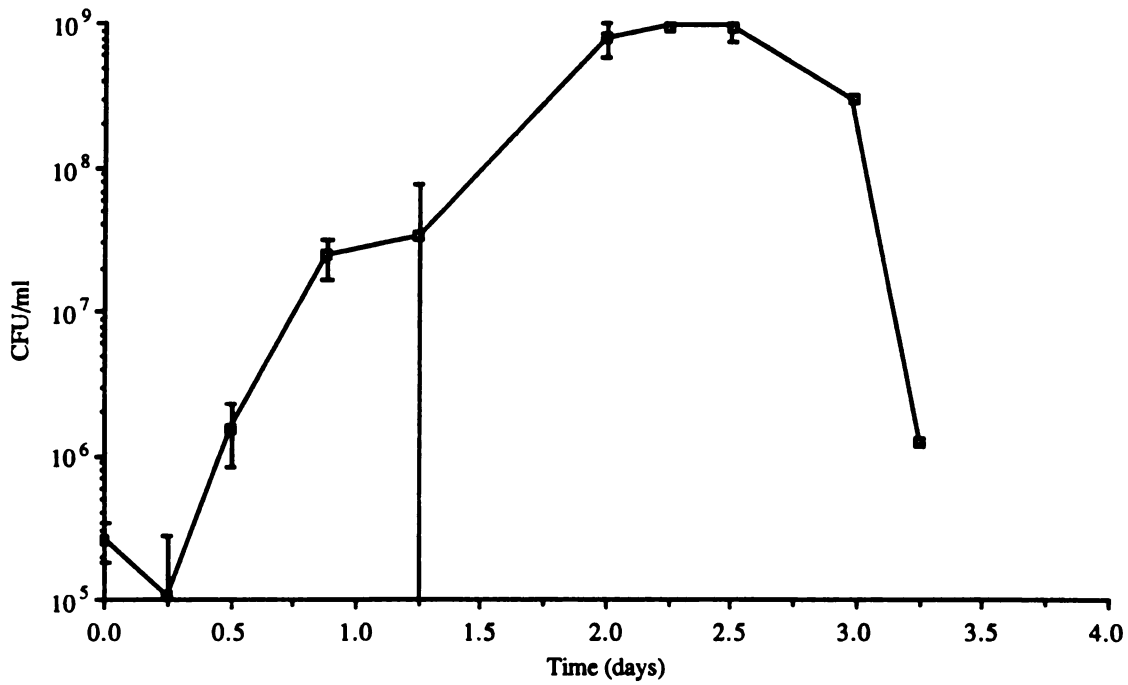

Figure 1. Growth curve of *Pseudomonas* sp. strain KC in Medium D. Error bars represent one standard deviation of three independently grown cultures.

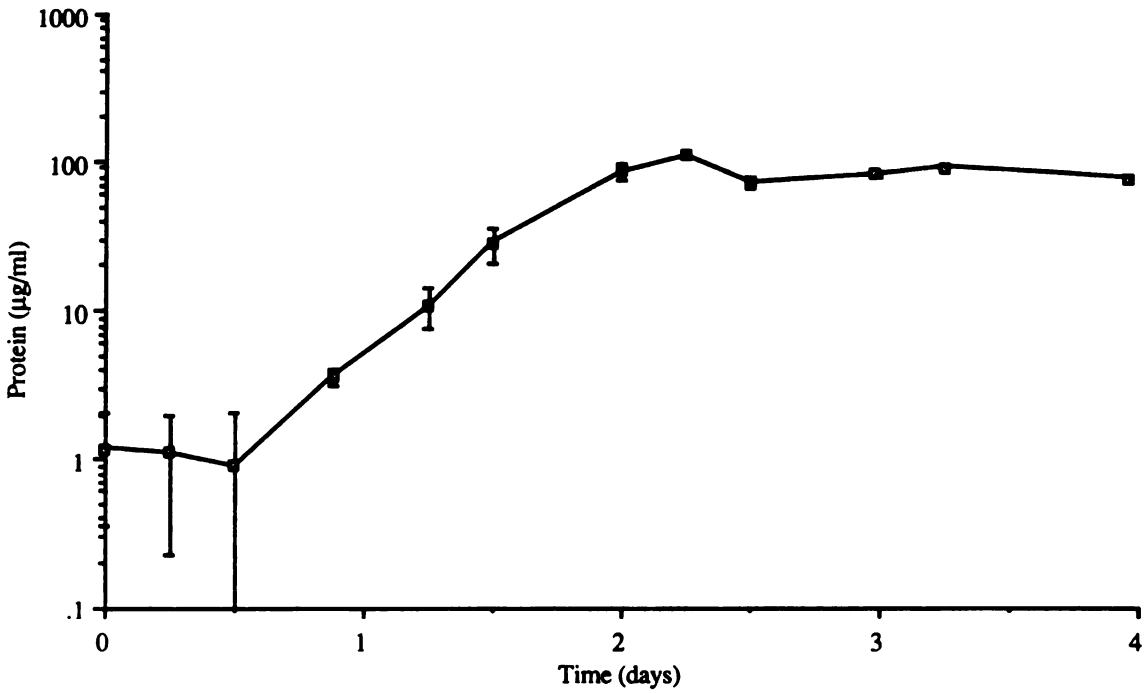

Figure 2. Accumulation of total protein of *Pseudomonas* sp. strain KC in Medium D. Error bars represent one standard deviation of three independently grown cultures.

Table 1. Effect of initial medium pH on protein production for *Pseudomonas* sp. strain KC and Schoolcraft aquifer microbial flora.[1]

Initial Culture pH	Final Culture pH[2]		Protein concentration (μg /ml)[2]	
	Strain KC	Schoolcraft consortium	Strain KC	Schoolcraft consortium
7.03	7.06 ± 0.01	7.17 ± 0.03	32.3± 0.5	19.7± 3.3
7.39	7.40 ± 0.01	7.64 ± 0.08	69.5± 1.9	35.2± 4.8
7.74	9.17 ± 0.01	7.76 ± 0.04	134± 2	11.0± 1.3
8.12	9.42 ± 0.03	8.19 ± 0.10	126± 11	14.4± 3.8
8.33	9.36 ± 0.01	8.36 ± 0.04	125± 4	12.99± 6.12
8.62	9.38 ± 0.04	8.53 ± 0.01	110± 9	7.4± 0.2
9.04	9.07 ± 0.29	8.99 ± 0.02	71.4± 14.3	6.2± 0.2
9.93	9.93 ± 0.03	9.91 ± 0.05	4.2± 0.8	5.7± 1.6

1. grown 92 hours in medium D.

2. average ± one standard deviation for triplicate cultures.

Highest protein concentrations for strain KC were obtained in the moderately alkaline range (pH 8-9), indicating that its growth is optimal under these conditions. Schoolcraft microbial flora showed a pH optimum for growth in the neutral range (pH 7-7.4). Reduced growth of strain KC at pH levels less than 8.0 are likely related to speciation changes that increase the toxicity of copper (Criddle *et al.*, 1990). Changes in the speciation and solubility of iron and cobalt may also have affected protein production (Criddle *et al.*, 1990, Tatara *et al.*, 1993). Denitrification by strain KC caused the pH to increase to 9.2 to 9.4, at which point no additional growth was observed.

Kinetic parameters for strain KC obtained from the results shown in Figures 1 and 2 are presented in Table 2. Assumptions underlying the calculations are complete nitrate conversion to gaseous end products and balanced stoichiometric equations. The amount of protein per strain KC cell was obtained by using plate counts and protein data throughout the first 54 hours. This limited data was used in order to avoid inclusion of dead cell protein in the calculations.

The rapid decay of strain KC shown in Figure 1 and Table 2 may have been the result of nitrite toxicity. Presumably, strain KC reduces nitrate to nitrite throughout its utilization of acetate. If the 23.5 mM nitrate in medium D is converted to nitrite before nitrite utilization begins, the nitrite concentrations may reach as high as 23.5 mM nitrite. Figure 3 displays possible toxic effects of 35 mM nitrite which may potentially be exhibited at 23.5 mM. As a control, strain KC was subjected to relatively low nitrate and nitrite concentrations in Schoolcraft aquifer water supplemented with 10 mM acetate. The growth under these conditions is shown in Figure 3.

Table 2. Kinetic parameters for *Pseudomonas* sp. strain KC in medium D.

μ_m, max specific growth rate ($days^{-1}$)	10.7
b, decay coefficient ($days^{-1}$)	8.92
Y_N, Yield mg protein/mg NO_3^-	0.076
Y_A, Yield mg protein/mg Acetate	0.093 (theoretical value)
fe, Fraction of electrons for energy	0.66 (theoretical value)
fs, Fraction of electrons for synthesis	0.34 (theoretical value)
µg protein/P. KC cell	1.72 E-07 ± 1.01 E-07
Ratio of mmol Acetate/mmol NO_3^-	0.868 (theoretical value)
km, max specific rate of substrate util. [µm/YN]=(mg NO3-/mg protein*day)	141

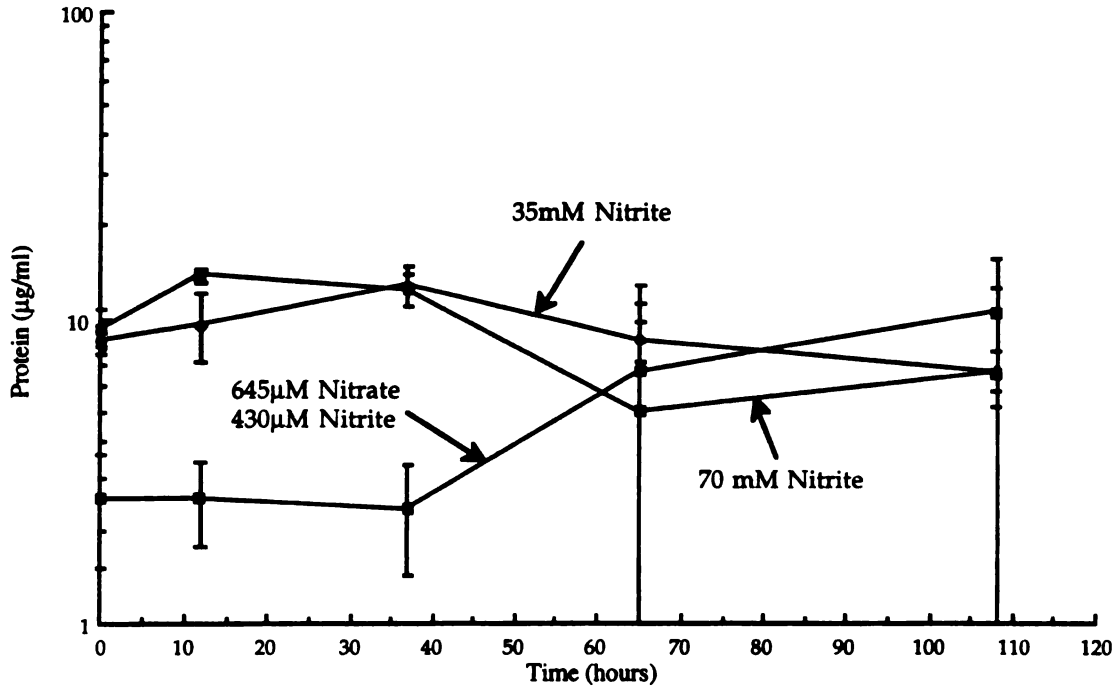

Figure 3. Effect of various concentrations of nitrite on *Pseudomonas* sp. strain KC in medium D with specified nitrite and/or nitrate concentrations.

Because CT is cometabolized by strain KC only under anoxic conditions, it became a concern as to whether or not the Schoolcraft aquifer is anoxic. One method of addressing this issue is to determine whether the indigenous organisms prefer aerobic or anaerobic conditions. If the resulting growth is much greater under aerobic conditions it is possible that the percentage of denitrifying bacteria is low. This would be an indication that anoxic conditions are not the norm for this aquifer. A comparison of aerobic and anaerobic growth for Schoolcraft aquifer organisms is shown in Figure 4. It is possible that the greater yield under aerobic conditions is due to the greater amount of energy obtained from oxygen versus nitrate as the terminal electron acceptor. A related explanation is that the Schoolcraft organisms have a higher growth rate when using oxygen. Enumeration of denitrifying

bacteria in the Schoolcraft aquifer is recommended as an area of future study. This will serve as an aid in narrowing down the relevant factors influencing the competitiveness of strain KC.

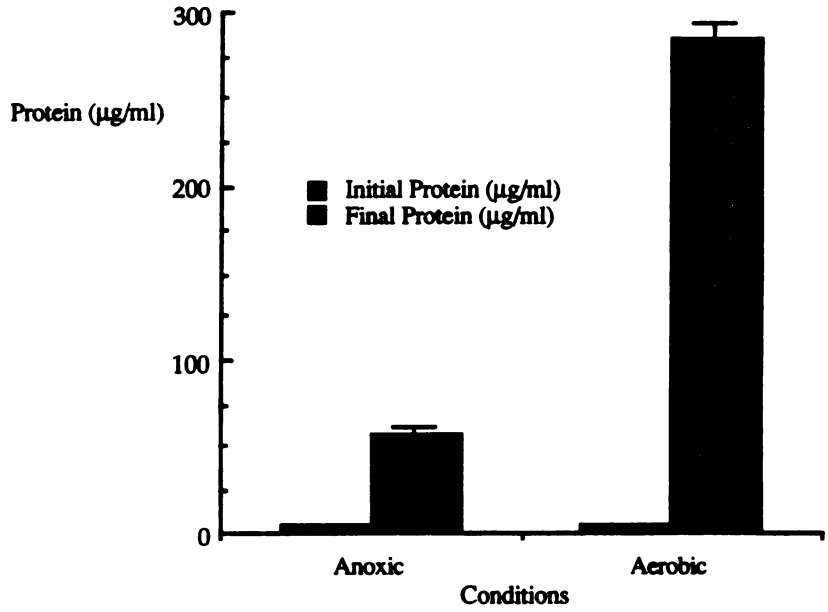

Figure 4. Comparison of growth of Schoolcraft aquifer consortium in medium D in aerobic and anoxic conditions. (Time = 92 hours).

