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Protistan predation and TCE biodegradation in a fractured rock aquifer

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PROTISTAN PREDATION AND TCE BIODEGRADATION IN A

FRACTURED ROCK AQUIFER

BY

JOSEPH J CUNNINGHAM III

Bachelors of Science, University of New Hampshire, 2003

THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Master of Science

in

Civil Engineering

May 2008

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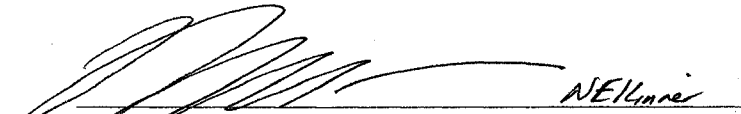
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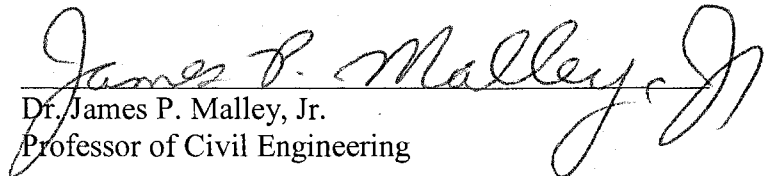
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ACKNOWLEDGEMENTS

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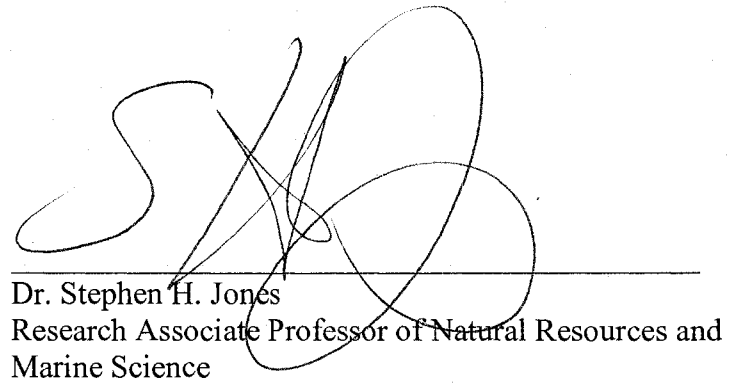
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ABSTRACT

PROTISTAN PREDATION AND TCE BIODEGRADATION

IN A BEDROCK AQUIFER

By

Joseph Cunningham III

University of New Hampshire, May 2008

Despite extensive research on the resources required to initiate dechlorination of trichloroethene (TCE), slow rates and stalling continue to be observed *in situ*. The majority of research on biodegradation of TCE has focused resource availability, while predation is poorly understood. Predation has the potential to significantly alter bacterial abundance, and can play an important role in selecting what species are present, and determine if the community is capable of mineralizing TCE. The impact of protistan predation on TCE biodegradation rates, and occurrence and length of stalls was measured in this experiment. When protists were inhibited, TCE was mineralized. Protistan predation appeared to impact the success of reductive dechlorination by selecting for bacterial morphology and community composition. There was a dual threshold effect: no predation limited the ability of the dechlorinators to become dominant, while too much predation resulted in a system where the dechlorinators were grazed to extremely low levels, inhibiting reductive dechlorination.

CHAPTER 1

INTRODUCTION

1.1 <u>Introduction</u>

After it was first produced in the early 1920's, the chlorinated compound

trichloroethene (TCE) (C_2HCl_3) became one of the most widely used industrial solvents

(Russell et al., 2002). As the use of TCE increased, so did the incidence of accidental and

intentional releases to the environment. TCE contamination in groundwater was first

discovered in 1977, and since then TCE has become one of the most frequently detected

subsurface contaminants (Russell et al., 2002). TCE is denser than water, and when

released into the ground migrates downwards until it reaches an impervious barrier. Due

in part to its relatively low aqueous solubility (1200 mg/L @ 25°C, 1 atm), TCE often

exists as a dense non-aqueous phase liquid (DNAPL), and slowly diffuses into the

surrounding groundwater until the DNAPL has been exhausted (US EPA, 2000; Norris,

1994).

TCE contamination in fractured bedrock is of particular concern due to the

difficulty associated with its cleanup. In cases where DNAPL has migrated into an

extensive fracture network, it may be impossible or economically infeasible to extract the

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free product using conventional methods (e.g., pump and treat). Even when the DNAPL is extracted, the residual dissolved and sorbed phases are difficult to treat, and often energy intensive methods (e.g., granular activated carbon (GAC) adsorption, volatilization) must be used to reduce the TCE to acceptable levels (US EPA, 2000; Bradley, 2003; Norris, 1994). These methods have the disadvantage of requiring the contaminated water to be pumped to the surface for treatment, simply transferring the contamination to another medium, rather than eliminating it.

An alternative treatment method used to reduce the dissolved and sorbed phases of TCE contamination is microbially-mediated reductive dechlorination. This process results in the sequential dechlorination of chlorinated compounds and, under the correct conditions, can fully mineralize TCE to CO_2 (Vogel and McCarty, 1985; Vogel et al., 1987; Bradley, 2003). Microbially-mediated reductive dechlorination of TCE can result from many anaerobic processes, however, the two most common are dehalogenation and co-metabolism. In dehalogenation, (i.e., direct anaerobic reductive dechlorination), the bacteria are able to directly consume the contaminant (e.g., TCE) as an electron acceptor and gain energy from the reaction (US EPA, 2000). In co-metabolism, (i.e., indirect anaerobic reductive dechlorination), the bacteria do not directly consume the contaminant as an electron acceptor, and thus do not gain energy from the reaction. The bacteria produce non-specific enzymes (co-factors) through the consumption of a primary substrate (i.e, a degradable organic carbon compound), which aid in the dechlorination of chlorinated compounds (US EPA, 2000).

The reductive pathway for the dechlorination of TCE is usually: Trichloroethene (TCE) →cis-Dichloroethene (cDCE) → Vinyl Chloride (VC) → Ethene (Figure 1.1). Under the correct conditions, TCE can be fully degraded to ethene, which can be easily mineralized to CO_2 by a variety of heterotrophic microorganisms.

TCE
$CHCl=CCl_2$
H_2 HCl

cis-DCE
$CHCl=CHCl$
H_2 HCl

VC
$CH_2=CHCl$
H_2 HCl

Ethene
$CH_2=CH_2$
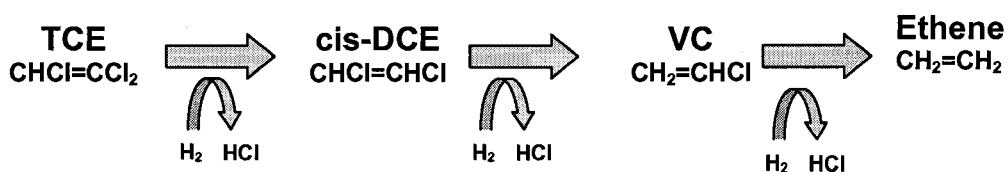
Figure 1.1: Microbial Reductive Pathway of TCE (Created from Bradley, 2003)

Three treatment strategies are typically available when biodegradation is chosen as a TCE remediation strategy; monitored natural attenuation (MNA), enhanced bioremediation, and bioaugmentation. MNA relies on natural conditions to promote biodegradation of TCE, and typically, there are no amendments to the subsurface. Enhanced bioremediation involves the addition of one or more chemical amendments to the groundwater to ensure that the bacteria have adequate resources available to degrade the chlorinated solvents. Bioaugmentation involves the injection of dechlorinating bacteria, often along with one or more chemical amendments. Bioaugmentation is frequently used when the indigenous bacterial community does not contain microbes that can fully mineralize TCE.

Complete mineralization to CO_2 using dehalorespiration typically requires specific electron donors (i.e., acetate, H_2), sulfate reducing or methanogenic conditions, adequate nutrients and mineral content, as well as the presence of bacteria capable of

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performing dehalorespiration (Bradley, 2003). *Dehalobacter restrictus,, Dehalospirillum multivorans, Desulfitobacterium strain PCE1, Desulfuromonas chloroethenica, Desulfuromonas michiganenis, and Dehalococcoides ethenogenes* are among the many species able to dechlorinate TCE by sequentially replacing the chlorine atom(s) with hydrogen (Lu and Kampbell, 2006). While many species are capable of degrading TCE to cDCE and VC, only members of one species, *Dehalococcoides ethenogenes*, appear capable of completing the last step in the reductive dechlorination pathway: VC to ethene (Lu and Kampbell, 2006; Duhamel and Edwards, 2006; Duhamel and Edwards, 2007). Therefore, if *Dehalococcoides ethenogenes* is not indigenous, degradation will likely stall at cDCE or VC unless the system is bioaugmented.

Stalling is a significant problem because it results in the presence of a compound that is more toxic (e.g., VC, cDCE) than the parent compound (TCE) (Lu and Kambpell, 2006; Euro Chlor, 1999; National Academy of Sciences, 2006). Although stalls can occur for many reasons, they are most often attributed to electron donor limitation or inadequate abundance of the appropriate dechlorinating species (Becker, 2006; Bradley, 2003). A stall due to electron donor deficiency can often be remedied through the addition of an electron donor (e.g., biodegradable organic carbon), which can be degraded by indigenous bacteria, creating the preferred electron donor, H_2.

A stall can also be caused by the lack of bacteria capable of performing microbially-mediated reductive dechlorination. In many TCE-contaminated systems, indigenous dechlorinators such as *Geobacter* or some sulfate reducers are present, and

capable of degrading TCE to cDCE or VC. In many of these systems, *Dehalococcoides ethenogenes* may be present in small numbers, however, their low abundance results in negligible degradation unless they can become predominant (Bradley, 2003; Becker, 2006; Duhamel and Edwards, 2007; Voytek, 2007; Naser, 2005).

There has been a growing consensus that in order for effective reductive dechlorination to occur, a consortium of bacteria must be present and undergo a community shift as the ratio of parent compound to progeny changes. Becker (2006) found that when put into competition with other common dechlorinating species under electron-limiting conditions, *Dehalococcoides ethenogenes* was competitively eliminated from the system, while the species able to use the electron acceptors (cDCE, VC) more effectively flourished. However, as the preferred electron equivalents were eliminated through consumption, the abundance of *Dehalococcoides ethenogenes* began to increase because there was less competition for the remaining electron equivalents. For example, *Dehalobacter restrictus* and *Dehalococcoides ethenogenes* are both capable of degrading TCE to cDCE, however, *Dehalobacter* are able to use TCE more efficiently. Therefore, when adequate TCE is available, *Dehalobacter restrictus.* will competitively exclude *Dehalococcoides ethenogenes*. *Dehalococcoides ethenogenes* is more efficient at degrading cDCE, and will therefore competitively exclude *Dehalobacter restrictus.* This suggests that indigenous *Dehalococcoides ethenogenes* will be competitively excluded unless the microbial community is capable of undergoing a community shift that reduces the intraspecific competition associated with a shift in the electron acceptor (chlorinated ethene) availability. This was corroborated by Duhamel and Edwards (2007), who

reported that *Geobacter spp.* and sulfate reducers are responsible for the transformation of TCE to VC, while *Dehalococcides ethenogenes* is responsible for the transformation from VC to ethene. As the electron acceptor (i.e., TCE) was transformed to a new compound, the dominant species was displaced by another that was able to use the newly formed compound most efficiently (i.e., electron equivalents basis). Although *Dehalococcoides ethenogenes* was present throughout the experiment, it did not become dominant until VC was the primary electron acceptor, suggesting that other species were energetically more efficient at reducing TCE to cDCE than *Dehalococcoides ethenogenes.*

The discovery that competition between microbial populations influences the ability of a system to completely and effectively undergo microbially-mediated reductive dechlorination raises some questions about the influence of other ecological processes. Basic ecological theory suggests that there are two fundamental forces in any system; a bottom-up force which represents resources, and a top-down force which represents predation (Foster et al., 2006; Muylaert, 2002; Psenner, 1992). The bulk of the biodegradation research on the subsurface has focused on the resource side (e.g., availability of and competition for nutrients and electron donor). It is possible that top-down forces from predation also play a significant role in the microbially-mediated reductive dechlorination process, and may influence the overall degradation rate and/or stalling.

The role of predation in fractured bedrock aquifers has only been examined by a handful of researchers, so little is known about the potential effects it has on microbially-mediated reductive dechlorination of TCE. Although an established link exists for a predator-prey relationship between protists-bacteria in sandy aquifers (Kinner et al., 1998) and surface water systems (Simek et al., 1997; Fenchel, 1987; Lawler, 1993), a literature review yielded no published instances where this connection was established in fractured-bedrock systems. Research at the University of New Hampshire's Bedrock Bioremediation Center (BBC) has shown that the ratio of bacteria to protists in fractured bedrock systems is sometimes similar to that of other systems where a predator-prey relationship exists. In addition, the nanoflagellates present are big enough to consume the unattached bacteria. Although the microbial abundances are often lower than in porous media, protists are likely the primary predator of bacteria in fractured bedrock aquifers.

Research in surface water aquatic systems has shown that protists play an important ecological role and influence nutrient and organic carbon cycling; bacterial biomass, composition and cell size; and overall community structure (Corno et al., 2006; Novarino et al., 1997). In systems where organic carbon (electron donor) is limiting (e.g., fractured bedrock aquifers), the presence of protists is important to ensure that the organic carbon is recycled and not stored in cell biomass. Flagellated protists tend to be "transect feeders", propelling themselves in a straight line and consuming any cells of the correct size they encounter (Novarino et al., 1997). Protists can: consume up to 74% of the unattached bacterial biomass daily; cause bacteria to undergo morphological changes to avoid predation; and selectively prey upon bacterial species in a specific size-class,

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resulting in a significant influence on community composition and cell size (Kinner et al., 1998).

