BIOTRANSFORMATION OF TRICHLOROETHENE BY A MIXED CULTURE OF NITRIFYING BACTERIA

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Trichloroethene (TCE) is a colorless, volatile liquid that is extensively used in the metals processing, electronics, printing, pulp and paper, textiles and aerospace industries. Its broad spectrum of use results from its ability to cleanse more thoroughly and efficiently than alkaline cleansers, its low flammability, and its high flashpoint, which render it relatively safe compared to other solvents [Mahaffey *et al.*, 1992].

TCE has been used as an industrial solvent worldwide for about fifty years. TCE has contaminated the environment by accidental spillage, leaking storage tanks, improper disposal, and landfill leachates [Alvarez-Cohen and McCarty, 1991b]. The Environmental Protection Agency has found TCE in at least 614 sites out of the 1300 sites on its National Priorities List (NPL) [ATSDR, 1991]. Exposure to high levels of TCE can lead to dizziness or sleepiness. Breathing high concentrations of TCE can lead to unconsciousness. It may cause nerve damage and leukemia upon prolonged exposure. TCE is a known carcinogen in mice and a suspected carcinogen in humans [Alvarez-Cohen and McCarty, 1991a]. Because of its toxicity, TCE waste and wastes containing TCE have been classified as hazardous wastes according to the provisions of the Resource Conservation and

Recovery Act [Mahaffey *et al.*, 1992]. The physical and chemical properties of TCE are listed in the Appendix.

Drinking Water Standards for TCE

TCE is one of the most frequently detected organic contaminants found in ground water [Alvarez-Cohen and McCarty, 1991a]. Based on available federal and state surveys, between 9% and 34% of the drinking water supply sources that have been tested in the United States have some TCE contamination. Since about one-half the population of the United States relies on ground water as a source of drinking water, concern about contamination of this resource has grown considerably in the last 20-25 years [Beeman and Suflita, 1987]. TCE has been assigned a maximum contaminant level (MCL) - the level designated by the Safe Drinking Water Act as allowable in public drinking waters - of 5 μg/L [Mahaffey *et al.*, 1992]. Waters contaminated with TCE in concentrations greater than the MCL are considered unsafe and must be treated before use as a drinking water source.

Fate of TCE in the Environment

Most of the TCE used in the United States is released into the atmosphere by evaporation, primarily from vapor degreasing operations. The dominant TCE degradation process in the atmosphere is reaction with hydroxyl radicals. The estimated half life for this process is 7 days. When TCE is broken down in the air, phosgene, a

lung irritant, is formed [ATSDR, 1991]. TCE present in surface waters or on soil surfaces will predominantly volatilize into the atmosphere. TCE is also highly mobile in soil. In subsurface soils, TCE is only slowly degraded and may be relatively persistent [ATSDR, 1991].

The discovery of such a large number of sites contaminated with TCE and the concern about its effects on human health have led researchers to seek novel methods to remediate these sites. Biotransformation can be a significant process affecting the fate of organic contaminants in the subsurface. *In-situ* bioremediation which attempts to facilitate biotransformation of pollutants in place, in the subsurface, is a promising technique currently under investigation.

Nitrifying Bacteria

Among the organisms which have shown promise for the degradation of TCE are the class of bacteria known as nitrifying bacteria. Nitrifying bacteria are ubiquitous soil and water dwelling organisms. They are autotrophic in nature and require oxygen for their survival. These bacteria grow lithotrophically at the expense of reduced inorganic nitrogen compounds. No lithotrophic organism is known that carries out the complete oxidation of ammonia to nitrate. Thus nitrification of ammonia in nature results from the sequential action of two separate groups of organisms, the ammonia-oxidizing bacteria and the nitrite-oxidizing bacteria

[Brock and Madigan, 1988]. The ammonia oxidizers (eg. *Nitrosomonas europaea*) derive their energy for growth exclusively from the oxidation of ammonia to nitrite [Rasche *et al.*, 1991]. The oxidation of ammonia in the ammonia-oxidizers is initiated by the enzyme ammonia monooxygenase (AMO) [Wood, 1986]. *Nitrobacter* is an example of nitrite-oxidizing bacteria. The oxidation of nitrite to nitrate is carried out by the enzyme nitrite oxidase.

TCE Biotransformation by Nitrifying Bacteria

Recent evidence indicates that many of the nitrifiers, specifically the ammonia oxidizers, are also capable of cooxidizing hydrocarbons and aliphatic halogenated hydrocarbons, including industrial pollutants such as TCE [Arciero *et al.*, 1989]. Because many halogenated hydrocarbons are suspected human carcinogens, increasing concern about the presence of these chemicals in soil and groundwater supplies has stimulated interest in characterizing the activity and physiology of bacteria which exhibit biodegradative potential. Nitrifying bacteria are excellent candidates for study because it may be possible to enhance the biodegradative capacity of these ubiquitous soil bacteria with the simple addition of ammonia and oxygen to support halocarbon cometabolism [Rasche *et al.*, 1991].

Research Objectives

Based on these facts, a research project was initiated to investigate the fate of TCE when exposed to a mixed culture of nitrifying bacteria and to discern the various parameters that could affect the rate of TCE biotransformation. The primary objectives of this research were the following:

1. To investigate the effects of conditions of a mixed culture of nitrifying bacteria on the rate of biotransformation of TCE, including:
   - (i) - effects of different ammonia concentrations
   - (ii) - effects of different bacterial concentrations
   - (iii) - effects of different initial TCE concentrations
2. To determine the effects of toxicity on the nitrification process, including:
   - (i) - effects of TCE toxicity
   - (ii) - effects of methanol toxicity

A review of literature pertinent to this study is presented in Chapter II. Chapter III gives a description of the materials used and the experimental and analytical methods employed in this study. The results obtained from this study are discussed in Chapter IV. The conclusions that can be drawn from these experiments are presented in Chapter V. Also in Chapter V, suggestions for advancement of the study are offered.

CHAPTER II

LITERATURE REVIEW

Biodegradation

Grady [1985] has defined biodegradation as "the biological transformation of an organic chemical to another form." The extent of transformation could vary from a single step to complete mineralization (the conversion to carbon dioxide, water, and various other inorganic forms). The ultimate removal of hazardous contaminants like TCE may be accomplished by converting these organic pollutants into biomass, harmless intermediates, or byproducts of microbial metabolism like water, carbon dioxide, methane and inorganic salts. This process may take place in a single step or in a series of discrete smaller steps where the compound is progressively mineralized by microbial activity into simpler fractions [Alexander, 1981].

One of the most critical factors affecting the fate of a chemical after its release into the environment is microbial degradation. Biodegradation can be accomplished by any living organism, however higher organisms tend to excrete chemicals that do not fit into their normal metabolic pathways, and plants usually convert chemicals to neutral, water-insoluble forms for easy storage. The high catabolic

versatility, species diversity and metabolic efficiency of microorganisms, suggests that they play a major role in the ultimate degradation of synthetic chemicals that enter the environment [Howard and Banerjee, 1984].

An important factor that determines the susceptibility of an organic compound to microbial attack is the length of time that it has been on earth [Grady, 1985]. Naturally occurring (biogenic) compounds are degradable by some organism that has evolved or adapted to use it as food. Most modern chemicals are similar to biogenic compounds, so they can perhaps be degraded. However there are some chemicals known as xenobiotics which are unlike any naturally occurring compounds and are difficult to degrade [Grady, 1985]. Despite these difficulties, numerous mechanisms exist which allow xenobiotic compounds to be microbially degraded [Alexander, 1981].

Modes of Biodegradation

Bacteria possess a wide variety of mechanisms to metabolize chemicals. The mechanism used for a given chemical depends upon the nature of the chemical, the environment, the type of organism and the specific metabolic capabilities of the organism. For example, oxygen is a vital part of the organic food sources for animals and microorganisms. Oxidation reactions are the means for the animals and microorganisms to obtain energy. There are also many organic compounds such as alkanes which are devoid of oxygen. Bacteria possess a unique biochemical characteristic of being able

to catalyze oxidations using molecular oxygen and are capable of degrading such compounds [Grady, 1985].

Biodegradation can also occur in anaerobic environments. The most common byproducts of anaerobic metabolism are carbon dioxide and methane. Kobayashi and Rittmann [1982] have shown that some halogenated organics require aerobic conditions for dehalogenation to occur but others require anaerobic conditions. This shows that the success of biodegradation depends on the environment in which it is attempted.

There are diverse populations of microorganisms in water and soil that have considerable metabolic capabilities for degrading natural and xenobiotic organic chemicals. When a chemical is introduced into a microenvironment, Howard and Banerjee [1984] report that one of the following three conditions could occur: (a) one or more of the microorganisms present has the required enzymes and is present in a high enough concentration to effect immediate biodegradation, or (b) acclimation of the microorganisms may be necessary, and this could be signalled by a lag period between addition of a chemical and the onset of biodegradation. The acclimation period could represent enzyme induction, gene transfer or mutation, or, where the necessary enzymes are available, growth in population of the responsible microorganisms, the third case, being (c) a microorganism capable of degrading the chemical is absent. The initial species present, their relative concentrations, the condition of their enzymes and their ability to acclimate once exposed to a

chemical are likely to vary considerably depending upon the existing environmental parameters. The concentrations of individual species and the enzymes (requisite or inducible) that they contain vary considerably with the type of microenvironment [Howard and Banerjee, 1984].

Biodegradation and Cometabolism

Enzymes are very specific catalysts. However this specificity is with respect to their catalytic function. They catalyze only particular types of reactions. They are much less specific with respect to substrate binding, although the degree of specificity depends on the enzyme in question [Grady, 1985]. Enzymes may bind to analogs of the natural substrate, including functional groups on xenobiotic compounds. If the functional groups on the xenobiotic compound do not appreciably alter the charge makeup of the active sites, then the enzyme can catalyze its specific reaction. This process where an existing enzyme happens to have a suitable catalytic activity toward a novel substrate, has been called gratuitous metabolism [Grady, 1985].

Cometabolism is another term which is used to describe gratuitous metabolism, and it has been defined by Dalton and Stirling [1982] as "the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound." In this process, the way in which microorganisms can effect continual biodegradation of the xenobiotic compound is through the

use of additional carbon and energy sources supplied from the medium or from the action of other organisms in a mixed microbial community [Alexander, 1981].

Aerobic Biotransformation of Chlorinated Aliphatics

Cometabolism is one of the major mechanisms by which aerobic biotransformation of chlorinated aliphatics takes place. Reactions between organic compounds and oxygen cannot usually take place since they exist in different ground states. Bacteria contain classes of enzymes called monooxygenases and dioxygenases which can catalyze the cometabolic transformations of chlorinated aliphatics [Wood, 1982]. Two of the important oxygenase enzymes that cause aerobic transformation of chlorinated aliphatics are ammonia monooxygenase (AMO) [Hyman *et al.*, 1988] and methane monooxygenase (MMO) [Oldenhuis *et al.*, 1991]. These enzymes are produced by the bacteria in response to inducing agents which are also their growth substrates. Ammonia induces the production of the enzyme AMO and methane induces the production of the enzyme MMO. Though the enzyme AMO is produced to oxidize ammonia and MMO is produced to oxidize methane, they exhibit relaxed substrate specificity which results in the oxidation of many chlorinated aliphatics as well [Hyman *et al.*, 1988 and Oldenhuis *et al.*, 1989]

One potential approach to aerobic biological treatment of chlorinated aliphatics is to develop a population of organisms expressing high levels of oxygenase activity by adding that

substrate which induces the synthesis of oxygenase enzymes. While many compounds may serve to induce oxygenase activity, based on cost and environmental acceptability, methane and ammonia appear to be two favorable oxygenase inducers for use in bioremediation processes.

Methane Monooxygenase (MMO) Systems

Methane monooxygenase (MMO) is an enzyme produced by methanotrophic bacteria. Methanotrophs derive both energy and carbon from the oxidation of methane by the broadly nonspecific enzyme MMO, with NADH or NADPH as an intermediate energy source [Alvarez-Cohen and McCarty, 1991a]. Oldenhuis and coworkers [1990] have observed the capability of soluble MMO produced by methanotrophic bacteria to degrade halogenated aliphatics. They have found that methanotrophic bacteria can degrade halogenated organic compounds that are not utilized by organisms as carbon sources. Methane-oxidizing bacteria could therefore become increasingly important for the application of biological techniques for soil cleanup and groundwater treatment, if suitable treatment technologies for employing their cometabolic degradative capacity can be developed. Important factors to consider include substrate specificity, reaction rates, and stability of the organisms. Oldenhuis and coworkers [1990] have also observed the inactivation of MMO due to its reaction with the products formed from the degradation of TCE. Alvarez-Cohen and McCarty [1991b] have found that MMO inactivation results from TCE oxidation rather than from exposure to TCE itself. They call this phenomenon product toxicity.

Ammonia Monooxygenase (AMO) Systems

Ammonia monooxygenase (AMO) is an enzyme produced by nitrifying bacteria. Ammonia oxidizing nitrifiers derive their energy from the oxidation of ammonia to nitrite by the enzyme AMO. In addition to oxidizing ammonia, these nitrifiers are capable of cooxidizing a broad range of hydrocarbon substrates, including alkanes and alkenes. These oxidations are mediated by ammonia monooxygenase (AMO) [Rasche *et al.*, 1990]. One factor which potentially limits biodegradative capacity is the effect of halocarbon cometabolism on the physiology of the microorganism. Arciero and coworkers [1989] have reported that ammonia oxidation in the nitrifying bacterium *N. europaea* is not inactivated during short term exposure to TCE (15-min incubation of cells with 1 mM ammonium and a nominal TCE concentration of 11 μM). The absence of a toxic effect on the cells as a result of TCE oxidation would make nitrifiers unique among bacteria known to cooxidize TCE and as such would represent a considerable advantage of nitrifiers in bioremediation schemes [Rasche *et al.*, 1991].

However, Rasche and coworkers [1991] have found that inactivation of ammonia oxidation occurred during biodegradation of TCE. Cells incubated with TCE under conditions which supported AMO turnover resulted in progressive, irreversible loss of ammonia-oxidizing activity, as measured by the ability of cells to convert ammonia to nitrite. Rasche and coworkers [1991] further report that the extent of inactivation depended on the length of time the cells were

exposed to TCE as well as the initial TCE concentration. These results are in contrast with a previous report [Arciero *et al.*, 1989] which indicated that loss of ammonia-oxidizing activity did not accompany TCE biodegradation by *N. europaea*. Rasche and coworkers [1991] speculate that the discrepancy between their results and those of Arciero and coworkers [1989] may be accounted for by differences in the experimental system, such as reductant concentration, cell densities, and TCE concentrations. Vannelli and coworkers [1990] have reported that *N. europaea* catalyzed the ammonia-stimulated aerobic transformation of various halogenated aliphatics including TCE.

Toxicity Effects on the Nitrification Process

Previous experiments have studied the effects of toxicity on the nitrification process. Though the effects of toxic organics on the nitrification process have not been studied extensively, various studies have been conducted to determine the effects of other contaminants, such as heavy metals on the nitrification process. Bagby and Sherrard [1981] have studied the effect of cadmium and nickel on the nitrification process, and they conclude that nitrifying organisms are very susceptible to the toxic effects of heavy metals. They found almost complete inhibition of nitrification due to the presence of cadmium (5.25 mg/L as Cd^{2+}) and nickel (1.15 mg/L as Ni^{2+}). Randall and Buth [1984] reported a correlation between temperature and toxicity. They found that the inhibitory effects of nickel on nitrification was greater at 14°C than at 17°C or 30°C.

This indicates the existence of a synergistic inhibitory effect between temperature and nickel toxicity for nitrification. Randall and Buth [1984] also say that the toxic compounds have a stronger inhibitory effect on nitrifying bacteria than on heterotrophic bacteria because of the slower growth rates of the nitrifiers.

Differences between MMO and AMO systems

While the reaction mechanisms of oxygenases such as MMO and AMO are similar, there are significant differences between methanotrophs and nitrifiers that may affect their applicability to a full scale bioremediation processes. These differences include:

1. Methanotrophs produce significant amounts of soluble MMO only under conditions of copper-stress [Oldenhuis *et al.*, 1991]. If copper is readily available, the organisms produce a particulate MMO that does not degrade chlorinated aliphatics. AMO, produced by nitrifiers, does not appear to be affected by growth conditions such as copper-stress.

2. AMO appears to have lower substrate specificity than soluble MMO [Rasche *et al.*, 1990]. The ability to transform a larger number of compounds may be an advantage at some sites.

3. Pumping ammonia and oxygen into the ground for the stimulation of nitrifiers has a potential adverse side effect of increasing the concentration of nitrate (the product of ammonia metabolism) in

groundwaters that may be used as drinking water sources. This may not be a major concern since the presence of TCE makes the groundwater unfit to drink anyway.

4. In contrast, methane, propane etc., which are added to stimulate the MMO systems, are explosive gases that are potential hazards if improperly handled and makes this approach more risky.

As such, utilization of either monooxygenase system appears to have significant advantages and disadvantages.

Summary

TCE is a priority pollutant of surface waters and groundwater. Nitrifying bacteria using the enzyme AMO are capable of cometabolically degrading TCE. All published studies focussing on AMO systems have used a pure culture of *N. europaea*. In the studies described below, a mixed culture of nitrifiers is investigated, a condition that is more likely representative of environmental conditions. The focus of this study is on the breakdown of TCE by mixed culture nitrifying systems that express AMO.

CHAPTER III

MATERIALS AND METHODS

This chapter describes the various analytical and experimental techniques used to conduct this study and the materials that were used in the study.

Materials

All reagents used in this study were commercially available and were used without further purification. Methanol and TCE were HPLC grade and were obtained from Fisher Scientific (Fair Lawn, NJ). The serum bottles (volume = 120 mL) used to run the experiments, Teflon-lined rubber septa, and aluminum crimp caps used to seal the bottles were obtained from Supelco (Bellefonte, PA). The chemicals used to prepare the feed solution were all of an analytical grade or better.

Analytical Methods

Measurement of DO, pH, VSS and NH_3

The dissolved oxygen content (DO) was measured using a "YSI" Model 5739 DO probe connected to a "YSI" Model 54A DO meter as described in "Standard Methods (Method #4500 O)" [APHA, 1991]. Measurement

of pH was carried out with a Fisher Scientific "Accumet 900" pH meter and probe as described in "Standard Methods (Method #4500 H)" [APHA, 1991]. In this study volatile suspended solids (VSS) concentration was used as a means to estimate biomass concentration. VSS analysis was carried out as described in "Standard Methods (Method #2540 E)" [APHA, 1991]. Since the analysis destroys the sample, the VSS concentration could not be measured directly in every reactor. Instead the following method was used. When the reactors were being prepared, triplicate samples were taken simultaneously for VSS analysis. These samples were then individually analyzed and their average is reported as the initial VSS concentration. The final VSS concentration was measured for each set of samples at the end of the analysis and is reported. The ammonia (NH_3-N) was measured using the distillation procedure outlined in "Standard Methods (Method #4500-NH_3 E)" [APHA, 1991] using a Hach ammonia distillation apparatus. In tests conducted with samples having a known ammonia concentration the results obtained were within an average of 10% of the predicted values.

Measurement of TCE

Assays for biotransformation of TCE were conducted in clear glass serum bottles (volume = 120 mL) sealed with Teflon-lined rubber septa and secured with aluminum crimp caps to obtain an airtight fit [Hughes and Parkin, 1992]. These bottles were also used to make up the calibration curve for TCE. Sixteen (16) bottles were used for

this. In each case the reactors were filled with 50 mL of distilled water leaving a headspace of 70 mL.