CHAPTER 4

DEVELOPMENT OF SCHOOLCRAFT GROUNDWATER PASTEURIZATION PROCESS

Materials and Methods

Growth of strain KC in Schoolcraft groundwater with varying concentrations of acetate. Schoolcraft groundwater was adjusted to pH 8.2 by addition of NaOH. It was previously shown by M. Dybas (personal communication) that a final concentration of 0.18 mM NaOH produced a final pH of 8.2 in Schoolcraft groundwater. One hundred microliters of a 100 mM phosphate buffer was added to each 100 ml sample to produce a 0.100 mM phosphate concentration to serve as a phosphorus source. An appropriate amount of a concentrated acetate stock solution was added to yield the desired acetate concentrations. The resulting solution was sterilized by filtration through a 0.22 µm pore vacuum filtration unit pressurized to approximately 15 psi. Each sample was transferred to a sterile 250 ml Erlenmeyer flask and passed through the anaerobic vacuum chamber for three full cycles to ensure anaerobic conditions. Once in the anaerobic hood, 10 ml aliquots were aseptically transferred to 4 sterile test tubes and sealed with sterilized heavy black rubber septa. Common crimp tops were used to seal the test tubes. Strain KC inocula consisted of a 48 hour 1% aerobic medium D inocula that had been washed and resuspended in Schoolcraft groundwater with nutrients added that had been sterilized by filtration. Starter cultures were centrifuged at 14,000 rpm for 5 minutes, resuspended and centrifuged again at 8,000 rpm for 10 minutes. This was to eliminate trace carryover of nutrients from the starter cultures into the growth tubes.

Effect of groundwater filtration on strain KC growth. The experimental procedure used was identical to the above with the exception of filtration. Half the samples were filtered before addition of phosphate, NaOH, and acetate; the other half were filtered after the addition of nutrients.

Effect of various environments on strain KC and the Schoolcraft organisms. Several different environments were prepared in order to determine the reason for minimal growth of strain KC in Schoolcraft groundwater. Aerobic conditions were established for the first set of samples by following the procedure used to test growth at various acetate concentrations. Samples were sterilized by filtration before addition of nutrients and sealed under aerobic conditions. Excess nitrate was added to a second set of samples in the event that the nitrate present in Schoolcraft water (about 55 ppm) was insufficient to support adequate growth. The nitrate added stoichiometrically balanced the 10 mM acetate concentration. Samples were sealed in the anaerobic hood to produce denitrifying conditions. A third set was autoclaved previous to inoculation with strain KC to determine if trace nutrients were locked in the precipitate that formed from the autoclaving process. A fourth set consisted of Schoolcraft groundwater adjusted to pH 8.2 with NaOH and supplemented with phosphate and acetate. No strain KC cells were added. Samples in the fifth set were prepared in identical fashion as those in the experiment testing the varying acetate concentrations except that the nutrients were added after sterilization by filtration. The final or sixth set consisted of Schoolcraft groundwater with all nutrient additions and pH adjustment with a 2% washed strain KC inoculation. No sterilization technique was used on sample set six.

Pasteurization of Schoolcraft groundwater using 65° C incubation. Schoolcraft groundwater was dispensed in triplicate sterile serum bottles (20 ml aliquots)

then incubated at 20° C and 65° C. Serial dilution and nutrient agar plate counts were used to determine the effectiveness of 65° C pasteurization.

Results and Discussion

There are many experimental methods which can be used to measure the kinetic parameters of bacteria. Before any of these can be successfully implemented, certain basic parameters must first be established. The minimum concentration of the electron donor (acetate) required to achieve maximum growth was the first parameter to be determined. Obviously, as acetate concentration is increased, growth should increase as long as adequate electron acceptor and trace nutrient concentrations are available. This trend will continue until the acetate concentration is no longer limiting growth. If acetate is supplied at concentrations that stoichiometrically balance the bacterial demand for nitrate, then no additional growth will occur as acetate concentrations are increased further. This upper limit for acetate was evaluated to determine an appropriate concentration to be used in future experiments aimed at obtaining kinetic parameters for strain KC. In an initial experiment, acetate was added at different concentrations to Schoolcraft groundwater and growth was monitored by optical density. The results shown in Figure 5 indicate that growth of strain KC in Schoolcraft groundwater is insufficient for measurement by optical density, regardless of acetate concentration. Note the optical density scale on the vertical axis. Typically, adequate growth measurements are an order of magnitude greater than those shown here.

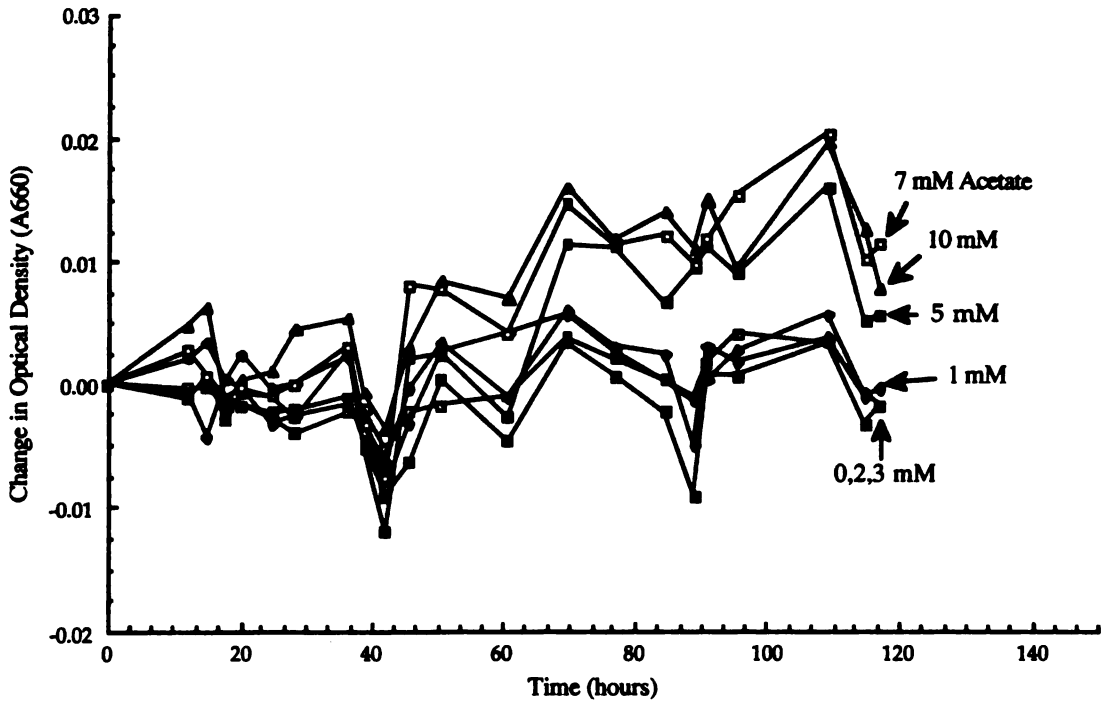

Figure 5. Growth of *Pseudomonas* sp. strain KC in Schoolcraft groundwater sterilized by filtration with various initial values of acetate. Initial phosphate concentration and pH was 0.100 mM and 8.2, respectively.

The results in Figure 5 were obtained by addition of sodium hydroxide, phosphate, and acetate to Schoolcraft groundwater before sterile filtration (no additional nitrate was provided) and inoculation under anaerobic conditions. The lack of growth of strain KC under these conditions was unexpected and puzzling. It was hypothesized that the cause for this may have been related to the sterile filtration process preceding inoculation of strain KC. Trace nutrients, such as iron, may have adhered to the filter membrane and caused growth limiting conditions. To test this hypothesis, growth in samples that were filtered before nutrient addition was compared to growth results in samples that had been sterilized by filtration after nutrient addition. Protein measurements over a period of six days showed that filtration before or after nutrient addition had no apparent effect on growth of strain KC. Samples from both sets again showed very little growth.

A possible explanation for the lack of growth of strain KC was inadequate electron acceptor concentration. Oxygen and nitrate can both be utilized by strain KC as the terminal electron acceptor. Accordingly, the next experiment tested growth of strain KC under aerobic and excess nitrate conditions in Schoolcraft groundwater that had been sterilized by filtration before addition of nutrients. As a control, strain KC was also subjected to identical conditions as the previous experiment in which nutrients were added after sterilization by filtration. Autoclaved Schoolcraft groundwater containing phosphate and acetate at pH 8.2 was also used as the medium for strain KC. Results are provided in Table 3.

Autoclaving caused precipitate to form that may have converted trace nutrients to inaccessible forms. The decrease in CFU/ml of strain KC in the samples sterilized by filtration and autoclaving indicates that these processes have some adverse effects on the growth of strain KC in Schoolcraft groundwater. Results for sample number 4 show a significant increase in indigenous organisms in Schoolcraft groundwater adjusted to pH 8.2 with nutrient addition. Strain KC was not inoculated into sample 4 and the Schoolcraft groundwater was neither filter sterilized nor autoclaved. Sample number 6 consisted of identical conditions as sample 4 except that strain KC was inoculated in sample 6. Strain KC and Schoolcraft organisms were differentiated by colony morphology in sample 6 which may have been a source of some inaccuracy. Be that as it may, the results of samples 4 and 6 show significant growth of both strain KC and the Schoolcraft organisms. These results give further credibility to the hypothesis that filter sterilization and autoclaving cause adverse effects on bacterial growth in Schoolcraft groundwater. Another interesting conclusion which can be made from the results of samples 4 and 6 is that Schoolcraft organisms show significant growth at pH 8.2. Previous to this experiment it was believed that alkaline conditions were inhibitory to indigenous organisms in Schoolcraft groundwater. This issue will be addressed again in the results of later experiments.

Table 3. *Pseudomonas* sp. strain KC and native aquifer consortium growth/decay after 69 hours in Schoolcraft groundwater under various conditions. Initial acetate, phosphate, and pH was 10 mM, 0.100 mM, and 8.2, respectively.

Amendments	Sterilization Method	Initial population (10^4 CFU/ml)	Final population (10^4 CFU/ml)
1. Oxygen	Filtration	4.10 ± 1.40	1.04 ± 9.20
2. Excess nitrate	Filtration	2.11 ± 3.66	0.64 ± 3.50
3. None	Autoclaving	142.72 ± 53.66	7.19 ± 4.48
4. None	Non-Sterile (Schoolcraft organisms only)	8.88 ± 2.34	779.17 ± 101.29
5. None	Filtration	9.65 ± 4.59	9.93 ± 6.27
6a. None	Non-Sterile (*P.* KC, identified by colony morphology)	3.11 ± 1.55	58.28 ± 41.70
6b. None	Non-Sterile (Schoolcraft organisms, identified by colony morphology)	8.24 ± 8.88	257.05 ± 106.00

Because of the results discussed above, it became evident that a sterilization method other than filter sterilization or autoclaving would be needed in order to eliminate indigenous flora from Schoolcraft groundwater. An experiment was designed to determine whether the organisms in the Schoolcraft groundwater were all psycrophilic or mesophilic bacteria. If so, then incubation at higher temperatures without the excessive pressure and temperature of an autoclave may provide adequate removal of indigenous flora. This pasteurization process was tested, and the results are shown in Figure 6. Incubation of Schoolcraft groundwater at 65° C for 8 hours removed all indigenous organisms that were capable of forming colonies on nutrient agar plates. This process was used for all the following experiments to determine growth of strain KC in Schoolcraft groundwater.

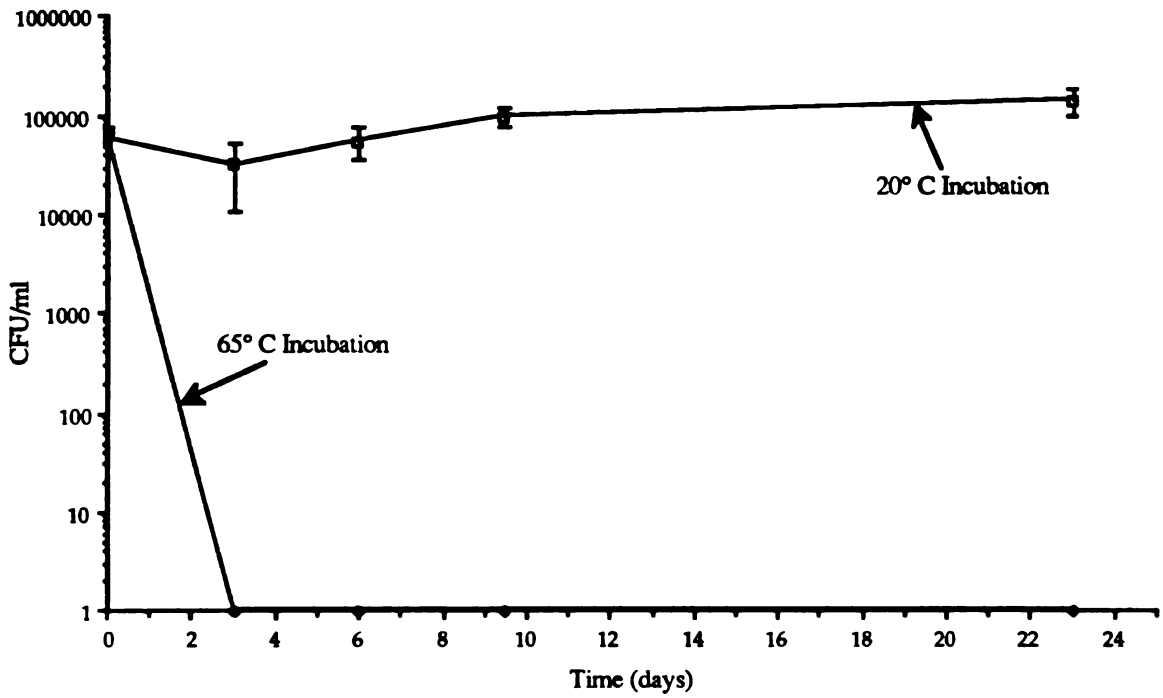

Figure 6. Pasteurization effect on indigenous organisms by Schoolcraft groundwater at 65° C.