In a fractured bedrock aquifer, the size and specific growth rate of the dechlorinatoring bacteria will likely be very important. If they are at a competitive disadvantage relative to other species in the community, they may become eliminated from the system (Becker, 2006). In order for a community shift to occur, the bacteria must have a specific growth rate greater than the grazing rate of protists, hence a slow-growing species (e.g., *Dehalococcoides ethenogenes)* may be at a competitive disadvantage, preventing it from reaching an abundance that allows for effective conversion of cDCE and VC to ethene.

Protists in fractured bedrock systems are likely to play a similar role as they do in surface water systems: regulating bacterial abundance and community structure, and facilitating organic carbon and nutrient recycling. These influences can also be manifested as a change in the contaminant biodegradation rate. If protistan predators are excluded from a TCE contaminated system (e.g., as a result of contaminant toxicity), the bacterial abundance and community composition may be different than a system in which they are present. If community composition and the ability to undergo compositional shifts are critical in order to achieve complete mineralization and avoid stalls (Duhamel and Edwards, 2007), then the presence of protistan predators should significantly impact the ability of a system to dechlorinate TCE.

1.2 <u>Objectives</u>

The goal of this study was to increase the TCE biodegradation rate and decrease the occurrence of cDCE and VC stalls through a better understanding of the predator-prey relationships that exist in TCE contaminated fractured bedrock systems. In order to understand the specific roles protists play in microbially-mediated reductive dechlorination, the following questions were posed:

1) How is the biodegradation rate of TCE influenced by the presence of protists under ambient and organic carbon amended systems?

2) Does the presence of protists influence the occurrence of stalls and their duration?

3) How does the presence of protists influence the abundance and composition of the bacterial community?

4) How does the presence of protists influence the TCE degradation rate, bacterial abundance and community structure in a bioaugmented system?

Two continuously-stirred 2 L reactors were constructed to mimic conditions at Site 32 at the former Pease Air Force Base in Portsmouth, NH. Site 32 is an historically-contaminated fractured metasandstone/metashale aquifer in which TCE and cDCE have migrated into underlying weathered and competent bedrock. Dechlorinators, including *Dehalococcoides ethenogenes* and sulfate reducers, have been identified at this site using fluorescence in-situ hybridization (FISH), and polymerase chain reaction (PCR)/denaturing gradient gel electrophoresis(DGGE) (Naser, 2005). Three experiments

were conducted to examine the TCE biodegradation rate and occurrence of stalls using ambient (1 – 10 mg TOC/L) and elevated (~120 mg TOC/L) organic carbon concentrations (electron donor = sodium lactate). The first and second experiments examined the TCE degradation rates in the absence and presence of protists, respectively. In the third experiment, the dechlorinating bacterial culture KB-1 (SiREM Labs; Guelph, ON) was added with protists present.

1.3 Thesis Organization

This thesis is organized by chapters and includes a manuscript to be submitted for publication in *Environmental Science and Technology*. The manuscript contains its own introduction, materials and methods, and results and discussion sections.

- Chapter 1- Introduction,

- Chapter 2 – Literature Review,

- Chapter 3- Materials and Methods,

- Chapter 4 – Manuscript to be submitted for publication in *Environmental Science and Technology,* and

- Chapter 5 – Conclusions and Recommendations.

CHAPTER 2

LITERATURE REVIEW

2.1 Trichloroethene

Tricholoroethene (TCE) is a chlorinated organic solvent that was first commercially produced in the early 1920s, primarily for agricultural purposes. By the late 1930s, the popularity of TCE had increased exponentially, with it becoming the chemical of choice for dry cleaning and metal degreasing operations (Doherty, 2000). TCE use peaked in 1970 as numerous pieces of legislation, including the Clean Air Act, took effect and regulated it as a volatile organic compound (VOC). TCE was placed on the US EPA's hazardous waste substance list in 1976 in part because the National Cancer Institute (NCI) reported it caused tumors in laboratory mice. It became a fully regulated hazardous waste in 1980. By 1989, a Safe Drinking Water Act (SDWA) maximum contaminant limit (MCL) of 5 µg/L was in effect, and the Occupational Safety and Health Administration (OSHA) had reduced the permissible exposure limit (PEL) in air to 25 ppm (Doherty, 2000). Despite the tight regulation, the mid 1990s saw an increase in TCE use as a replacement for solvents banned under the 1990 Clean Air Act amendment, including trichloroethane (TCA) and chlorofluorocarbon 113 (CFC-113).

TCE (C_2HCl_3) is a halogenated aliphatic compound that is heavier than water (ρ = 1.46 g/cm^3 @ 20°C, 1 atm), and has a low aqueous solubility (1200 mg/L @ 25°C, 1 atm) (Lide, 1997). Exposure to TCE can potentially lead to serious health effects, including cancer of the liver and kidneys, reproductive and developmental abnormalities, and neurotoxicity (National Academy of Sciences, 2006). The most common exposure pathway is ingestion or inhalation through direct contact (i.e., bathing, drinking) with contaminated water (Lu and Kampbell, 2006).

When released into the subsurface, TCE will sink, because of its density relative to water, until it reaches an impervious barrier, oftentimes easily passing through unconsolidated zones and only stopping at low permeability clay or solid, minimally-fractured rock. When TCE pools on top of an impervious layer, the dense non-aqueous phase liquid (DNAPL) will slowly undergo dissolution. This will produce a large contaminant plume in the groundwater that has the potential to exist long after the DNAPL source is removed (Parker et al., 1994).

Because of its widespread use and instances of accidental and intentional releases into the environment, TCE has become one of the most frequently observed groundwater pollutants in the U.S. (Russell et al., 2002). Contamination typically occurs in three forms: DNAPL, dissolved, and adsorbed phases. In most cases, it is advantageous to remove the DNAPL phase in order to eliminate the source and prevent additional

dissolution or sorption. This can be accomplished through groundwater extraction (*ex situ* treatment) or in some cases *in situ* treatment (Norris et al., 1994).

Ex situ treatment of the dissolved phase can be accomplished through either volatilization or activated carbon adsorption, however, this is typically costly and extraction may take several years depending on the mass to be removed. In addition, pumping primarily removes fluid from high yielding formations, not those with low permeability. The adsorbed phase, especially that associated with a rock matrix, is the most difficult to treat because it cannot be economically extracted, and will slowly diffuse back into the groundwater once the dissolved phase has been removed.

2.2 TCE and Fractured Bedrock

Remediation of TCE contamination in fractured bedrock is often extremely difficult. Some of the water-bearing fractures connect to other fractures, producing veins, while others are essentially "dead-ends". This results in a unique hydrology that is difficult to predict and makes treatment of TCE contamination using conventional methods difficult and expensive (Eighmy et al., 2006). Fractures are typically classified according to size: fractures with a width of < 1 mm and > 1 cm are classified as microfractures and macrofractures, respectively (Eighmy et al., 2006). The fracture size is important because it dictates the volume of fluid transported, thus influencing the potential to remove contaminants using *ex situ* methods, as well as to distribute amendments (e.g., organic carbon) during *in situ* bioremediation. If the DNAPL phase of

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TCE is in a microfracture, an extraction well will have to be precisely placed and pumped at a very slow rate in order to recover it, resulting in a very long and costly cleanup. The unique hydrology of fractured bedrock systems also makes it difficult to perform *in situ* treatment for the dissolved and adsorbed phases of TCE because it can be difficult to control the water flow and ensure that every fracture has been assessed.

2.3 Biodegradation of TCE

Until the early 1980s, TCE was considered recalcitrant and it was generally accepted that volatilization and adsorption were the only treatment methods available. However, Bouwer and McCarty (1983), Vogel and McCarty (1985) and Vogel *et al.* (1987) reported that TCE could be degraded to cDCE by dehalorespiration. In this process, the chlorine in the compound is replaced with a hydrogen by specialized bacteria that are able to use the chlorinated compound as an electron acceptor.

Within a few years, Freedman and Gossett (1989) reported that Tetrachloroethene (PCE) could be fully degraded to ethene, a nontoxic plant hormone, when a suitable electron donor was provided. Ethene is readily mineralized to CO_2 by numerous indigenous bacteria, resulting in the potential for TCE to be completely mineralized (Bradley, 2003) (Figure 2.1).

TCE   cis-DCE   VC   Ethene

$CHCl=CCl_2$ $CHCl=CHCl$ $CH_2=CHCl$ $CH_2=CH_2$

H_2 HCl H_2 HCl H_2 HCl
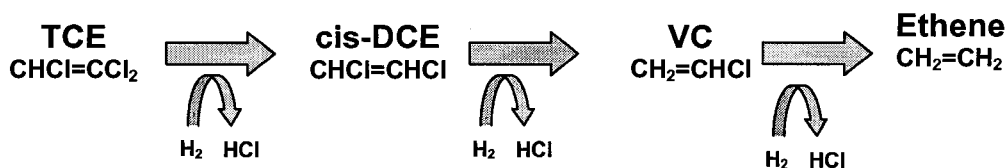
Figure 2.1: Reductive pathway of TCE using microbially-mediated dehalorespiration
(Created from Bradley, 2003)

Biodegradation of TCE occurs through the process of microbially-mediated

reductive dechlorination, during which chlorine is sequentially removed from the

molecule. Anaerobic microbially-mediated reductive dechlorination can result from

numerous processes, however, the two most common are co-metabolism and

dehalorespiration. Co-metabolism is a process by which a contaminant (e.g., chlorinated

compound) is gratuitously degraded by enzymes or co-factors produced by bacteria

during the metabolism of a primary substrate. The bacteria are unable to use the

contaminant as an electron donor or acceptor, and gain no energy from the dechlorination

(Chapelle, 2001; US EPA, 2000). In the case of chlorinated compounds, usually the

non-specific enzyme is often a mono-oxygenase, and may not fully degrade TCE to

ethene. In contrast, dehalorespiration, (e.g., direct anaerobic reductive dechlorination) is a

process where bacteria are able to use the contaminant in an energy-generating process

(i.e., in the case of a chlorinated compound as an electron acceptor) (Bradley, 2003; US

EPA, 2000). The chlorinated compound (e.g., TCE) can be fully mineralized to ethene

using this method. In practice, it is difficult to determine what process, co-metabolism or

dehalorespiration, is responsible for biodegradation at field sites, and oftentimes both

may be occurring simultaneously (US EPA, 2000).

The bacteria that are responsible for microbially-mediated dehalorespiration require an electron donor and a terminal electron acceptor (TEA) for energy generation. The energy generated is used to synthesize organic carbon to build and maintain cellular mass. In addition, they need macro and micronutrients and minerals to maintain cellular health. During energy generation, organic carbon or H_2 donates an electron, which is transferred through a series of redox reactions (Chapelle, 2001). Most bacteria use TEAs such as oxygen, nitrate, iron (III), manganese (IV), sulfate, and CO_2. It is the more specialized bacteria that are able to use xenobiotics, including chlorinated ethenes (e.g., PCE, TCE, cDCE, VC). For chlorinated compounds, energy generation results in production of hydrochloric acid production. PCE, TCE, cDCE and VC contain chlorine atoms, and thus can be used as electron acceptors, and theoretically allow dehalorespiration to sequentially degrade TCE to ethene.

The Gibbs free energy of the dechlorination via dehalorespiration decreases with the number of chlorine atoms. The more dechlorinated a molecule is, the less potential energy exists for the cell to capture and therefore, more chlorinated molecules (i.e., PCE, TCE) are preferentially dechlorinated because of the higher energy potential. Electron acceptors that result in microbial reactions with a higher Gibbs Free Energy must be consumed before reductive dechlorination can begin. As Table 2.1 illustrates, the microbial reactions associated with aerobic respiration, denitrification, and sulfate reduction all yield higher Gibbs Free Energy of reaction values than chlorinated ethene reduction. This suggests that under ideal conditions, the electron acceptors O_2, and NO_3^-,

and even SO_4^{-2} would need to be consumed before the chlorinated ethene reduction reaction would become energetically favorable and proceed.

Table 2.1. Microbial reactions and corresponding Gibbs free energy changes

(Adapted from Druschel, 2007 and He et al, 2002, Claypool and Kaplan, 1974)

Microbial Reactions	Electron Acceptor	Gibbs Free Energy, ΔG (kJ)*
Aerobic Respiration	O_2	-686
Denitrification	NO_3	-579
Sulfate Reduction	SO_4	-220
Chlorinated Ethene Reduction	TCE, cDCE, VC	-184
Carbonate Reduction	CO_2	-99
Fermentation (heterolactic)		-49
* At 25°C and pH 7.0, with 1 mM of organic substrates, 30 mM of HCO_3^-, 1000 ppmv of CH_4, 10 ppmv of H_2, 1 mM Cl^-, 5 ppmv ethene, and all chloroethenes at 5 mg/L.		

Significant research has gone into finding an electron donor that results in the most efficient transfer of electrons, thus yielding the most efficient dehalorespiration process. Carr and Hughes (1998), Gerritse et al. (1999), and Aulenta et al. (2005) compared numerous potential electron donors including H_2, methanol, formate, butyrate, acetate, and acetic acid. H_2 and methanol were found to be the most efficient donors, and produced the most biomass per μeq of electron acceptor in a controlled monoculture. However, due to competition between indigenous species and dehalorespiring species under *in situ* conditions, as well as environmental and safety concerns, pure H_2 and methanol are rarely used in field work. Instead, organic carbon, usually sodium lactate, is preferred. The organic carbon is degraded by indigenous bacteria into small quantities of

an electron donor, such as H_2, allowing the dehalorespiring bacteria to function with limited competition (Bradley, 2003).