A TCE standard was made by dissolving a known mass of TCE (5 mg) in a known volume of methanol (100 mL). The resulting concentration of TCE in methanol was 50 mg/L. Specific volumes of this standard were injected into the serum bottles through the septa to obtain seven different initial aqueous concentrations of TCE (0.001 mg/L, 0.005 mg/L, 0.01 mg/L, 0.05 mg/L, 0.1 mg/L, 0.5 mg/L and 1 mg/L) in the bottles. The solubility of TCE in water at 20°C is 1100 mg/L [Horvath, 1982]. The same volumes were injected into seven other bottles to get duplicate samples of these standards. The remaining two bottles served as blanks (0 mg/L TCE) for the experiment. The bottles were allowed to equilibrate in an incubator maintained at 20°C. The equilibration time of one hour was determined in a previous study by Gossett [1987].

After equilibration, headspace samples (20 μL) were withdrawn from these bottles and injected into the gas chromatograph. The GC system included a DB-5 fused silica capillary column with a film thickness of 0.25 μm, inner diameter of 0.25 mm and a length of 30 m (J & W Scientific, Folsom, CA) in a model 5890 Hewlett-Packard Series II gas chromatograph (GC) equipped with an electron capture detector (ECD).

Injections were made in the split mode (ratio 1:45) at an injector temperature of 150°C and a column temperature of 40°C. Helium

was the carrier gas, with a flow rate of 45 mL/min and a head pressure of 25 psi. A 95% argon/5% methane mixture was used as the ECD make-up gas. The run time for each injection was 5 min and the column temperature was held constant at 40°C throughout. Quantification was achieved by injecting standards, treated like samples, and comparing relative areas under each separated peak recorded by a model 3396 Hewlett-Packard Series II integrator. The minimum detectable concentration of TCE by this method was 0.01 mg/L (initial aqueous phase concentration) [Gossett, 1987].

Concentration of TCE present in the headspace is proportional to the aqueous concentration of TCE and the volume of the headspace (Henry's Law). Since Henry's constant is a function of temperature, the bottles were kept in a water-bath during the gas chromatography analysis. The water-bath maintained the temperature of the reactor bottles very close to 20°C even though the room temperature varied widely. It was very crucial to maintain the reactor temperatures, since the equilibrium between the aqueous and gaseous state of TCE is very temperature dependent. When the water-bath was not used, reproducible results were not obtained. Also in the early stages of the study various gastight syringes were tried for sampling the reactors. The syringe that was subsequently chosen (Hamilton 1802N) gave consistent and reproducible results. The distinguishing features that made this syringe superior were its twin valve and the double plunger assembly. A Hewlett-Packard 3396 Integrator received the output from the GC. A calibration curve was developed

from the reactors with known concentrations of TCE. A typical curve is included in the Appendix.

TCE Analysis During the Experiment with Nitrifying Bacteria

Headspace analysis was used during the kinetic experiments to determine the TCE concentration. This method was used because it was quick, reliable and involved no loss of sample. It is based on the premise that a volatile compound exists in equilibrium between its aqueous state and its gaseous state in a closed system at a constant temperature [Gossett, 1987]. When the bacteria degrade the TCE in its aqueous state, the equilibrium is disturbed and TCE in the gaseous state goes into solution until a new equilibrium is established. The headspace concentration is therefore an accurate indicator of the aqueous concentration of TCE and it can be determined with the help of a calibration curve.

According to Gossett [1987], the total moles (M) of a volatile solute added to a sealed serum bottle will be partitioned at equilibrium according to:

$$M = C_w V_w + C_g V_g = C_w V_w + C_w V_g H_c$$

Where C_w = concentration of solute in water (mol/L)

C_g = concentration of solute in the gas (mol/L)

V_w = volume of water in the bottle (L)

V_g = volume of headspace in the bottle (L)

H_c = Henry's constant (dimensionless)

the peak areas of the corresponding controls for each run to determine the fraction of the initial TCE concentration remaining in solution. This procedure allowed normalization of each day's data to the initial concentration of TCE. This eliminated the need to prepare a calibration curve during every sampling run. However, the absolute concentration of the controls were confirmed by periodic calibration. The data for each sampling time is thus automatically normalized to the reactor's initial concentration (C_0), and it is presented as such.

Experimental Methods

This section outlines the various experimental methods used during this study.

Establishment of a seed culture reactor

An inoculum of nitrifying bacteria was obtained from the municipal wastewater treatment plant, Stillwater, OK. It was used to seed a 10 liter reactor. This reactor was fed a growth medium for nitrifying bacteria at regular three day intervals. The seed culture reactor was kept continuously aerated keeping the cells in suspension. This was done to increase contact between organisms and substrates, thereby facilitating nutrient uptake.

The growth medium consisted of 10 mM ammonium sulfate, 3 mM potassium phosphate (monobasic), 750 μM magnesium sulfate,

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The growth medium consisted of 10 mM ammonium sulfate, 3 mM potassium phosphate (monobasic), 750 μM magnesium sulfate,

200 μM calcium chloride, 10 μM ferrous sulfate. The medium was buffered with the addition of a phosphate solution of pH = 8.0 consisting of 43 mM potassium phosphate (dibasic) and 4 mM sodium phosphate [Rasche *et al.*,1991]. Final pH of the feed solution was usually about 8.1.

A fill-and-draw technique was employed to feed the nitrifiers. This operation was carried out in the following manner. Aeration of the reactor would be shut down for two to three hours. This caused the nitrifying bacteria to settle to the bottom of the reactor. The supernatant fluid was decanted from the reactor using a peristaltic (Masterflex) pump. This was done very carefully to ensure that the nitrifiers settled at the bottom of the reactor were not disturbed. The growth medium was made up in another vessel. The peristaltic pump was again used to transfer the growth medium to the reactor vessel. The volume of growth medium added was equal to the volume of the supernatant fluid removed from the reactor.

After five months the nitrifiers were transferred to a 20 liter glass reactor. Feeding was continued as before. The reactor was maintained this way for about six months before starting the kinetic experiments. The status of the reactor was monitored regularly with measurements of ammonia, pH, and dissolved oxygen. The initial concentration of ammonia was approximately 300 mg/L and the initial pH varied from 7.9 to 8.2. The concentration of ammonia in the wasted supernatant was about 50 mg/L and the pH of the supernatant varied from 6.5 to 6.7.

mixtures were allowed to equilibrate in the dark in a 20°C incubator. After equilibration, headspace samples were taken and analyzed to determine the initial concentration of TCE as described below. Subsequently, headspace samples were taken and analyzed approximately every twelve hours to track the disappearance of TCE. This analysis was continued until most of the TCE had disappeared. Care was taken to ensure that the seed cultures fed to each reactor were as uniform as possible. As such, variations among the cultures in each reactor should have been minimized.

Specific Culture Conditions Tested

The effects of a number of factors on the rate and extent of TCE transformation by mixed nitrifying cultures were not studied previously. This study investigated several of these factors. The specific conditions investigated include the effect of the concentration of ammonia fed, the initial TCE concentration, and the biomass concentration.

(i) Effect of Ammonia Concentration

An experiment was conducted to determine the effect of different initial ammonia concentrations on the rate of biotransformation of TCE. Earlier research had shown that the degradation of TCE was proportional to the addition of ammonia [Vannelli *et al.*, 1990]. Arciero and coworkers [1989] had previously found that with aged nitrifier cells, the addition of ammonia stimulated the rate of TCE degradation. However since ammonia is the intended substrate of the enzyme AMO, TCE is expected to be a competing substrate. The

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following experiments were conducted to investigate the relationship that exists between the initial concentration of ammonia and the rate of biotransformation of TCE. Three different initial concentrations of ammonia were used for this experiment.

Preparation of a starved cell culture:

A small batch of nitrifiers were separated from the parent culture and kept without feeding for two months. This culture was not aerated but was open to the atmosphere. This slowed down the nutrient uptake. Two hours prior to the experiment, this culture was fed 20 mg/L of ammonia and the reactor was shaken to distribute the nutrients.

Experimental technique used to set up the reactors:

50 mL aliquots from the seed culture solutions were poured into identical serum bottles (120 mL). Initial measurements of pH, DO, NH_3-N and VSS concentrations were taken. The bottles were capped and sealed using Teflon-lined rubber septa and aluminum crimp seals leaving a headspace of 70 mL in each of the bottles. A specific volume of a TCE standard was injected into the bottles to make the aqueous concentration of TCE in the bottle equal to 1 mg/L.

Three bottles were prepared using the starved seed culture solution (20 mg/L of NH_3-N) with a VSS concentration of approximately 1071 mg/L. These samples were referred to as "Starved Hi" (since the starved culture was used, and Hi since 1 mg/L concentration of TCE used).

Another three bottles were prepared using the main seed culture solution before the feeding operation. As such it had an ammonia concentration near 100 mg/L of NH_3-N. These samples had a VSS concentration of approximately 856 mg/L. These samples were referred to as "Prefed Hi" (since the culture was taken prior to the feeding operation).

A third set of triplicate samples were prepared using the main seed culture solution immediately after the feeding operation and so, contained 300 mg/L of NH_3-N. These samples had a VSS concentration of approximately 877 mg/L. These samples are referred to hereafter as "Postfed Hi" (since the culture was taken after the feeding operation).

The setup used in the experiment with different ammonia concentrations is described in a matrix form in Table 1.

Table 1. Experimental setup with different ammonia concentrations

Reactor Name	NH_3-N* (mg/L)	TCE (mg/L)	VSS** (mg/L)
Starved Hi	20	1	1071
Prefed Hi	100	1	856
Postfed Hi	300	1	877

* ±10% precision in previous tests conducted

** Average of 3 samples prepared identical to reactors for VSS measurements

(ii) Effect of Biomass Concentration

This experiment was conducted to determine the effect of the concentration of nitrifying bacteria on the rate of degradation of TCE and to investigate the reaction order. It was discovered from the previous experiment that TCE degraded fastest in the reactors with the highest initial concentration of ammonia. Therefore nitrifiers from the main seed culture were used in this experiment just after the feeding operation (that is, with the highest initial ammonia concentration of 300 mg/L).

Experimental technique used to set up the reactors:

50 mL aliquots from the seed culture solution were poured into identical serum bottles (120 mL). Initial measurements of pH, DO, NH_3-N and VSS concentrations were taken. The bottles were capped and sealed using Teflon-lined rubber septa and aluminum crimp seals leaving a headspace of 70 mL in each of the bottles. A specific volume of a TCE standard was injected into the bottles to make the aqueous concentration of TCE in the bottle equal to 1 mg/L.

Three samples were prepared using the main seed culture solution immediately after the feeding operation (300 mg/L of NH_3-N). These samples had a VSS concentration of approximately 3754 mg/L. These samples were referred to as "X=1.0 Hi" (X=1.0 signifies 100% of initial VSS and Hi since 1 mg/L concentration of TCE was used).

Another three samples were made with a 50% dilution of the main seed culture solution immediately after the feeding operation

(300 mg/L of NH_3-N). These samples had a VSS concentration of approximately 2036 mg/L. These samples were referred to as "X=0.5 Hi" (X=0.5 signifies 50% of initial VSS).

A third set of triplicate samples were made with a 80% dilution of the main seed culture solution immediately after the feeding operation (300 mg/L of NH_3-N). These samples had a VSS concentration of approximately 2036 mg/L. These samples were referred to as "X=0.2 Hi" (X=0.2 signifies 20% of initial VSS).

The setup used in the experiment with different biomass (VSS) concentrations is described in a matrix form in Table 2.

Table 2. Experimental setup with different biomass concentration

Reactor Name	VSS** (mg/L)	TCE (mg/L)	NH_3-N* (mg/L)
X=1.0 Hi	3754	1	300
X=0.5 Hi	2036	1	300
X=0.2 Hi	876	1	300

* ±10% precision in previous tests conducted

** Average of 3 samples prepared identical to reactors for VSS measurements

(iii) Effect of TCE Concentration

This experiment was done to determine the effect of the initial concentration of the TCE on the rate of its degradation. This experiment would also indicate if the TCE is toxic to the nitrifying bacteria.

Experimental technique used to set up the reactors:

These experiments were performed by repeating those just described, except that 0.1 mg/L of TCE was the initial concentration instead of 1 mg/L. The bottles were similarly analyzed to determine the rate of degradation of TCE. The bottles with 0.1 mg/L of TCE were referred to as "Lo" instead of "Hi" and the other terms defined in the nomenclature system developed above were used with the same connotations. The setups used in the kinetic experiments are summarized in a matrix form in Table 3 and Table 4.

Table 3. Experimental setup with varied NH_3 and TCE concentrations

Reactor Name	NH_3-N* (mg/L)	TCE (mg/L)	VSS** (mg/L)	Number of Bottles
Starved Hi	20	1	1071	3
Prefed Hi	100	1	856	3
Postfed Hi	300	1	877	3
Controls	0	1	0	3
Starved Lo	20	0.1	1071	3
Prefed Lo	100	0.1	856	3
Postfed Lo	300	0.1	877	3
Controls	0	0.1	0	3

* ±10% precision in previous tests conducted

** Average of 3 samples prepared identical to reactors for VSS measurements

Table 4. Experimental setup with varied VSS and TCE concentrations

Reactor Name	VSS** (mg/L)	TCE (mg/L)	NH_3-N* (mg/L)	Number of Bottles
X=1.0 Hi	3754	1	300	3
X=0.5 Hi	2036	1	300	3
X=0.2 Hi	876	1	300	3
Controls	0	1	0	3
X=1.0 Lo	3754	0.1	300	3
X=0.5 Lo	2036	0.1	300	3
X=0.2 Lo	876	0.1	300	3
Controls	0	0.1	0	3

* ±10% precision in previous tests conducted

** Average of 3 samples prepared identical to reactors for VSS measurements

Experimental Controls

When the kinetic experiments were being conducted, there were controls set up to check for any abiotic removal of TCE. Triplicate blanks were set up for each experiment and for each concentration of TCE used. Blanks were made by taking 50 mL of distilled water in a serum bottle (no bacteria), sealing using Teflon-lined rubber septa and aluminum crimp caps. These bottles were injected with the same amounts of TCE as the reactor bottles. This ensured that the aqueous concentration of TCE was the same in both the reactors and the blanks. The blanks were devoid of bacteria but they were treated like the sample reactors in every other respect. Headspace

samples from the blanks were injected into the GC during every run and the concentration of TCE was analyzed. It was found that the concentration of TCE in these bottles remained constant over the course of each experiment, thus ruling out the occurrence of any significant abiotic removal.

Toxicity Experiments

Preliminary toxicity studies were conducted to gauge the effect of methanol and TCE on the nitrifying bacteria and on the nitrification process. Since the TCE injected into the reactors was dissolved in a methanol medium, it was necessary to determine if the presence of methanol had any significant effect on the nitrifying bacteria. Methanol could potentially affect the rate of TCE degradation in the reactors if it was toxic to the organisms or had an effect on the nitrification process.

Toxicity studies were conducted with TCE to get a preliminary understanding of the effect of TCE on the nitrification process, specifically, the rate of ammonia oxidation. The reactors for the toxicity experiments were set up in the following manner:

Eighteen identical serum bottles (120 mL volume) were each filled with 100 mL of well mixed seed culture, such that the VSS concentration in each of the bottles was approximately the same. This was done immediately after the feeding operation, so the level of ammonia was high. The bottles were sealed with Teflon-lined

rubber septa and capped using aluminum crimp caps. TCE was injected into six of the bottles such that the final concentration of TCE in the bottles was 1 mg/L (the highest concentration used in the kinetic experiments). Similarly methanol was injected into six bottles such that the final concentration of methanol in the bottles was 158 mg/L (which reflects the highest concentration of methanol injected into the reactors). Neither TCE nor methanol was added to the six remaining bottles. Initial measurements were taken of the following parameters: ammonia (NH_3-N), VSS, DO (dissolved oxygen), and pH. All eighteen bottles were placed on a shaker table. After 24 hours two bottles containing TCE, two bottles containing methanol and two bottles containing blanks were opened and measurements were made of ammonia (NH_3-N), DO, and pH. This procedure was repeated after another 24 hours. The same procedure was repeated after another 24 hours except that this time the VSS reading were also taken for all samples along with other measurements. The setup used for the toxicity experiments is summarized in a matrix form in Table 5.

Table 5. Experimental setup for toxicity experiments

Reactor Name	Conc. in Reactor	NH_3-N* (mg/L)	VSS** (mg/L)	Number of Bottles
MeOH	158 mg/L	280	661	3
TCE	1 mg/L	280	661	3
Controls	-	280	661	3

* ±10% precision in previous tests conducted

** Average of 3 samples prepared identical to reactors for VSS measurements

CHAPTER IV

RESULTS AND DISCUSSION

In this chapter the results obtained from the kinetic experiments and the toxicity experiments are discussed and analyzed. The results are also compared with previously published findings.

The parameters that were measured before the start of the kinetic experiments were the VSS concentrations, the ammonia-nitrogen concentrations, the dissolved oxygen content and the pH. The initial values of the parameters measured are given in Table 6.

Table 6. Initial values for the kinetic experiments

Reactor Description	VSS** (mg/L)	NH_3-N* (mg/L)	DO (mg/L)	pH
Starved	1071	20	6.2	7.2
Prefed	856	100	6.4	7.8
Postfed	877	300	7.3	8.2
X = 0.2	876	300	7.5	8.1
X = 0.5	2036	300	7.5	8.1
X = 1.0	3754	300	7.5	8.1

* ±10% precision in previous tests conducted

** Average of 3 samples prepared identical to reactors for VSS measurements

At the conclusion of the kinetic experiments the same parameters were measured again. The final values of the parameters measured are given in Table 7.

Table 7. Final values for the kinetic experiments

Reactor Labels	VSS+ (mg/L)	NH_3-N* (mg/L)	DO (mg/L)	pH
Starved Hi	797	2	1.5	6.7
Prefed Hi	598	2	2.2	7.2
Postfed Hi	693	112	1.1	7.2
Starved Lo	801	2	4.5	6.8
Prefed Lo	613	2	4.3	7.5
Postfed Lo	666	110	0.8	7.2
X=0.2 Hi	859	149	1.5	7.1
X=0.5 Hi	2039	92	1.1	7.3
X=1.0 Hi	4094	81	1.1	7.2
X=0.2 Lo	788	92	2.3	7.0
X=0.5 Lo	1969	87	2.3	7.1
X=1.0 Lo	4099	53	2.0	7.2

* ±10% precision in previous tests conducted

+ Actual values measured at the conclusion of the experiment

The results obtained from the kinetic experiments can be found in Figures 1 through 10. A more quantitative analysis of these results follows the description of these figures. Figure 1 is a plot showing the disappearance of TCE with respect to its initial concentration (C/C_o) versus time in cultures having different initial ammonia concentrations (20 mg/L, 100 mg/L and 300 mg/L NH_3-N). In these experiments, the initial concentration of TCE was high (1 mg/L). Figure 2 shows the result of an identical experiment but with a lower initial concentration of TCE (0.1 mg/L).

A few initial observations can be made from these figures. For example, in both cases there appears to be a lag period, in which there is no transformation of TCE, at each of the ammonia (NH_3-N) concentrations tested. However, this lag period appears to be considerably smaller for the solutions with the highest concentrations of ammonia (the "Postfed" reactor, with 300 mg/L NH_3-N). In the "Starved" reactors, which had been without ammonia for an extended period, it seems possible that the amount of AMO in the culture was low. Therefore, a lag before active ammonia oxidation (and hence, TCE transformation) could be expected. AMO should have been plentiful and active in the "Prefed" and "Postfed" cultures, so the significant lag time in the "Prefed" reactor is difficult to explain.