CHAPTER 5

EFFECTS OF pH ON STRAIN KC AND THE INDIGENOUS ORGANISMS IN SCHOOLCRAFT GROUNDWATER

Materials and Methods

Effect of various pH values on indigenous organisms in Schoolcraft groundwater. Schoolcraft groundwater was adjusted to the desired pH values using a sterile 100 mM Na_2CO_3 stock solution. This served to adjust the pH and as a carbonate buffer which is the natural buffering system of the Schoolcraft aquifer. Amounts to be added were determined by trial and error on test samples. Colony forming units were measured using serial dilutions and nutrient agar plate counts.

Persistence of strain KC in groundwater. To evaluate long-term survival of strain KC in Schoolcraft groundwater, strain KC ($2x10^6$-$1x10^7$ cells/ml) was added to pasteurized Schoolcraft groundwater that had been adjusted to a pH of 8.2 using sterile 100 mM sodium carbonate solution and sterile CO_2 gas. Pasteurized groundwater was used to allow reliable enumeration of strain KC by plate counts. The water contained no added carbon source or electron acceptor. Samples were removed at one to four day intervals and assayed for strain KC using the serial dilution and bacterial plate count procedure described above. After 14 days, the pH was adjusted to pH 7.5 by addition of carbon dioxide, and levels of *Pseudomonas* sp. strain KC were monitored for an additional 7 days.

Results and Discussion

As previously discussed, alkaline pH values were suspected to be inhibitory to indigenous organisms in Schoolcraft groundwater. This was thought to be a primary factor in the competitive advantage of strain KC exhibited in previous model aquifer column studies. Preliminary experiments (samples 4 and 6 of Table 3), however, indicated growth of indigenous organisms in Schoolcraft groundwater at pH 8.2. To confirm this result, Schoolcraft groundwater was adjusted to various pH values using a sterile Na_2CO_3 stock solution, and the effect on the indigenous organisms was monitored using plate counts. Results shown in Figure 7 exhibit little or no effect of various initial pH values on an existing population of indigenous Schoolcraft organisms. Alkaline niche adjustment does not reduce the concentration of indigenous organisms which will be in direct competition with strain KC. The slight increase in organism concentration may have been due to cryptic growth or to growth on carbon already present in the groundwater since none was added.

The persistence and containment of strain KC after release into an alkaline niche is of interest for application purposes. Figure 8 illustrates long-term persistence of strain KC under no-growth conditions (no electron acceptor or donor) and demonstrates a pH dependence. A stable population of stationary phase strain KC (10^3-10^5 CFU/ml) rapidly decayed upon a shift of pH from 8.2 to 7.5, resulting in a drop of three orders of magnitude in the population levels of strain KC in 7 days. This experiment simulated what would happen after substrate and/or base is no longer used to maintain the alkalinity of a niche. Without substrate addition, denitrification will no longer occur and the increase in pH caused by denitrification will also cease. If base is not added, carbonated groundwater will eventually titrate the alkalinity. As shown in Figure 8, once the pH was decreased to 7.5, the concentration of strain KC decreased dramatically. This decrease may have been caused by the toxicity of copper to strain KC at near neutral pH values (Criddle *et al.*,

1990). This must not be taken to conclude that all strain KC cells will eventually die. Replicate 2 shows persistence of a significant concentration of strain KC cells well after pH adjustment. It is highly likely that mutations may allow strain KC to survive at pH 7.5 and continue to persist in the aquifer (Lenski, 1992). This should not be a serious concern as the pathogenicity and toxicity of strain KC to plant and animal life has been tested and no adverse effects were reported (M. Dybas, personal communication).

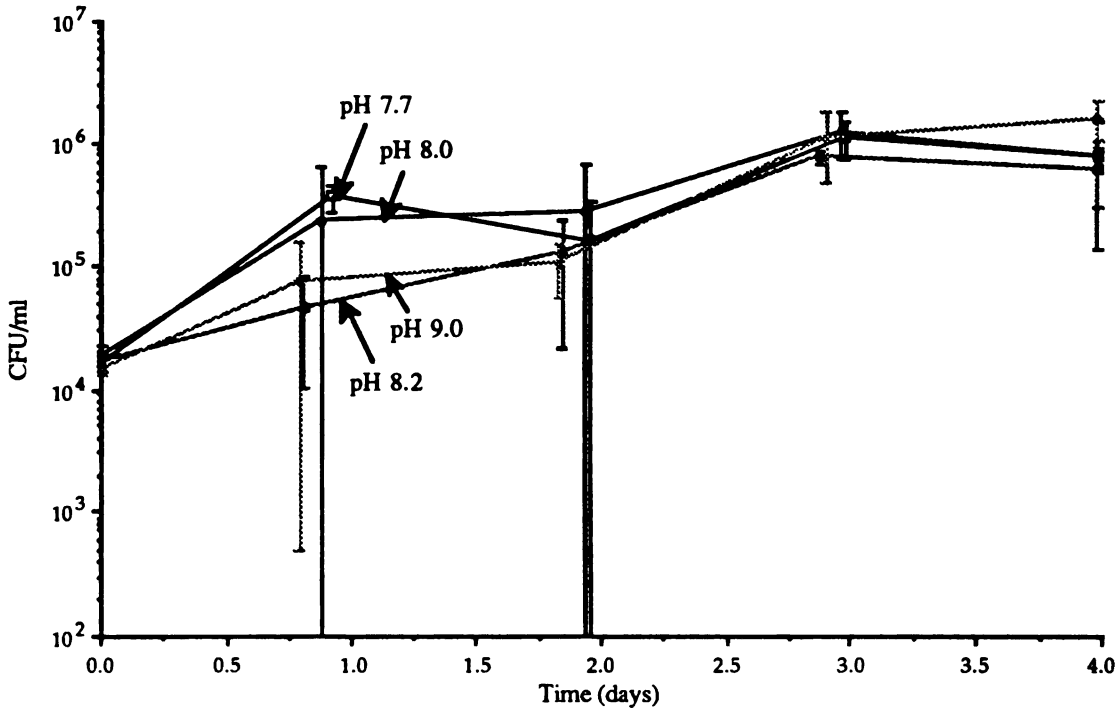

Figure 7. Effect of various initial pH values on an existing population of indigenous aquifer organisms in Schoolcraft groundwater.

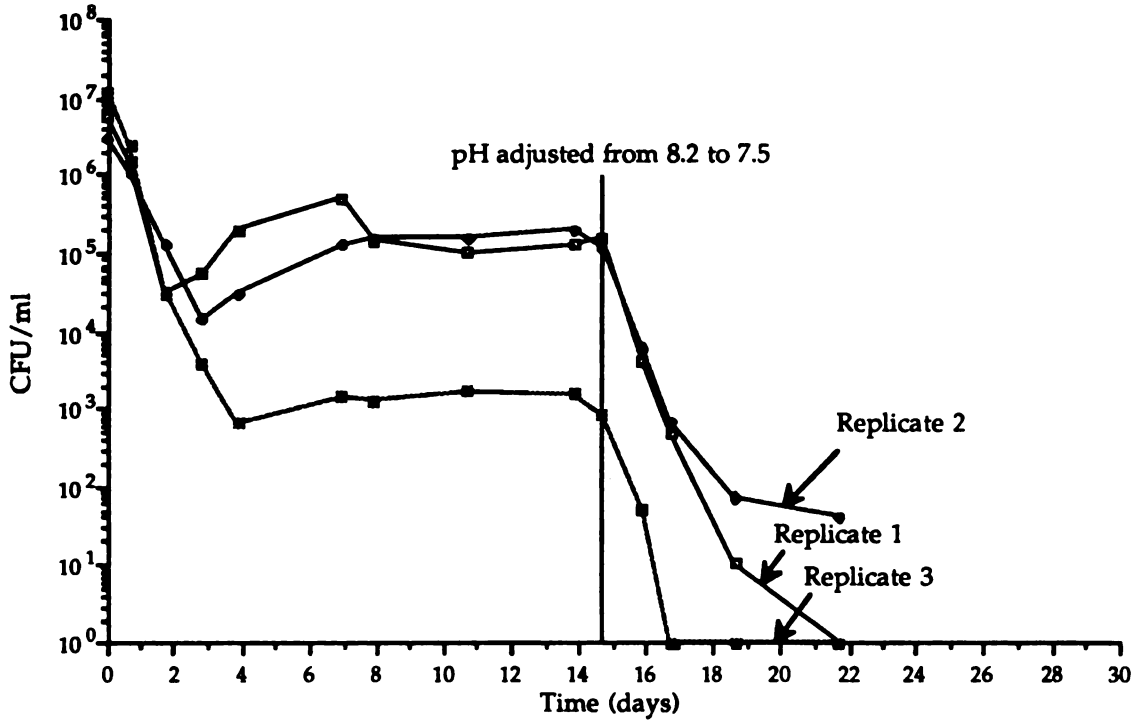

Figure 8. Persistence of strain KC under no-growth conditions in Schoolcraft groundwater at pH 8.2. After 2 weeks of incubation, the pH was reduced to 7.5.

CHAPTER 6

KINETIC PARAMETER CHARACTERIZATION OF STRAIN KC AND THE INDIGENOUS ORGANISMS IN SCHOOLCRAFT GROUNDWATER

Materials and Methods

Kinetics of growth of strain KC and indigenous groundwater organisms in niche adjusted groundwater. In order to evaluate the kinetics of growth of strain KC and the indigenous Schoolcraft groundwater organisms and their specific rate of utilization of acetate, nitrate, and nitrite, 500 mL Wheaton bottles containing pasteurized Schoolcraft groundwater (not pasteurized for the Schoolcraft organism samples) were supplemented with 30 mM acetate, 12 mM nitrate, 0.1 mM phosphate, and 10 g $NaHCO_3$/L to give an initial pH of 8.2. The groundwater was previously pasteurized by heating at 65°C for 8 hours. Pasteurization was used to remove indigenous flora because autoclaving produced a precipitate that interfered with organism growth and filter-sterilization had a similar adverse effect on organism growth (Chapter 4). Strain KC was added as a 1% inoculum from an aerobic culture grown for 72 hours in medium D and washed in pasteurized Schoolcraft aquifer water. Growth of both strain KC and Schoolcraft flora was followed by optical density measurements, plate counts, and dry weight. Acetate, nitrate, and nitrite utilization were followed by ion chromatography.

The maximum specific growth rate μ_m was obtained for log growth phase cells using optical density measurements at a wavelength of 660 nm and the relationship: $\mu_m = [\ln(X_f/X_i)] / (t_f-t_i)$, where X_f and X_i represent the final and initial optical density,

respectively, and t_f and t_i are the final and initial time, respectively. The observed yield on nitrate and nitrite for both cultures was calculated using dry weight measurements obtained by filtering a known volume through a 0.2 μm pore filter membrane (Gelman Sciences) and washing the collected cells with phosphate buffer adjusted to pH 5. The filter membranes were then dried overnight at 104° C and weighed. The filters were dried in this fashion and weighed before the cells were filtered allowing for an accurate measure to be obtained. Ion chromatography was used to measure the nitrate and nitrite consumption. The dry weight biomass concentration of each sample was corrected for the slight precipitate present in uninoculated, pasteurized groundwater. The observed yield was calculated by dividing the corrected dry weight biomass concentration increase by the total nitrate or nitrite consumed over the identical time period. Actual substrate data at specific time points were used but dry weight measurements were obtained from the plot of dry weight versus time. This was necessary due to the fact that substrate and dry weight data time points were taken at different times throughout each day. The maximum specific rate of nitrate and nitrite utilization k_m was estimated by dividing the instantaneous rates of nitrate or nitrite consumption by the dry weight biomass concentration at that time. See Appendix B for raw data and supporting calculations.

To determine the half-velocity coefficient Ks cells were grown as in the determination of μ_m, Y, and k_m but were harvested anaerobically during log phase. Cells were centrifuged and washed with Schoolcraft groundwater supplemented with 10 mM acetate and 0.1 mM phosphate. The final cultures contained approximately twice the biomass concentration of the original cultures to ensure rapid nitrate utilization. Schoolcraft groundwater was supplemented with additional nitrate to give a final nitrate concentration of approximately 1.3 mM. Samples were taken about every half hour, frozen, and later analyzed for nitrate and nitrite using ion chromatography.

Results and Discussion

As shown in Figures 9.1 through 14, both strain KC and the Schoolcraft groundwater flora exhibited similar biphasic growth patterns at an initial pH of 8.2: in each case, nitrate was first converted to nitrite, and the nitrite was subsequently converted, presumably to NO, N_2O, and N_2 although these gaseous products were not quantified. A longer lag phase occurred with the Schoolcraft flora, but this is probably due to differences in the number of the organisms initially present (10^5/mL for Schoolcraft flora vs. 10^6/mL for strain KC). The increase in pH exhibited for KC and for the Schoolcraft organisms is expected due to denitrification. This effect is dependent on the buffering capacity of the system. Schoolcraft groundwater was amended with 10 grams sodium bicarbonate per liter, but the buffering capacity of the carbonate system is at a minimum around pH 8. Highly buffered groundwater may prevent this increase in pH. This increase in pH may be advantageous for field scale niche adjustment. With KC producing alkaline conditions as a by product of growth, no supplemental base may be needed to produce alkaline conditions. Note the higher pH values obtained in the KC samples coinciding with the higher values for optical density and dry weight.

Results of kinetic characterization under alkaline conditions reveal a competitive advantage for *Pseudomonas* sp. strain KC over the indigenous Schoolcraft organisms. As shown by the optical density, plate count, and dry weight data in Figures 9.1 through 14 and by the growth parameters in Table 4, a critical difference between strain KC and Schoolcraft flora was in the observed yield: for the first phase of growth (nitrate to nitrite), the observed yield for strain KC was four times the observed yield for the Schoolcraft flora. This translated into a fourfold higher maximum specific growth rate for strain KC. Because of its high yield, strain KC also has a higher value for f_S, the fraction of electrons diverted for cell synthesis. Strain KC is able to utilize over half the available energy for cell synthesis.