The redox of a system is a good measurement of what electron acceptor is being consumed, and it is generally accepted that for the conversion of TCE→cDCE→VC, a minimum of sulfate-reducing conditions are required, while the conversion of VC to ethene is thought to be most effective under methanogenic conditions where the energetics are more favorable (Bradley and Chapelle, 1999). These findings illustrate the difficulty that can sometimes occur when trying to use reductive dechlorination in oxic or anoxic groundwater systems. In order to fully reduce TCE to ethene by reductive dechlorination, O_2, iron(III), manganese, and nitrate must be consumed, as well as a proportional amount of electron donor, in order to generate sulfate reducing conditions needed to convert TCE to VC by dehalorespiration. This can usually be accomplished through the addition of the electron donor, allowing indigenous bacteria to sequentially consume electron acceptors until the desired redox is reached.

Many species are able to degrade PCE and TCE to cis-DCE, while only *Dehalococcoides ethenogenes* has been shown to fully dechlorinate PCE to ethene (Grostern and Edwards, 2006; Holmes and He, 2006; Lu and Kampbell, 2006). This suggests that if *Dehalococcoides ethenogenes* is not present in the environment, biodegradation will stall at cis-DCE or VC.

There has been a growing consensus that in order for effective dehalorespiration

to occur, a consortium of bacteria must be present and undergo a community shift that

allows *Dehalococcoides ethenogenes* to become dominant. Becker (2006) reported that

competition between common indigenous bacterial species may significantly restrict the

dehalorespiration of TCE. She examined competition in three scenarios (Figure 2.2); two

dehalorespiring species (*Dehalococcoides ethenogenes and Dehalobacter restrictus*)

competing for H_2, a organotrophic dehalorespirer and dehalorespiring bacteria

(*Desulfitobacterium sp. Strain PCE1 and Dehalococcoides ethenogenes*) competing

indirectly for lactate and H_2, and a dehalorespirer that can only use acetate as a electron

donor and a dehalorespirer that can only use H_2 (*Desulfuromonas michiganensis* and

Dehalococcoides ethenogenes).
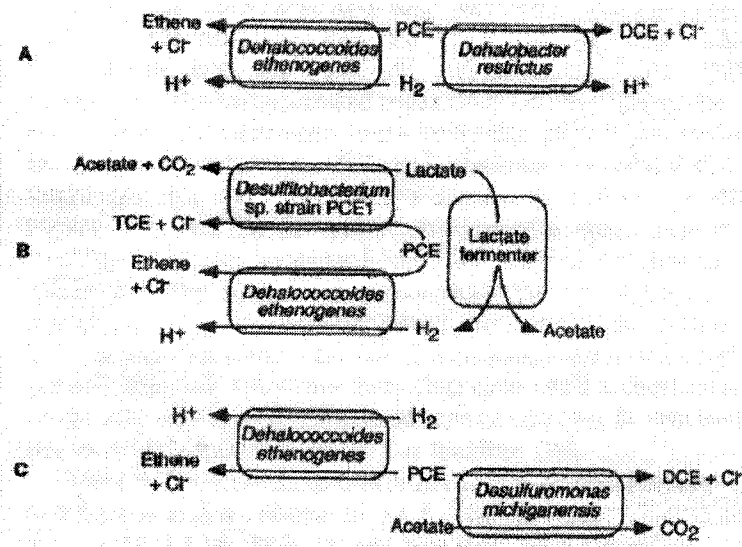
Figure 2.2: Conceptual models of competition between representative PCE-
respiring populations. Reprinted with permission from
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In the first scenario, Becker reported that under ambient conditions, *Dehalococcoides ethenogenes* is competitively excluded from the system in favor of the faster substrate (H_2) uptake kinetics of *Dehalobacter restrictus*. However, when the abundance of *Dehalococcoides ethenogenes* is increased to model a bioaugmentation scenario, it is able to become dominant despite its lower substrate uptake kinetics. In the second scenario, the faster uptake kinetics of *Desulfitobacterium sp. Strain PCE1* diverts the available lactate away from fermenters, and thus prevents the production of a suitable electron donor (i.e., H_2) for *Dehalococcoides ethenogenes*, which is eliminated from the system. However, in engineered situations where lactate is provided in excess, *Desulfitobacterium spp.* is not able to consume all of the available lactate and thus some is available for the fermenting species, which produces H_2. In the third scenario, *Desulfuromonas michiganensis* outcompetes *Dehalococcoides ethenogenes* for PCE and TCE in part because of its faster substrate uptake kinetics and its ability to use acetate as an electron acceptor, while *D. michiganesnis* cannot. Under augmented conditions, *Dehalococcoides ethenogenes* outcompetes *Desulfuromonas michiganensis* for TCE and PCE.

Becker's results show that competition resulting from different environmental conditions can play an important role in determining which species become dominant, and small changes in concentration of electron donor, or bacterial abundance, have the ability to influence community composition. Under natural conditions, *Dehalococcoides ethenogenes* is unlikely to become dominant if species that can compete for limited electron equivalents are present. In some cases it can coexist with other dehalorespirers

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and act as a cDCE to ethene specialist. Overall, the presence of multiple dehalorespiring

bacterial species (Table 2.2) is ideal because it allows for the most efficient chlorinated

ethene degradation (Becker, 2006).

Table 2.2: Bacteria that can reductively dechlorinate chlorinated ethenes
(Adapted from Lu and Kampbell, 2006)

Species	Dechlorination Steps Performed
Dehalobacter restrictus	PCE to *cis*-DCE
Dehalospirillum multivorans	PCE to *cis*-DCE
Desulitobacterium strain PCE1	PCE to TCE
Desulfuromonas chloroethenica	PCE to *cis*-DCE
Strain MS-1	PCE to *cis*-DCE
Strain TEA	PCE to *cis*-DCE
Desulitobacterium sp. Strain PCE-S	PCE to *cis*-DCE
Dehalococcoides ethenogenes ethenogenes strain 195	PCE to ethene
Desulitobacterium frappieri TCE1	PCE to *cis*-DCE
Desulitobacterium sp. Strain Y51	PCE to *cis*-DCE
Desulitobacterium metallireducens	PCE to *cis*-DCE
Desulfuromonas michiganenis	PCE to *cis*-DCE
Dehalococcoides ethenogenes sp. Strain BAV1	*cis*-DCE to ethene

Although competition between bacterial species can occur for any required

resource (e.g., nutrients, electron donor/acceptor, organic carbon, minerals, habitat), it is

likely to be the most severe and have the largest influence on community composition

and abundance in situations where the electron donor or acceptor is limiting.

2.4 Role of Predation

Basic theories of ecology suggest that there are two fundamental forces present in any natural system; a top-down force which represents predation, and a bottom-up force which represents resource availability (Power, 1992). The top-down force can be anything that causes the direct removal of individuals from a system, however, it is most commonly due to predation. The bottom-up force is much broader, and can include nutrients, electron acceptor or donor, substrate, and habitat availability (Billen et al., 1990; Foster et al., 2006). In a balanced system, these forces will oppose each other and reach equilibrium where the community composition and abundance is regulated by resource availability and predation (Billen et al., 1990; Foster et al., 2006).

When the balance is disrupted, the community composition and abundance is often severely altered and a new equilibrium is reached, resulting in a different distribution of organisms within the community. The classic example of this is collapse of the sea kelp community on the California coast in the late 20th century (Foster, 2006). For several years, the kelp forests had been disappearing at an alarming rate without an obvious cause. The sea urchins, the primary predator of sea kelp, had exponentially increased, exerting a dominating top-down force (Konar, 2000). The sea urchin thrived because its primary predator, the California sea otter (*E. lutris kenyoni*) was on the verge of extinction after decades of heavy hunting for their pelts (Foster, 2006).

In balanced systems, predation plays several vital roles including: carbon and nutrient recycling, limitation of resource consumption, and control of prey abundance and community composition. In closed systems (i.e., those without significant inputs) predation is critical to ensure that organic carbon and nutrients are recycled, alleviating resource limitation. Predation can influence prey abundance through direct consumption. In fact, community composition can be influenced through selective predation, producing a balanced ecosystem (Corno and Jurgens, 2006). Many species of bacteria have evolved mechanisms to limit predation's effects including: (1) higher specific growth rates (i.e., bacterial production > grazing), and (2) and morphological shifts that produce cells that exceed the predator's ideal prey size range (Jurgens et al., 1999; Pfandl et al., 2004; Shikano et al., 1990).

2.5 Predation in Fractured Bedrock Systems

Two of the most important categories of species in aquatic systems are bacteria and protists. Bacteria are critical in the trophic structure of aquatic systems and form the base of the food chain, ensuring that energy and biomass are created that can be transferred into higher levels of the food web. Protists, a broad category that includes many different types of single-celled eukaryotic organisms, are important not only because they are the primary predators on bacteria, but because they are prey for many planktonic species. Without bacteria and protists, the trophic structure of many systems would be vastly different (Curds, 1992).

The role of predation in fractured bedrock aquifers has only been examined by a handful of researchers. An established link exists for a predator-prey relationship between protists and bacteria in sandy aquifers (Kinner et. al., 1998. Kinner et al., 2002) and surface-water systems (Muylaert et al., 2002). Research at the University of New Hampshire Bedrock Bioremediation Center Site 32 has shown that the ratio of bacteria to protists in fractured bedrock systems is similar to that of other systems where a predator-prey relationship between the two has been established. Although the actual abundances are often lower in fractured bedrock than in freshwater and terrestrial systems, it is reasonable to hypothesize that protists are the primary predator of bacteria in the non-porous media, as no other true predators exist in these systems. Hence, there is no top-down force on the protistan predators.

Indeed, Kinner et al. (1998) examined protist predation in an organically contaminated sandy groundwater aquifer and concluded that protists preyed on the bacterial size class with the highest dividing frequency (0.8 μm to 1.5 μm length). This gives the predator a significant influence on the productivity and biodegradation rate of these bacteria and has the potential to influence the potential *in situ* degradation of organic contaminants.

Due to pore size limitations inherent in fractured bedrock aquifers, it is likely protists are the sole predators, and not subject to significant top-down predation pressure by zooplankton as they would be in a surface water system (Eighmy et al., 2006, Kinner et al., 1998, Billen and Servais, 1990). This likely creates a system in which protistan abundance is not controlled by predation, but rather by the availability of prey.

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Protists can exhibit top-down control on bacterial abundance in nutrient rich surface water systems, and play a key role in nutrient recycling in nutrient poor systems (Sharon, 1993). In experiments conducted by Kinner et al., (1998), protists consumed between 12 – 74% of the unattached bacterial population daily. This study also showed that the protists selectively prey on unattached bacteria and have the greatest impact on the most productive size of bacteria. These protists are capable of exerting a strong top-down force on bacteria, selecting for those that maintain a high growth rate. Flagellated protists in groundwater systems tend to be "straight-line" feeders, propelling themselves in a straight line and any bacteria of the correct size and type they encounter will be consumed (Novarino et al.; Sherr and Sherr, 1993). This results in the most productive bacteria in the system becoming the most preyed upon because there is a higher probability of encountering them. Because the protists do not actively "hunt" for one particular species, numerous species of bacteria can coexist in the same system because the probability of being grazed to extinction is reduced with increasing diversity (Jurgens et al., 1998).

2.6 Bacterial Response to Predation

In order to reduce the effects of predation, many species have evolved avoidance traits, including high growth rates and the ability to undergo a morphological change to an inedible form (Shikano et al., 1990; Simek et al., 1997). High microbial growth rates allow for a certain portion of the population to be grazed by predators without causing them substantial negative effects overall. Chrzanowski et al. (1990) reported a positive

correlation between increasing protistan predation rate and increasing specific bacterial growth rate. Hence, protistan predation helps maintain them in a log growth phase. Others have used this same phenomenon to argue that predation increased growth rates because fewer bacteria mean that there are more nutrients and organic carbon available per individual, resulting in higher growth rates (Kinner et al., 1998).

Morphological shifts in some bacterial populations have been suggested as an alternate method to avoid protistan predation. A shift from a coccoidal to thread-like (elongated) morphology was observed in experiments conducted by Simek et al. (1997). Under heavy predation, some variants of Proteobacteria were able to shift to an elongated form (> 3 μm long) within two days. Inspection of food vacuoles in the bacterivorous protists suggested that the upper limit of digestible particles was ~ 3 μm, suggesting that the elongation was selected through predation. Three days after protists were introduced to the system, ~ 60 to 75% of the bacteria were elongated. Simek et al. (1997) reported a significant reduction in the growth rate of the elongated bacteria. This is not surprising as more energy is needed for cell maintenance and hence less is available for reproduction. Because this study was conducted under laboratory conditions with the bacterial and protistan species carefully controlled, it is possible that under *in situ* conditions the net benefit to the bacteria would be reduced if larger protists were present that could consume the enlarged bacteria.

Research by Shikano et al. (1990) examined morphological and metabolic changes in bacteria that occur in the presence of protists and also observed a

morphological shift. They concluded that, although the elongation of the bacteria resulted

in a reduction in the number of bacterial cells grazed, it also corresponded with a

reduction in the metabolic and growth rate of the bacteria. It is unclear why a reduction in

metabolic rate was observed, as an elongation of the cell will result in a greater surface

area to volume ratio, theoretically increasing the amount of material transferred across

the cell membrane. This suggests that the cell may be less efficient in the elongated form,

and in order to maintain the elongated form to avoid predation, it may need to sacrifice

growth or metabolic rate.

If a predator is removed from a bacterial system at equilibrium, the overall effect

on the community varies with the genetic diversity of the system (Shikano and

Luckinbill, 1990). In systems with low genetic diversity (e.g., groundwater), the removal

of one bacterial species is likely to cause a dramatic shift in the community and the

specific roles of all. Under equilibrium conditions, the bacteria and protists have evolved

into a community where niches are filled and a balance between predation and bacterial

growth has been reached. When the predator (i.e., protists) is removed, there is no longer

a balance. Bacterial species once competitive because of the mechanisms they had

evolved to survive predation may no longer be competitive and can diminish in

significance as other species become dominant (Shikano and Luckinbill, 1990; Simek and

Vrba, 1997).