Figures 3, 4 and 5 redisplay this data for comparison of the effects of TCE concentrations under each ammonia feeding condition tested

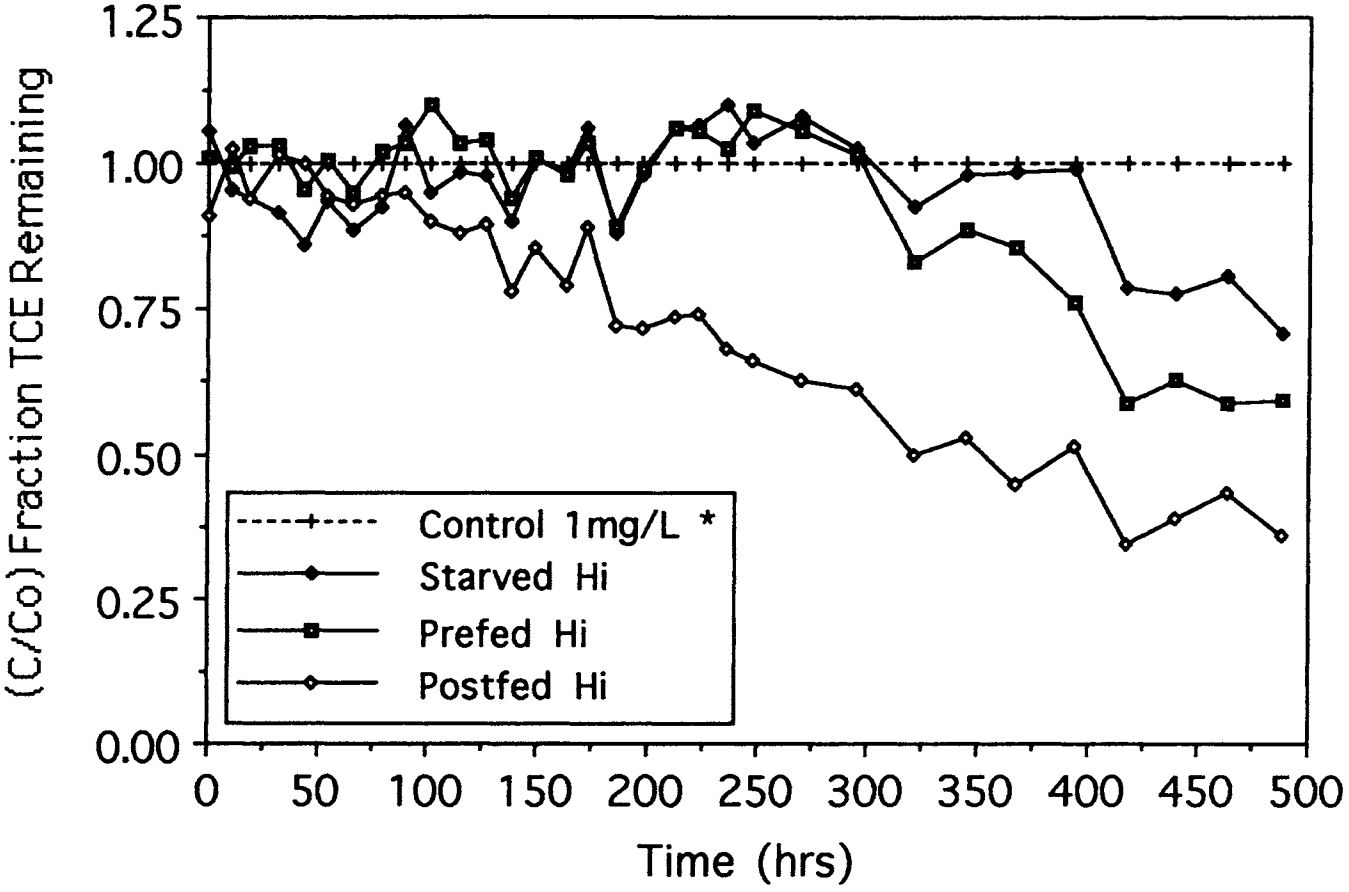

Figure 1. TCE (1mg/L) degradation with different ammonia concentrations.
* normalized line with s.d. < 5%

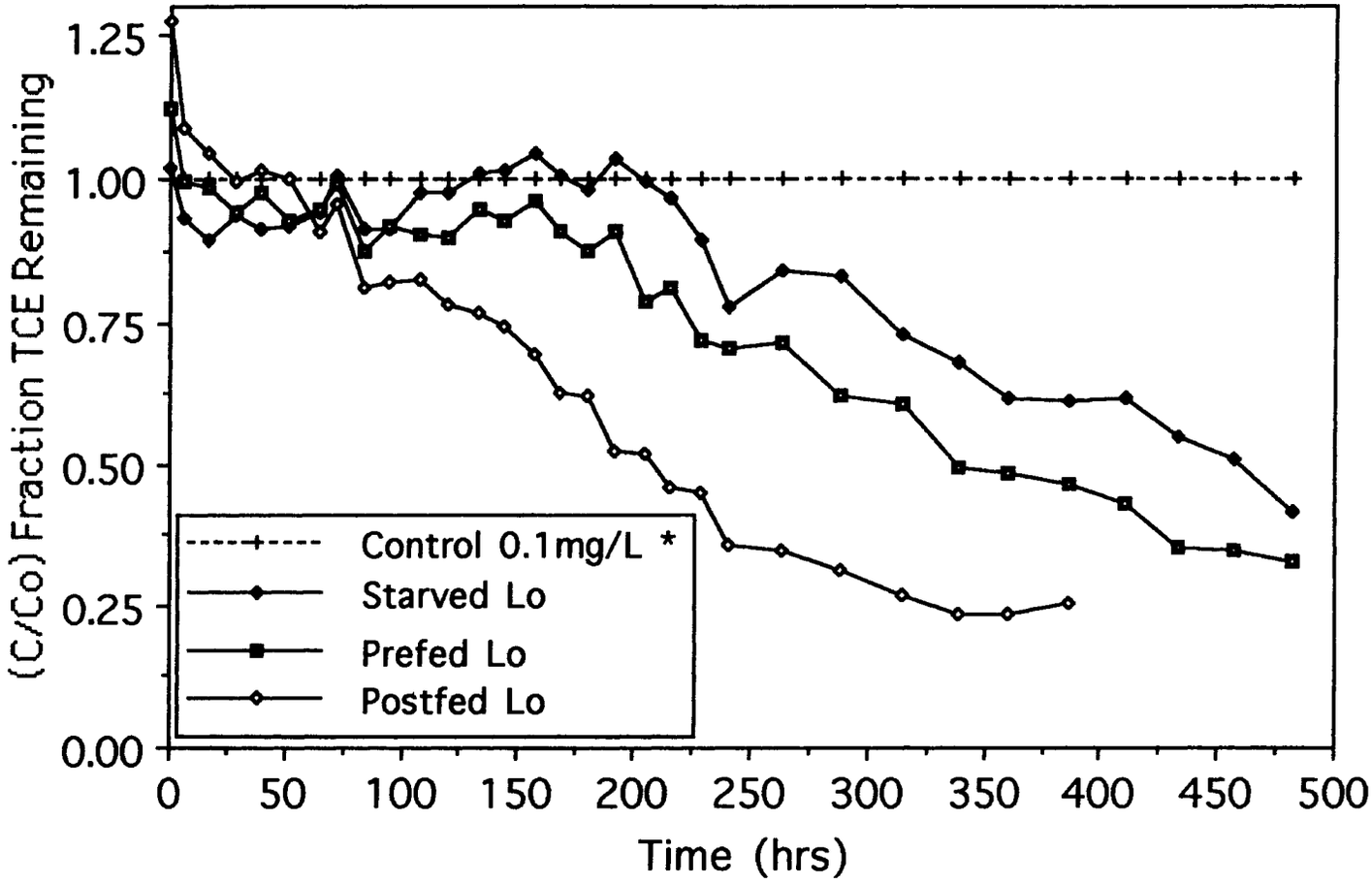

Figure 2. TCE (0.1mg/L) degradation with different ammonia concentrations.
* normalized line with s.d. < 5%

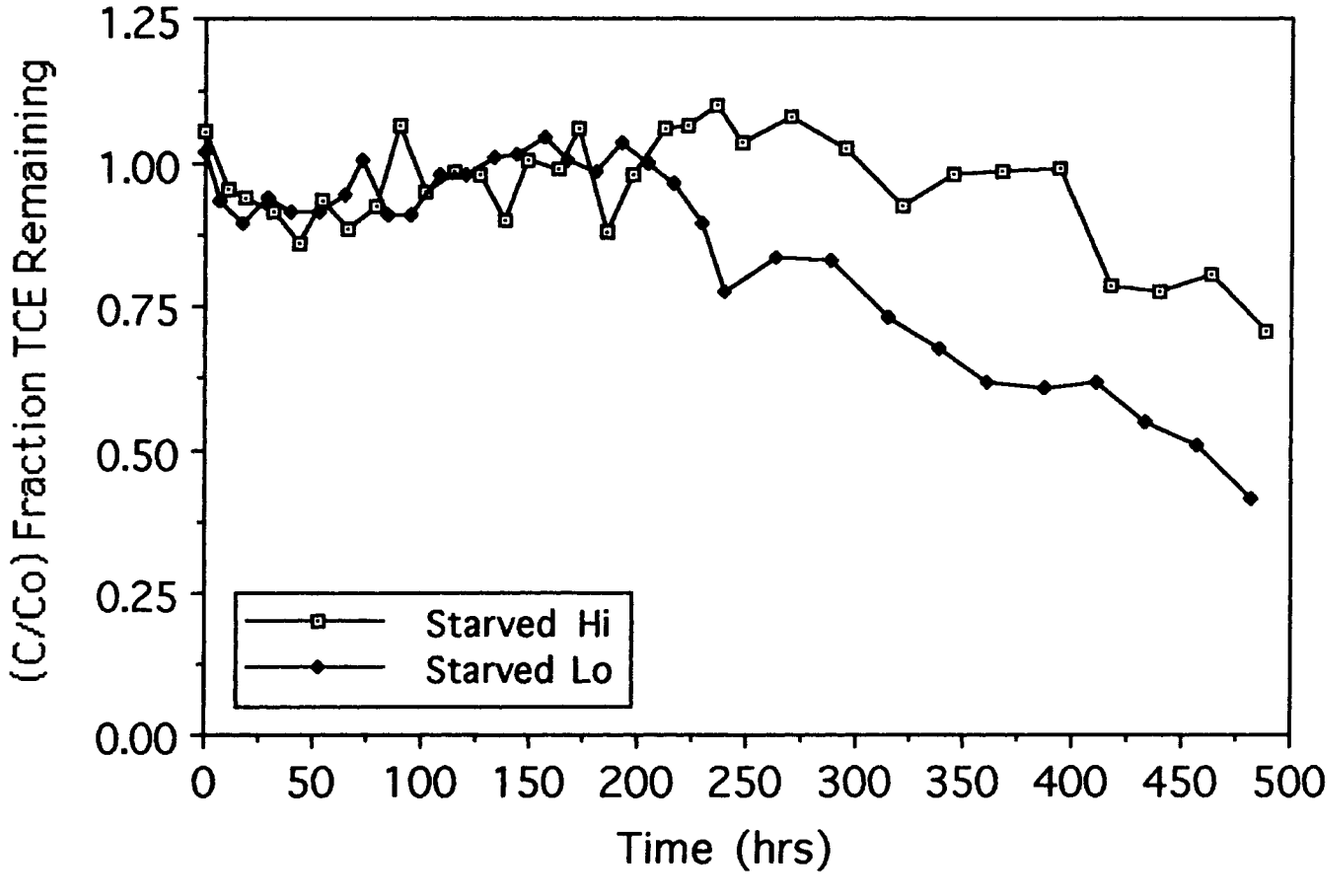

Figure 3. TCE (1 & 0.1mg/L) degradation with 20 mg/L ammonia.

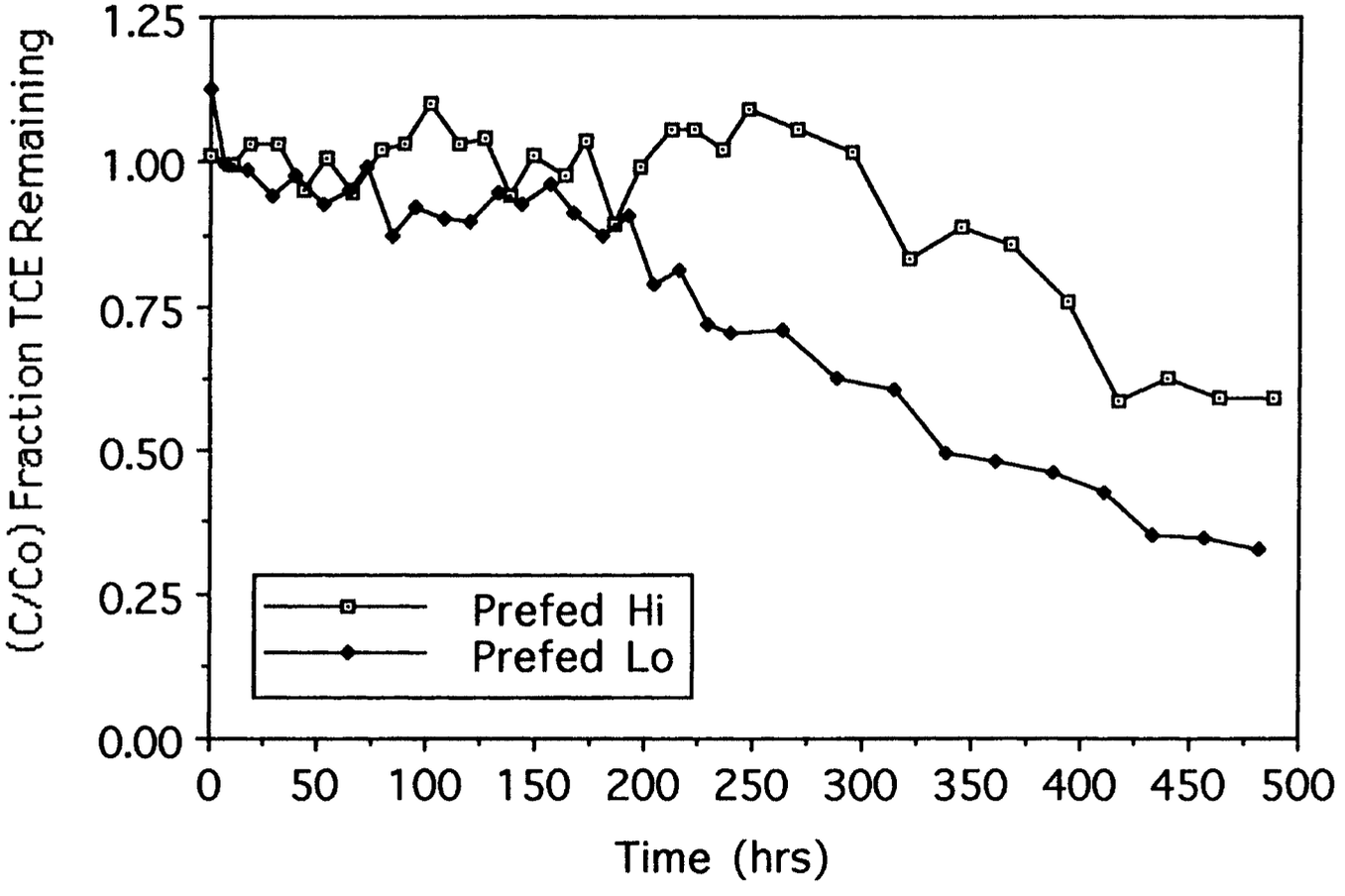

Figure 4. TCE (1 & 0.1mg/L) degradation with 100 mg/L ammonia.

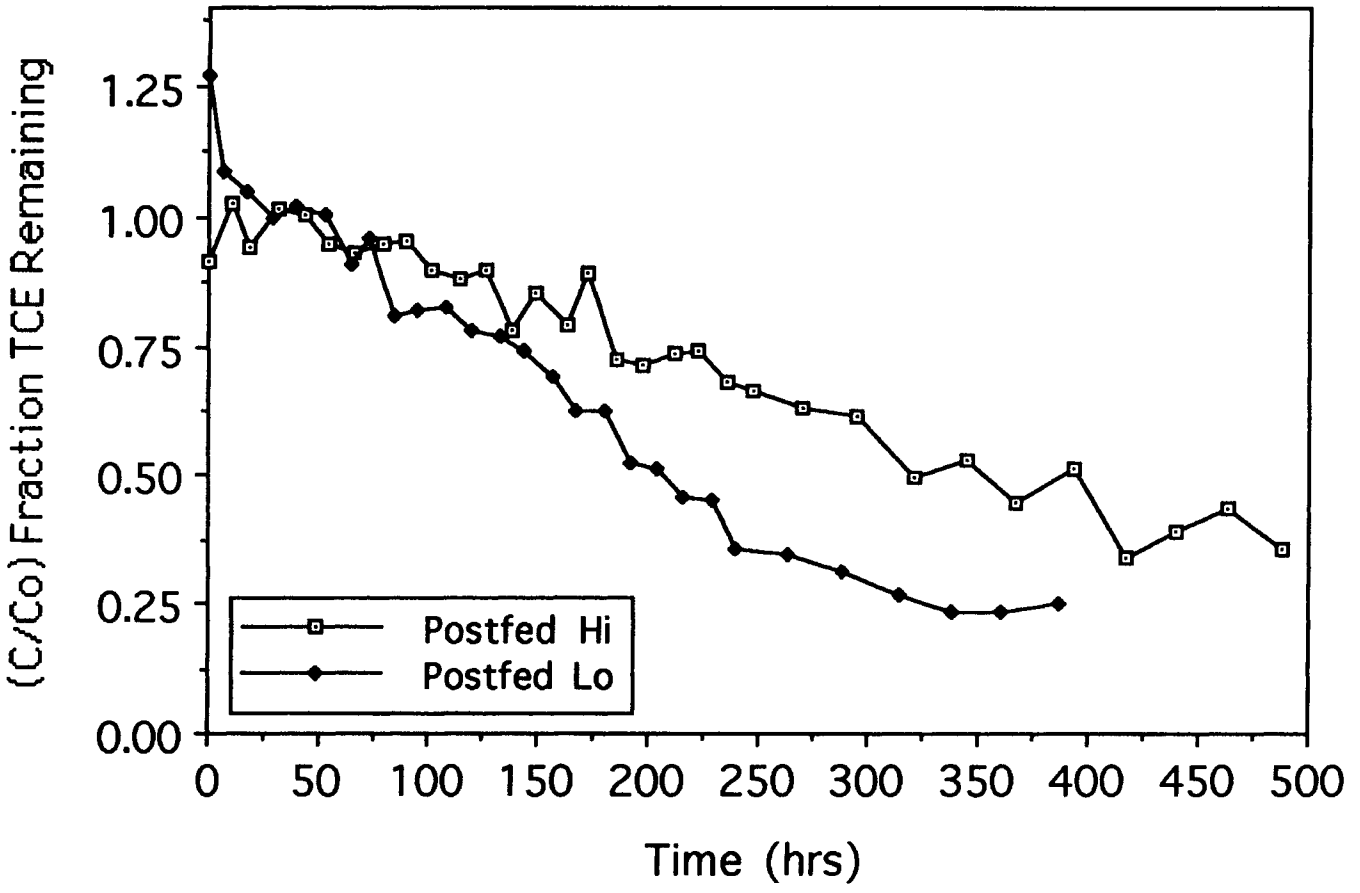

Figure 5. TCE (1 & 0.1mg/L) degradation with 300 mg/L ammonia.

(20 mg/L, 100 mg/L and 300 mg/L NH_3-N). From the figures it can also be seen that the lag periods are smaller when the initial concentration of TCE was lower (0.1 mg/L TCE), for the "Starved" and "Prefed" reactors. The effect of depressed AMO concentrations or activities on TCE transformation in these cultures would be less noticeable on a ten-fold lower TCE concentration. There was no lag period observed for the "Postfed" reactor with the lower initial TCE concentration (0.1 mg/L TCE). Quantitative analysis of these results appears below.