Supporting data for the parameters in Table 4 are located in Appendix B. A possible explanation for this yield difference is the competitiveness of strain KC under iron limiting conditions. Dybas *et al.* (1994 a) have shown that strain KC produces a wide spectrum of iron-binding activities at pH 8.2.

In this thesis, kinetic parameters were determined for *Pseudomonas* sp. strain KC and the indigenous microorganisms in Schoolcraft groundwater. As previously mentioned, μ_m for *P.* KC was four times larger than the μ_m for the indigenous organisms. The K_S's for the two organism types were virtually indistinguishable. Decay rates were not obtained and thus S_{min} values could not be accurately calculated. However, in light of the large μ_m for *P.* KC, a relative value for S_{min} significantly lower than that for the indigenous flora can be predicted. Assuming relatively equal decay rates, resource-based competition theory predicts that if the strains also have equal K_S's, the strain with the higher μ_m outcompetes all other species (Hansen *et al.*, 1980). Classical competition theory asserts that two-species outcomes are independent of the intrinsic rates of increase of the two species, a claim that the more mechanistic resource-based approach shows to be incorrect (Hansen *et al.*, 1980). *Pseudomonas* sp. strain KC will outcompete the indigenous microorganisms in Schoolcraft groundwater under alkaline conditions on the grounds of resource-based competition theory and the assumptions above.

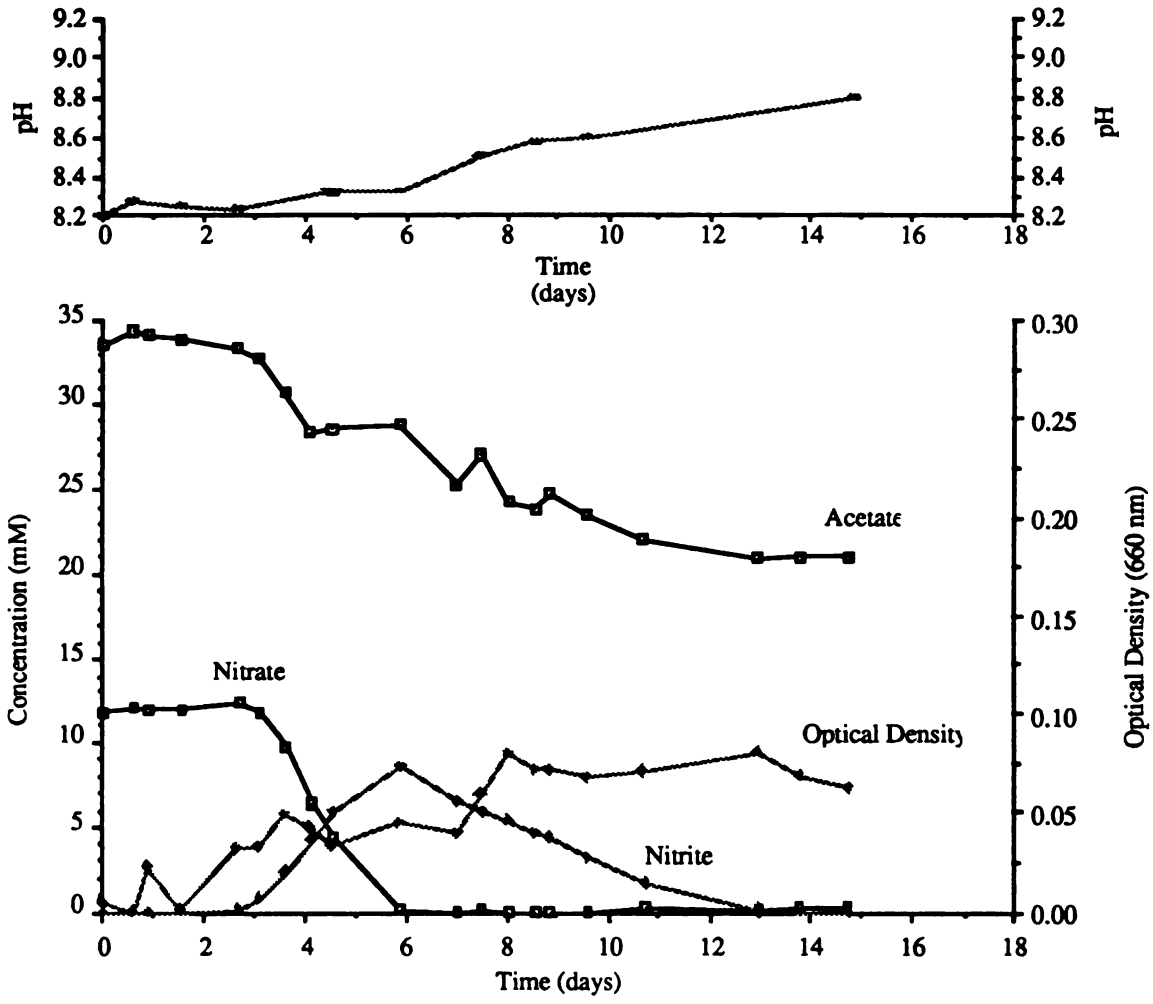

Figure 9.1. Growth pattern, substrate utilization, and pH increase for indigenous aquifer organisms in Schoolcraft groundwater adjusted to an initial pH of 8.2. (Replicate #1)

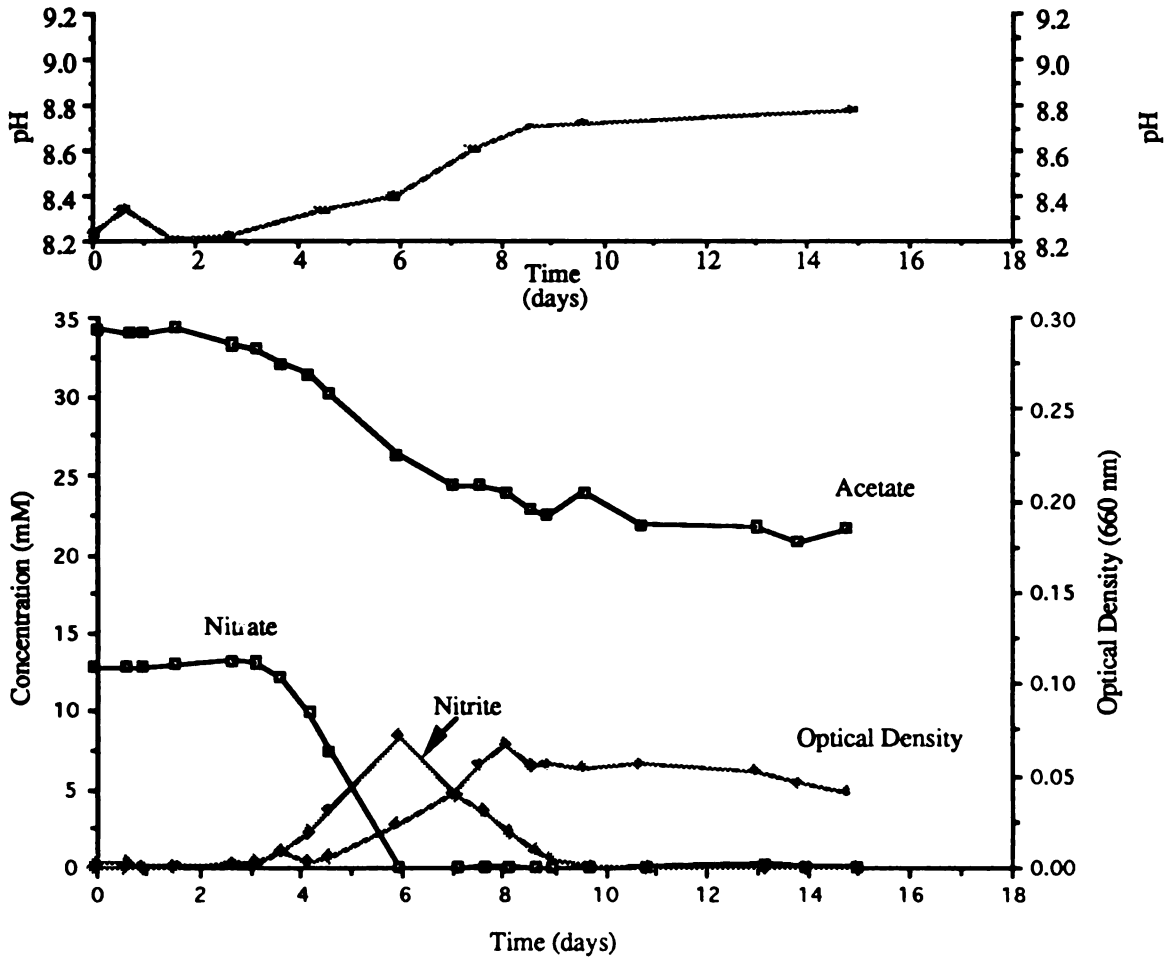

Figure 9.2. Growth pattern, substrate utilization, and pH increase for indigenous aquifer organisms in Schoolcraft groundwater adjusted to an initial pH of 8.2. (Replicate #2)

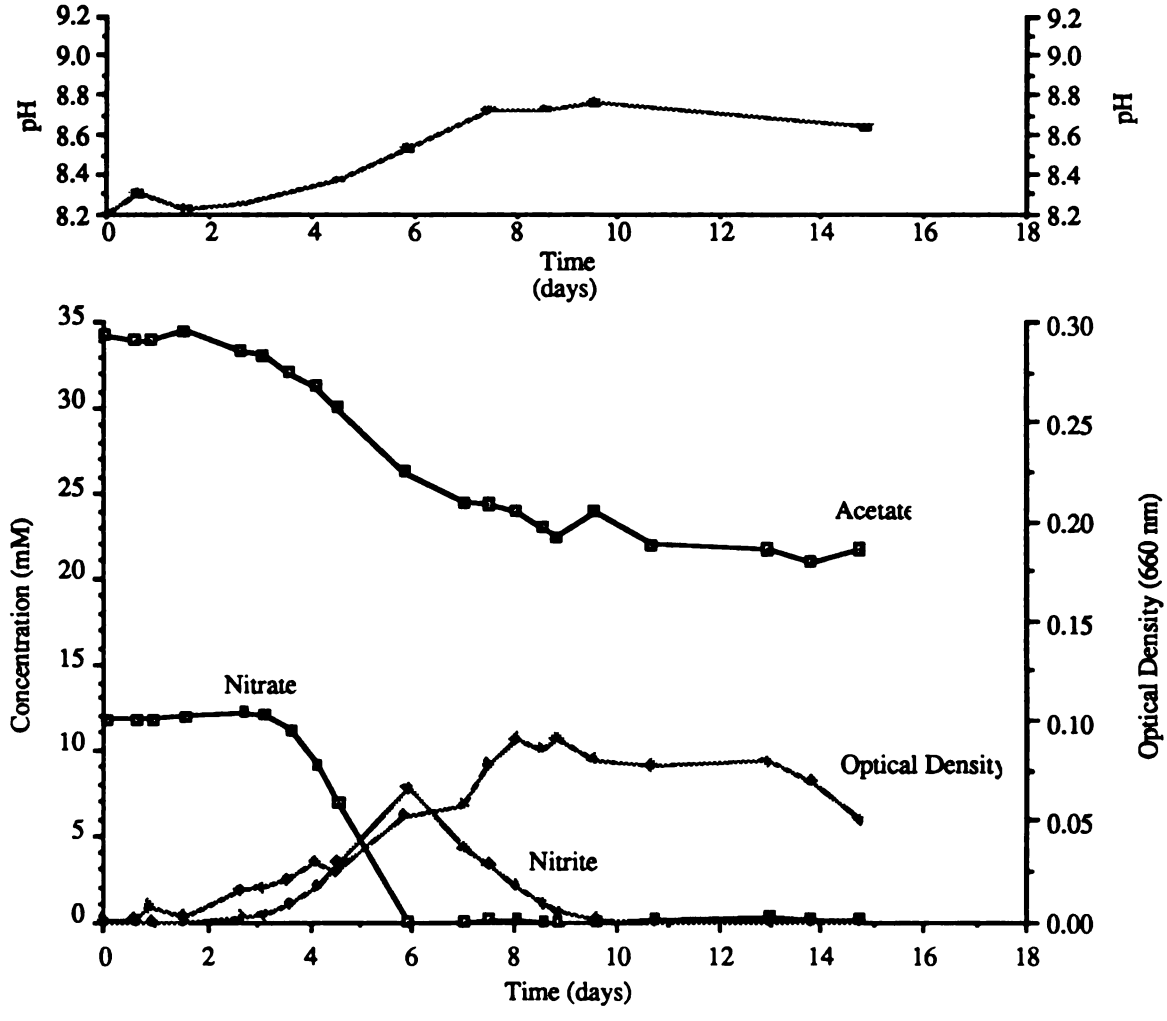

Figure 9.3. Growth pattern, substrate utilization, and pH increase for indigenous aquifer organisms in Schoolcraft groundwater adjusted to an initial pH of 8.2. (Replicate #3)

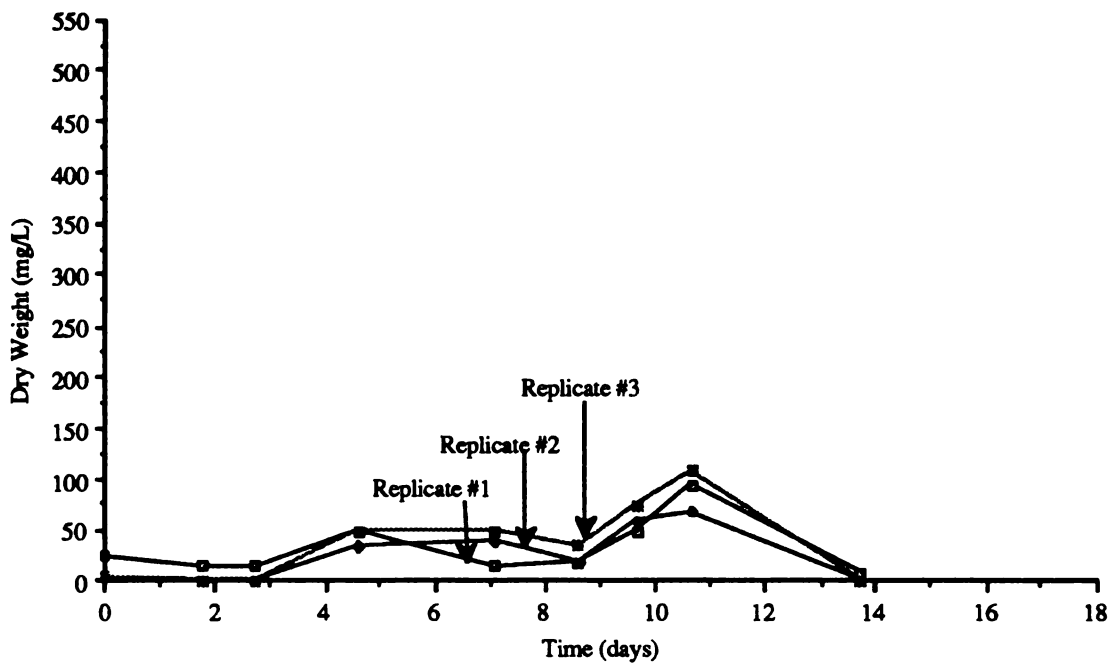

Figure 10. Biomass accumulation of indigenous organisms in Schoolcraft groundwater measured as dry weight.