2.7 Bacterial Community Composition and Biodegradation

Flynn et al. (2000) studied enrichment cultures from three different river sediments capable of complete PCE mineralization, and reported that each site had different dechlorinating bacterial communities. Communities that specialized in cis-DCE and VC dechlorination were different than those responsible for PCE dechlorination. This suggests that at least two distinct bacterial communities are responsible for the degradation of PCE to ethene, with a community shift occurring as the system transitions from cDCE to VC. Flynn et al. (2000) also reported significantly different dechlorination rates in the cultures, suggesting that despite the presence of similar resources, the composition of the bacterial community influences degradation.

Duhamel and Edwards (2007) conducted a microcosm experiment to examine the population dynamics in a bioaugmented system and confirmed that a bacterial consortium was responsible for affecting complete mineralization of TCE. Their findings suggest that while *Dehalococcoides ethenogenes* alone is theoretically capable of degrading TCE to ethene, a mixed bacterial community is able to accomplish the mineralization more efficiently than a monoculture. For example, under ideal conditions, *Geobacter spp.* is capable of degrading TCE to cDCE more efficiently than *Dehalococcccoides etheneogenes*. However, *Geobacter spp.* dominated systems will stall because this species is not physically capable of degrading cDCE. *Sporomusa spp.*, which comprise 17% of the population in the Duhamel and Edwards study, are inhibited by cDCE and VC. This species ferments methanol to acetate and H_2, which is the preferred electron

donor for *Dehalococcoides ethenogenes.* Hence, the addition of organic carbon may have limited usefulness if it cannot be fermented to H_2 by *Sporomusa spp.* due to the presence of cDCE and VC. This may also be a partial explanation for the prevalence of stalls at cDCE and VC.

These findings suggest that shifts in the bacterial community from *Geobacter spp.* to *Dehalococcoides ethenogenes* must occur, as well as the presence of adequate amounts of bacteria capable of converting organic carbon to H_2 (e.g., the fermenter *Sporomusa sp.*). The role predation has on the population dynamics is not clear. Protists could preferentially prey upon some of the dominant bacterial species, changing the abundance and community structure of the system and hence interfere or enhance the balance of TCE degradation reactions.

2.8 Effect of Predation on Biodegradation

Predation can hinder or promote biodegradation of xenobiotics. Predation can promote nutrient and organic carbon recycling (Wright, 1988), thereby promoting bioremediation by reducing any resource limitations the bacteria are experiencing (i.e., the "exponential" growth effect). Conversely, predation may inhibit bioremediation by reducing the abundance of bacteria, especially if they selectively ingest species that play a crucial role in contaminant degradation (e.g., *Geobacter spp., Sporomusa sp., Dehalococcoides ethenogenes*).

Modeling by Travis and Rosenberg (1997) suggested that protistan predation will decrease the TCE degradation rate in a Savannah River aquifer by as much as 25%. In a separate system, the aerobic biodegradation of benzene, toluene, xylenes and ethylbenzene was significantly higher in the absence of protists. Although two different processes were examined, anaerobic microbially-mediated reductive dechlorination and aerobic co-metabolic degradation, their models suggest that predation may be very process and site specific, and sensitive to the initial microbial community composition and abundance.

A significant amount of work has gone into identifying what bacterial species are capable of using reductive dechlorination to degrade TCE, as well as various ways to introduce or promote growth of these species in contaminated systems (Bradley, 2003; Grostern and Edwards, 2006). However, predation's effect(s) on the efficiency, occurrence, and length of stalls remains unknown. Most *in situ* bioremediation methods require an amendment, which will disrupt the equilibrium conditions that have been reached in the microbial community. This disruption could result in negative or positive effects in terms of bioremediation (Travis and Rosenberg, 1997), and is highly dependant on the initial environmental conditions (Bradley, 2003), microbial composition and protistan predation.

2.9 TCE Predation Pilot Study

In 2005, Maureen Lewis, an undergraduate researcher at the University of New Hampshire, conducted a microcosm experiment to examine TCE degradation in a simulated fractured bedrock aquifer. The microcosm was designed to mimic conditions present at Site 32 of the BBC field laboratory. Ambient (1 – 10 mg TOC/L) and organic carbon amended (120 mg TOC/L from sodium lactate) conditions with protists present were simulated. Chlorinated ethene, TOC, and protistan and bacterial abundances were analyzed every three to seven days. No significant changes in TCE or cDCE concentration were observed, suggesting that biodegradation was not occurring. Lewis concluded daily sampling was required to resolve changes in bacterial and protistan abundances. In addition, the experimental design needed to include conditions where protists were removed to examine how a predator-free bacterial community would behave. My thesis research was a continuation of Lewis' research.

2.10 Conclusion

The literature clearly shows that the top-down force of predation has a significant potential to influence bacterial composition and abundance, and therefore, biodegradation, through selective grazing. A limited number of bacterial species acting in concert are able to perform reductive dechlorination of chlorinated solvents. Hence, selective protistan predation on the bacterial community has the potential to influence the success of *in situ* bioremediation in fractured rock aquifers. My thesis research used

Lewis' reactors, designed to mimic a fractured bedrock aquifer, to determine whether protists exhibit a predator-prey relationship with bacteria, and how that predation affects reductive dechlorination and mineralization of TCE.

CHAPTER 3

MATERIALS AND METHODS

Two draw and fill, continuously-stirred, 2 L reactors were constructed to mimic conditions at the BBC field site. Site 32 is an historically-contaminated fractured metasandstone/metashale aquifer containing between 10 – 250 µg/L TCE, 5 – 200 µg/L cDCE, < 14 µg/L of VC, and TOC from 1 to 10 mg C/L. Each reactor was of sufficient size so that ~100 mL of liquid could be removed daily for analysis without exchanging > 10% of the total volume.

A pilot study conducted in 2005 by Lewis used similar reactors. Her research suggested that over ~7 weeks, the presence of protists inhibited TCE degradation, and the concentration of TCE and cDCE, as well as the abundances of protists and bacteria, were variable. Sampling during the pilot study occurred every 3-7 days, and was too infrequent to capture any predator-prey cycling that was occurring. The methods and reactors in my thesis were similar to hers, however, sampling occurred daily. The experiments were also allowed to run much longer, ~ 14 weeks, in order to resolve long acclimation periods and slow degradation rates. In addition, the impact of using a specialized dechlorinating microbial culture (KB-1; Sirem Labs; Ontario, Canada) was assessed.

The study consisted of three experiments designed to examine the impact protists have on fractured bedrock systems under *in situ* (unamended), organic carbon amended, and KB-1 amended conditions. In the first experiment, protists were excluded from the reactors and the TCE degradation rate was determined under ambient organic carbon concentrations and amended organic carbon conditions (~120 mg/L). In the second experiment, protists were allowed to inhabit the reactors, and the TCE degradation rate was determined under unamended and amended organic carbon conditions. Due to a leak in one of the reactors, as well inability to obtain groundwater, this experiment was repeated (N.B., identified as Runs 1 and 2, respectively).

The third experiment was designed to examine the TCE degradation rate in a bioaugmented system in the presence of protists. This was accomplished by adding KB-1. The rate was examined under *in situ* conditions, unamended and amended organic carbon conditions, with protists initially present, but later eliminated.

3.1 Reactor Construction

Two reactors were constructed using 2 L silica glass commercially-available canning jars with tin sealable lids and screw caps (Ball Canning Corporation; Broomfield, CO) The lids were modified by drilling four pairs of 0.2 cm holes into them, and placing a sheet of teflon on the bottom side and silicone rubber on the top side cut to the same size as the lid (Figure 3.1, 3.2). The silicone rubber in contact with the tin lid provided a water-tight seal and prevented water from escaping the reactor. The teflon seal

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prevented the chlorinated ethenes in the water from sorbing to the silicone rubber. A strand of nylon fishing line (Stren Original; Clear 20lb test weight; Spirit Lake, IA), cut to approximately 60 cm, passed through the teflon seal, the lid, the silicone rubber seal, and the second drilled hole and was knotted off, leaving approximately 55 cm of fishing line inside the reactor. This process was repeated for the remaining three pairs of holes so that four lengths of fishing line were attached. These were used to suspend bedrock in the reactor (Figure 3.1a, c, 3.2d, f).

Two 1.3 cm holes were drilled through the silicone rubber/metal/teflon, and a stainless-steel 1 cm compression x 1.3 cm male pipe thread (MPT) fitting was threaded into the lid. A 1 cm teflon silicone septum was inserted into the compression side of the fitting to form a sampling port. This process was repeated for the second 1.3 cm hole. The installation of two sampling ports allowed for simultaneous effluent sampling and refill water injection and prevented the formation of headspace within the reactor.

Rock obtained from a local outcropping in Portsmouth, NH with similar geologic properties to the BBC Site 32 bedrock (fractured metasandstone and metashale of the Silurian or Ordovician Kittery formation) was split into fragments between 2 and 6 cm long, 2 to 5 cm wide, and 2 to 5 cm thick using a hammer. One hole was drilled into each fragment using a 0.2 cm masonry bit. The rock was cleaned (scrubbing with a brush in a stream of laboratory reverse osmosis (RO) water), autoclaved and sorted according to size.

Figure 3.1: (a) Rock line construction, (b) detail view of rock attachment to fishing line, and (c) rock line passed attached to metal lid
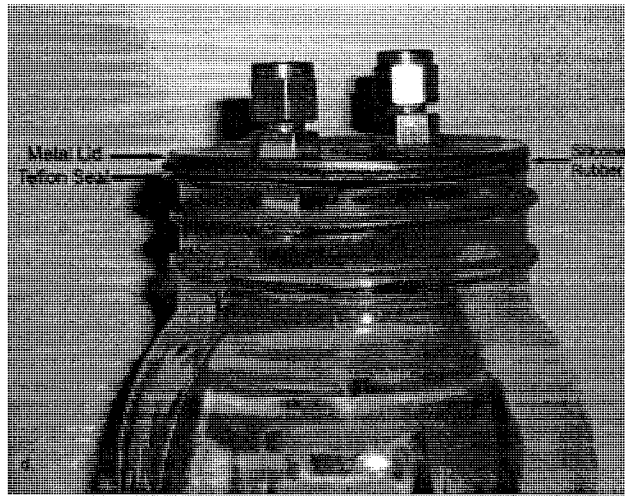
Figure 3.1: (d) Detail view of teflon seal, silicone rubber seal, and metal lid, (e) completed lid, and (f) completed reactor

A total of ~ 500 g of fragments were attached to the fishing lines in the reactors by threading the line through each rock fragment and knotting it between each one (Figure 3.1b). The rocks were suspended so that there was a 2.5 cm clearance from the bottom of the reactor. Efforts were made to ensure that all four lines contained an equal mass of rock (~ 125 g). 500 g of rock was chosen because that represented a compromise between a similar surface area to volume ratio to fractures at Site 32 and the volume of the reactors.

Once the rock had been suspended from the lid, each reactor was assembled by carefully placing the rock lines inside (Figure 3.2f). A 1.9 cm magnetic stir bar was also inserted into the reactor. The stir bar was used to slowly mix the reactors' contents. The liquid volume of each reactor was measured by filling it with RO water and allowing it to sit at room temperature for 24 h. This allowed water to penetrate the pore space in the rock. The reactors were sealed with a screw cap and topped off with additional RO water using the sampling ports to ensure no headspace existed. The lid was removed, and the water was drained into multiple 1 L graduated cylinders. This was repeated two times, and the mean volume of liquid recovered was considered the wet volume of each reactor. This volume was used to calculate the TCE spikes required to yield a given concentration and to ensure the rock volumes in the duplicate reactors were similar. Small fragments of rock were added to the reactors until the liquid volumes were similar. 1.52 L ± 5% of water were in the reactors. The empty, unsealed reactors and rocks were then autoclaved at 120° C for 45 minutes at 15 psi in order to ensure sterility.

3.2 **Reactor Startup**

Sample water was obtained from Well #6 (BBC6) at Site 32 using a multi-packer system that isolated a fracture at a depth of ~ 36 m bgs. The water contained: 10 – 250 μg/L TCE, 5 – 200 μg/L cDCE, < 14 μg/L VC, as well as indigenous populations of bacteria and nanoflagellates that are assumed to be acclimated to the presence of chlorinated ethenes. Numerous bacterial species have been identified, including sulfate reducers and *Dehalococcoides ethenogenes* in water collected at BBC6 (Naser, 2005). Sample water was extracted from the packed interval (~ 20 – 40 m bgs) using a stainless-steel Redi-Flow sampling pump (Grundfos USA; Olathe, KS). Approximately one well volume (210 L) was purged (~ 4 L/min flowrate) prior to sampling to ensure the sample water collected was recovered from the fracture (not the borehole). Purge water was disposed at the Pease Wastewater Treatment Facility (Site 8) during the first two experiments, however, due to reduced activity and resources at the site during the third experiment, the water was discharged at the surface and allowed to infiltrate the soil away from the well. The Grundfos sampler was decontaminated prior to installation in the well by pumping an Alconox/water (1 cup powdered Alconox to ~50 L of water)(Alconox; White Plains, NY) solution through the system for approximately 5 min, then rinsing with clean water (obtained from off-site bedrock well) for 15 min. The Grundfos sampling pump was left in BBC6 for the duration of the study, but purged before each use.

For the initial reactor startup, two 4 L amber glass containers with teflon caps were filled with groundwater to zero headspace and transported to a nitrogen-filled glovebox. During the first two experiments, the glovebox was located at Site 32 in a portable laboratory setup inside a trailer. For the third experiment, a glovebox was used in an environmentally-controlled room at UNH. During the first two experiments when the glovebox was at Site 32, no ice was required to transport the water as the total time to processing < 5 min. During the third experiment, the 4 L amber glass containers with water were placed in a cooler with ice-packs and transported to UNH; typical travel time was < 45 min. During the first two experiments, the glovebox was flushed with nitrogen daily prior to sampling. This was accomplished by using compressed ultra-high purity (UHP) nitrogen gas to apply a pressure of approximately 10 psi at the inflow valve, and opening an outflow valve on the glovebox. The glovebox was allowed to flush for 10 min. In the final experiment, the nitrogen was left on to allow a continuous flow into the glovebox because of the significantly smaller glovebox volume and liquid nitrogen was used as a nitrogen source.