Figure 6 is a plot showing the transformation (C/C_o) versus time of a high concentration of TCE (1 mg/L) in cultures having different concentrations of nitrifying bacteria (with VSS concentrations of 876 mg/L, 2036 mg/L and 3754 mg/L). Figure 7 shows the results of an identical experiment except with a low concentration of TCE (0.1 mg/L). From these figures it can be seen that there was no lag phase, and the TCE transformation began instantly for all the bacterial(VSS) concentrations tested. This was true for both the TCE concentrations used (1 mg/L and 0.1 mg/L). Since these experiments were conducted with the high concentration of ammonia (300 mg/L NH_3-N), this minimal lag time is consistent with the previous experiments. It can also be seen in these figures that, as expected, the greater the biomass concentration, the greater the rate of TCE disappearance. These results will be analyzed more quantitatively below.

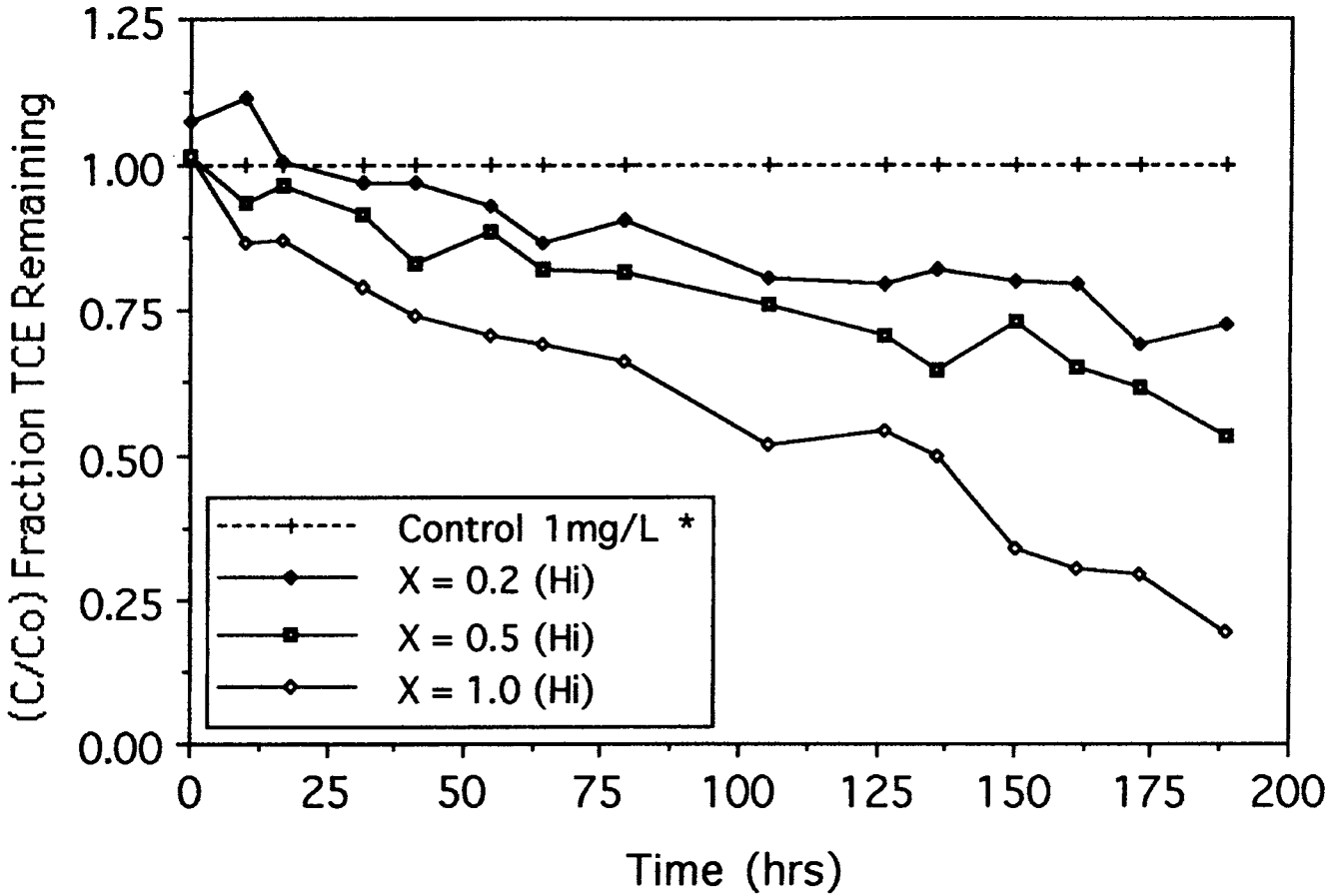

Figure 6. TCE (1mg/L) degradation with different VSS concentrations.
* normalized line with s.d. < 5%

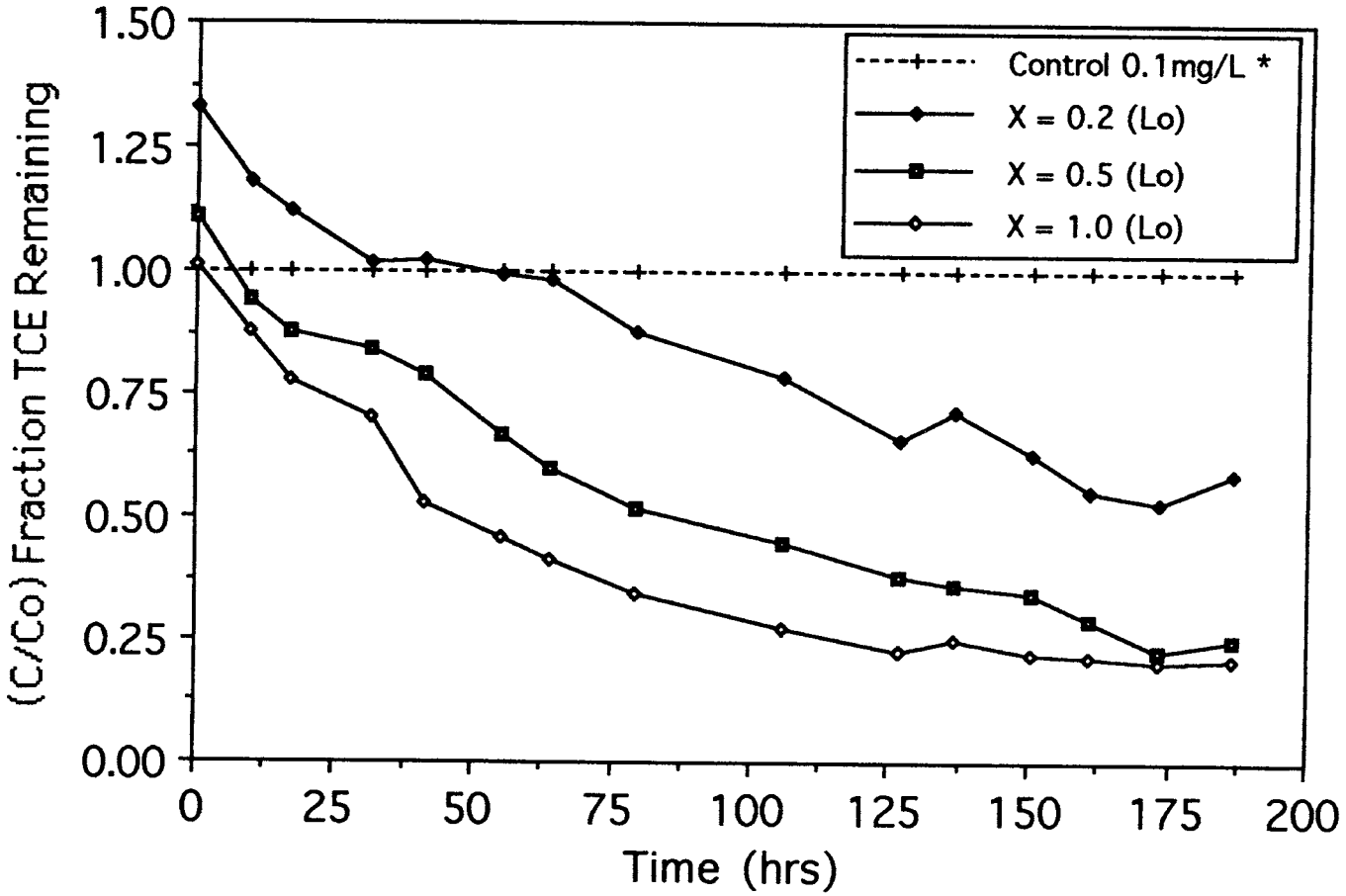

Figure 7. TCE (0.1mg/L) degradation with different VSS concentrations.
* normalized line with s.d. < 5%

Figures 8, 9 and 10 redisplay this data for comparison of the effects of TCE concentration for each biomass concentration tested (VSS concentrations of 876 mg/L, 2036 mg/L and 3754 mg/L). It does appear that the higher concentration of TCE seems to slow down its transformation. While 1 mg/L of TCE was clearly not extremely inhibitory to the culture, it appears possible that some overall metabolic inhibition occurred. The toxicity of TCE to the culture is discussed in further detail below.

Analysis of Transformation Kinetics

There are numerous kinetic expressions used to describe the transformation of xenobiotic compounds by suspended microorganisms in aquatic environments. Schmidt and coworkers [1985] have described various kinetic models used to describe the metabolism of organic substrates that are not supporting bacterial growth. These models are variations of the Michaelis-Menten relationship, which can be expressed as:

$$dC/dt = -(V_{max}CX)/(K_m+C) \quad (1)$$

where, if TCE is the substrate of interest

C = TCE concentration at time t (mg_{TCE}/L)

X = Bacterial (VSS) Concentration (mg_{VSS}/L)

V_{max} = Maximum Specific Reaction Rate ($mg_{TCE}/mg_{VSS}\bullet h$)

K_m = half-saturation constant (mg_{TCE}/L)

t = Time (h)

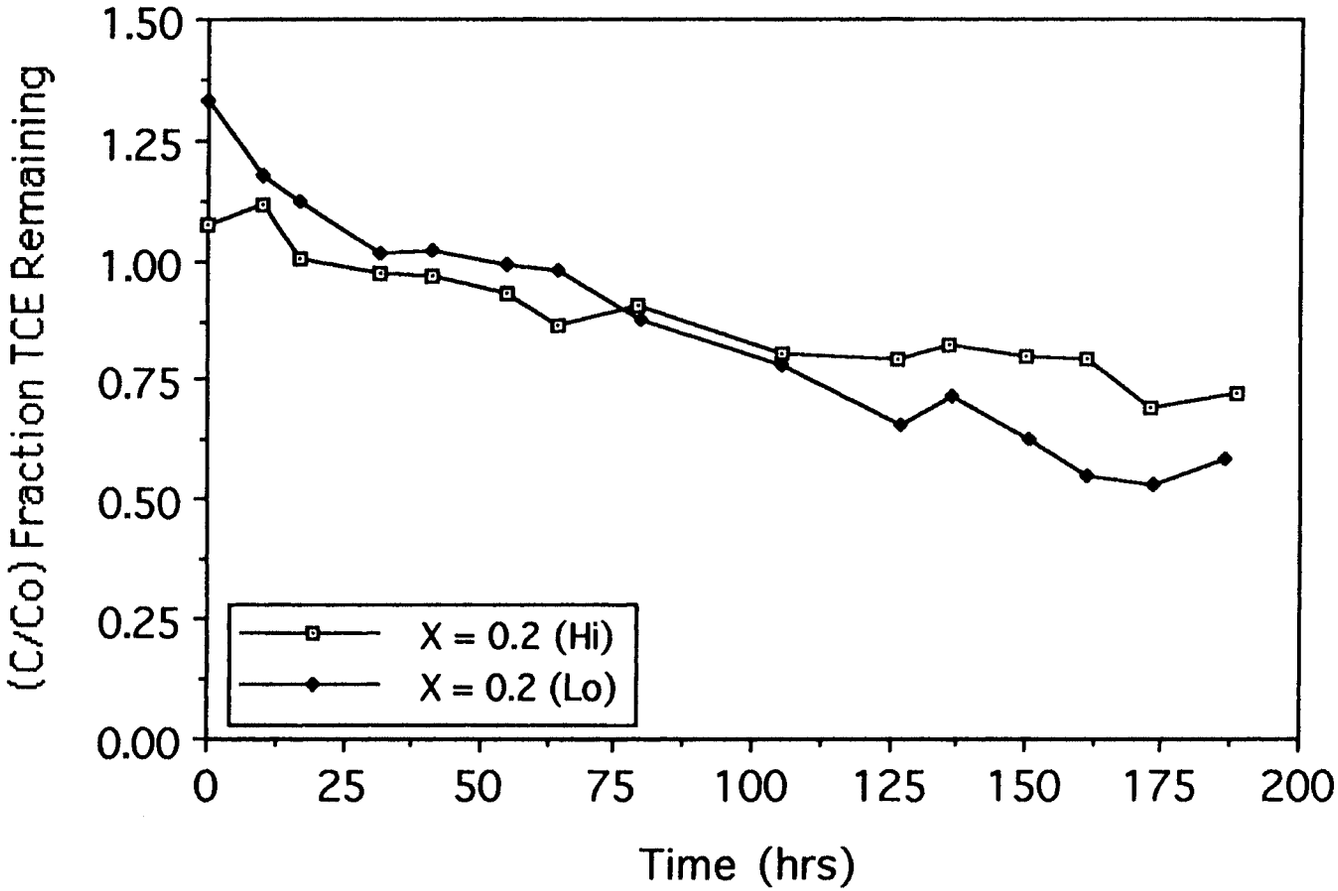

Figure 8. TCE (1 & 0.1mg/L) degradation with VSS = 876 mg/L.

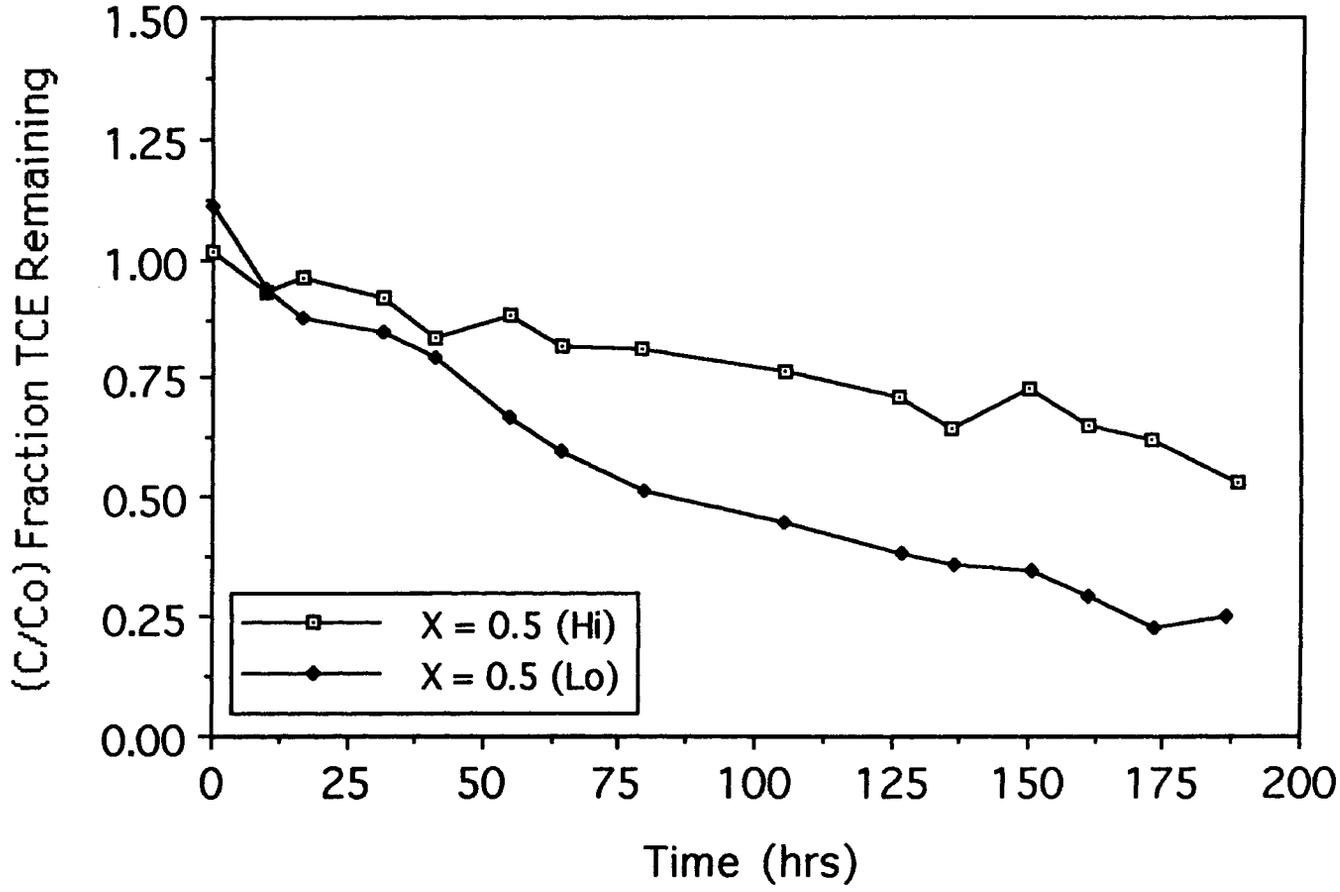

Figure 9. TCE (1 & 0.1mg/L) degradation with VSS = 2036 mg/L.

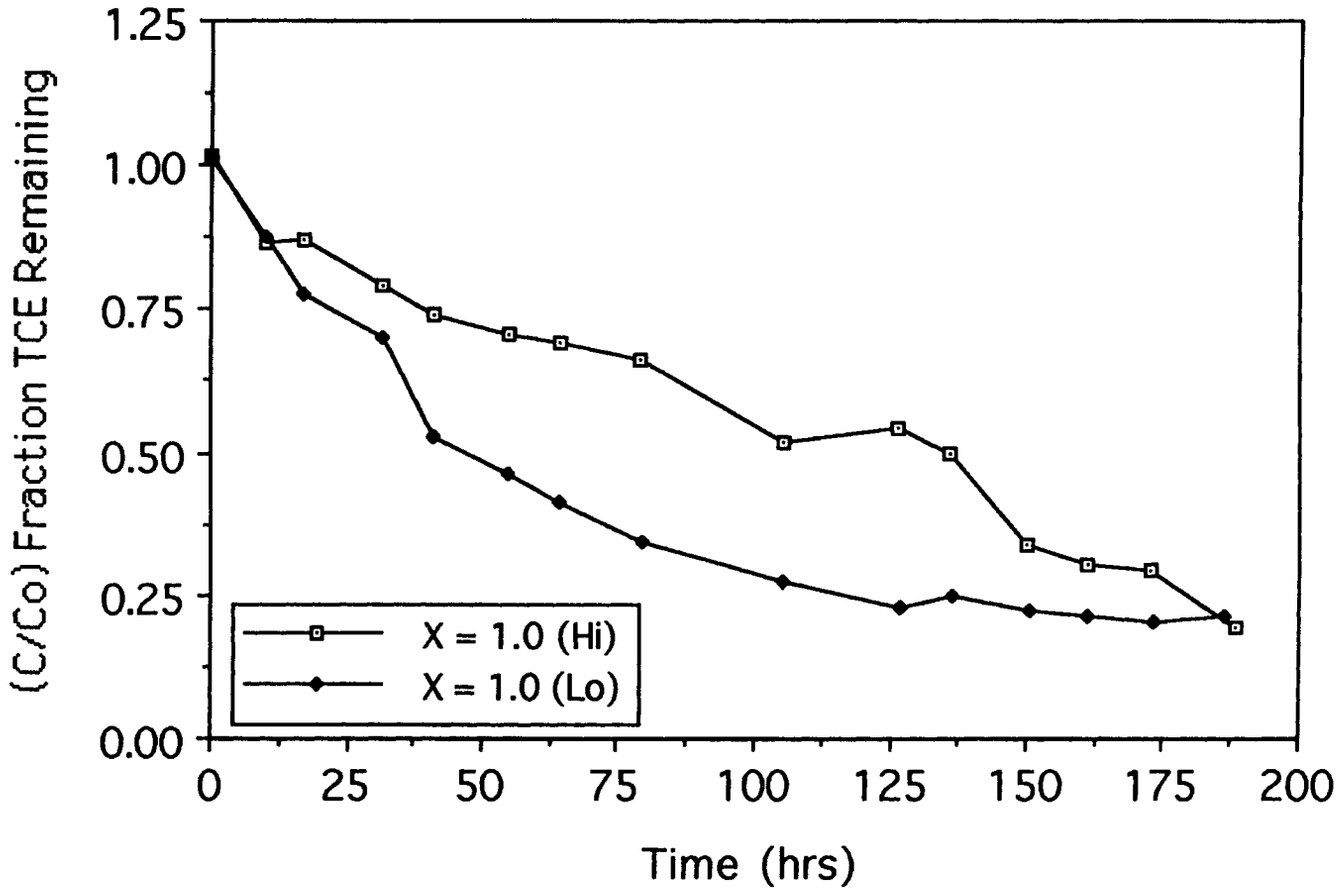

Figure 10. TCE (1 & 0.1mg/L) degradation with VSS = 3754 mg/L.