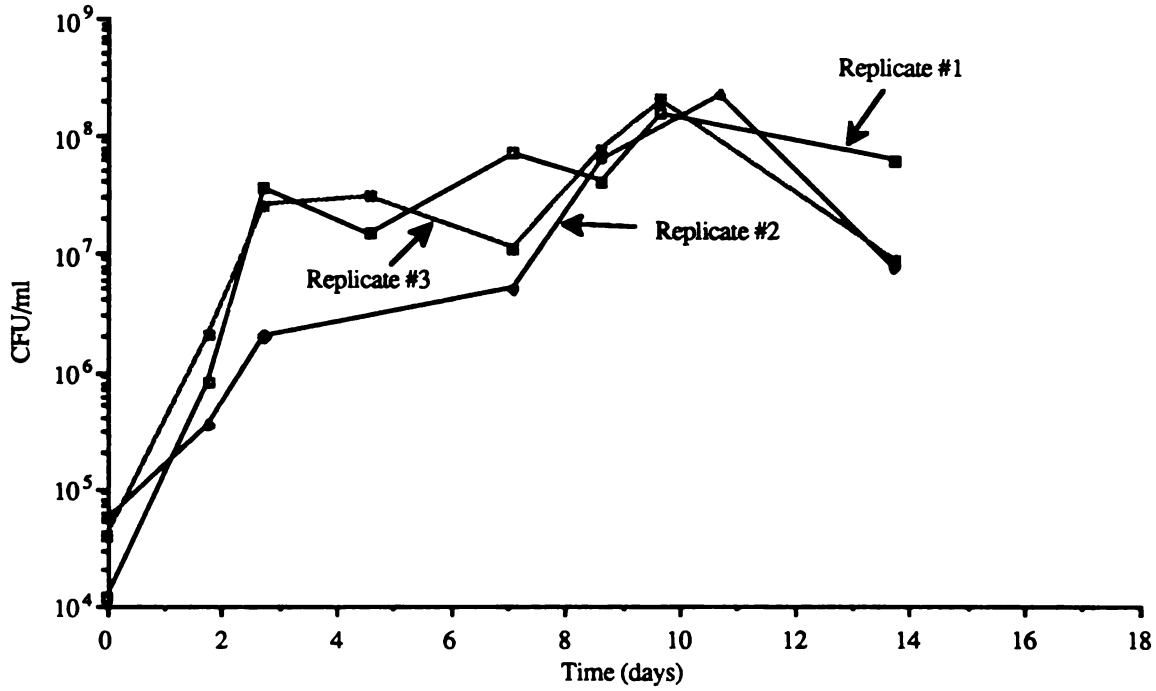

Figure 11. Growth of indigenous organisms in Schoolcraft groundwater measured by bacterial plate counts.

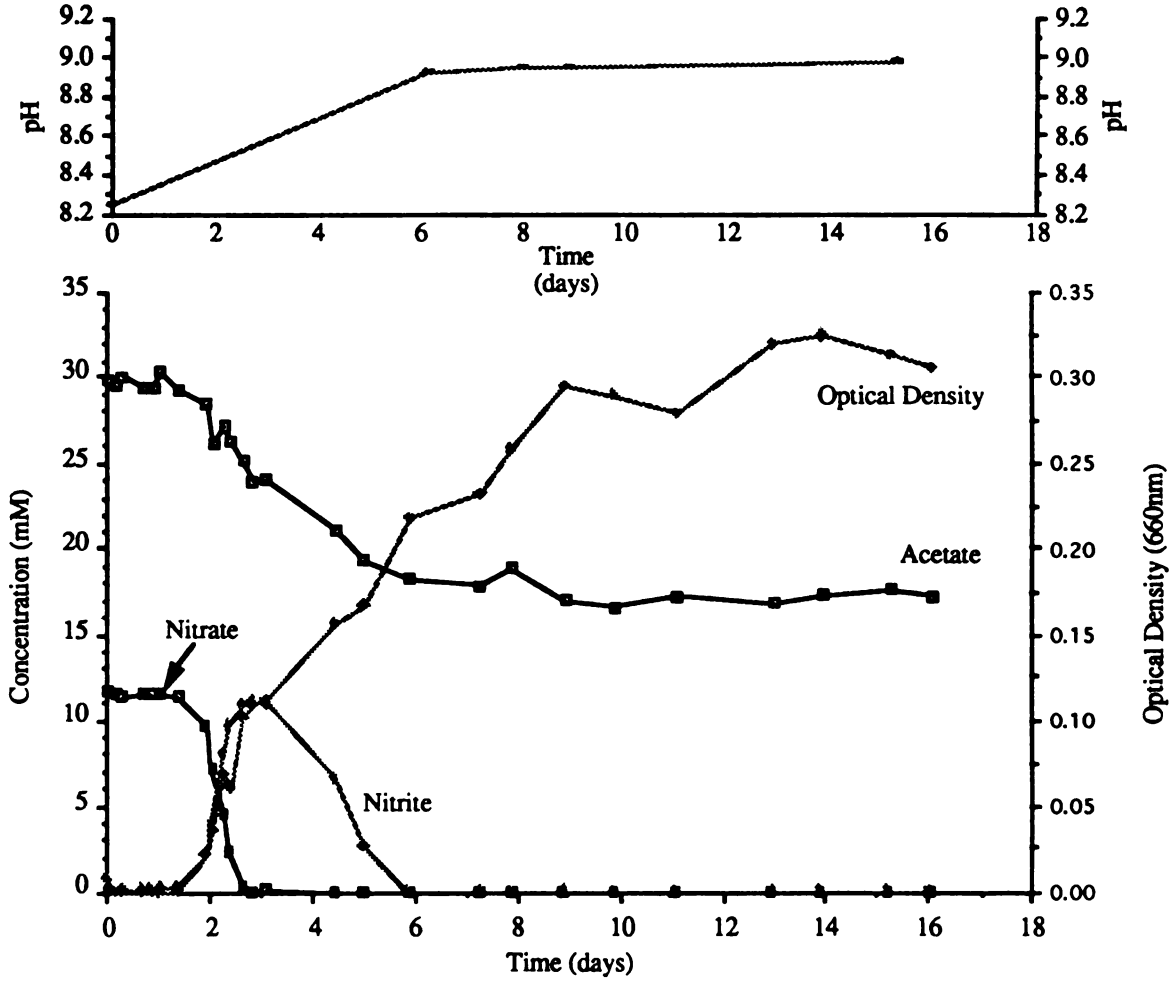

Figure 12.1. Growth pattern, substrate utilization, and pH increase for *Pseudomonas* sp. strain KC in Schoolcraft groundwater adjusted to an initial pH of 8.2. (Replicate #1)

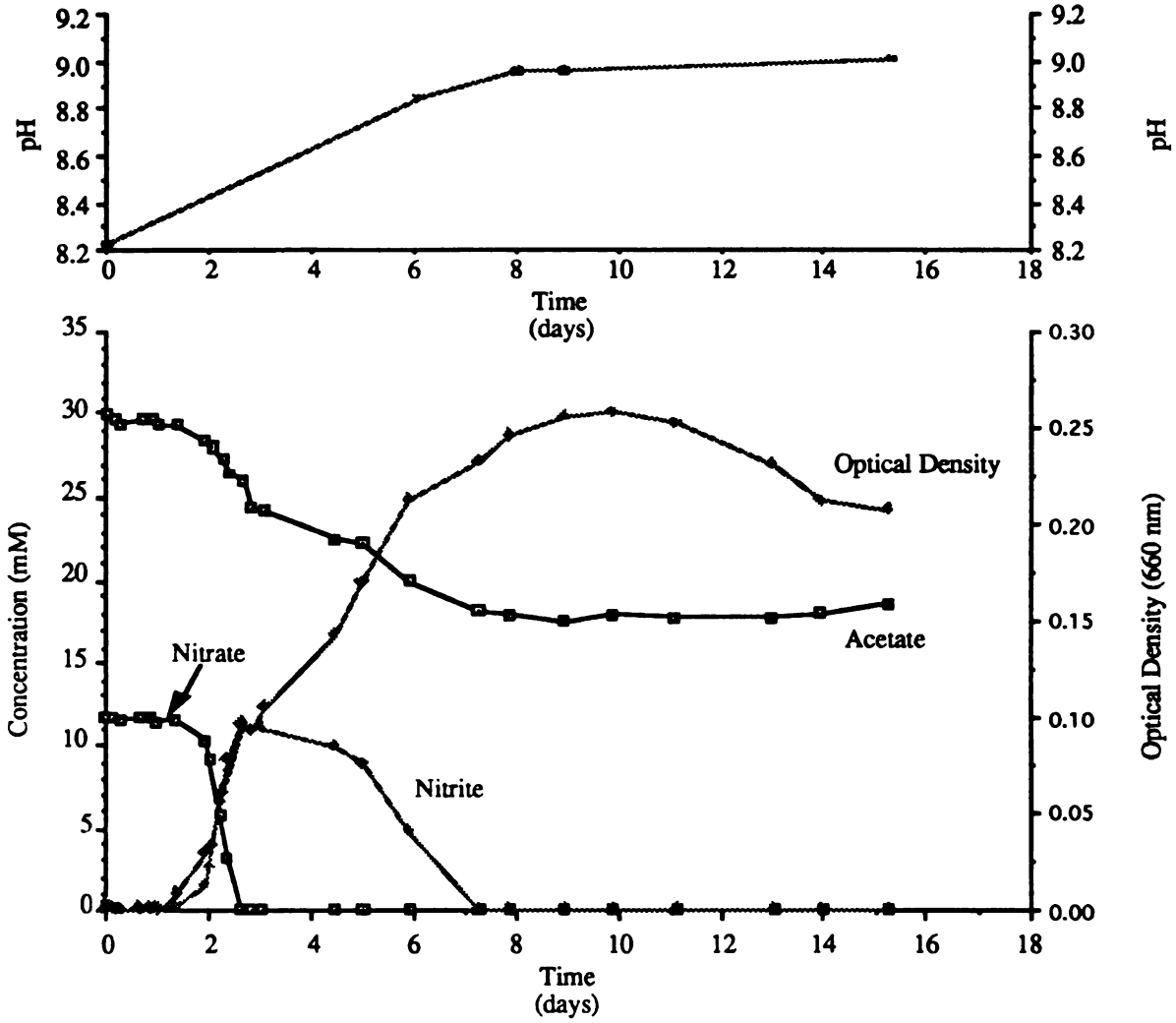

Figure 12.2. Growth pattern, substrate utilization, and pH increase for *Pseudomonas* sp. strain KC in Schoolcraft groundwater adjusted to an initial pH of 8.2. (Replicate #2)

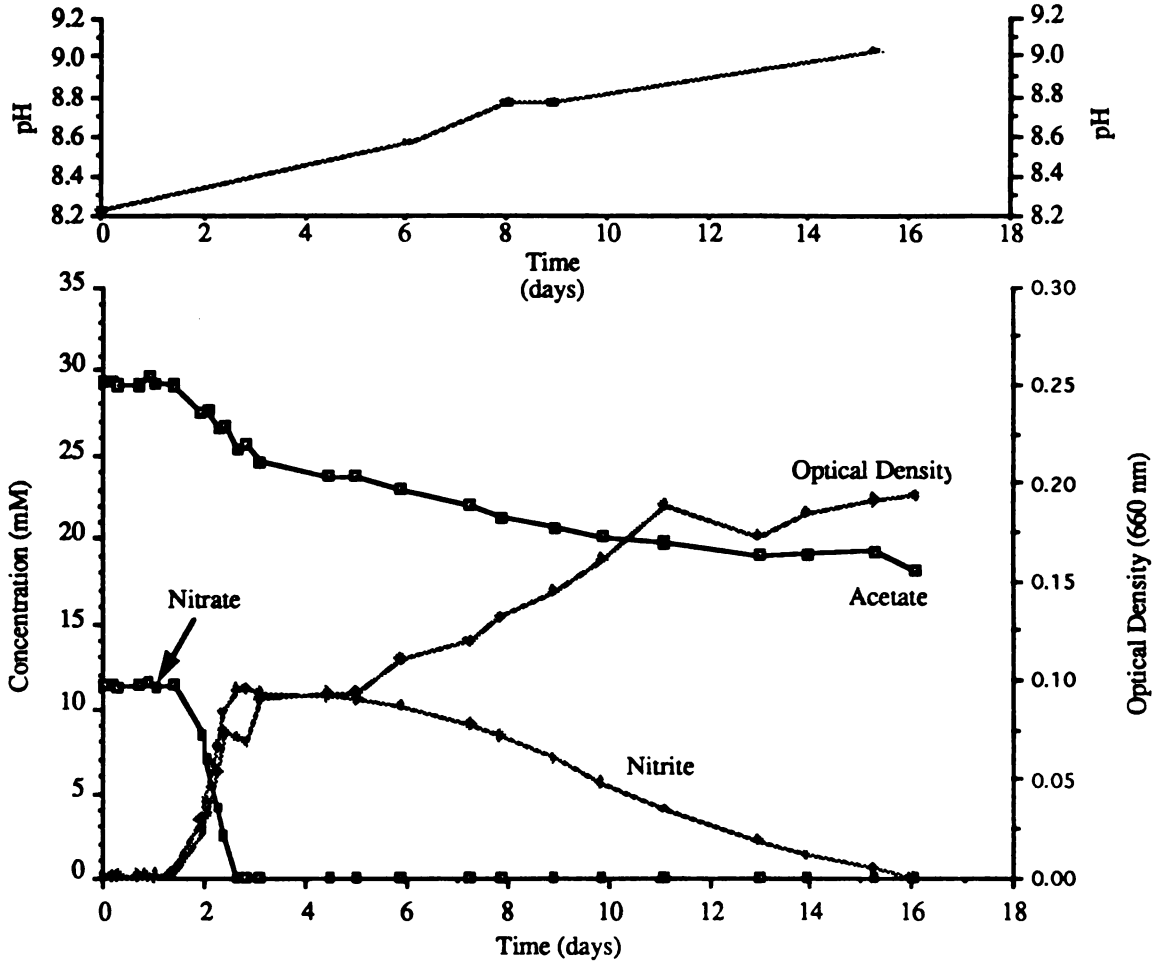

Figure 12.3. Growth pattern, substrate utilization, and pH increase for *Pseudomonas* sp. strain KC in Schoolcraft groundwater adjusted to an initial pH of 8.2. (Replicate #3)