3.3 Removal of Protists

In order to remove the protists, the groundwater was filtered through a 0.8 μm cellulose nitrate membrane filter (Cat #7184-002; Whatman International; Middlesex UK). Filtration removed the protists without significantly affecting the bacterial abundance. [N.B., Optimal pore size was determined prior to this experiment when the

groundwater was filtered using 0.8, 1.0, 1.2 and 3.0 μm filters and the protists and

bacteria present were analyzed using fluorescence microscopy. 0.8 μm filtration removed

the protists, but did not significantly impact bacterial abundance]. Protist-free water was

made by placing a 0.8 μm filter into a filter syringe tip and attaching it to a 60 mL sterile

syringe with the plunger removed. Groundwater water was slowly poured into the

syringe, and the plunger was replaced. Moderate pressure was then applied to the

plunger; during the initial reactor fill, the filtered groundwater was injected directly into

the sterile reactors. When refill water was being filtered, the filtered groundwater was

injected into sterile 250 mL amber-glass bottles with teflon caps. All filtration occurred in

the glovebox to prevent the aeration of the groundwater. During the second and third

experiments (protists present, KB-1 amended), groundwater was injected directly into the

reactors without filtration.

Once the reactors were filled, ~ 0.62 mL of a 1200 mg/L TCE solution was

injected to achieve a concentration of 540 μg/L (4 μM). The 1200 mg/L TCE solution

was created by injecting 5 mL of laboratory grade TCE (Cat #T341-500; Fisher

Scientific; Hampton, NH) into 55 mL of RO water in a 60 mL amber glass vial. The TCE

dissolved into the water from the DNAPL-phase to create the 1200 mg/L stock solution.

The reactors were placed on magnetic stir plates set at approximately 100 rpm,

allowing adequate mixing without causing excessive turbulence. The reactors were

maintained at $10 \pm 2\ ^\circ C$ to mimic groundwater conditions. In the first two experiments,

the reactors were kept inside 0.8 m^3 refrigerators (i.e., "dorm" sized) which were placed

inside of the glovebox. For the third experiment, the glovebox, in which the reactors were operating, was kept inside an environmentally-controlled room in a UNH laboratory.

Bacterial and protistan abundances were monitored by sampling every 72 hrs during the acclimation period (i.e., the first 15 days). Data from the pilot study showed that after approximately 10 days, the variability in microbial abundance was reduced. Sampling was then switched to every 24 hrs. TOC, chlorinated ethene concentrations, and bacterial and protistan abundances were monitored for ≤ 30 days to evaluate population interactions and degradation under *in situ* conditions with no TOC added. The TOC was then increased by injecting approximately 3 mL of a 19,200 mg/L sodium lactate solution (Cat #NC9262715; Fisher Scientific) into the reactors to yield a final concentration of 120 mg TOC/L. Sodium lactate was used as a carbon source because it is readily degraded by bacteria, and is commonly used in the field in enhanced bioremediation treatments. This concentration was significantly higher than what occurs in most natural groundwater systems. The high concentration insured organic carbon was not limiting to the bacteria. In the third experiment, 3 mL of the commercially-available bacteria culture KB-1 was injected into each reactor along with sodium lactate amendment.

3.4 Refill Water

In order to ensure that the TCE and TOC concentrations in the reactors were not significantly altered by sampling, the refill water contained ~ 540 µg/L (4 µM) of TCE

and ~ 120 mg TOC/L during the amended phases. To accomplish this, groundwater from BBC 6 was collected in 250 mL amber glass bottles with zero headspace using the same procedure used to collect the initial water. The 250 mL amber glass vials were opened in the glovebox under nitrogen and spiked with 0.1 mL of the 1200 mg/L TCE solution. During the later stages of the experiment when TOC was injected into the reactors, 1 mL of the sodium lactate sodium lactate was also injected into the refill water to maintain a TOC concentration of 120 mg TOC/L. A 3.2 mm teflon stir bar was placed into each vial, which was located on a stir plate set to ~ 300 rpm. The reactor contents were allowed to mix for 5 min.

3.5 Sampling/Analysis

Two 25 mL gastight glass/teflon syringes (Model #1025; Hamilton Co; Reno, NV) were equipped with 4 cm long, 18 gauge septum-piercing needles; one syringe was used to add refill water, while the other removed sample from the reactors. The plunger for the effluent syringe was fully depressed and then placed in the sampling port, while the plunger for the influent syringe was removed. Approximately 25 mL of refill water was poured into the influent syringe, and the plunger replaced. As water was withdrawn from the reactor with the effluent syringe, the water was injected using the influent syringe, ensuring a balance, so that minimal headspace formed. This was repeated 3 – 4 times per reactor so that each day approximately 100 mL of water was removed as sample and an equal amount was injected as refill water. For the third experiment, this method was modified to prevent short-circuiting. ~100 mL of sample was removed by

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syringe, and then refill water was injected using the procedure above. Although this allowed headspace to form, the short period over which it existed caused negligible volatilization or aeration (because the glovebox was flushed with nitrogen).

3.6 TOC Sampling

TOC samples were collected in 20 mL amber glass VOA vials with teflon/silicone septum caps. The vials were muffled using a Lindberg/Blue Box Furnace, (Model #BF51828C-1; Riverside ,MI), (5 min 0-100°C, 60 min at 100°C, 45 min from 100°C to 550°C, and 90 min at 550°C). The vials were then spiked with 2 mL of 1 N H_2SO_4 as a TOC preservative. 20 mL of sample water was injected into each VOA vial, taking care to prevent contamination by avoiding contact between the needle and acidified water. The vial was then sealed and the water analyzed with a TOC V CSH Total Organic Carbon Analyzer (Shimadzu; Kyoto, Japan) using the non-purgeable organic carbon (NPOC) method. Samples were held $\leq$ 60 days at 10 $\pm$2 °C prior to analysis. Calibration curves were created approximately every 3 months using 1, 10, 25, 50, 100, 200, and 500 C/L Potassium Hydrogen Phthalate (KHP) standards (Cat # AC41795-5000, Fisher Scientific) made with RO water. Calibration standards and RO water blanks were analyzed after every tenth sample for quality control (QC).

3.7 Chlorinated Ethenes

Chlorinated ethene samples were collected in 20 mL amber glass VOA vials with teflon/silicone septum caps. They were filled by inserting the needle of the syringe to the bottom of the vial and injecting sample until there was no headspace. The vial was sealed, checked for bubbles, and then sent to Resource Laboratories, Inc. (RLI; Portsmouth, NH). The samples were analyzed for chlorinated ethenes using a gas chromatograph equipped with an ECD (Electron Capture Detector) in accordance with USEPA SW-846, Method 5030B/8260B, within 14 days of sampling.

3.8 Microbial Abundance

The bacterial and protistan samples were collected using a 60 mL sterile centrifuge tube (Part #14-375-150; Fisher Scientific). Approximately 50 mL of sample from each reactor were injected into a centrifuge tube, and after redox (Eh) and pH measurements were taken, 3 mL of 37% 0.2 μm filter-sterilized formalin (Cat #F79-1; Fisher Scientific) was injected as a preservative. Preserved samples were stored ≤ 21 days at $10 \pm 2°C$.

The bacteria were enumerated using epifluorescence microscopy. 5 mL of sample were passed through a 0.2 μm x 25 mm sterile membrane filter (Cat #110656; Whatman International) under ~ 5 mm Hg of vacuum and then stained with a 0.01% acridine orange solution (Part #212536; BD Chemical, Sparks, MD), as described in Kinner et al.

(1998), using a Millipore 12-port filtration manifold (Model #1125; Millipore; Billerica, MA). The stained slides were examined using a 60x or 100x oil immersion lens and 10x oculars on a Nikon Optiphot-2 fluorescence microscope equipped with a B2H filter cube and an external high-pressure mercury vapor lamp. The bacteria were enumerated by randomly selecting seven fields and counting the number fluorescing green or orange within a Whipple grid (Whipple grid dimensions at 1000x magnification = 4.5μm x 4.5μm). The seven microscope field counts were arithmetically averaged and adjusted to a 1 L volume. Seven fields was chosen as a balance between adequate representation of the slide and time required to perform the field counts. The total number of bacteria counted ranged from 75 – 300 cells per field.

The protists were also enumerated using fluorescence microscopy; ≤ 35 mL of sample was passed through a 0.8 um sterile filter (Catalog #E08BP02500; Osmonics Inc, Trevrose, PA) and stained with primulin (Direct Yellow 59; Color Index 49000; Aldrich Chemical; St. Louis, MO) as described in Caron (1983) and Kinner et al. (1998). The stained slides were examined using a 40x objective and 10x oculars using the Nikon Optiphot-2 microscope equipped with a UV-2A filter cube. The protists were enumerated by focusing on the upper edge of the filter and scanning horizontally until the opposite edge was reached. The slide was then moved on the y-axis, and scanned horizontally in the opposite direction. This process was repeated until the entire filter had been scanned. The total abundance of cells observed on the filter was expressed as the number of protists present per volume of sample filtered.

35 mL was selected as the target sample size because it balanced filtering enough sample to allow a reasonable quantity of protists to be present, and prevented the filter from clogging. As the reactors aged, the filters began to clog more quickly and the amount filtered was adjusted accordingly ($\geq$ 10 mL). Protists were identified as circular cells (5 - 20 μm) that fluoresced a yellow-green color (the color of primulin associated with eukaryotes).

3.9 <u>Redox and pH</u>

After completion of the first experiment, it became evident that collecting redox and pH data was important. The redox (Eh) was measured by immersing an epoxy-platinum redox probe (Sure-Flow Combination Redox-ORP probe, Model #9179BNMD; Thermo-Electron; Waltham, MA) with a silver chloride reference electrode solution into the 60 mL centrifuge tube containing the bacteria and protist sample (prior to fixing it with formalin). During the second and third experiment, this was done inside of the glovebox under nitrogen and on the bench open to the atmosphere, respectively. The probe was left in the sample until the redox reading had stabilized, approximately ~5 min. Calibration occurred weekly using 0 mV and +200 mV standards (Cat #6595-32, NC9262580; Fisher Scientific). A two point calibration curve was created and was manually derived using values given from the meter.

pH measurements were collected by immersing a probe (Model #14002-754; VWR; West Chester, PA) into the sample immediately after removal of the redox probe and prior to injection of the formalin. The pH probe was calibrated weekly using a three point curve (pH 4, 7 and 10 standards, Cat #SB85-1, SB108-1, SB116-1; Fisher

Scientific). The pH and redox probes were rinsed with sterile RO water and dried prior to and between samples to reduce the potential for contamination.

3.10 Glassware Preparation

All glassware used in the experiments was soaked overnight in an Alconox solution (1 cup of powdered Alconox to ~ 8 L of RO water), scrubbed with a nylon bottle brush, and rinsed three times with RO water. All glass sample vials were muffled using a Lindberg/Blue Box Furnace, (Model #BF51828C-1; Riverside ,MI), with a temperature program of 5 min ramp from 0-100°C, 60 min ramp to 100°C, 45 min ramp from 100°C to 550°C, and 90 min at 550°C. Syringes and needles were cleaned weekly by immersion in an Alconox solution and rinsing three times with RO water and then autoclaving at 120° C and 15 psi for 30 min.

3.11 PCR and DGGE Analysis (USGS)

Samples sent to the USGS laboratory for PCR and DGGE analysis were bacteria and/or protists samples that were collected but not analyzed (i.e., they were not specifically collected for PCR/DGGE analysis). Samples were centrifuged at 10,000 RPM for 10 min until a pellet was formed. The water was decanted, and replaced with a 0.1% NaCL/reverse-osmosis water solution and centrifuged again until a pellet was formed. The water was decanted, and the pellet was shipped on dry ice to the USGS laboratory for analysis.

3.12 Data Analysis

An arithmetic mean of the data from the two reactors was calculated and used for all reaction coefficient calculations and data analysis. The reaction coefficients were calculated by graphing the mean chlorinated ethene concentration versus time and visually identifying areas of significant reductive declorination (experimental decay); defined as a decrease in concentration of TCE and an associated increase in cDCE. This method was repeated for each of the progeny. Once an area of dechlorination was identified, that section of data was plotted on a semi-log scale and a linear regression was performed. The slope of the linear regression line was the reaction constant (k, (time^{-1}) and its half life ($t_{1/2}$). This method only takes into account active reduction, and does not consider acclimation.

When protists were allowed to return to the reactors at the end of the first experiment, a morphological change was observed as the bacteria increased in length (length:width > 10:1). Bacterial size was identified using the microscope Whipple grid. Data were collected on the percent of bacteria that had undergone the morphological shift (L:W $\geq$ 10:1); this new form was designated "elongated". The percentage of elongated bacteria was determined by counting the number of bacteria in the elongated form vs. the coccoidal form separately (percent elongated = [number elongated / number elongated + number coccoidal] x 100).