This equation can be adapted to reflect specific circumstances, such as a change in biomass concentration over time, or significant differences between K_m and C. Two such adaptations will be explored here.

Schmidt and coworkers [1985] report that reactions with no growth of active organisms and a high concentration of test substrate can be modelled using zero-order kinetics. Previous researchers who studied the degradation of TCE by nitrifying bacteria have used zero-order kinetics to describe the process [Arciero *et al.*, 1989 and Vannelli *et al.*, 1990]. When the substrate concentration, C, is much larger than K_m, Equation (1) reduces to zero order kinetics as shown:

$$dC/dt = -V_{max}X \qquad (2)$$

It can be noted from the VSS values in Tables 6 and 7, that there is no significant growth of biomass over the course of these kinetic experiments. Therefore considering X a constant, the expression for a zero-order model can be written as:

$$dC/dt = -k_0 \qquad (3)$$

where

k_0 = zero-order rate constant ($mg_{TCE}/L \bullet h$) = $V_{max}X$

Thus the zero-order rate constant can be determined from a plot of TCE concentration versus time. Arciero [1989], Vannelli [1990] and

their coworkers have focussed on the maximum TCE transformation rate achieved. The maximum TCE transformation rates achieved during this study were determined in the following manner. The fraction of TCE remaining was plotted against time, using only the steepest initial sections. These sections represented the time when the transformation rates were the highest. Linear regressions were applied to these plots and the slopes of the lines were calculated. The slopes of the lines represented the respective maximum zero-order transformation rates.

Figure 11 displays such a plot for the cultures having different ammonia concentrations (20 mg/L, 100 mg/L and 300 mg/L of NH_3-N). This plot is for the experiment where the initial concentration of TCE was high (1 mg/L). Figure 12 shows the result of the identical experiment with the lower initial concentration of TCE (0.1 mg/L).

Figures 13, 14 and 15 permit comparison of the effect of TCE concentration on the maximum zero-order transformation rates for each initial ammonia concentration.

Figure 16 is a similar plot showing the maximum zero-order transformation rate of a high concentration of TCE (1 mg/L) in cultures having different concentrations of nitrifying bacteria (with VSS concentrations of 876 mg/L, 2036 mg/L and 3754 mg/L). Figure 17 shows the results of an identical experiment except with a low initial concentration of TCE (0.1 mg/L).

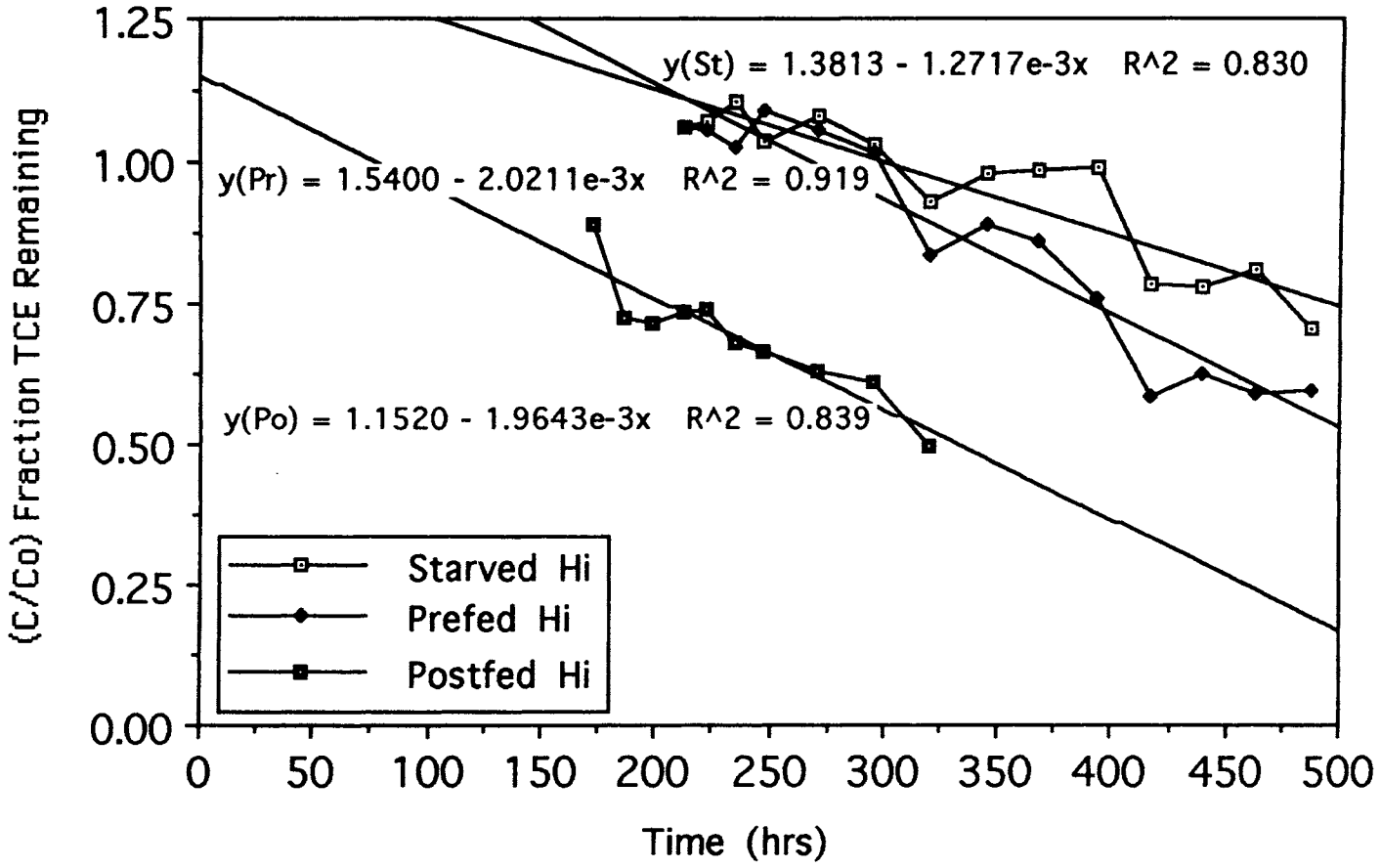

Figure 11. Zero-order plot to determine maximum TCE transformation rate: Comparison of different ammonia concentrations for 1mg/L TCE.

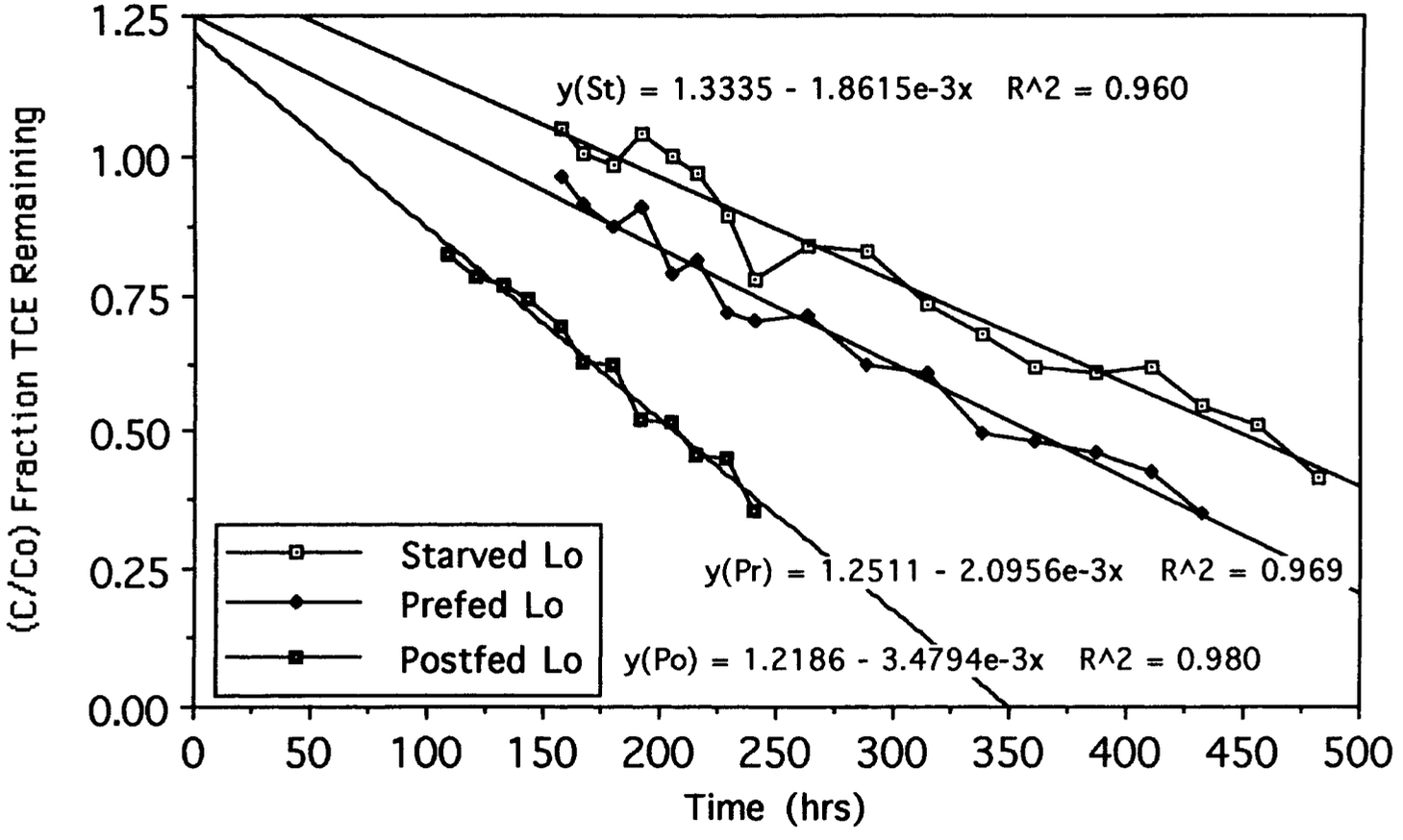

Figure 12. Zero-order plot to determine maximum TCE transformation rate: Comparison of different ammonia concentrations for 0.1mg/L TCE.

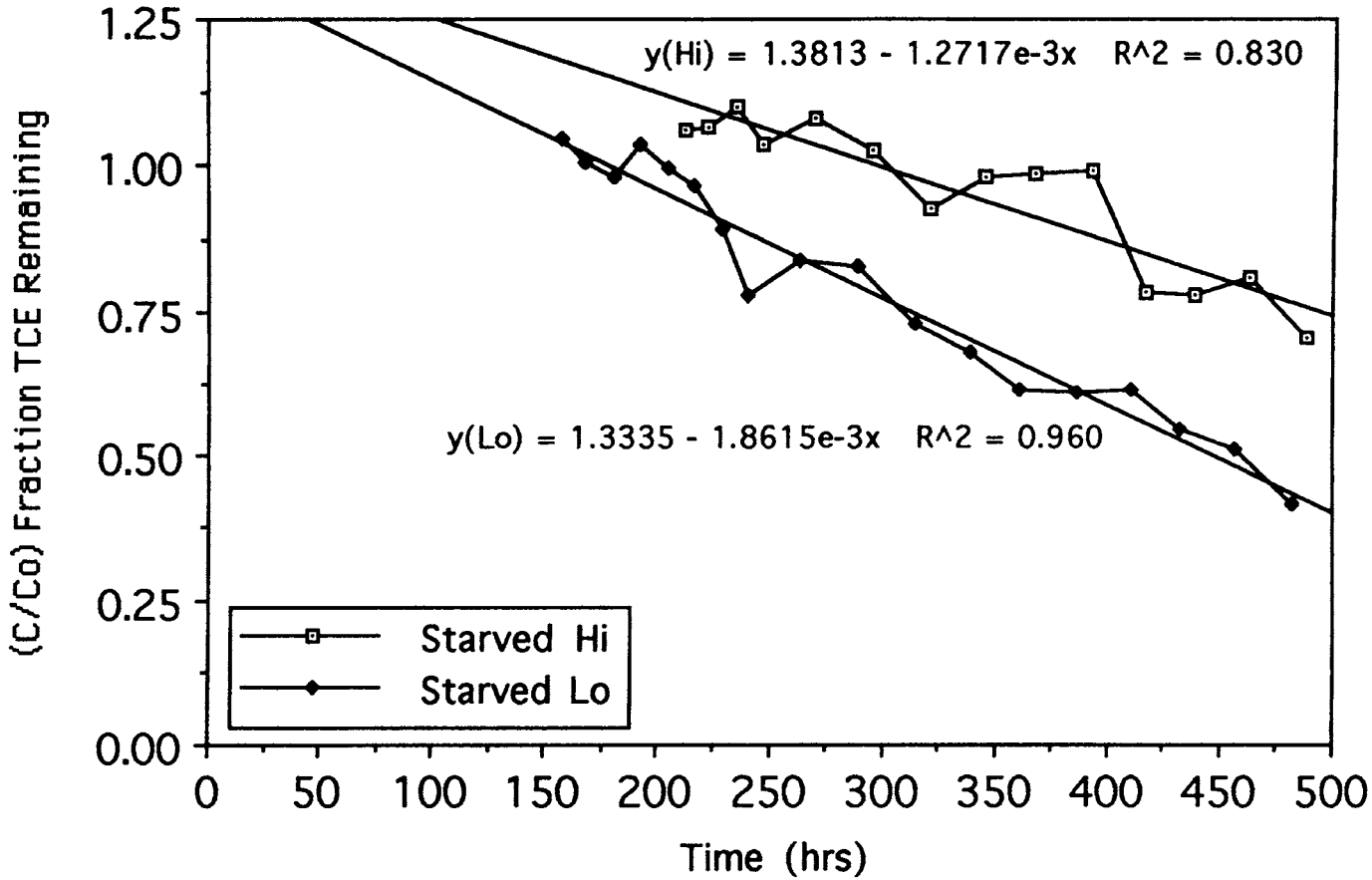

Figure 13. Zero-order plot to determine maximum TCE transformation rate: Comparison of different TCE concentrations for 20mg/L ammonia.

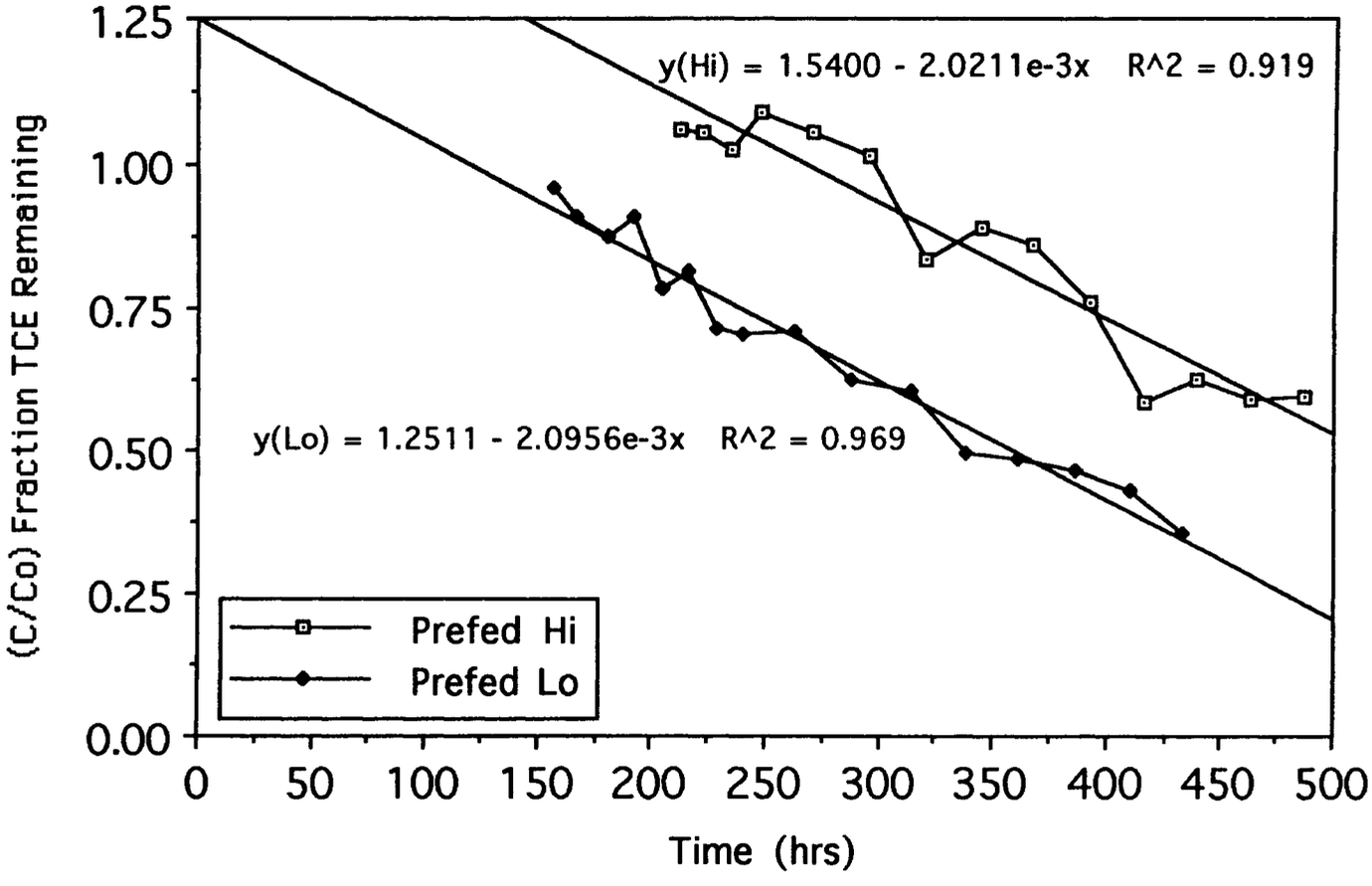

Figure 14. Zero-order plot to determine maximum TCE transformation rate: Comparison of different TCE concentrations for 100mg/L ammonia.