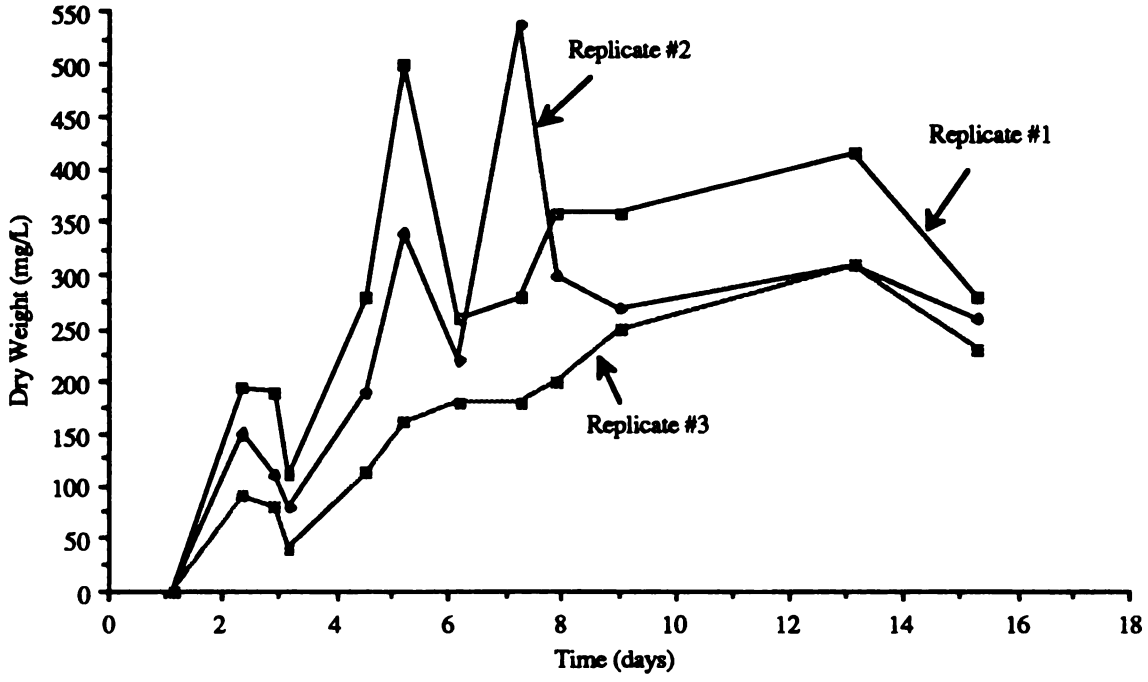

Figure 13. Biomass accumulation of *Pseudomonas* sp. strain KC in Schoolcraft groundwater measured as dry weight.

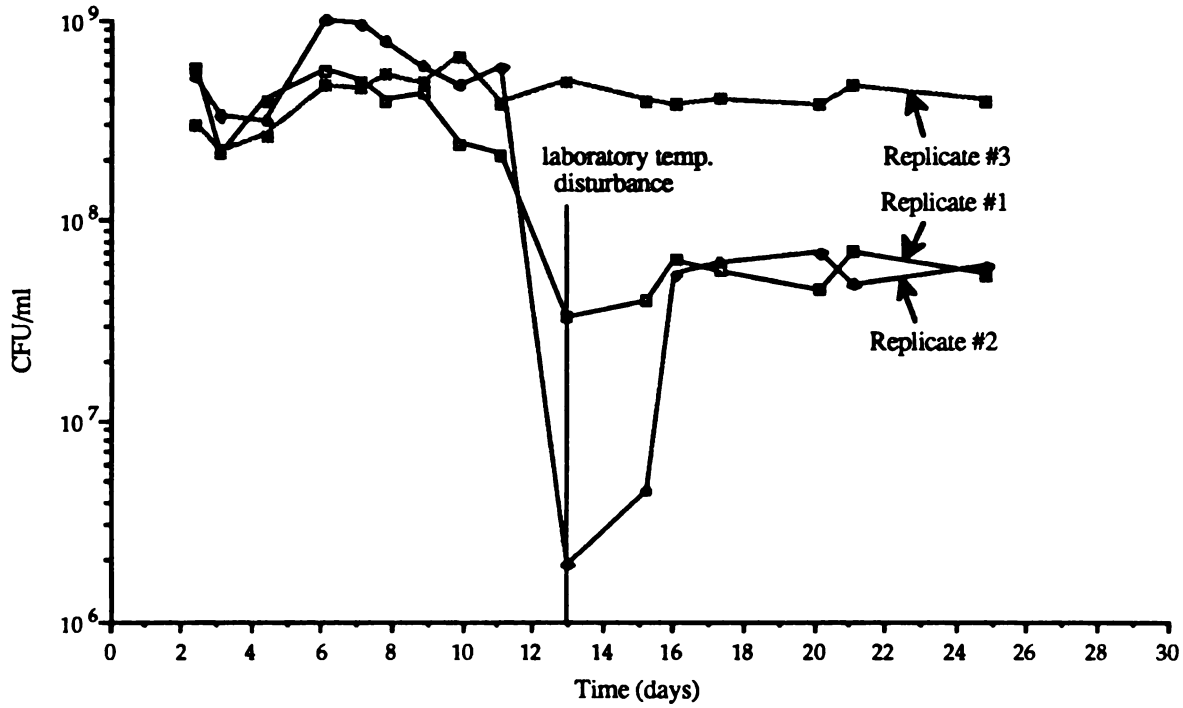

Figure 14. Growth of *Pseudomonas* sp. strain KC in Schoolcraft groundwater measured by bacterial plate counts.

Table 4. Kinetic parameters and yield coefficients for strain KC and Schoolcraft aquifer flora in Schoolcraft groundwater adjusted to an initial pH of 8.2.

Kinetic Parameter	KC[1]	Schoolcraft Flora[1]
Nitrate to nitrite		
μ_m, max. specific growth rate during NO_3^- conversion to NO_2^- (d^{-1})	3.12± 0.69	0.81± 0.21
Observed yield (mg cell dry weight per mg NO_3^-converted to NO_2^-)	0.21± 0.04	0.05± 0.01
Observed yield (10^9 CFU per mg NO_3^- converted to NO_2^-)	0.64 ± 0.19	0.03 ± 0.02
km, Maximum specific rate of nitrate removal (mg NO_3^- per mg cell dry weight per day)	12.1± 1.8	11.7± 0.9
Ratio Acetate to NO_3^- consumed	1.01 ± 0.03	1.05 ± 0.01
Nitrite to gaseous end products		
μ_m, max. specific growth rate during NO_2^- conversion to gaseous end products (d^{-1})	0.23± 0.09	0.67± 0.08
Observed yield (mg cell dry weight per mg NO_2^-reduced)	0.46± 0.18	0.18± 0.07
Observed yield (10^9 CFU per mg NO_2^-reduced)	1.51 ± 0.78	0.65 ± 0.21
km, Maximum specific rate of nitrite removal (mg NO_2^-per mg cell dry weight per day)	0.59± 0.26	3.07± 0.50
Nitrate to gaseous end products		
Overall observed yield (mg cell dry weight per mg NO_3^-)	0.40± 0.04	0.12± 0.03
Overall observed yield (10^9 CFU per mg NO_3^-)	1.02 ± 0.31	0.26 ± 0.05
f_s, fraction of electrons diverted for synthesis	0.61 ± 0.03	0.28 ± 0.05
f_e, fraction of electrons used for energy	0.39 ± 0.03	0.72 ± 0.05
Dry weight per cell (10^{-7} µg per CFU)	4.08 ± 1.52	3.09 ± 1.10

[1] Average ± one standard deviation for three independently grown cultures at 21.1°C.

The half-velocity coefficient Ks is another microbial kinetic parameter effecting competition between microbial groups. It is numerically equal to the nutrient concentration at which the specific growth rate is half of its maximum value (Brock *et al.*, 1991). The approximate values can be estimated by identifying the points at which the plots in Figures 15 and 16 change from first order to zero order. As shown in theses figures, the nitrate half-velocity coefficients for strain KC and the indigenous Schoolcraft organisms are essentially indistinguishable.

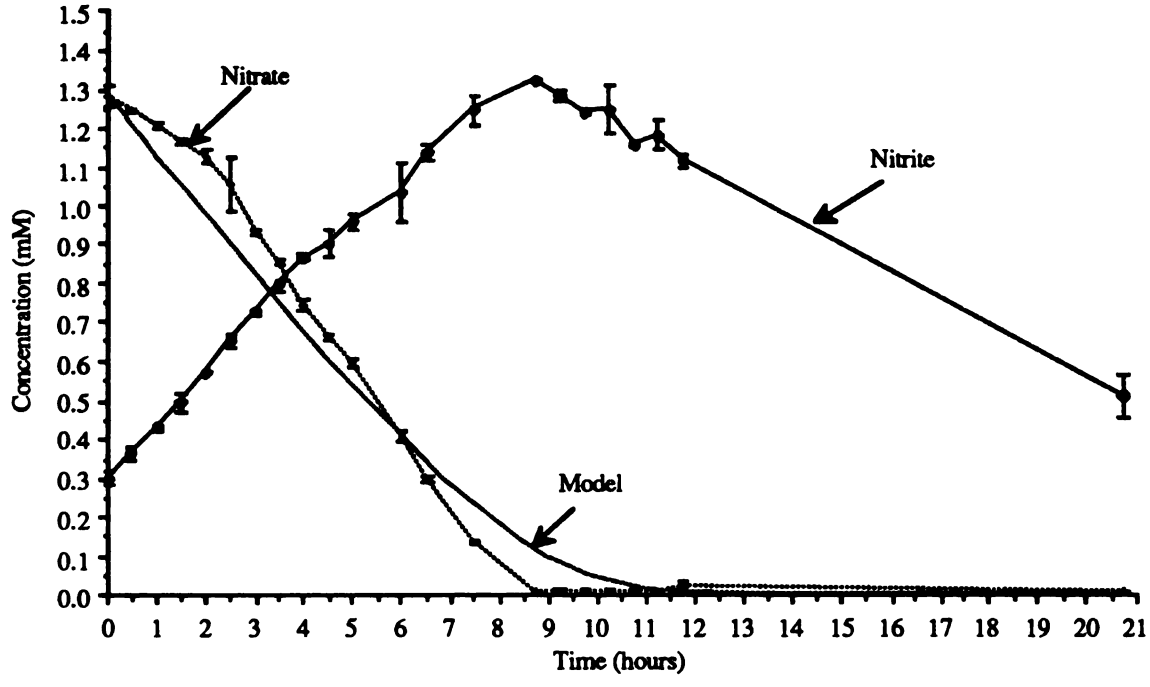

Figure 15. Nitrate and nitrite utilization by indigenous aquifer organisms in Schoolcraft groundwater adjusted to an initial pH of 8.2.

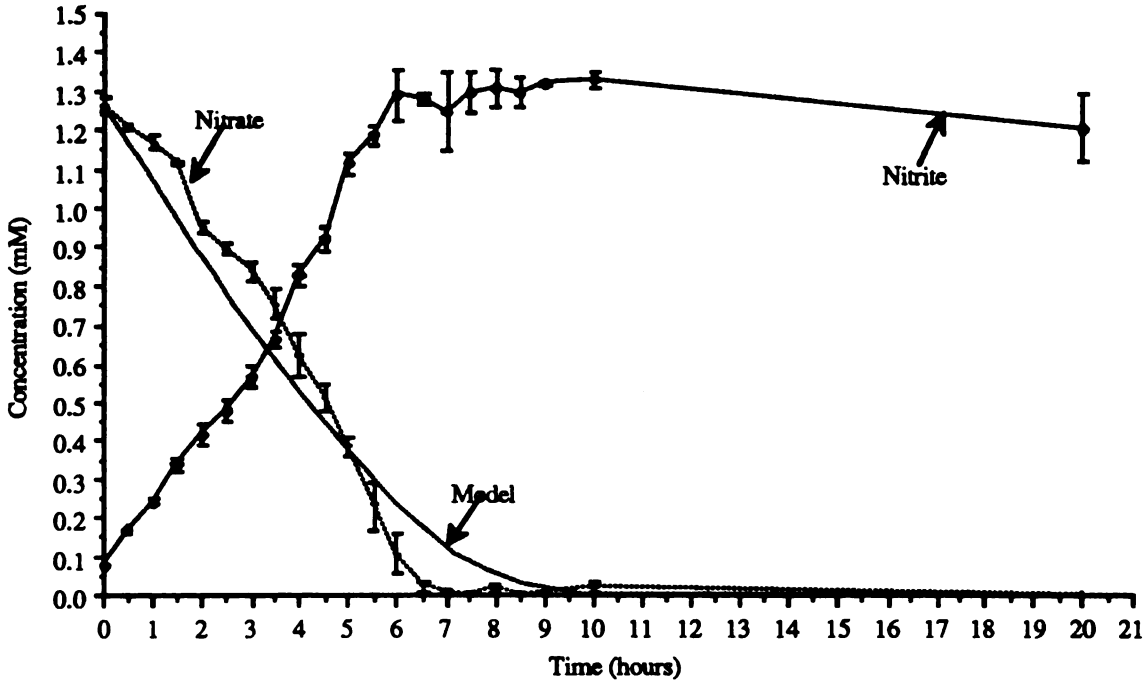

Figure 16. Nitrate and nitrite utilization by *Pseudomonas* sp. strain KC in Schoolcraft groundwater adjusted to an initial pH of 8.2.