CHAPTER 4

PROTISTAN PREDATION AND TCE BIODEGRADATION

IN A BEDROCK AQUIFER

4.1 Abstract

Despite extensive research on the resources required to initiate effective

microbially-mediated reductive dechlorination of trichloroethene (TCE), slow

degradation rates and stalling continue to be observed *in situ*. Thus far, the majority of

research on *in situ* biodegradation of TCE has focused on the bottom-up force of resource

availability (e.g., electron donors and acceptors, nutrients), while the top-down force of

predation is poorly understood. Predation has the potential to significantly alter bacterial

abundance, and can play an important role in selecting what species are present, thus

determining if the community is capable of mineralizing TCE. The impact of protistan

predation on TCE biodegradation rates, and occurrence and length of stalls was measured

in microcosms mimicking ambient and organic carbon amended conditions with and

without protists present, and with a commercially-available dechlorinating bacterial

culture. When only indigenous bacteria from a TCE contaminated fractured rock aquifer

were present, the indigenous protists inhibited reductive dechlorination. Without protists,

reductive dechlorination stalled at cDCE. The presence of protists under organic carbon

amended conditions coincided with a morphological shift in the bacteria to an elongated form (Length:Width $\geq$ 10:1), but no reductive dechlorination was observed. Similarly, when a commercially-available dechlorinating bacterial culture was added in the presence of protists, the elongated bacteria predominated and reductive dechlorination stalled at cDCE. When protists were inhibited, TCE was mineralized. Protistan predation appeared to impact the success of reductive dechlorination through selection of bacterial community composition. There was a dual threshold effect: no predation limited the ability of the dechlorinators to become dominant, while too much predation resulted in a system where the dechlorinators were grazed to extremely low levels, inhibiting reductive dechlorination. In order for bioremediation to fully and quickly result in TCE mineralization, protistan predation may need to be controlled *in situ*.

4.2 <u>Introduction</u>

The microbially-mediated process of reductive dechlorination is often proposed as the most cost-effective *in situ* treatment to remediate aquifers contaminated with the cleaning solvent trichloroethene (TCE). Biodegradation results in the sequential dechlorination of TCE and its progeny, and under the correct conditions, can fully mineralize the chlorinated solvents to CO_2 (Bradley, 2003). Microbially-mediated reductive dechlorination of TCE generally results from two processes; dehalorespiration and co-metabolism. In dehalorespiration, also called direct anaerobic reductive dechlorination, the bacteria use TCE as an electron acceptor and gain energy from the reaction (US EPA, 2000). Complete mineralization to CO_2, under the correct

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environmental conditions, is possible. In contrast, during co-metabolism, the bacteria do not use the contaminant in an energy generating process. Rather, degradation occurs gratuitously catalyzed by non-specific enzymes (e.g., mono-oxygenases) or co-factors produced while the bacteria degrade a primary, energy-generating, substrate (i.e, organic carbon). These non-specific enzymes dechlorinate TCE and sometimes its progeny, however, complete mineralization is unlikely (US EPA, 2000). The literature contains numerous studies that explore reductive dechlorination and how biodegradation rates can be increased by amending the subsurface with electron donor and nutrients to stimulate bacteria using either process (Bradley, 2003; Aulenta and Gossett, 2005; Zinder and Gossett, 1995; Vogel and McCarty, 1985).

The overall sequence for reductive dechlorination (dehalorespiration or co-metabolism) of TCE is usually the same: TCE → cDCE → VC → Ethene. Once created, ethene can be easily mineralized to CO_2 by a wide variety of heterotrophs (Bradley, 2003; US EPA, 2000; Lu and Kampbell, 2006).

Complete mineralization to CO_2 using dehalorespiration typically requires specific electron donors (i.e., acetate, H_2), sulfate reducing or methanogenic (i.e., strongly reducing) conditions, and adequate nutrients, as well as the presence of bacteria capable of performing dehalorespiration (Bradley, 2003; Lu and Kampbell, 2006). *Dehalobacter restrictus, Dehalospirillum multivorans, Desulfitobacterium strain PCE1, Desulfuromonas chloroethenica, Geobacter spp., Desulfuromonas michiganenis, and Dehalococcoides ethenogenes* are among the species able to dechlorinate TCE by

replacing chlorine atoms with hydrogen (Lu and Kambpell, 2006). While many species are capable of degrading TCE to cDCE and VC, members of one genera, *Dehalococcoides ethenogenes,* have been identified as able to complete the last step in the reductive dechlorination pathway; VC to ethene (He et al., 2002). This means that if an indigenous population of *Dehalococcoides ethenogenes* is not present, the system will likely stall at cDCE or VC. Stalling at cDCE or VC is problematic because it greatly increases the remediation time, and can result in the presence of VC, which is more toxic than TCE (Lu and Kampbell, 2006). Although stalls can occur for many reasons, they are most often attributed to electron donor limitation or inadequate abundance of the appropriate dechlorinating species (Becker, 2006; Bradley, 2003). A stall due to electron donor deficiency can be remedied by adding biodegradable organic carbon which is degraded by indigenous bacteria (e.g., usually by fermentation) to create the preferred electron donor, H_2. If the stall is caused by the absence of bacteria capable of degrading VC to ethene (e.g., only *Geobacter spp.* and sulfate reducers are present) bioaugmentation with *Dehalococcoides ethenogenes* may be helpful. In many subsurface ecosystems, *Dehalococcoides ethenogenes* may be present in such low abundance that there is negligible VC degradation (Bradley, 2003; US EPA, 2000; Eighmy et al., 2006; Naser, 2005).

Aerobic respiration, denitrificiation, iron and manganese reduction, and to a certain extent sulfate reduction have significantly higher Gibbs free energies than the reactions associated with dehalorespiration (Claypool and Kaplan, 1974). It is generally accepted that for the conversion of TCE→cDCE→VC, a minimum of sulfate-reducing

conditions are required, while the conversion of VC to ethene is thought to be most effective under methanogenic conditions. Producing such reducing conditions can usually be accomplished through the addition of organic carbon, which provides an electron donor, allowing indigenous bacteria to sequentially consume electron acceptors until the desired redox is reached.

There has been a growing consensus that in order for effective reductive dechlorination to occur, a consortium of bacteria must be present and undergo a community shift as the ratio of TCE to its progeny changes. Becker (2006) reported that under ambient *in situ* conditions, *Dehalococcoides ethenogenes* is unlikely to become to the dominant species under electron donor limiting conditions as it is out-competed by other dehalorespirers with faster substrate utilization kinetics. The dehalorespirers that become dominant are often only capable of degrading TCE to cDCE or VC, potentially resulting in a stall. In contrast, under engineered (bioaugmented) conditions, adding a large number of *Dehalococcoides ethenogenes* relative to other dehalorespirers allows them to control an increasingly larger percentage of the electron donor, eventually out-competing other species despite slower substrate utilization kinetics. This strongly suggests that presence of *Dehalococcoides ethenogenes* in contaminated aquifers may not inherently produce complete mineralization of TCE unless they are able to become dominant once the TCE has been exhausted and cDCE or VC predominates.

Duhamel and Edwards (2007) examined the bacterial community composition of mixed microbial cultures used to dechlorinate TCE and found despite the presence of

Dehalococcoides ethenogenes, Geobacter spp. was responsible for between 60 – 96% of the conversion of TCE to cDCE. When the TCE was eliminated, *Dehalococcoides ethenogenes* became the dominant dehalorespirer and converted the remaining cDCE to ethene. The hierarchy of species associated with TCE biodegradation appears to be, not surprisingly, a function of energetics (i.e., the amount of energy generated by a species in using different chlorinated ethenes as electron acceptors to degrade an electron donor). This seems to suggest that as the distribution of available electron acceptors (i.e., chlorinated ethenes) shifts, the composition of the bacterial community will shift as well in order to use the electron donors most efficiently. Duhamel and Edwards also reported that the growth of *Sporomusa sp.*, responsible for production of H_2 required by *Dehalococcoides ethenogenes,* appeared to be inhibited by cDCE and VC, perhaps resulting in H_2 limiting conditions and reducing biodegradation potential through electron donor limitation.

Becker (2006) and Duhamel and Edwards (2007) suggest that a mixed microbial community, capable of undergoing compositional shifts as the dominant chlorinated ethene changes, is required for effective microbially-mediated dehalorespiration. Fermenters and other beneficial bacteria are required to ensure that adequate resources (i.e., H_2) are available to the dehalorespiring bacteria. However, competition between bacteria for resources may significantly reduce the abundance of required species, potentially limiting the biodegradation potential. Because competition between bacterial populations affects the extent of reductive dechlorination achieved, the top-down force of selective predation on bacteria must be examined to fully understand what is occurring *in*

situ. The role of predation in the subsurface has only been examined by a few researchers, so little is known about the potential effects it has on microbially-mediated reductive dechlorination of TCE. Further, almost no research has been done on protistan predation on bacteria in fractured bedrock systems. Although an established link exists for a predator-prey relationship between protists and bacteria in sandy aquifers (Kinner et al, 2002) and surface water systems (Curds, 1992; Jurgens et al., 1992), a literature review yielded no published instances where this connection was established in fractured bedrock systems. Research at the University of New Hampshire's Bedrock Bioremediation Center (BBC) has shown that the ratio of bacteria to protists in fractured bedrock systems is similar to that of other systems where a predator-prey relationship exists (unpublished data). Contrary to popular belief, protists are not strict aerobes, and have been found in anaerobic environments, including fractured bedrock aquifers (Kinner et al., 1998 and 2002). Protistan predation usually influences nutrient and organic carbon cycling, and bacterial composition and cell size (Corno et al., 2006; Novarino et al., 1997). In systems where electron donor is limiting (e.g., many fractured bedrock aquifers), protistan predation ensures that the organic carbon is recycled and not stored in cell biomass. Flagellated protists, the small species that predominate in the subsurface, tend to be "transect feeders" propelling themselves in a straight line and consuming any cells of the preferred size they encounter (Novarino et al., 1997). They can consume > 70% of the unattached bacterial biomass daily (Kinner et. al., 1998), and often selectively prey on bacterial species in a specific size class, influencing bacterial distribution and cell size (Kinner et al., 1998; Novarino et al., 1997). In TCE contaminated aquifers, such size selective predation on *Dehalococcoides ethenogenes*

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could prevent conversion of cDCE to ethene even if an energy generating bottom-up (i.e., resource dominated) bacterial hierarchy appeared to favor it.

Two continuously stirred reactors, constructed to mimic conditions at the BBC's research site at the former Pease Air Force Base (Portsmouth, NH) were used to examine how microbially-mediated reductive dechlorination is influenced by protistan predation. The site is a fractured bedrock aquifer in which TCE and cDCE have migrated into underlying competent bedrock. Dechlorinating bacteria, including *Dehalococcoides ethenogenes,* have been identified at this site using fluorescence *in situ* hybridization (FISH), and PCR/DGGE (Naser, 2005). Three experiments were conducted to examine the degradation rate of TCE and occurrence of stalls using ambient (≤ 10 mg TOC/L) and amended (~ 120 mg TOC/L) organic carbon concentrations. The first two experiments examined the TCE degradation rate in the absence and presence of protists. In the third experiment, the bacterial culture KB-1 (SiREM Labs; Guelph, ON) was added with protists present. The following research questions were addressed: 1) How is the degradation rate of TCE influenced by the presence of protists under organic carbon limiting (ambient) and amended conditions? 2) Does the presence of protists influence stalling at cDCE? 3) How does the presence of protists influence the abundance and composition of the bacterial community? 4) Does the presence of protists influence the TCE degradation rate, bacterial abundance, and community structure in a bioagumented system?

4.3 **Materials and Methods**

The study consisted of three experiments designed to examine the impact protists have on fractured bedrock systems under ambient (unamended), organic carbon amended, and KB-1 amended conditions. In the first experiment, protists were excluded from the reactors by 0.8 μm filtration and the TCE degradation rate was determined under ambient organic carbon concentrations and amended organic carbon conditions. In the second experiment, protists were allowed to inhabit the reactors, and the degradation rate of TCE was examined under ambient and amended organic carbon conditions. Due to a leak developing in one of the reactors, as well as difficulty controlling the TCE concentration, this experiment was repeated (i.e., Runs 1 and 2). The third experiment was designed to examine the TCE degradation rate in a bioaugmented (i.e., KB-1) system in the presence of protists with amended organic carbon conditions.

The reactors were 2 L silica glass canning jars with tin sealable lids and screw caps (Ball Canning; Broomfield, CO) (Figure 3.1). The lids had four pairs of 0.2 cm holes, and sheets of silicone rubber and teflon on the top and bottom sides, respectively. This prevented water from leaking out and the chlorinated ethenes from sorbing to the silicone rubber, respectively. A strand of nylon fishing line (Stren Original; Clear 20lb test; Spirit Lake, IA), cut to approximately 60 cm, passed through the teflon, tin, silicone rubber, and the second drilled hole and was knotted off, leaving approximately 55 cm of fishing line inside the reactor. Four lengths of fishing line, attached in this manner, suspended the rock, in the reactor.

Two stainless steel 1 cm compression x 1.3 cm male pipe thread (MPT) fittings were threaded into the lid, each with 1 cm teflon silicone septa on the compression side of the fitting to form sampling ports. These allowed simultaneous effluent sampling and refill water injection to prevent headspace formation.

Rock obtained from a local outcropping in Portsmouth, NH with similar geologic properties to the BBC site (fractured metasandstone and metashale of the Silurian and Ordovician Kittery formation) was split into fragments between 2 and 6 cm long, 2 to 5 cm wide, and 2 to 5 cm thick using a hammer. A 0.2 cm hole was drilled into each fragment. The rock was cleaned (scrubbing with brush in laboratory reverse osmosis (RO) water), autoclaved and sorted according to size. Approximately 500 g of fragments were attached to the fishing lines in the reactors by threading it through the holes and knotting it. There was a 2.5 cm clearance between the rock and the bottom and sides of the reactor. 500 g of rock represented a similar surface area to volume ratio to the bedrock fractures.

Water for the reactors was obtained from BBC Well #6 (BBC6) using a multi-packer that isolated a fracture system at a depth of approximately 36 m below ground surface (bgs) and a Grundfos Redi-flow pump (Olathe, KS) purged for one well volume (~ 210 L). The water contained: 10 – 250 µg/L TCE, 5 – 200 µg/L cDCE and < 14 µg/L VC, as well as an indigenous community of bacteria and flagellated protists (3 – 5 µm) that are acclimated to the presence of chlorinated ethenes. Site water used to fill the

reactors was pumped into 4 L amber glass containers with teflon caps and zero headspace and transported to a nitrogen-filled glovebox. All work was conducted in a nitrogen filled glovebox to prevent the addition of oxygen into the system, as the BBC site is anoxic.