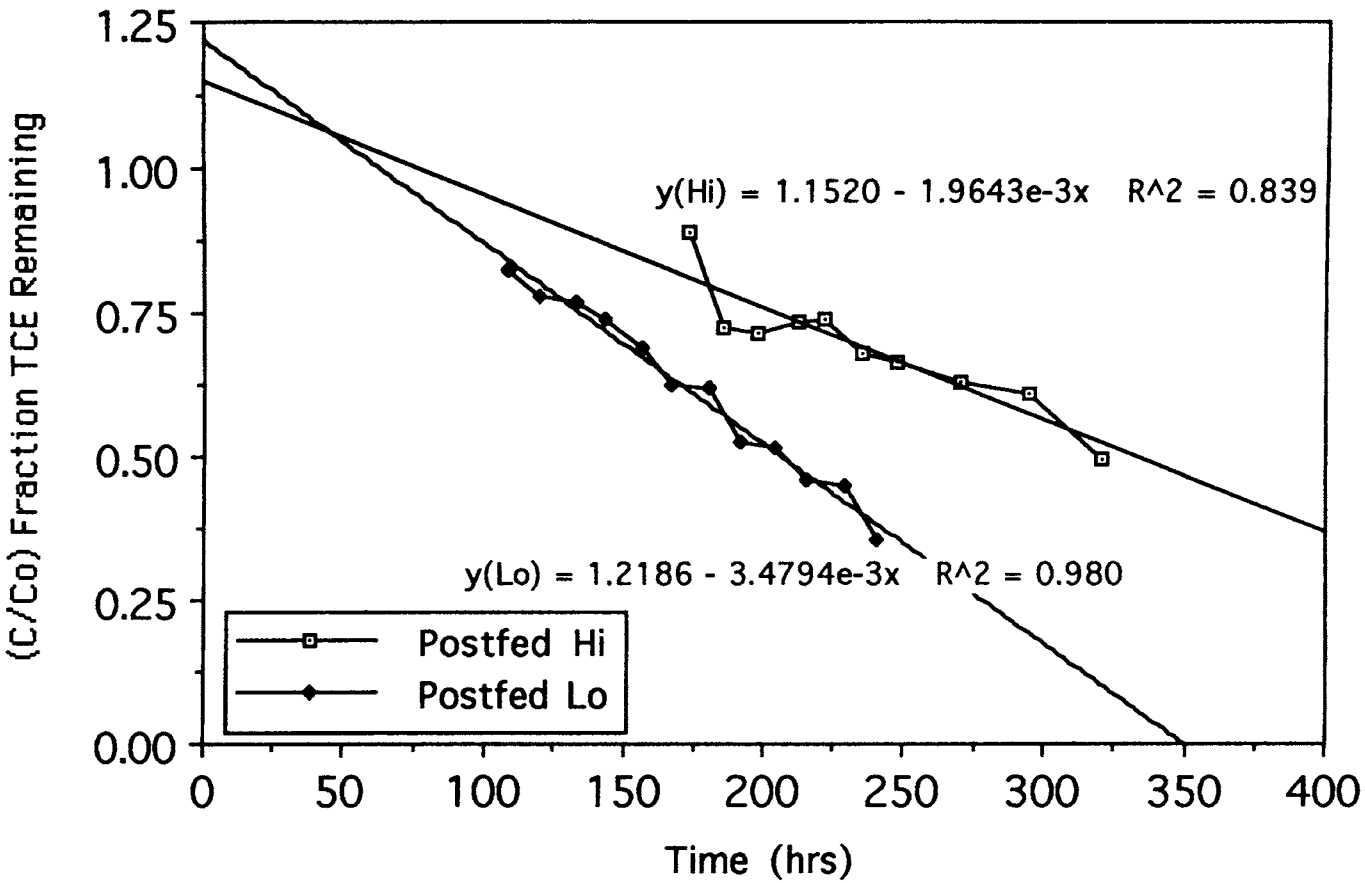

Figure 15. Zero-order plot to determine maximum TCE transformation rate: Comparison of different TCE concentrations for 300mg/L ammonia.

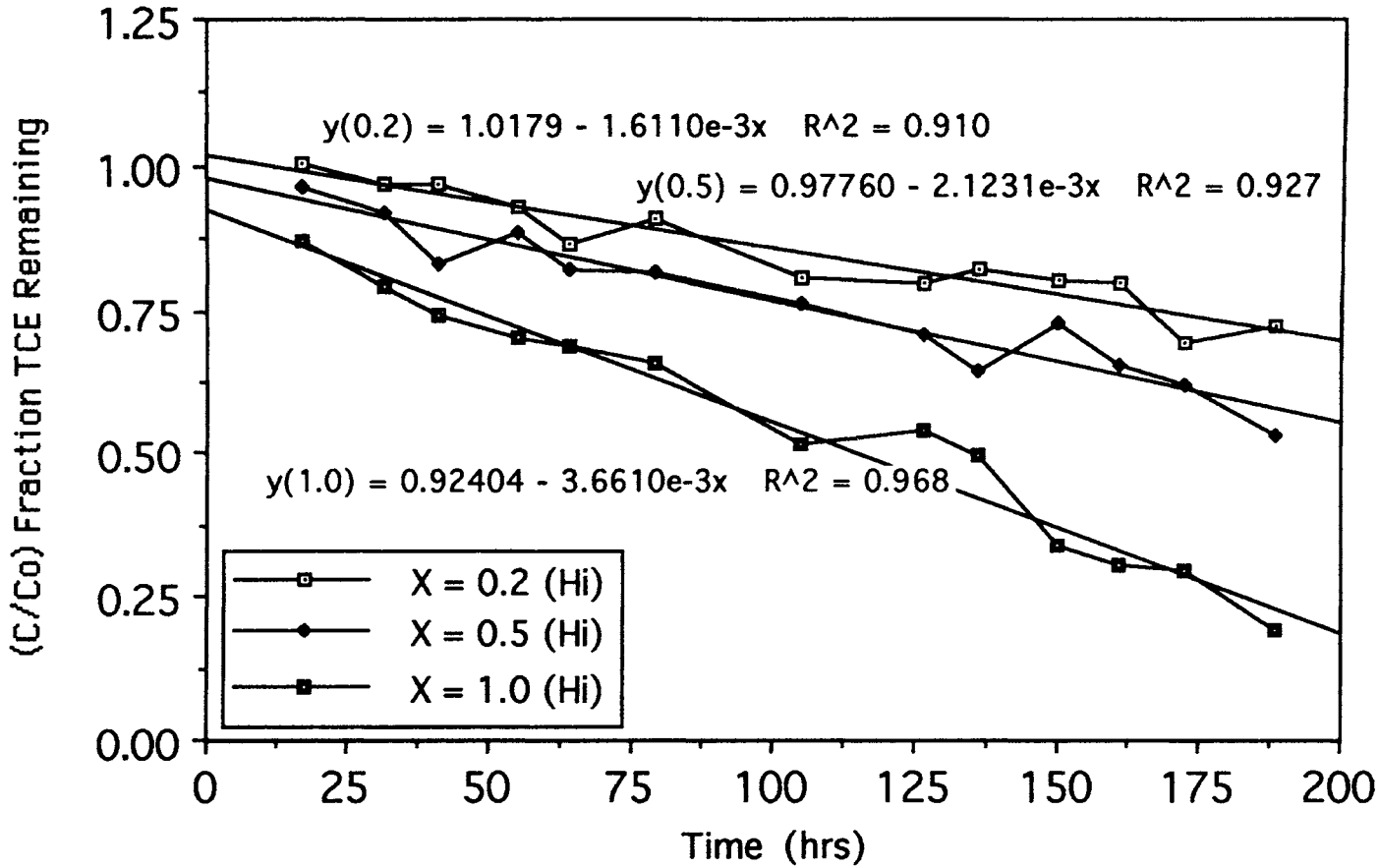

Figure 16. Zero-order plot to determine maximum TCE transformation rate: Comparison of different VSS concentrations for 1mg/L TCE.

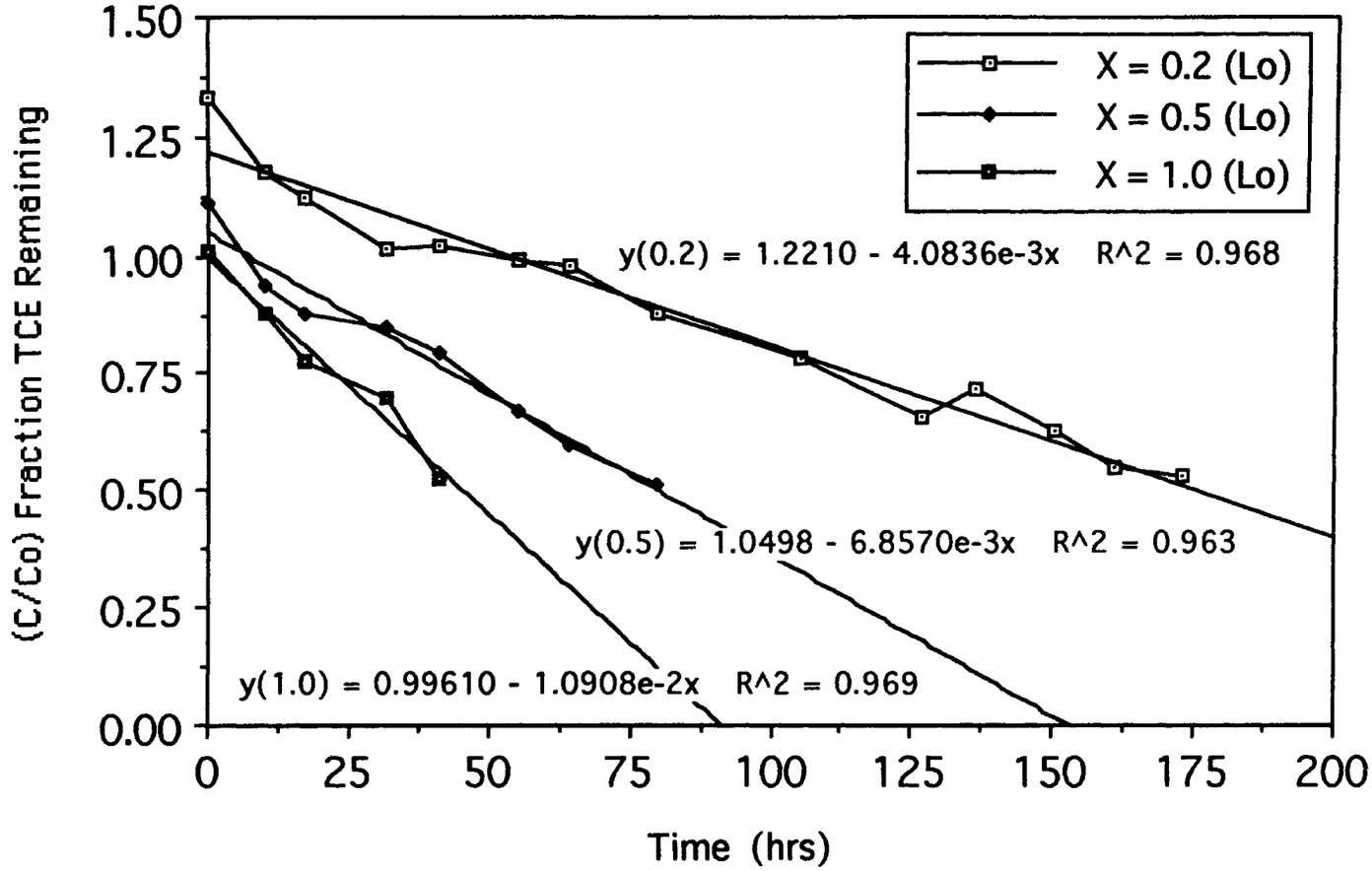

Figure 17. Zero-order plot to determine maximum TCE transformation rate: Comparison of different VSS concentrations for 0.1mg/L TCE.

Figures 18, 19 and 20 permit comparison of the effect of TCE concentration on the maximum zero-order transformation rates for each concentration of nitrifying bacteria (VSS) used.

The maximum TCE transformation rates represented by the slope of the lines were calculated for all the cases described above. They are listed in Table 8.

From Table 8 it can be observed that both k_0 and V_{max} values were higher when the initial ammonia concentration was higher, except in case of the "Postfed Hi" samples, where both values drop slightly. This contradicts the assumption that competitive inhibition is the mechanism responsible for the degradation of TCE. Furthermore, the k_0 values increased when higher biomass concentrations were used. This was an expected trend. However V_{max} values appear to decrease with increased biomass concentration when they should in fact remain constant regardless of initial biomass concentration. This was probably caused by inefficient mass transfer in the reactors. Both k_0 and V_{max} values are higher when the initial TCE concentration is lower (0.1 mg/L). This indicates the possibility of an inhibitory mechanism at work at the higher TCE concentration.

Vannelli and coworkers [1990] use a zero-order kinetic model to describe the degradation of TCE by *Nitrosomonas europaea* and give a V_{max} value of 2.8 μmoles per hour per gram (wet weight) of cells for cells without ammonia. Similar calculations were made with the

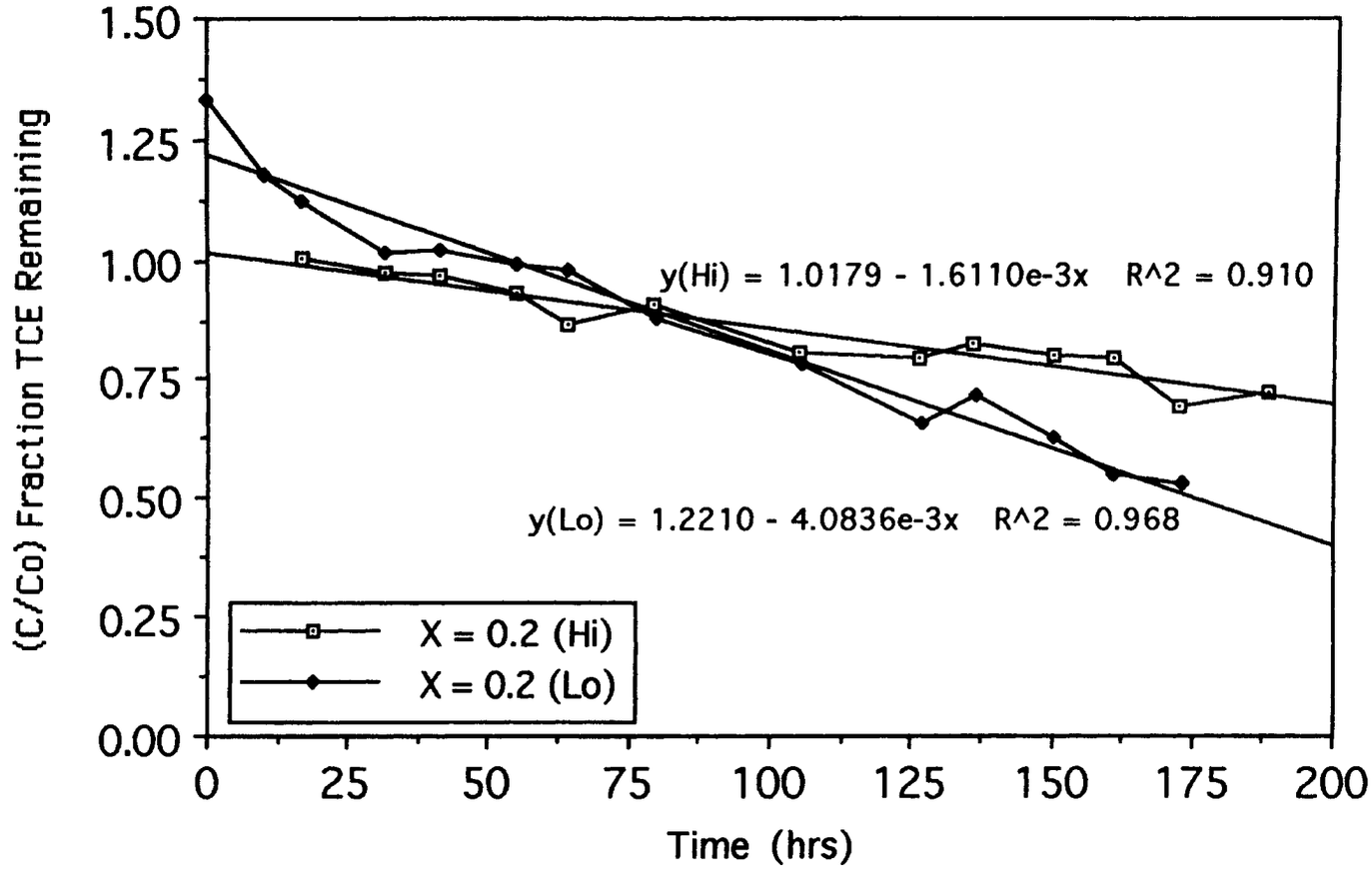

Figure 18. Zero-order plot to determine maximum TCE transformation rate: Comparison of different TCE concentrations for VSS = 876mg/L.

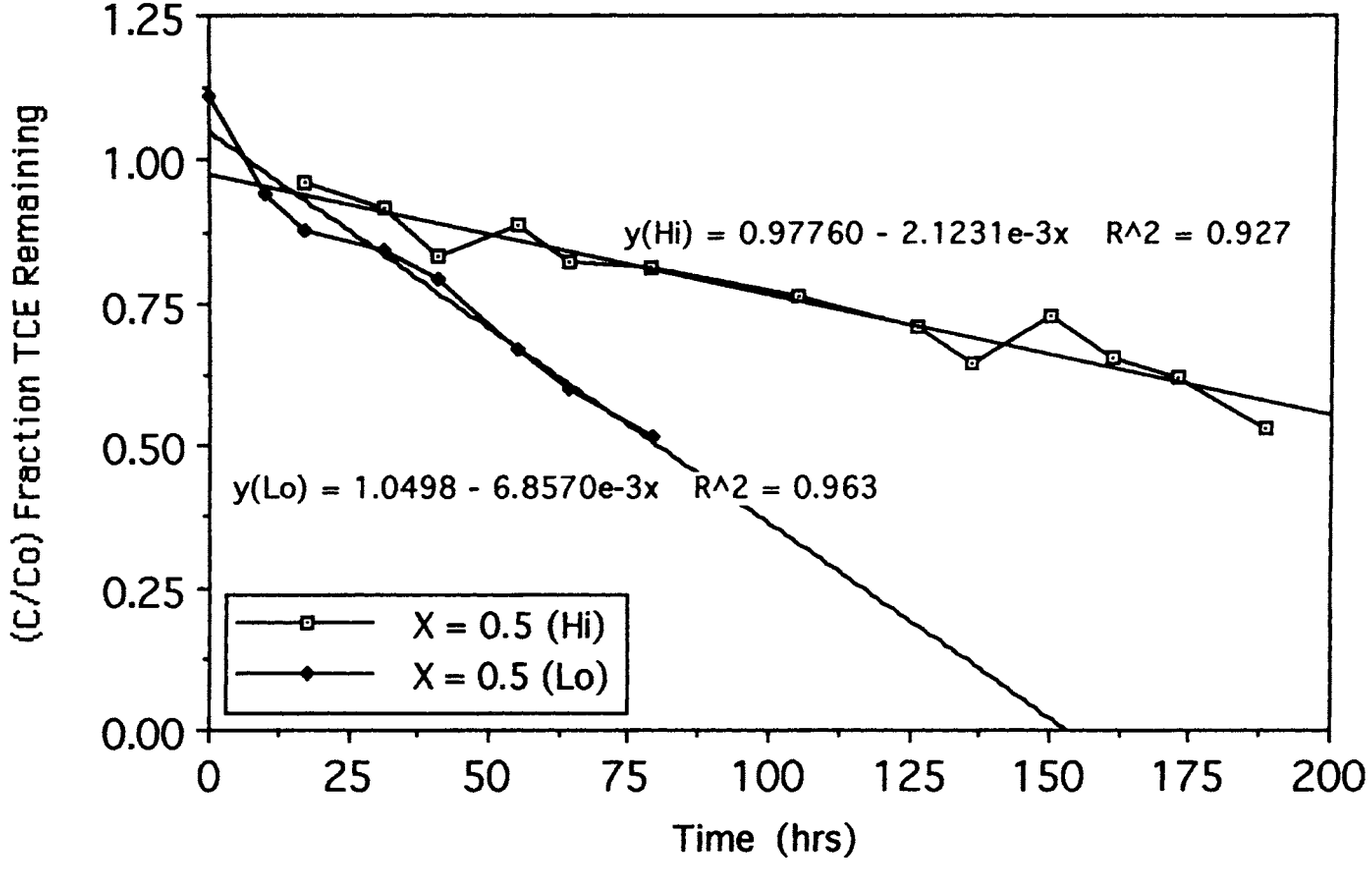

Figure 19. Zero-order plot to determine maximum TCE transformation rate: Comparison of different TCE concentrations for VSS = 2036mg/L.