Calculated values for the half-velocity coefficients (Ks) for strain KC and the Schoolcraft organisms can be found in Table 5. These values were calculated using the computer modeling software package Systat®. This model computes the best fit value for Ks using time and nitrate depletion data. The maximum specific rates of substrate utilization k_m shown in Table 5 are comparable to approximately half the value of those shown in Table 4. This is under the assumption that protein represents half the dry weight of each cell. See Appendix C for raw data and calculations supporting the parameters in Table 5. The model plots in Figures 15 and 16 were developed by using the average Ks, k_m, protein, and nitrate data for triplicate samples in the relationship below:

$$T = (K_S * \ln(S_O/S)/(k_m * X) + (So-S)/(k_m * X)$$

where:

Ks = the half-velocity coefficient

So = the initial nitrate concentration

S = time dependent nitrate concentration

km = the maximum specific rate of substrate utilization (mg NO3-/mg dry weight *hour)

X = average protein concentration

The above equation is based on the theory that substrate utilization generally follows a similar kinetic model as that for bacterial growth (Lawrence *et al.*, 1970). It has the same form as the well-known Michaelis-Menton expression for enzymatic degradation, but it is an empirical relationship based on observed patterns of substrate consumption by whole cells, and its coefficients may or may not be related to the activity of a specific enzyme. As shown in Figures 15 and 16, the model does not fit the actual data perfectly. This may be caused by conditions in which a nutrient or nutrients other than nitrate are limiting growth. The initial lag period of the data could be the result of cell acclimation to a new environment (Brock *et al.*, 1991).

As discussed in the introduction of this thesis and the beginning of this chapter, it is apparent that strain KC has the competitive advantage on the grounds of resource-based competition theory. Assuming approximately equivalent decay rates for strain KC and the Schoolcraft organisms, strain KC has a much lower S_{min} and hence would be expected to outcompete the indigenous organisms at alkaline pH levels (>8).

Table 5. Half-velocity coefficients and maximum specific rates of substrate utilization for *Pseudomonas* sp. strain KC and the indigenous organisms in Schoolcraft groundwater.

Kinetic Parameter	KC[1]	Schoolcraft Flora[1]
Nitrate to nitrite		
Maximum specific rate of nitrate removal, km (mg NO_3^- per mg protein per day)	19.51 ± 2.41	21.68 ± 0.49
Half velocity coefficient, Ks (mg NO_3^-/L)	11.97 ± 1.34	9.40 ± 0.32
Nitrite to gaseous end products		
Maximum specific rate of nitrite removal, km (mg NO_2^- per mg protein per day)	0.77 ± 0.58	5.60 ± 0.22

[1] Average ± one standard deviation for three independently grown cultures at 21.1°C.

CHAPTER 7

ENGINEERING APPLICATION

There has been much discussion in the literature concerning the introduction of genetically engineered microorganisms into the environment. *Pseudomonas* sp. strain KC is not a genetically engineered microorganism, but many of the concerns surrounding its introduction are addressed in such reports. The introduction of novel organisms into a new environment is termed bioaugmentation. This process has much untapped potential for remediation of many environmental contaminants in a variety of treatment schemes.

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 in the environment, and effects of the introduced organisms on ecosystem function (Tiedje *et al.*, 1989). When considering the environmental application of some microorganism, one of the most important series of questions to ask concerns the opportunity for persistence of the population after it has been introduced into the target environment (Lenski, 1992). Is it desirable for the introduced population to be self-sustaining? Or is it better if the introduced population performs its intended function and then dies out, being reintroduced only as need arises? The answer will depend, of course, on a comparison of the magnitude of the additional benefits that may derive from prolonged persistence with the possible costs, if any, that might arise from potential adverse effects caused by persistence (Lenski, 1992). Once this comparison has been made, it is appropriate to ask: What efforts, if any, have been made to enhance or limit the persistence

of the introduced population, as so desired? Results contained herein have shown that persistence and related containment of strain KC can be controlled to a certain degree by alkaline niche adjustment. Alkaline conditions have been shown to support a stable population of strain KC which rapidly decreased upon lowering the pH to 7.5. Copper toxicity is the suspected cause for the rapid decay of strain KC under near neutral conditions (Criddle *et al.*, 1990). However, this effect is not immune to mutations within strain KC which may allow it to persist.

Another necessary question to ask is: What empirical data are there concerning the indefinite persistence of the introduced microbial population in the target environment (Lenski, 1992)? Figure 8 provides evidence for the ability to contain strain KC through pH adjustment, but there is also evidence for the lack of ability to completely eliminate its persistence. It is quite likely that strain KC will not completely die out but will continue to persist. Lewis and Crawford (1993) observed persistence of strain KC for one year in aquifer materials stored at 4°C. This may be the result of genotype differences between generations. A key element in determining the likelihood of persistence of any introduced organism is its *fitness* in the new environment (Lenski, 1992). In most cases, a target environment will be supporting an indigenous population that is closely related to the organism proposed for introduction. Interactions between these two organism types is likely to be quite significant for the fate of the introduction; even slight differences in kinetic parameters or the ability to withstand various conditions may affect the opportunity for persistence of the introduced population. It becomes clear that the fitness of an introduced organism *relative* to a closely related indigenous population is likely to be especially useful in predicting the fate of an introduced population (Lenski, 1992). This thesis has addressed the fitness of *Pseudomonas* sp. strain KC in comparison to the indigenous organisms in Schoolcraft groundwater. Inherently the Schoolcraft groundwater contains organisms that are somewhat related to strain KC, but it is possible that the most competitive organisms

are fixed to soil particles in the Schoolcraft aquifer and not present in the groundwater that was used for the experiments in this thesis .

A textbook definition of fitness is 'The average contribution of one allele or genotype to the next generation or to succeeding generations, compared with that of other alleles or genotypes' (Lenski, 1993). The Darwinian fitness of an organisms therefore refers to its capacity for survival and reproduction, which depends on its environmental circumstances as well as on its genotype (Lenski, 1993). Fitness of an organism is dependent upon environmental conditions. Therefore, fitness is best regarded as a relative property, not an absolute one (Lenski, 1992). The results contained in this thesis have shown that under alkaline conditions, strain KC has the competitive advantage over the indigenous organisms (as a group) in Schoolcraft groundwater. This is not to infer that strain KC is the best competitor under all environmental conditions.

The concept of niche adjustment has broad implications for bioaugmentation efforts, where competition with native microorganisms is a major hurdle. Addition of alkalinity is a simple procedure that may be effective at certain sites. Of course, many other niche adjustment strategies can be envisioned. The optimal choice of strategies will depend upon the physiology of the organism to be introduced, the nature of the indigenous organisms, and prevailing environmental conditions at a targeted site.

CHAPTER 8

CONCLUSIONS

1. *Pseudomonas* sp. strain KC has a higher yield than Schoolcraft aquifer organisms at all pH values in medium D.

2. Alkaline pH values are optimal for growth of strain KC in medium D.

3. Niche adjustment does not reduce the concentration of indigenous organisms.

4. Strain KC will not persist at significant concentrations in a post niche-adjusted environment.

5. Higher maximum specific growth rate and yield are the primary reasons for the competitive advantage of strain KC under moderately alkaline conditions.

6. The half-velocity coefficient (Ks) for nitrate is insignificant in the assessment of strain KC's growth advantage.

7. Using resource-based competition theory, a lower estimated S_{min} is evidence that strain KC would outcompete the indigenous organisms in Schoolcraft groundwater.

The competitiveness of strain KC and its ease of transport through columns packed with Ottawa sand and Schoolcraft aquifer material (Mayotte *et al.*, 1994) indicate that field-scale

studies are justified. It may be possible to establish and maintain a CT-degrading zone or biofence by colonizing a pH-adjusted region in front of a migrating CT plume. Considering the diverse environments that strain KC was able to colonize and remediate (Dybas *et al.*, 1994 b), it can be concluded that alkali niche-adjustment is a useful means of maintaining a competitive population of strain KC under non-sterile operating conditions.

FUTURE WORK RECOMMENDATIONS

1. Complete kinetic growth parameter characterization of strain KC and the indigenous organisms in Schoolcraft groundwater at pH 7.5.

2. Determination of the kinetic growth parameters of strain KC and the indigenous organisms in Schoolcraft groundwater with nitrite as the sole initial electron acceptor.

3. Determination of the minimum *P.* KC dose needed for adequate colonization in the Schoolcraft aquifer

4. Enumeration of denitrifying bacteria in the Schoolcraft aquifer.

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APPENDIX A

APPENDIX A

ORIGINAL DATA AND CALCULATIONS FOR *PSEUDOMONAS* SP. STRAIN KC KINETIC PARAMETERS IN MEDIUM D.

Table A-1. Original data used for determination of μ_m, b, and protein per cell for *P.* KC in medium D.

Time (hr)	Average µg Protein /ml	Standard Dev. µg Protein/ml	Goemean P.KC Cells/ml	Geo Std. Dev. P.KC Cells	µg Prot./Cell
0	1.20	0.85	263810	84080	4.53E-06
6	1.12	0.90	105910	167596	1.06E-05
12	0.90	1.17	1550295	723635	5.79E-07
21	3.59	0.45	24489931	8148639	1.46E-07
30	10.61	3.14	33019272	42342687	3.21E-07
36	28.03	7.86	*NA	NA	NA
48	85.13	11.39	795811442	202469401	1.07E-07
54	111.21	6.70	981427532	184247047	1.13E-07
60	71.41	6.92	980446832	216859304	7.28E-08
71.5	80.91	3.73	303973683	391972074	2.66E-07
78	90.42	4.72	1216553	2614244	7.43E-05
95	75.63	4.66	NA	NA	NA

*NA denotes data that was not available.

Average µg Protein/Viable P.KC Cell = 1.72 X 10-7 ± 1.01 X 10-7
The correlation for the above relationship is shown by $R^2 = 0.996$

Time points 21 - 54 hours used to ensure that cells were in log growth phase and to avoid including dead cell protein in calculations.

Table A-2. Data used for calculations of μ_m and b for Pseudomonas sp. strain KC.

Time (hours)	μ (days^-1)	Time (hours)	b (days^-1)
6-12	10.73	71.5	2.44
12-21	7.36	78	20.38
21-30	0.80		
30-48	4.24		
48-54	0.84		

μ_m (days^{-1}) = 10.73

Overall b (days^{-1}) = 8.92

Time range for calculation of overall b = 60 to 78 hours.

Balanced stoichiometric equations (shown below) using f_s and f_e were used for the theoretical determination of the ratio of acetate to nitrate for cells (Criddle *et al.*, 1991).

R_d: $1/8\ CH_3COO^- + 1/4\ H_2O = 1/4\ CO_2 + 7/8\ H^+ + e^-$

f_sR_c: $f_s * [5/28\ CO_2 + 1/28\ NO_3^- + 29/28\ H^+ + e^- = 1/28\ C_5H_7O_2N + 11/28\ H_2O]$

f_eR_a: $f_e * [1/5\ NO_3^- + 6/5\ H^+ + e^- = 1/10\ N_2 + 3/5\ H_2O]$

where:

R_d = half reaction for the oxidation of an electron donor normalized by the moles of electrons removed from the donor.

R_a = half reaction for the reduction of an electron acceptor used for energy normalized by the moles of electrons added to the acceptor.

R_c = half reaction for the reduction of an electron acceptor used for synthesis normalized by the moles of electrons added to the acceptor, and $f_s + f_e = 1$.

Using the balanced equations above and yield data, the following relationship can be made:

$$\text{Yield} = \frac{f_s\,(1/28)\ 113\ \text{g cells/mole e}^-}{[f_e\,(1/5) + f_s\,(1/28)]\ 62\ \text{g } NO_3^-/\text{mole e}^-}$$

Using the relationship $f_e = 1\text{-}f_s$, the values for f_e and f_s were calculated. It was assumed that all the nitrate was converted to nitrogen gas and that the dry weight of each cell was equal to twice the measured protein. The maximum overall protein measurements were used in the above calculations. The assumed formula for cells was $C_5H_7O_2N$ which has not been proven to for *Pseudomonas* sp. strain KC.

APPENDIX B

APPENDIX B

ORIGINAL DATA AND CALCULATIONS FOR KINETIC PARAMETERS IN SCHOOLCRAFT GROUNDWATER.

Table B-1. Data used for determining the maximum specific growth rate μ_m of Schoolcraft organisms in Schoolcraft groundwater.

μ_m on NO_3^- $=(\ln X_2/X_1)/(t_2-t_1)$ ($days^{-1}$)				μm on NO_2^- $=(\ln X_2/X_1)/(t_2-t_1)$ ($days^{-1}$)			
Time (days)	S-1	S-2	S-3	Time (days)	S-1	S-2	S-3
3.083-3.604	0.76			7.021-8.042	0.69		
4.542-5.875		1.04		7.021-7.500		0.74	
3.604-4.125			0.62	7.021-7.50			0.59
Average	0.81			Average	0.67		
Standard Dev.	0.21			Standard Dev.	0.08		

Table B-2. Data used for determining the maximum specific rate of substrate utilization (k_m) of Schoolcraft organisms in Schoolcraft groundwater.