Preliminary experiments indicated (data not shown) 0.8 µm cellulose nitrate membrane filter (Cat #7184-002; Whatman International; Middlesex UK) filtration allowed the indigenous protist population to be removed from the sample water without significantly affecting the abundance of native bacteria.

Once the reactors were filled with the appropriate water, approximately 0.62 mL of a 1200 mg/L TCE solution was injected (final concentration ~ 540 µg/L (4.0 µM)). The 1200 mg/L TCE solution was created by injecting 5 mL of laboratory grade TCE (Cat #T341-500; Fisher Scientific; Hampton, NH) into 55 mL of RO water in a 60 mL amber glass vial. The reactors were placed on magnetic stir plates (~100 rpm) in the glovebox and were maintained at $10 \pm 2\ ^\circ$ C to mimic groundwater conditions.

Sampling occurred every three days during an initial 14 day acclimation period, then became daily. TOC, chlorinated ethene concentrations, and bacterial and protistan abundance were monitored for ≤ 30 days to evaluate population interactions and degradation under ambient (*in situ*) conditions. 3 mL of a 19,200 mg/L sodium lactate solution were injected into the reactors to yield a final concentration of 120 mg TOC/L, so that organic carbon would not be limiting. For the bioaugmented experiment, 3 mL of

KB-1 were injected into the reactor along with sodium lactate as recommended by SiREM Labs.

The TCE and TOC concentrations in the reactors were maintained by injecting ~100 mL of fresh BBC6 water spiked with 0.1 mL of the 1200 mg/L TCE solution and, as appropriate, 1 mL of the sodium lactate stock to achieve TCE and TOC concentrations of 540 µg/L and 120 mg/L, respectively, to replace water removed by sampling.

Two 25 mL gastight glass/teflon syringes (Model #1025; Hamilton Co; Reno, NV), equipped with 4 cm long, 18 gauge septum-piercing needles were used to add refill water and remove sample, respectively. The plunger for the effluent syringe was fully depressed and then placed in the sampling port, while the plunger for the influent syringe was removed. Approximately 25 mL of refill water was poured into the influent syringe, and the plunger is replaced. As water was withdrawn from the reactor with the effluent syringe, it was replaced using the influent syringe, minimizing headspace formation. This was repeated 3 – 4 times per reactor so that each day approximately 100 mL of water was removed as sample and an equal amount was injected as refill water. In the third experiment, to prevent short-circuiting removing KB-1, ~100 mL of sample was removed by syringe, and then refill water was injected. Although this allowed headspace to form, the short period over which the headspace existed yielded negligible volatilization losses and no addition of oxygen as the reactors were under a nitrogen atmosphere in the glovebox.

The TOC samples were stored in 20 mL amber glass VOA vials with teflon/silicone septum caps and spiked with 2 mL of 1 N H_2SO_4 as a preservative with a maximum hold time of 21 days. Analysis was conducted using a TOC V CSH Total Organic Carbon Analyzer (Shimadzu; Kyoto, Japan) and a non-purgeable organic carbon combustion method. Calibration occurred monthly using 10, 25, 50, 100, 120, and 150 mg TOC/L calibration standards, and blanks and standards were included as every 10th and 11th sample, respectively.

The chlorinated ethene samples were collected in 20 mL amber glass VOA vials capped with a teflon/silicone septum caps. The vials were checked for bubbles and sent to RLI Laboratories (Portsmouth, NH) for analysis using gas chromatography with an electron capture detector (ECD) in accordance with USEPA SW-846, Method 5030B/8260B, within 14 days of sampling.

The bacterial and protistan samples were collected using a 60 mL sterile centrifuge tube (Part #14-375-150; Fisher Scientific). Approximately 50 mL of sample from the reactor were injected into the centrifuge tube, and after redox and pH measurements were taken, 3 mL of 37% 0.2 μm filter-sterilized formalin (Cat #F79-1; Fisher Scientific) were injected as a preservative. Samples were stored ≤ 21 days at 10 $\pm 2°C$ prior to processing.

The bacteria were enumerated using epifluorescence microscopy. 5 mL of sample were passed through a 0.2 μm x 25 mm sterile membrane filter (Cat #110656; Whatman

International) under ~ 5 mm Hg of vacuum and then stained with a 0.01% acridine orange solution (Part #212536; BD Chemical; Sparks, MD) as described in Kinner et al. (1998) using a 12-port filtration manifold (Model #1125; Millipore; Billerica, MA). The stained slides were examined using a 100x oil immersion lens and 10x oculars on a Nikon Optiphot-2 microscope equipped with a B2H filter cube and an external high-pressure mercury vapor lamp. The bacteria were enumerated by randomly selecting seven fields and counting the number fluorescing green or orange within a Whipple grid (dimensions at 1000x magnification: 4.5μm x 4.5μm). The seven microscope field counts (containing 75 to 300 cells each) were arithmetically averaged and adjusted to a volume of 1 L.

The protists were also enumerated using fluorescence microscopy; ≤ 35 mL of sample were passed through a 0.8 um sterile filter (E08BP02500; Osmonics Inc; Trevrose, PA) and stained with primulin (Direct Yellow 59, Color Index 49000; Aldrich Chemical; St. Louis, MO) as described in Caron (1983) and Kinner et al. (1998). The stained slides were examined using a 40x objective and 10x oculars on a Nikon Optiphot-2 microscope (Garden City, NY) equipped with a UV-2A filter cube. The filter was scanned horizontally (x-axis) by focusing on the upper edge. The slide was then moved along the y-axis and scanned horizontally in the opposite direction. This process was repeated until the entire filter had been scanned. The total cells observed on the filter were considered the number of protists present per volume of sample filtered.

After completion of the first experiment (protists removed), it became evident that collecting redox and pH data would be advantageous. The redox was measured by immersing an epoxy-platinum redox probe (Sure-Flow Combination Redox-ORP probe, Model #9179BNMD; Thermo-Electron; Waltham, MA) with a silver chloride reference electrode solution into the 60 mL centrifuge tube containing the bacteria and protist sample, prior to fixing with formalin. During the second experiment, this was done inside of the glovebox; during the third experiment, KB-1 amended, this was done in the lab open to the atmosphere. The probe was left in the sample until the redox reading had stabilized (~5 min). Calibration occurred weekly using a 0 mV and +200 mV standards (Cat #6595-32 and NC9262580; Fisher Scientific). A two point calibration curve was created and manually applied to the values from the meter.

pH measurements were collected by immersing a pH probe (Model #14002-754; VWR; West Chester, PA) into the sample immediately after removal of the redox probe and prior to injection of the formalin. The pH probe was calibrated weekly using a three point calibration curve (pH 4, 7 and 10 standards; Cat #SB85-1, SB108-1, SB116-1; Fisher Scientific). The pH and redox probes were rinsed with sterile RO water and dried prior to and between samples to reduce potential contamination.

Samples sent to the USGS laboratory for PCR and DGGE analysis were bacteria and/or protists samples that were collected but not analyzed (i.e., they were not specifically collected for PCR/DGGE analysis). Samples were centrifuged at 10,000 RPM for 10 min until a pellet was formed. The water was decanted, and replaced with a

0.1% NaCL/RO solution and centrifuged again until a pellet was formed. The water was decanted, and the pellet was shipped on dry ice to the USGS laboratory for analysis.

An arithmetic mean of the data from the reactors was calculated and used for all reaction coefficient calculations and data analysis. The microbial degradation rates were calculated by graphing the chlorinated ethene concentration versus time and visually identifying areas of active reductive declorination; defined as an exponential decrease in concentration of TCE and an associated increase in cDCE. This method was repeated for each chlorinated ethene and its respective progeny. Once an area of active dechlorination was identified, that section of data was plotted on a semi-log scale and a linear regression was performed. The slope of the linear regression line was the reaction constant (k, time^{-1}) and half life ($t_{1/2}$ = time =ln 2/k). This method only takes into account active reduction, and does not account for acclimation time.

When protists were allowed to return to the reactors at the end of the first experiment, a morphological change was observed in the bacteria (length : width > 10:1). Bacterial size was determined using the Whipple grid. The percentage of bacteria that had undergone the morphological shift (elongated) was determined by counting the number of elongated and coccoidal bacteria (% elongated = [(number elongated / number elongated + coccoidal) x 100].

4.4 Results and Discussion

4.4.1 Protists Removed

For the first 26 days, the reactors simulated *in situ* conditions (< 10 mg TOC/L). No significant changes in chlorinated ethene concentrations, bacterial abundance or morphology were observed, and no protists were detected (Figure 4.1a). From Days 27 to 80, the reactors were amended with sodium lactate (120 mg TOC/L). An increase in cDCE (> 4 μM) was detected on Day 40 and reached its first peak on Day 67, coinciding with a proportional decrease in TCE (Figure 4.1a). No further change in cDCE concentration was observed nor did VC increase. The total molar concentration of chlorinated ethenes remained fairly constant through Day 80, further indicating that the decrease in TCE was due to biodegradation, not volatilization. The first-order degradation rate of TCE was 0.120 $\pm$0.018 d^{-1} (Table 4.1) (half-life $t_{1/2}$= 5.7 d). The production of cDCE also followed a first order reaction (+0.184 $\pm$0.023 d^{-1}).

Table 4.1: First Order Reaction Constants (d^{-1}).

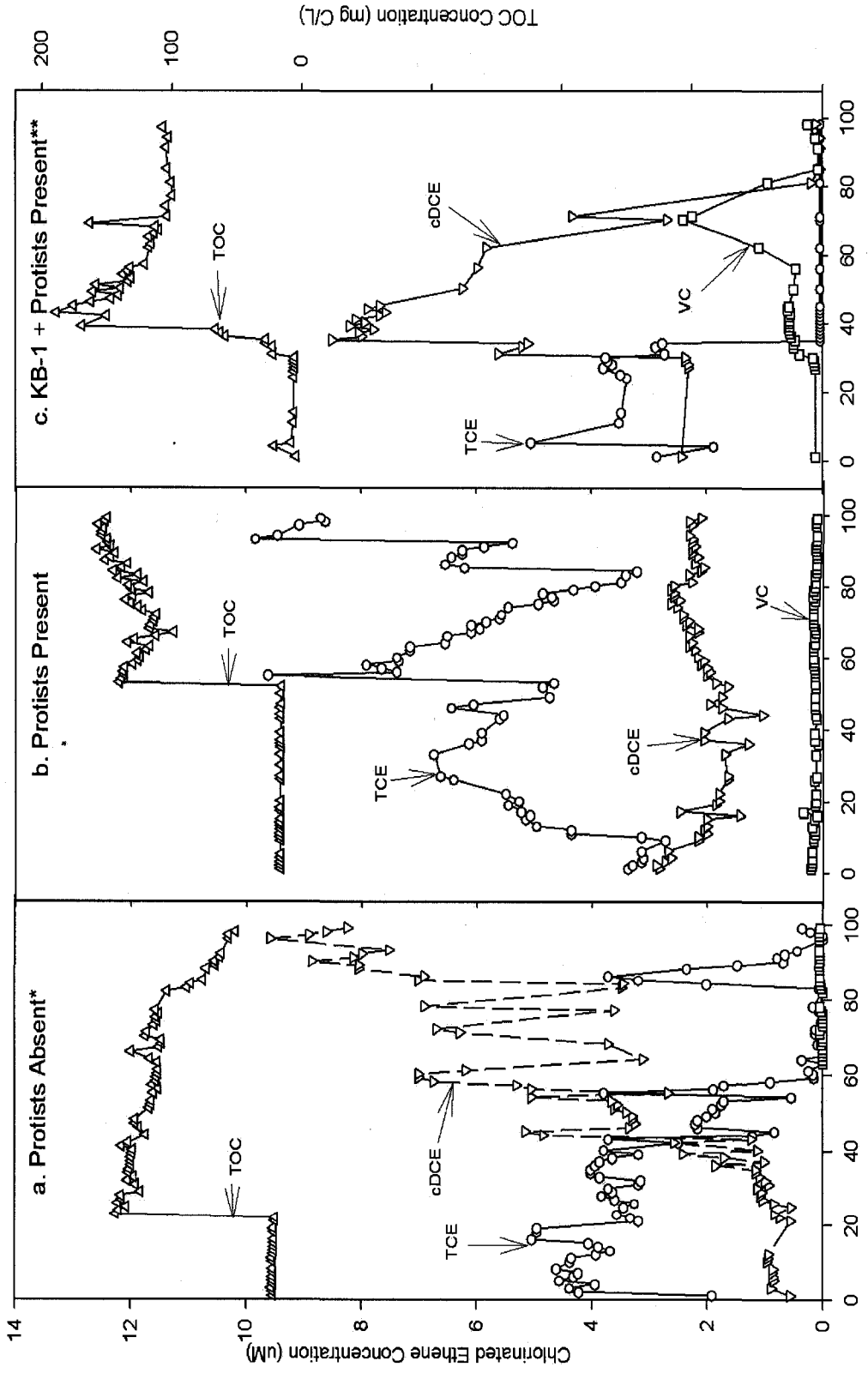| Treatment	TCE Reduction	cDCE Production	cDCE Reduction	VC Production
Protists Removed	-0.120 $\pm$0.018	+0.184 $\pm$ 0.024	ND	ND
Protists Present	ND	ND	ND	ND
KB-1 + Protists	-0.589 $\pm$0.190	+0.817 $\pm$ 0.263	-0.184 $\pm$ 0.018	+0.059 $\pm$ 0.013

ND – Not Detected

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Figure 4.1: TCE, cDCE, and VC Concentrations (uM) versus Time with a. Protists Absent, b. Protists Present, and c. KB-1 + Protists Present. * Protists Allowed to Return on Day 83 ** Protists absent from Day 70 to 100.