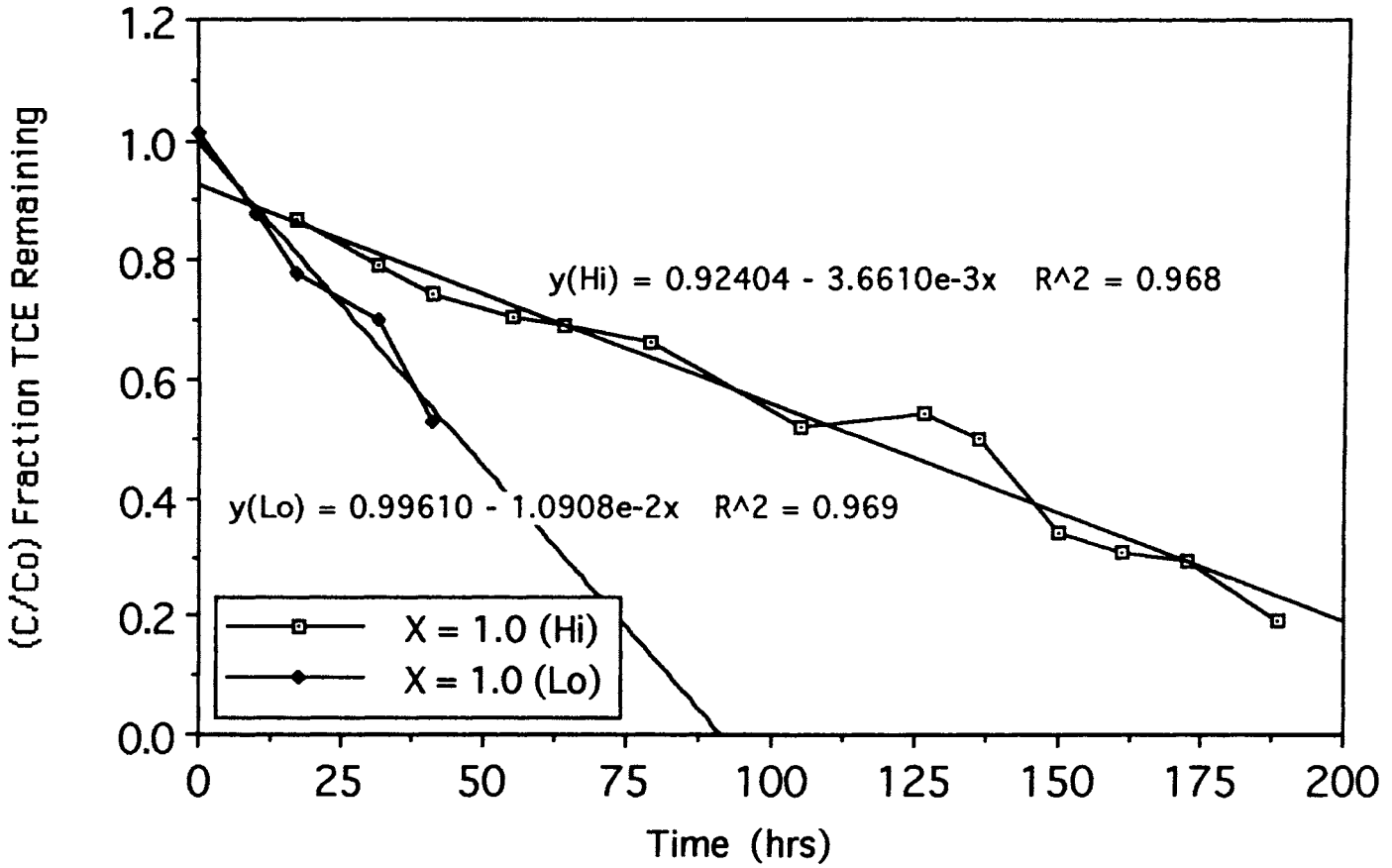

Figure 20. Zero-order plot to determine maximum TCE transformation rate: Comparison of different TCE concentrations for VSS = 3754mg/L.

Table 8. k_0 and V_{max} values for the kinetic experiments

Reactor Labels	k_0 ($mg_{TCE}/L \cdot h$)	VSS++ (mg/L)	V_{max} ($mg_{TCE}/mg_{VSS} \cdot h$)	R^2
Starved Hi	0.0013	934	1.4×10^{-6}	0.830
Prefed Hi	0.0020	727	2.8×10^{-6}	0.919
Postfed Hi	0.0019	785	2.5×10^{-6}	0.839
Starved Lo	0.0019	936	2.0×10^{-6}	0.960
Prefed Lo	0.0021	734.5	2.9×10^{-6}	0.969
Postfed Lo	0.0035	771.5	4.5×10^{-6}	0.980
X=0.2 Hi	0.0016	877.5	1.8×10^{-6}	0.910
X=0.5 Hi	0.0021	2037.5	1.0×10^{-6}	0.927
X=1.0 Hi	0.0037	3924	0.9×10^{-6}	0.968
X=0.2 Lo	0.0041	832	4.9×10^{-6}	0.968
X=0.5 Lo	0.0069	2002.5	3.4×10^{-6}	0.963
X=1.0 Lo	0.0109	3926.5	2.8×10^{-6}	0.969

++ Average of initial and final VSS values

data from Table 8. For example, for the "Starved Lo" experiment (i.e. 0.1 mg/L TCE, 20 mg/L NH_3-N) the zero-order V_{max} value was 2.0 $x10^{-6}$ mg_{TCE}/mg_{VSS}•h which is equivalent to 4.6 $x10^{-3}$ μmoles per hour per gram (wet weight) of cells assuming that water constitutes 70% of a cell [Brock and Madigan, 1988]. Vannelli and coworkers [1990] give a V_{max} value of 6.7 μmoles per hour per gram (wet weight) of cells for cells with ammonia present. The "Postfed Lo" sample had a V_{max} value of 4.5 $x10^{-6}$ mg_{TCE}/mg_{VSS}•h (from Table 8) and this is equivalent to 1.03 $x10^{-2}$ μmoles per hour per gram (wet weight) making the same assumption as above.

Arciero and coworkers [1989] also use a zero-order kinetic model to describe the degradation of TCE by *Nitrosomonas europaea* and give a V_{max} value of 0.42 nmoles per min per $mg_{protein}$. The "Postfed Lo" sample had a V_{max} value of 4.5 $x10^{-6}$ mg_{TCE}/mg_{VSS}•h (from Table 8) and this was equivalent to 9.5 $x10^{-4}$ nmoles per min per $mg_{protein}$ assuming that protein constitutes 60% of the dry weight of a cell [Brock and Madigan, 1988].

These differences are probably because both Vannelli [1990] and Arciero [1989] and their coworkers were using pure cultures of *Nitrosomonas europaea*, while the "VSS" measurements here include not only AMO-expressing organisms, but nitrite oxidizers, heterotrophic scavengers and possibly some dead cells as well. It is also not possible from their work [Vannelli *et al.* 1990] to determine the actual cellular concentrations present. Therefore a direct comparison of these V_{max} values may not be particularly meaningful.

Another widely used biotransformation model approach uses second-order kinetics. The second-order rate expression depends on the concentration of xenobiotic compounds and the active bacterial population [Paris and Rogers, 1986]. Paris and Rogers [1986] propose the following expression for a second-order kinetic model:

$$dC/dt = -k_bCX \quad (4)$$

where, if TCE is the substrate of interest

C = TCE concentration at time t (mg_{TCE}/L)

k_b = second-order rate constant ($L/mg_{VSS}\bullet h$)

X = Bacterial (VSS) Concentration (mg_{VSS}/L)

t = Time (h)

This equation corresponds to Schmidt and coworkers [1985] "first-order" model in which K_m is much larger than C (It is first-order with respect to both C and X, second-order overall).

Although in natural waters (and in these experiments) the xenobiotics may not be the sole carbon source, Paris and coworkers [1981] have found that a second-order approach can be used to describe microbial transformation of xenobiotics.

As a preliminary examination of the kinetics of these experiments, K_m is assumed to be much greater than C (TCE concentration). As will be discussed, more experiments need to be conducted to confirm

this hypothesis. If biomass concentration (X) is constant, the product k_bX in Equation (4) can be replaced by K_1, resulting in

$$dC/dt = -K_1C \quad (5)$$

where K_1 is the pseudo-first-order rate constant (h^{-1}). Rearranging and integrating yields

$$\ln(C/C_o) = -K_1t \quad (6)$$

where C_o is the TCE concentration at time t = 0. Thus, the pseudo-first-order reaction rate constant (K_1) can be obtained if a plot of $\ln(C/C_o$, fraction of TCE remaining) versus time yields linear results. The second-order overall reaction rate (k_b) is then calculated by dividing K_1 by the VSS (bacterial) concentration. This approach was used to analyze the data from the experiments in which initial biomass concentration was varied (with initial ammonia concentration of 300 mg/L).

The semilogarithmic plots of (C/C_o) versus time have been plotted for the studies with various biomass concentrations and are given in Figures 21 and 22. The plots yielded linear results, with the lowest correlation coefficient (R^2) being 0.900. The pseudo-first-order reaction rate constants (K_1) were obtained from the slope of the lines for all the experimental conditions and are listed in Table 9. The second-order rate constants (k_b) were calculated by dividing the pseudo-first-order reaction rate constant by the VSS concentration for all the samples. The k_b values are also listed in Table 9.

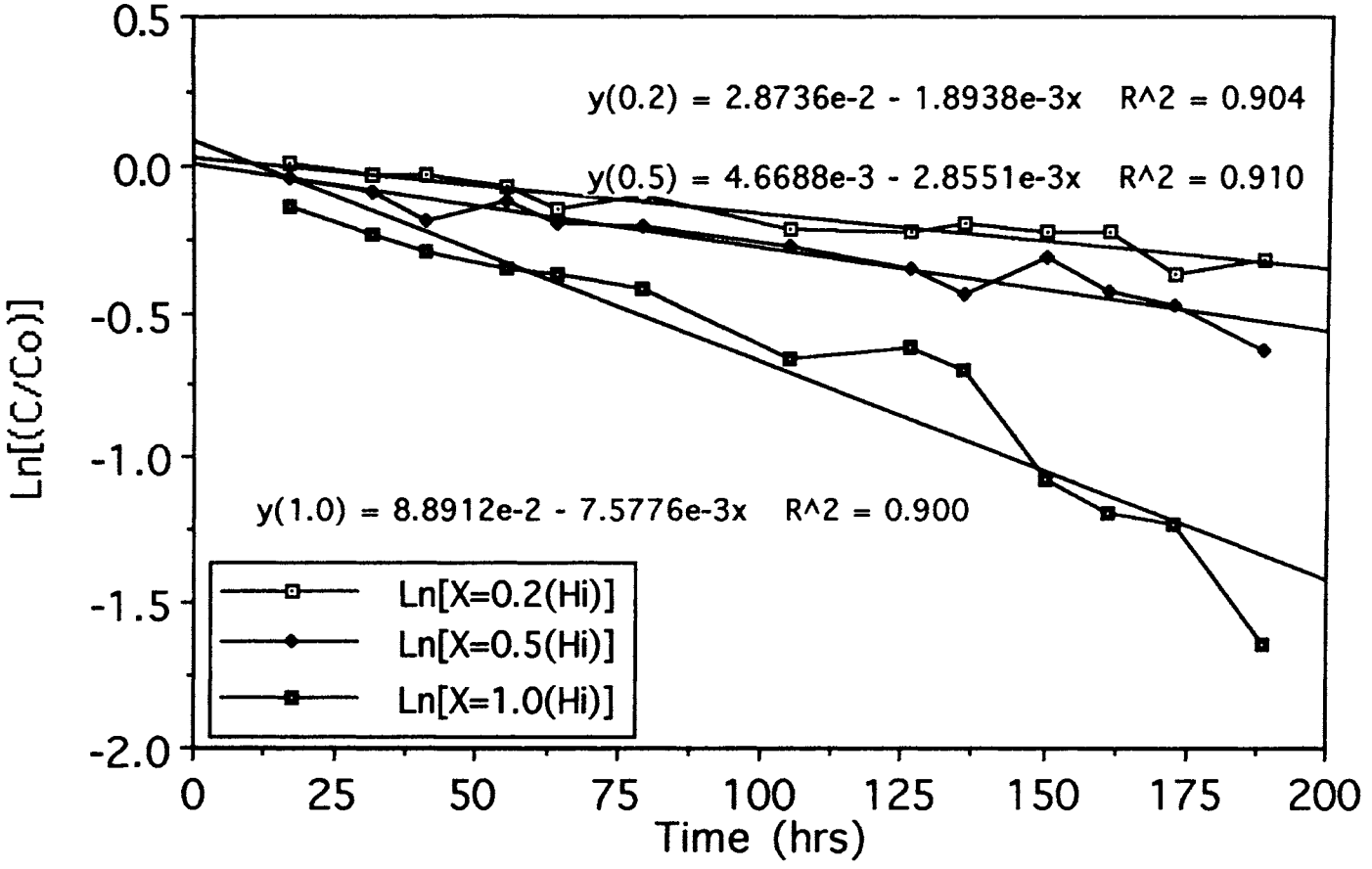

Figure 21. First-order plots to determine pseudo-first-order TCE transformation rate constants: Comparison of different VSS concentrations for 1mg/L TCE.

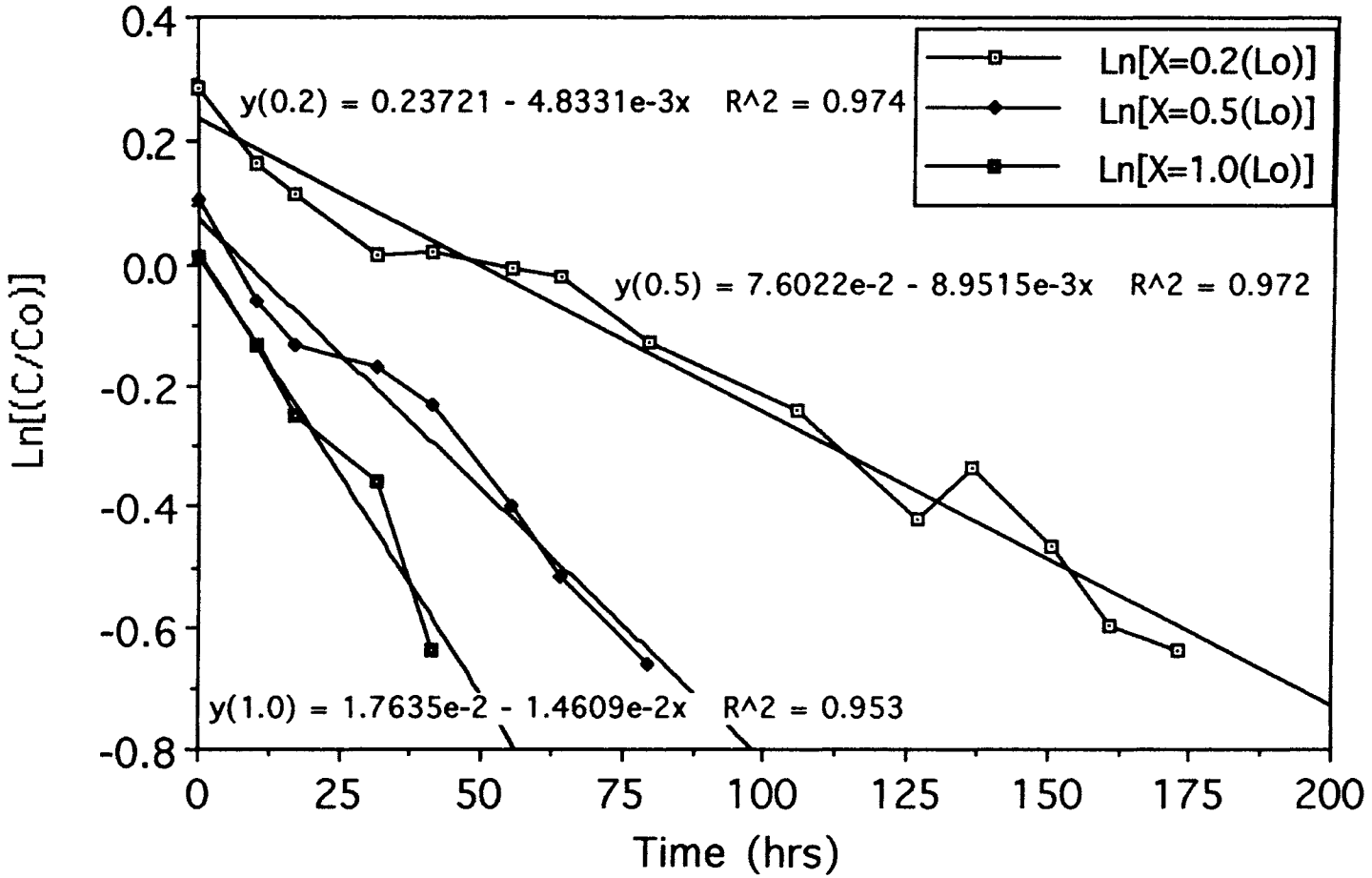

Figure 22. First-order plots to determine pseudo-first-order TCE transformation rate constants: Comparison of different VSS concentrations for 0.1mg/L TCE.

Table 9. K_1 and k_b Values for the experiments with different VSS

Reactor Labels	$-K_1$ (h^{-1})	VSS++ (mg/L)	k_b ($L/mg_{VSS} \cdot h$)	R^2
X=0.2 Hi	0.0019	877.5	2.2×10^{-6}	0.904
X=0.5 Hi	0.0029	2037.5	1.4×10^{-6}	0.910
X=1.0 Hi	0.0076	3924	1.9×10^{-6}	0.900
Average k_b			1.84×10^{-6}	
X=0.2 Lo	0.0048	832	5.8×10^{-6}	0.974
X=0.5 Lo	0.0090	2002.5	4.5×10^{-6}	0.972
X=1.0 Lo	0.0146	3926.5	3.7×10^{-6}	0.953
Average k_b			4.66×10^{-6}	

++ Average of initial and final VSS values

From Table 9 it is observed that the k_b values obtained for the samples with a low TCE concentration (0.1 mg/L) are all higher than the corresponding k_b values obtained for the same samples with a high TCE concentration (1 mg/L). This may be because TCE is inhibiting overall cellular metabolism, thereby inhibiting its transformation. This could also be due to the inhibition of the enzyme AMO by TCE. Toxicity of nitrification (and therefore, AMO) is discussed further below. The available literature discussing this subject deal primarily with only one concentration of TCE (1.4 mg/L) and pure cultures of *Nitrosomonas* species. Arciero and coworkers [1989] have reported that ammonia oxidizing ability of the cells was inhibited 98 % in presence of 1.1 mM (145 mg/L) TCE.

The data from Table 9 also show that the k_b values do not change appreciably with a change in the bacterial concentration (VSS) for a given initial concentration of TCE. This is also consistent with second-order reaction kinetics. There is a general downward trend noticed with increased biomass, which may be due to mass transfer limitations mentioned previously.

The data obtained from the kinetic experiments had good fits with both the models tested. More data are required to determine which of these is more appropriate for these particular systems.

Results of Toxicity Experiments

The results of the experiment to determine the effect of methanol and TCE on nitrification are presented here. The concentrations of methanol and TCE used in this experiment were equivalent to their highest concentrations in the kinetic experiments. TCE dissolved directly into water was used for this experiment, since the objective was to determine the toxicity of TCE alone. The residual ammonia concentration was plotted against time for the three cases to determine the effect of TCE and methanol on nitrification rates (and hence, AMO activity), and the results are shown in Figure 23. The values for the various parameters measured during the course of the experiment are listed in Table 10.