S-1 Max NO_3^- Util.			S-1 Max NO_2^- Util.		
Time (days)	NO_3^- (mM)	Rate (mg NO_3^-/L*d)	Time (days)	NO_2^- (mM)	Rate (mg NO_2^-/L*d)
4.13 - 3.60	9.739-6.401	397.31	10.71-7.02	6.527-1.686	60.349
Dry Weight (mg/L)	km (mg NO_3^- /mg cells*d)		Dry Weight (mg/L)	km (mg NO_2^- /mg cells*d)	
34	11.69		24	2.51	

S-2 Max NO_3^- Util.			S-2 Max NO_2^- Util.		
Time (days)	NO_3^- (mM)	Rate (mg NO_3^- /L*d)	Time (days)	NO_2^- (mM)	Rate (mg NO_2^- /L*d)
4.54 - 4.13	9.153-6.882	337.90	8.54-7.02	4.215-1.031	96.36
Dry Weight (mg/L)	km-2 (mg NO_3^- /mg cells*d)		Dry Weight (mg/L)	km-2 (mg NO_3^- /mg cells*d)	
27	12.51		30	3.21	

S-3 Max NO_3^- Util.			S-3 Max NO_2^- Util.		
Time (days)	NO_3^- (mM)	Rate (mg NO_3^- /L*d)	Time (days)	NO_2^- (mM)	Rate (mg NO_2^- /L*d)
4.13-3.60	9.703-7.118	302.4	7.02-5.88	4.422-0.284	167.00
Dry Weight (mg/L)	km-3 (mg NO_3^- /mg cells*d)		Dry Weight (mg/L)	km-3 (mg NO_3^- /mg cells*d)	
28	10.80		48	3.48	

Schoolcraft Flora Max. Specific Nitrate Utilization, km		Schoolcraft Flora Max. Specific Nitrite Utilization, km	
Average	11.67	Average	3.07
Standard Dev.	0.86	Standard Dev.	0.50

Table B-3. Data used for determining the yield of Schoolcraft organisms in Schoolcraft groundwater on nitrate reduced to gaseous end products .

Yield Data (Nitrate to gaseous end products)				
S-1 (using largest dry weight value)			(overall highest)	
NO_3^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_3^-)	Plate Counts (CFU/ml)	Yield (10^9 CFU/ mg NO_3^-)
12.003	93	0.12	1.50E+08	0.20

S-2 (using largest dry weight value)			(overall highest)	
NO_3^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_3^-)	Plate Counts (CFU/ml)	Yield (10^9 CFU/ mg NO_3^-)
11.931	68	0.09	2.20E+08	0.30

S-3 (using largest dry weight value)			(overall highest)	
NO_3^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_3^-)	Plate Counts (CFU/ml)	Yield (10^9 CFU/ mg NO_3^-)
11.938	108	0.15	2.00E+08	0.27

Table B-4. Data used for determining the yield of Schoolcraft organisms in Schoolcraft groundwater on nitrate reduced to nitrite.

Yield Data (nitrate to nitrite)				
S-1 (T=5.88 days)			(T=5.88)	
NO_3^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_3^-)	Plate Counts (CFU/ml)	Yield (10^9 CFU/mg NO_3^-)
12.003	30	0.04	3.00E+07	0.04

S-2 (T=5.88 days)				
NO_3^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_3^-)	Plate Counts (CFU/ml)	Yield (10^9 CFU/mg NO_3^-)
11.931	36	0.05	4.00E+06	0.01

S-3 (T=5.88 days)				
NO3- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_3^-)	Plate Counts (CFU/ml)	Yield (10^9 CFU/mg NO_3^-)
11.938	48	0.06	2.00E+07	0.03

Table B-5. Data used for determining the yield of Schoolcraft organisms in Schoolcraft groundwater on nitrite reduced to gaseous end products.

Yield Data (Nitrite to gaseous end products)					
S -1 (T=5.88-10.71 days)			(5.88-9.58 days)		
NO_2^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_2^-)	NO_2^- Consumed (mM)	Plate Counts (CFU/ml)	Yield (10^9 CFU/mg NO_2^-)
6.791	63	0.20	5.5	1.20E+08	0.47

S -2 (5.88-8.83 days)			(5.88-10.71 days)		
NO_2^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_2^-)	NO_2^- Consumed (mM)	Plate Counts (CFU/ml)	Yield (10^9 CFU/mg NO_2^-)
7.161	32	0.10	7.701	2.16E+08	0.61

S -3 (T=4.54-7.02 days)			(5.88-10.71 days)		
NO2- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_2^-)	NO2- Consumed (mM)	Plate Counts (CFU/ml)	Yield (10^9 CFU/mg NO_2^-)
5.352	60	0.24	4.422	1.80E+08	0.88

Table B-6. Data used for determining the maximum specific growth rate (μ_m) of *Pseudomonas* sp. strain KC in Schoolcraft groundwater.

μ_m on NO_3^- $=(\ln X_2/X_1)/(t_2-t_1)$ ($days^{-1}$)				μ_m on NO_2^- $=(\ln X_2/X_1)/(t_2-t_1)$ ($days^{-1}$)			
Time (days)	S-1	S-2	S-3	Time (days)	S-1	S-2	S-3
1.375-2.375			3.21	5.021-5.896	0.30	0.25	
1.938-2.250	3.76	2.38		9.875-11.125			0.13
Average	3.11			Average	0.23		
Standard Dev.	0.69			Standard Dev.	0.09		

Table B-7. Data used for determining the maximum specific rate of substrate utilization (k_m) of *Pseudomonas* sp. strain KC in Schoolcraft groundwater.

P. KC -1 Max NO_3^- Util.			P. KC -1 Max NO_2^- Util.		
Time (days)	NO_3^- (mM)	Rate (mg NO_3^-/L*d)	Time (days)	NO_2^- (mM)	Rate (mg NO_2^-/L*d)
2.04 - 1.94	9.587 - 7.157	1506.6	5.02-4.46	6.534-2.717	313.54
Dry Weight (mg/L)	km (mg NO_3^- /mg cells*d)		Dry Weight (mg/L)	km (mg NO_2^- /mg cells*d)	
129	11.68		390	0.80	

P. KC-2 Max NO_3^- Util.			P. KC-2 Max NO_2^- Util.		
Time (days)	NO_3^- (mM)	Rate (mg NO_3^- /L*d)	Time (days)	NO_2^- (mM)	Rate (mg NO_2^- /L*d)
2.38 - 2.25	5.595 - 3.061	1208.5	5.90-5.02	8.677-4.666	209.67
Dry Weight (mg/L)	km-2 (mg NO_3^- /mg cells*d)		Dry Weight (mg/L)	km-2 (mg NO_3^- /mg cells*d)	
114	10.60		310	0.68	

P. KC-3 Max NO_3^- Util.			P. KC-3 Max NO_2^- Util.		
Time (days)	NO_3^- (mM)	Rate (mg NO_3^- /L*d)	Time (days)	NO_2^- (mM)	Rate (mg NO_2^- /L*d)
2.25 - 2.04	7.233 - 4.185	899.89	9.88-8.92	7.284-5.681	76.81
Dry Weight (mg/L)	km-3 (mg NO_3^- /mg cells*d)		Dry Weight (mg/L)	km-3 (mg NO_2^- /mg cells*d)	
64	14.06		254	0.30	

Pseudomonas sp. strain KC Max. Specific Nitrate Utilization, km			Pseudomonas sp. strain KC Max. Specific Nitrite Utilization, km		
Average	12.11		Average	0.59	
Standard Dev.	1.77		Standard Dev.	0.26	

Table B-8. Data used for determining the yield of *Pseudomonas* sp. strain KC in Schoolcraft groundwater on nitrate reduced to gaseous end products .

Yield Data (Nitrate to gaseous end products)					
P. KC -1 (T=6.17 days)			(overall highest)		
NO_3^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_3^-)	Plate Counts (CFU/ml)	Yield (10^9 CFU/ mg NO_3^-)	
11.716	259	0.36	5.70E+08	0.78	

P. KC -2 (T=7.92 days)			(overall highest)		
NO_3^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_3^-)	Plate Counts (CFU/ml)	Yield (10^9 CFU/ mg NO_3^-)	
11.624	299	0.41	9.90E+08	1.37	

P. KC -3 (T=13.15 days)			(overall highest)		
NO_3^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_3^-)	Plate Counts (CFU/ml)	Yield (10^9 CFU/ mg NO_3^-)	
11.492	309	0.43	6.50E+08	0.91	

Table B-9. Data used for determining the yield of *Pseudomonas* sp. strain KC in Schoolcraft groundwater on nitrate reduced to nitrite.

Yield Data (nitrate to nitrite)				
P. KC -1 (Average between 2.38 and 2.94 days)			(T=2.46)	
NO_3^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_3^-)	Plate Counts (CFU/ml)	Yield (10^9 CFU/mg NO_3^-)
11.716	192	0.26	5.70E+08	0.78
P. KC -2 (Average between 2.38 and 2.94 days)			(T=2.46)	
NO_3^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_3^-)	Plate Counts (CFU/ml)	Yield (10^9 CFU/mg NO_3^-)
11.624	129	0.18	5.20E+08	0.72
P. KC -3 (Average between 2.38 and 2.94 days)			(T=2.46)	
NO3- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_3^-)	Plate Counts (CFU/ml)	Yield (10^9 CFU/mg NO_3^-)
11.492	145	0.20	2.98E+08	0.42

Table B-10. Data used for determining the yield of *Pseudomonas* sp. strain KC in Schoolcraft groundwater on nitrite reduced to gaseous end products.

Yield Data (Nitrite to gaseous end products)					
P. KC -1 (T=3.19-6.17 days)			(3.13-6.13 days)		
NO_2^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_2^-)	NO_2^- Consumed (mM)	Plate Counts (CFU/ml)	Yield (10^9 CFU/mg NO_2^-)
11.050	150	0.30	11.010	3.42E+08	0.68
P. KC -2 (3.19-7.92 days)			(3.13-6.13 days)		
NO_2^- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_2^-)	NO_2^- Consumed (mM)	Plate Counts (CFU/ml)	Yield (10^9 CFU/mg NO_2^-)
10.943	220	0.44	6.500	6.60E+08	2.21
P. KC -3 (T=3.19-13.15 days)			(3.13-9.96 days)		
NO2- Consumed (mM)	Dry Weight (mg/L)	Yield (mg cells/mg NO_2^-)	NO2- Consumed (mM)	Plate Counts (CFU/ml)	Yield (10^9 CFU/mg NO_2^-)
9.000	270	0.65	5.600	4.25E+08	1.65

The relationship below and the method outlined in Appendix B was used to calculate f_e and f_s. Yield on nitrate reduced to gaseous end products represented the yield for these calculations.

$$\text{Yield} = \frac{f_s\,(1/28)\,113 \text{ g cells /mole e-}}{[f_e\,(1/5) + f_s\,(1/28)]\,62 \text{ g NO3-/mole e}^-}$$

APPENDIX C

APPENDIX C

ORIGINAL DATA USED FOR DETERMINING THE HALF-VELOCITY COEFFICIENTS IN SCHOOLCRAFT GROUNDWATER.

Table C-1. Supporting data for the determination of the maximum specific rate of **nitrate** utilization k_m of Schoolcraft organisms in Schoolcraft groundwater.

Sample	Time (hours)	NO_3^- consumed (mM)	Average Protein (mg/L)	k_m (mg NO_3^-/mg protein*d)
S 1	3-7.5	0.802	12.09	21.93
S 2	3-7.5	0.784	11.79	21.99
S 3	3-7.5	0.788	12.34	21.11

Table C-2. Supporting data for the determination of the maximum specific rate of **nitrite** utilization k_m of Schoolcraft organisms in Schoolcraft groundwater.

Sample	Time (hours)	NO_2^- consumed (mM)	Average Protein (mg/L)	k_m (mg NO_2^-/mg protein*d)
S 1	8.75-20.75	0.851	13.91	5.63
S 2	8.75-20.75	0.776	13.30	5.37
S 3	9.25-20.75	0.879	13.91	5.81

Table C-3. Calculated half-velocity coefficients Ks and supporting data for Schoolcraft organisms in Schoolcraft groundwater.

Parameter	S 1	S 2	S 3	Average	Standard Dev.
K_S (mg/L)	9.05	9.5	9.66	9.40	0.32
k_m (hr^-1)	0.914	0.916	0.88	0.90	0.02
Protein (mg/L)	12.09	11.79	12.34	12.07	0.28

The above values for km and protein were used to calculate the half-velocity coefficients K_S for Schoolcraft organisms. The average K_S was then used in developing the model plot shown in Figure 15.

Table C-4. Supporting data for the determination of the maximum specific rate of **nitrate** utilization k_m of *Pseudomonas* sp. strain KC in Schoolcraft groundwater.

Sample	Time (hours)	NO_3^- consumed (mM)	Average Protein (mg/L)	km (mg NO_3^-/mg protein*d)
P.KC 1	1.5-5.5	0.940	18.25	19.16
P.KC 2	1.5-6	0.962	18.41	17.28
P.KC 3	1.5-6	1.01	15.12	22.08

Table C-5. Supporting data for the determination of the maximum specific rate of **nitrite** utilization k_m of *Pseudomonas* sp. strain KC in Schoolcraft groundwater.

Sample	Time (hours)	NO_2^- consumed (mM)	Average Protein (mg/L)	km (mg NO_2^-/mg protein*d)
P.KC 1	10 - 20	0.069	18.74	0.41
P.KC 2	10 - 20	0.219	16.77	1.44
P.KC 3	10 - 20	0.077	18.22	0.47

Table C-6. Calculated half-velocity coefficients Ks and supporting data for *Pseudomonas* sp. strain KC in Schoolcraft groundwater.

Parameter	*P*.KC 1	*P*.KC 2	*P*.KC 3	Average	Standard Dev.
K_S (mg/L)	13.52	11.13	11.26	11.97	1.34
k_m (hr^-1)	0.80	0.72	0.92	0.81	0.10
Protein (mg/L)	18.25	18.41	15.12	17.26	1.85

The above values for km and protein were used to calculate the half-velocity coefficients K_S for *Pseudomonas* sp. strain KC. The average K_S was then used in developing the model plot shown in Figure 16.