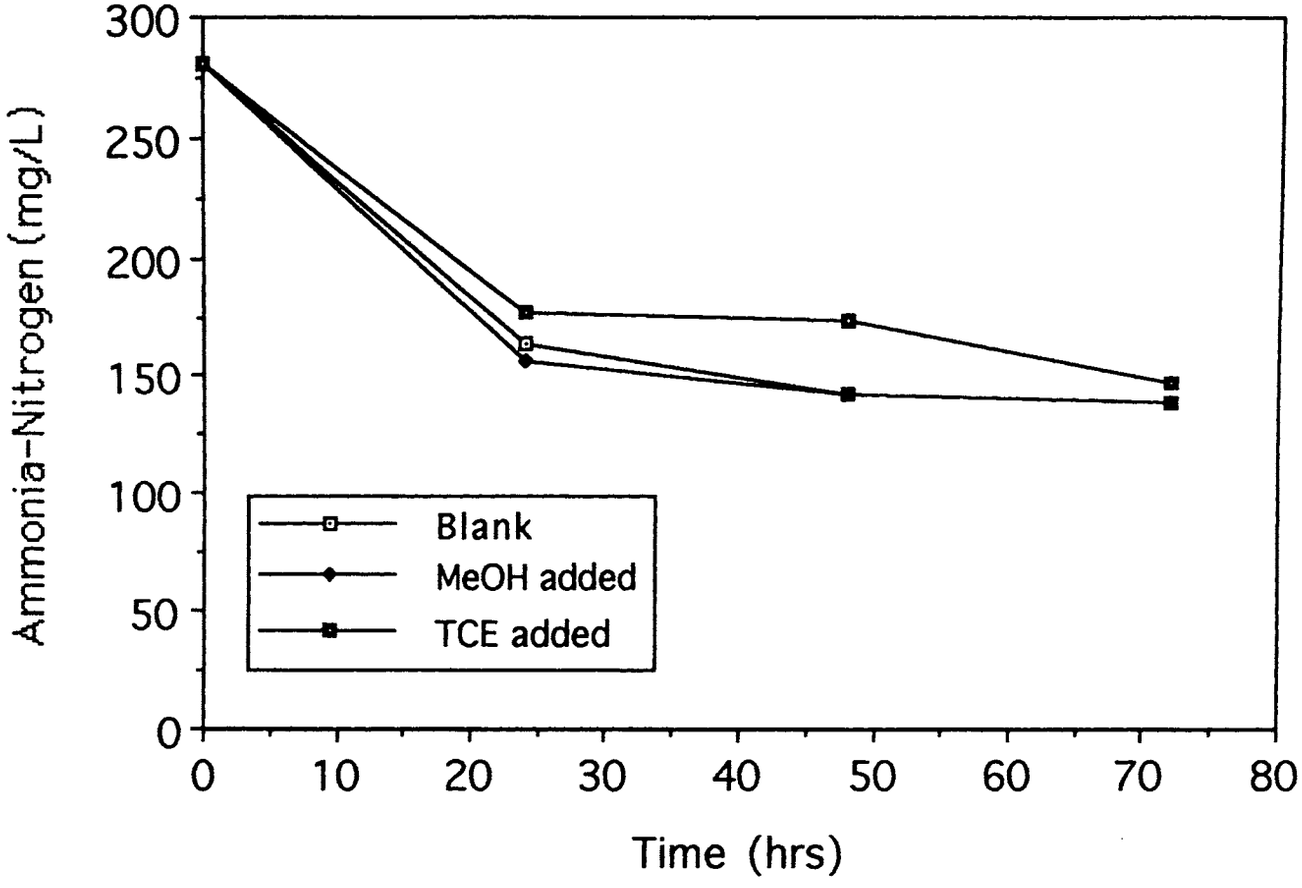

Figure 23. Nitrification in presence of methanol and TCE

Table 10. Values of parameters measured for toxicity experiments

Time (hours)	NH_3-N* (mg/L)	VSS (mg/L)	DO (mg/L)	pH
Reactor controls (Blanks with just nitrifying bacteria)				
Initial	280	661**	7.2	8.0
24	164	-	3.5	7.8
48	143	-	3.0	7.8
72	139	587+	2.5	7.85
Reactors with 158 mg/L MeOH added				
Initial	280	661**	7.2	8.0
24	157	-	3.6	7.8
48	143	-	3.3	7.8
72	138	654+	2.4	7.85
Reactors with 1 mg/L TCE added				
Initial	280	661**	7.2	8.0
24	176	-	3.65	7.9
48	173	-	3.4	7.8
72	147	651+	2.4	7.75

* ±10% precision in previous tests conducted

** Average of 3 samples prepared identical to reactors for VSS measurements

\+ Actual values measured at the conclusion of the experiment

From Figure 23 and Table 10 it can be observed that there was no significant suppression of nitrification by either TCE or methanol for the concentrations used in this experiment. The rate of nitrification for the samples with TCE were slightly lower than the others. Conclusive statements cannot however be made, since there is only limited data available. Arciero and coworkers [1989] report that the cells of *Nitrosomonas europaea* are not permanently inactivated by short term incubations with 1.1 mM TCE (145 mg/L). However, they also report that the presence of TCE has an inhibitory effect on the production of nitrite from ammonia. Vannelli and coworkers [1990] report that for *Nitrosomonas europaea*, the degradation of TCE decreased the rate of nitrite production from ammonia, consistent with competition for an active site on the AMO enzyme. However Rasche and coworkers [1991] report that the inactivation of ammonia oxidation by *Nitrosomonas europaea* occurred during biodegradation of TCE and the extent of inactivation depended on the time of exposure to TCE and the initial TCE concentration. This is consistent with the "product toxicity" observations in methane monooxygenase (MMO) systems [Alvarez-Cohen and McCarty, 1991b].

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The following conclusions can be drawn from the results of this research.

1. The rate of biotransformation of TCE by the nitrifying culture was found to be higher at the lower initial concentration of TCE (0.1 mg/L). Zero and second-order TCE transformation rates at low concentration of TCE (0.1 mg/L) were higher than the rates with high concentration of TCE (1 mg/L) regardless of the amount of ammonia present and the amount of bacteria present. This may be due to the toxicity of TCE or its metabolic products.

2. The biotransformation of TCE was fastest when there was a higher (300 mg/L) initial concentration of ammonia. This trend was observed for both of the initial concentrations of TCE used (1 mg/L and 0.1 mg/L). TCE transformation began immediately in these cultures, while a significant lag period was observed in those with lower (100 mg/L and 20 mg/L) initial ammonia concentrations.

3. TCE transformation rates in all cultures were proportional to biomass concentrations, as expected. Rate coefficients for zero and

second-order models, when normalized for biomass concentration, were relatively constant. However, mass transfer limitations may have been significant at the higher biomass concentrations.

4. The data from these experiments fit both zero and second-order equations well. For the experimental conditions tested, neither model was found to be preferable.

5. The concentrations of TCE and methanol used in the experiments did not appear to significantly inhibit the nitrification process.

Recommendations

1. Experiments need to be done with a wider range of TCE concentrations to learn more about the kinetics of its biotransformation.

2. More experiments are necessary with different bacterial concentrations to gain a better understanding of the reaction kinetics.

3. A better understanding of the interactions between ammonia, TCE, and oxygen, and their effects on ammonia monooxygenase systems is needed. Continuous culture experiments, in which oxygen is continuously fed, and hence, can not become limiting, are recommended.

4. Additional studies, including radiotracer and mass-spectral analyses are also needed to determine the byproducts of TCE cometabolism by these cultures.

Practical Implications

This study provides additional information about the possibilities of stimulating native nitrifying bacterial populations, or introduced nitrifiers, to enhance *in-situ* bioremediation of sites contaminated with TCE. Nitrifying bacteria may be viable alternatives to use in the biorestoration of TCE-contaminated sites if aerobic conditions are maintained and an adequate amount of ammonia can be supplied. Laboratory-scale tests would be required to determine the optimal conditions for TCE biotransformation in a particular site. Techniques like pumping nutrients (ammonia and oxygen) into the contamination zone need to be tested to see if they can help increase the transformation rate of TCE.

This study can also be applied by POTWs that have nitrification processes. The fate (and ultimate emission) of chlorinated aliphatics (and other toxic organics) is of particular concern in these systems, and the research presented here provides some insight into one important fate process. It is clear that TCE will be transformed in mixed nitrifying systems. Its net effect on these systems, and the fraction of TCE transformed by nitrifiers, relative to other fate processes, is yet to be determined.

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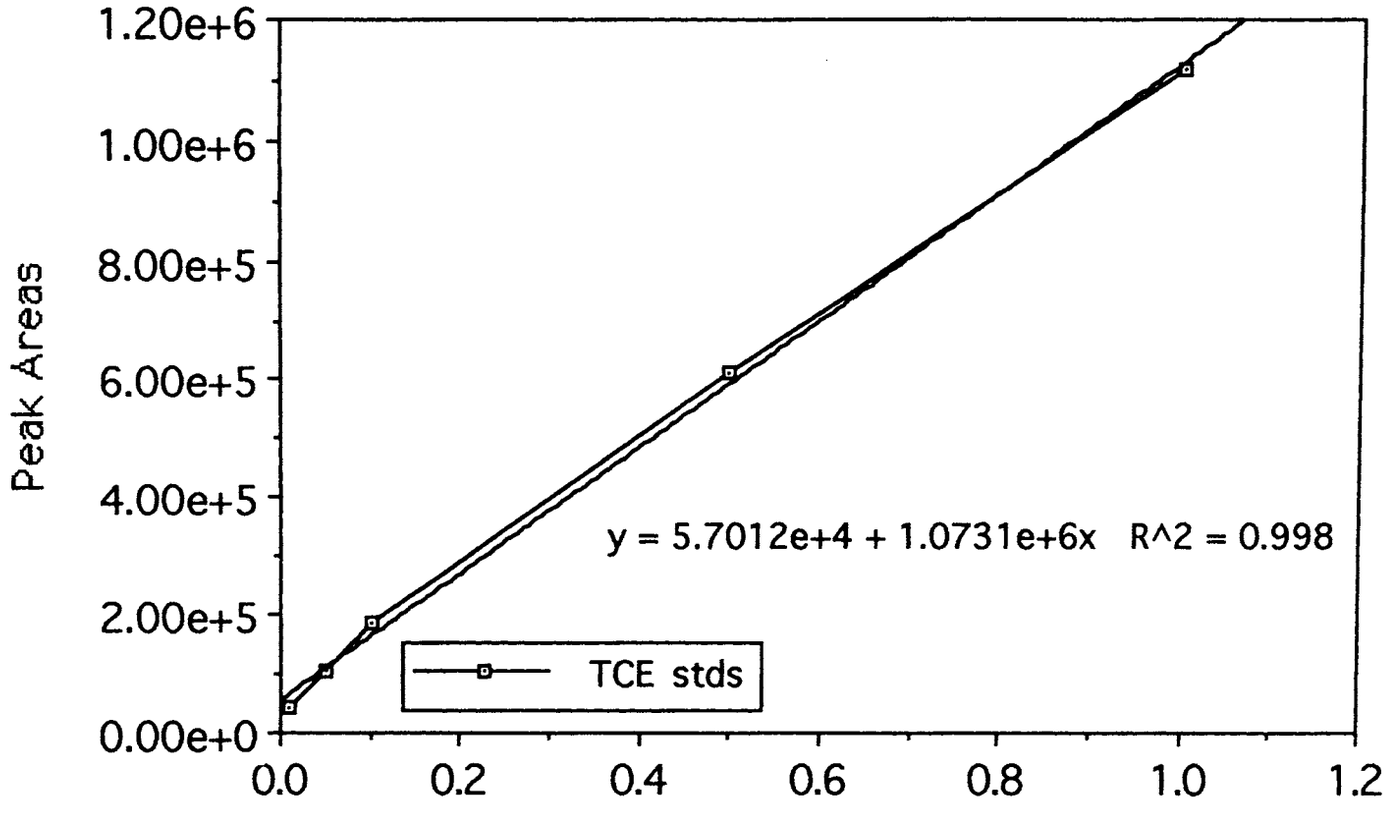
APPENDICES



APPENDIX A. TCE CALIBRATION CURVE

APPENDIX B

PHYSICAL AND CHEMICAL PROPERTIES OF TRICHLOROETHENE

Property	Information
Chemical formula	C_2HCl_3
Molecular weight	131.40
Color	Clear; colorless
Physical state	Liquid at room temperature
Melting point	-87.1°C
Boiling point	86.7°C
Density at 20°C	1.465 mg/L
Odor	Ethereal; sweet
Vapor pressure at 25°C	74 mm Hg
Henry's law constant at 20°C	0.020 atm-m^3/mol

Reference: [ATSDR, 1991]

APPENDIX C

DATA FROM KINETIC EXPERIMENTS WITH
DIFFERENT TCE AND AMMONIA CONCENTRATIONS (1 mg/L)

Ammonia and VSS Concentrations

Starved: Ammonia = 20 mg/L; VSS = 1071 mg/L
Prefed: Ammonia = 100 mg/L; VSS = 856 mg/L
Postfed: Ammonia = 300 mg/L; VSS = 877 mg/L

(C/Co) Normalized Peak Areas

Time (hrs)	Blank 1mg/L	Starved (Hi)	Prefed (Hi)	Postfed (Hi)
0	1	1.05596741	1.01131241	0.91249646
10	1	0.95734096	0.99567155	1.02689048
19	1	0.94343463	1.03310623	0.94329893
31	1	0.91485981	1.03094297	1.0134537
43	1	0.86382401	0.95458592	1.00289311
54	1	0.93415491	1.00562846	0.94756735
66	1	0.88545518	0.94961138	0.92962855
78.5	1	0.92751237	1.02317759	0.94718225
90	1	1.06784686	1.03400612	0.95353156
101.5	1	0.95195683	1.10190667	0.89922534
114.5	1	0.98393863	1.03377301	0.88044848
126	1	0.97949103	1.04003901	0.89522636
138	1	0.89912017	0.94108068	0.78181389
149	1	1.00682353	1.01281126	0.85436066
163	1	0.99202174	0.9794714	0.79215908
173	1	1.06090181	1.0371033	0.89014041
186	1	0.8830425	0.89196069	0.72329033
198	1	0.98062227	0.99240678	0.71655162
212	1	1.06209442	1.05860232	0.73538968
222	1	1.06791628	1.05531304	0.74157387
235	1	1.10265281	1.02414796	0.68120658
247	1	1.03648163	1.08933756	0.66264263
270	1	1.08166267	1.05498947	0.62771027
295	1	1.02802195	1.01524934	0.61225639
320.5	1	0.92760913	0.83317794	0.49662499
345	1	0.9821682	0.88838599	0.52902983
367	1	0.98515712	0.85814531	0.44645928
393	1	0.99200898	0.76047407	0.51046419
417	1	0.78581672	0.58564331	0.34187049
439	1	0.77858127	0.62721158	0.3881446
463	1	0.80908603	0.59001693	0.43232132
488	1	0.70543236	0.59258031	0.35808144

APPENDIX D

DATA FROM KINETIC EXPERIMENTS WITH
DIFFERENT TCE AND AMMONIA CONCENTRATIONS (0.1 mg/L)

Ammonia and VSS Concentrations

Starved: Ammonia = 20 mg/L; VSS = 1071 mg/L
Prefed: Ammonia = 100 mg/L; VSS = 856 mg/L
Postfed: Ammonia = 300 mg/L; VSS = 877 mg/L

(C/Co) Normalized Peak Areas

Time (hrs)	Blank 0.1mg/L	Starved (Lo)	Prefed (Lo)	Postfed (Lo)
0	1	1.020541	1.124096	1.274071
6	1	0.934976	0.995156	1.089607
17	1	0.895183	0.987837	1.046236
29	1	0.938791	0.94475	0.998723
40	1	0.914794	0.97556	1.018317
52	1	0.918418	0.928799	1.002732
64	1	0.945149	0.950417	0.910322
72	1	1.006557	0.992103	0.959962
84	1	0.912494	0.875261	0.810951
95	1	0.912482	0.920703	0.820258
108	1	0.978928	0.904715	0.824067
120	1	0.979791	0.897508	0.782145
132.5	1	1.010147	0.947703	0.768956
143	1	1.01659	0.927145	0.7421
157	1	1.048298	0.962413	0.692013
167	1	1.005356	0.911203	0.625363
180	1	0.983953	0.873204	0.622048
191.5	1	1.037966	0.909902	0.52301
204.5	1	0.999177	0.787119	0.515926
215.5	1	0.966642	0.812598	0.458624
228.5	1	0.894569	0.717062	0.449926
240	1	0.77717	0.704555	0.35571
263	1	0.838913	0.711355	0.34469
288.5	1	0.830576	0.622927	0.314094
314.5	1	0.730486	0.606627	0.268759
338.5	1	0.677282	0.494741	0.236849
360.5	1	0.61667	0.482717	0.235106
386.5	1	0.609439	0.463101	0.253504
410.5	1	0.617327	0.428888	
432.5	1	0.54557	0.353362	
456.5	1	0.510401	0.349051	
482	1	0.415591	0.329051	

APPENDIX E

DATA FROM KINETIC EXPERIMENTS WITH
DIFFERENT TCE AND BIOMASS CONCENTRATIONS (1 mg/L)

Ammonia Concentration = 300 mg/L
VSS Concentrations

X = 0.2, VSS = 876 mg/L
X = 0.5, VSS = 2036 mg/L
X = 1.0, VSS = 3754 mg/L

(C/Co) Normalized Peak Areas

Time (hrs)	Blank 1mg/L	X = 0.2 (Hi)	X = 0.5 (Hi)	X = 1.0 (Hi)
0	1	1.073352	1.015624	1.014952
10	1	1.117965	0.933924	0.865178
17	1	1.005944	0.964233	0.870928
31.5	1	0.971304	0.918581	0.791597
41	1	0.970014	0.833687	0.744032
55	1	0.930283	0.886622	0.706098
64	1	0.866308	0.821448	0.690455
79	1	0.908265	0.814972	0.660964
105	1	0.807413	0.762927	0.517235
126.5	1	0.795992	0.708428	0.540345
136	1	0.823949	0.647076	0.499211
150	1	0.80091	0.730297	0.340867
161	1	0.796055	0.652441	0.30519
172.5	1	0.693116	0.619252	0.29398
188.5	1	0.725112	0.53152	0.193455

APPENDIX F

DATA FROM KINETIC EXPERIMENTS WITH
DIFFERENT TCE AND BIOMASS CONCENTRATIONS (0.1 mg/L)

Ammonia Concentration = 300 mg/L
VSS Concentrations

X = 0.2, VSS = 876 mg/L
X = 0.5, VSS = 2036 mg/L
X = 1.0, VSS = 3754 mg/L

(C/Co) Normalized Peak Areas

Time (hrs)	Blank0.1mg/L	X = 0.2 (Lo)	X = 0.5 (Lo)	X = 1.0 (Lo)
0	1	1.330175	1.11313	1.011932
10	1	1.180124	0.941006	0.876481
17	1	1.124571	0.877281	0.778121
31.5	1	1.014697	0.845716	0.700688
41	1	1.023397	0.792895	0.527873
55	1	0.993814	0.67017	0.461831
64	1	0.98185	0.597907	0.412906
79.5	1	0.880184	0.516516	0.342297
105.5	1	0.784916	0.447737	0.271556
127	1	0.657781	0.379647	0.228583
136.5	1	0.714516	0.359657	0.249988
150.5	1	0.62602	0.344946	0.223146
161	1	0.550756	0.293397	0.212593
173	1	0.529333	0.226112	0.203331
186.5	1	0.584946	0.248745	0.211788

VITA 2

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Master of Science

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