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The increase in TOC did not result in a significant change ($p = 0.05$) in bacterial abundance (mean abundance = $3.5 \times 10^6 \pm 5.7 \times 10^5$ cells/L and $5.7 \times 10^6 \pm 1.8 \times 10^6$ cells/L, before and after TOC amendment), respectively (Table 4.2). No protists were detected during this stage of the experiment (Figure 4.2a).

Table 4.2: Mean Bacterial Abundance in Reactors (cells/L)

Treatment	In Situ		Amended	
	Mean Abundance	Standard Deviation	Mean Abundance	Standard Deviation
Protists Removed	3.5×10^6	5.7×10^5	5.7×10^6	1.8×10^6
Protists Present	2.3×10^6	8×10^5	7×10^6	1.7×10^6
KB-1 + Protists	2.5×10^6	1.8×10^6	11.5×10^6	1.9×10^6
Pilot Study (Lewis, 2005)	2.8×10^5	3.3×10^5	1.80×10^5	1.3×10^5
BBC6 Groundwater	1.8×10^5	1.3×10^5		

On Day 83, filtration of the injectate stopped and protists were allowed to return to the reactors, and on Day 85 TCE was injected into the reactors to raise the concentration to ~4 μM. The TCE was rapidly converted to cDCE and was eliminated by Day 90 (Figure 4.3). As the protistan abundance increased, a morphological change was observed in the bacteria with almost all becoming elongated by Day 95 (Figures 4.4a and 4.5a).

The results confirmed the conclusion of Eighmy et al. (2007) that the BBC site is electron donor (organic carbon) limited with respect to reductive dechlorination of TCE. The groundwater TOC at Site 32 is typically between 1 – 10 mg TOC/L, and there is competition for the limited amount of biodegradable organic carbon between many indigenous species. Within 13 days after sodium lactate addition, enough resources
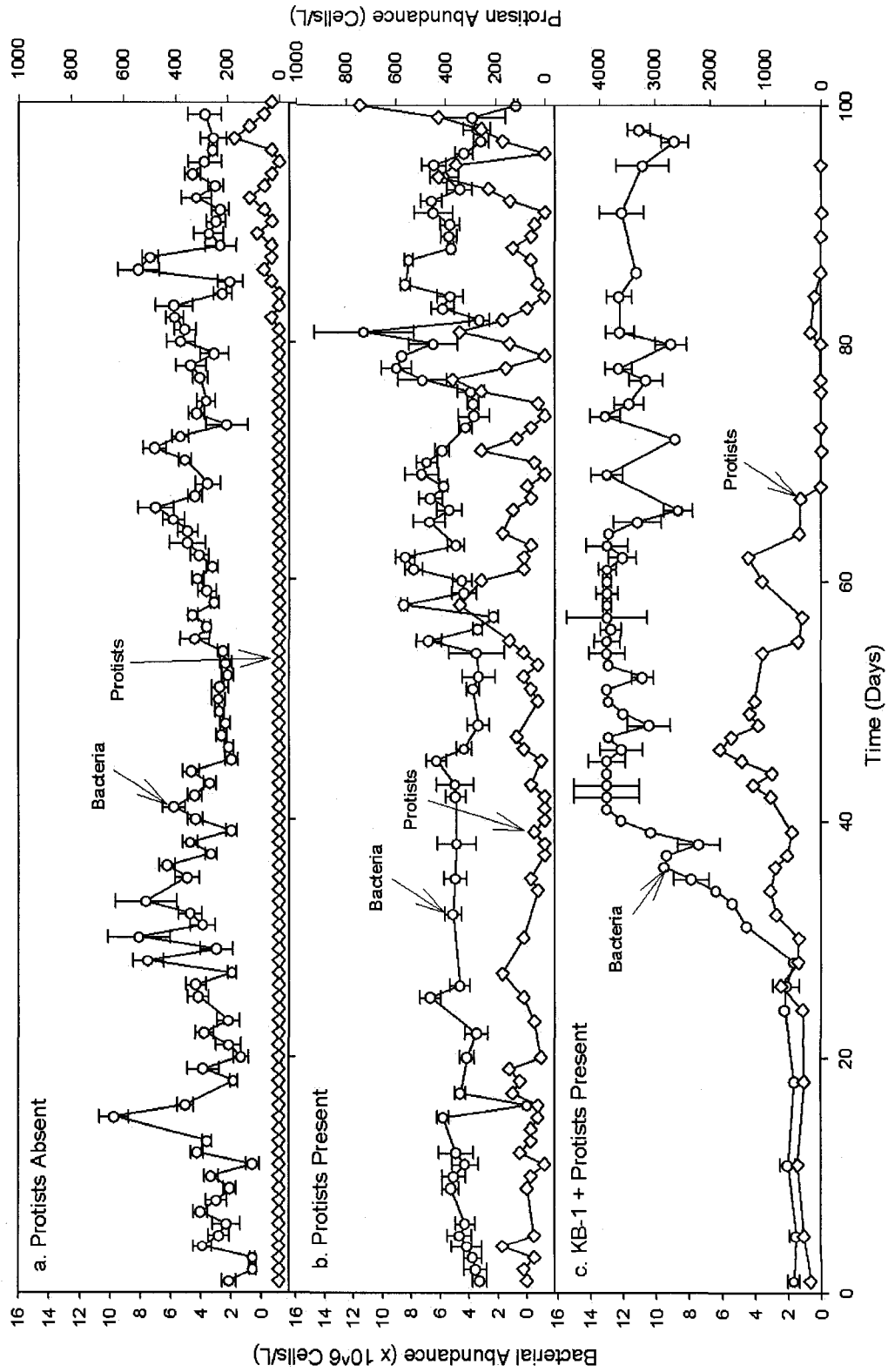
Figure 4.2: Relationship between Protistan and Bacterial Abundance.
Note: Protistan Abundance Scale Different for c. KB-1 + Protists
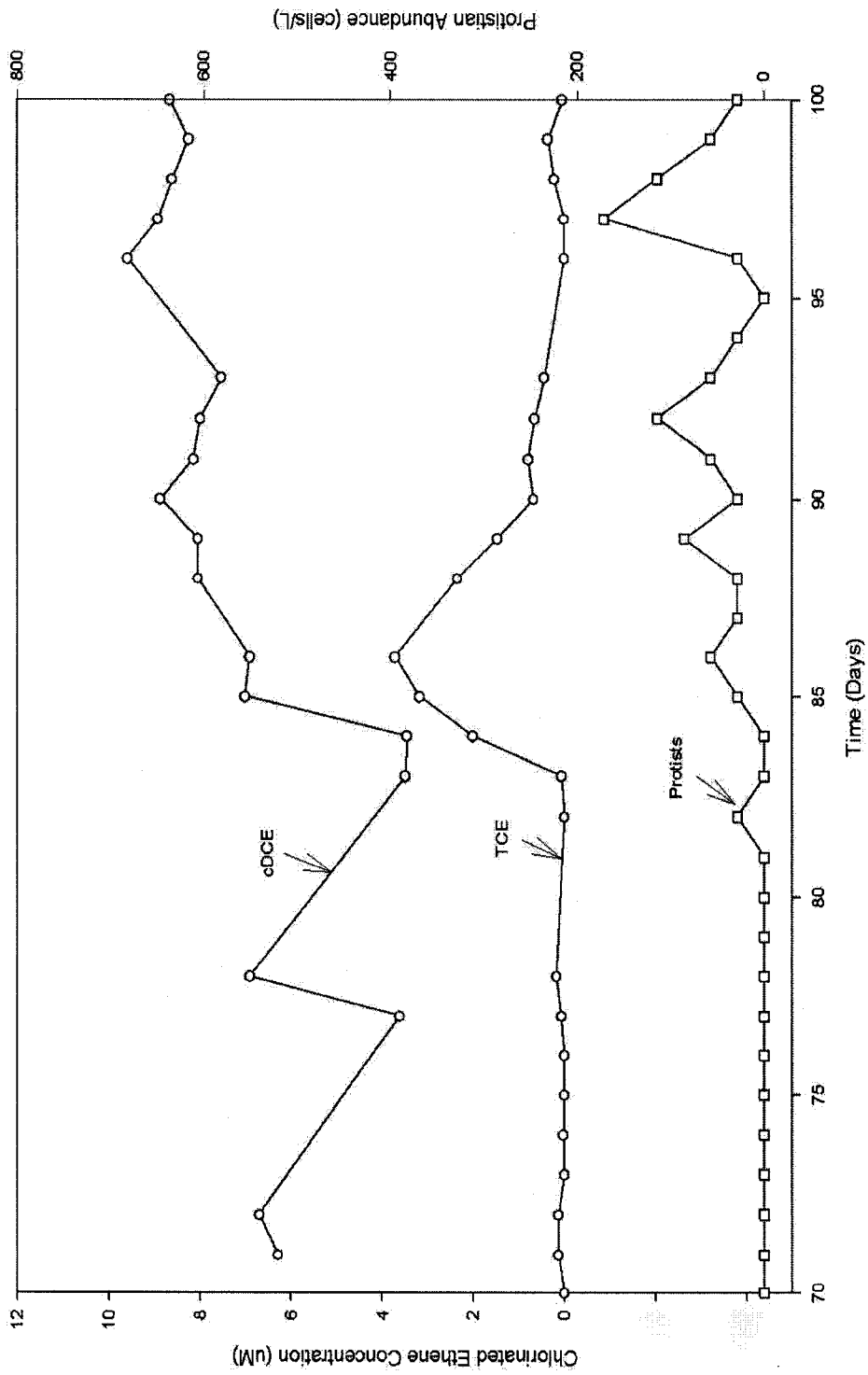
Figure 4.3: TCE, cDCE and Protistan Abundance After Protists Were Allowed to Return to Reactors in Protists Removed Experiment; Day 80 = Protist Filtration Stopped, Day 83 = TCE spike. Note Offset Scales for Y-Axis
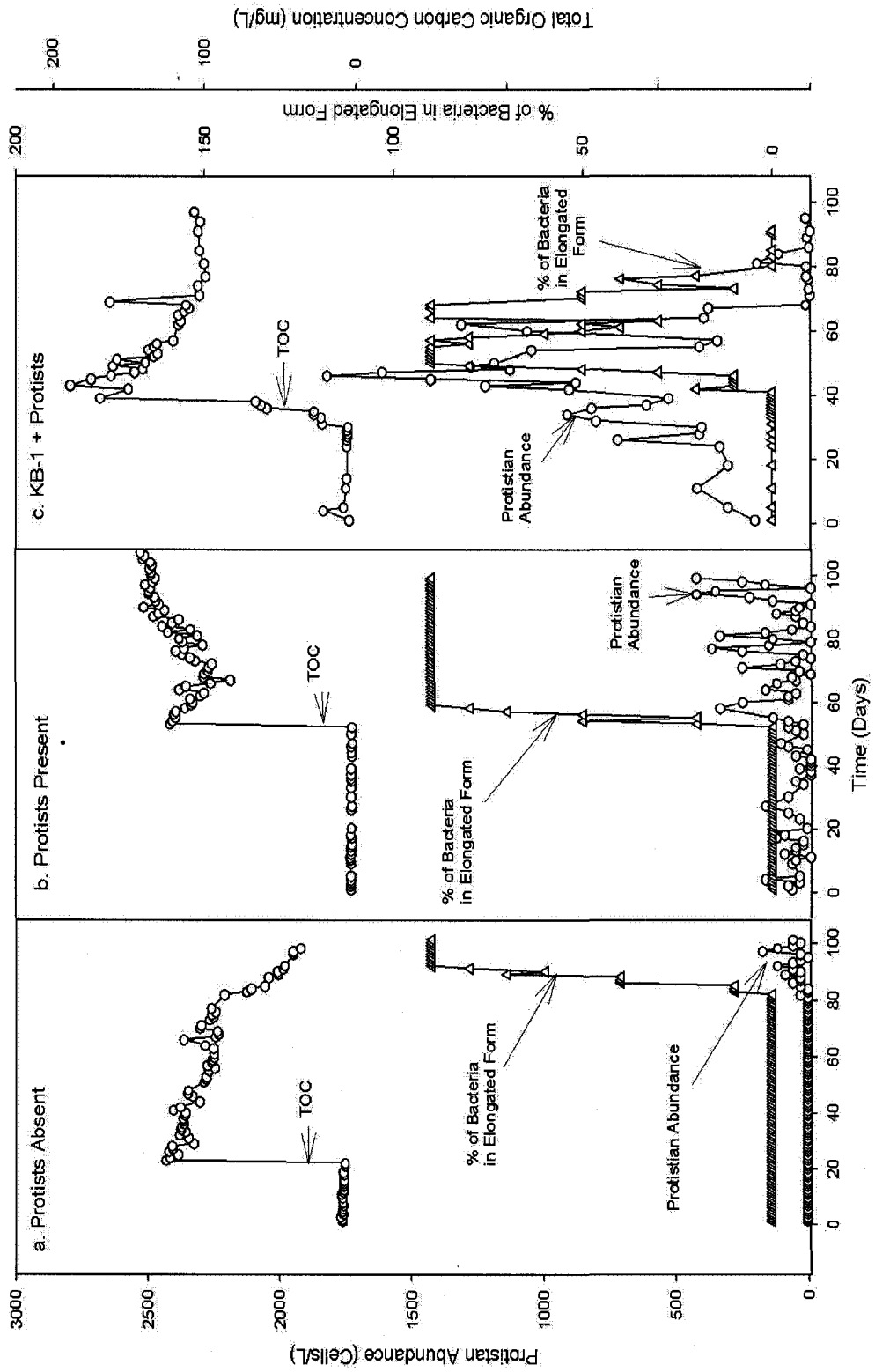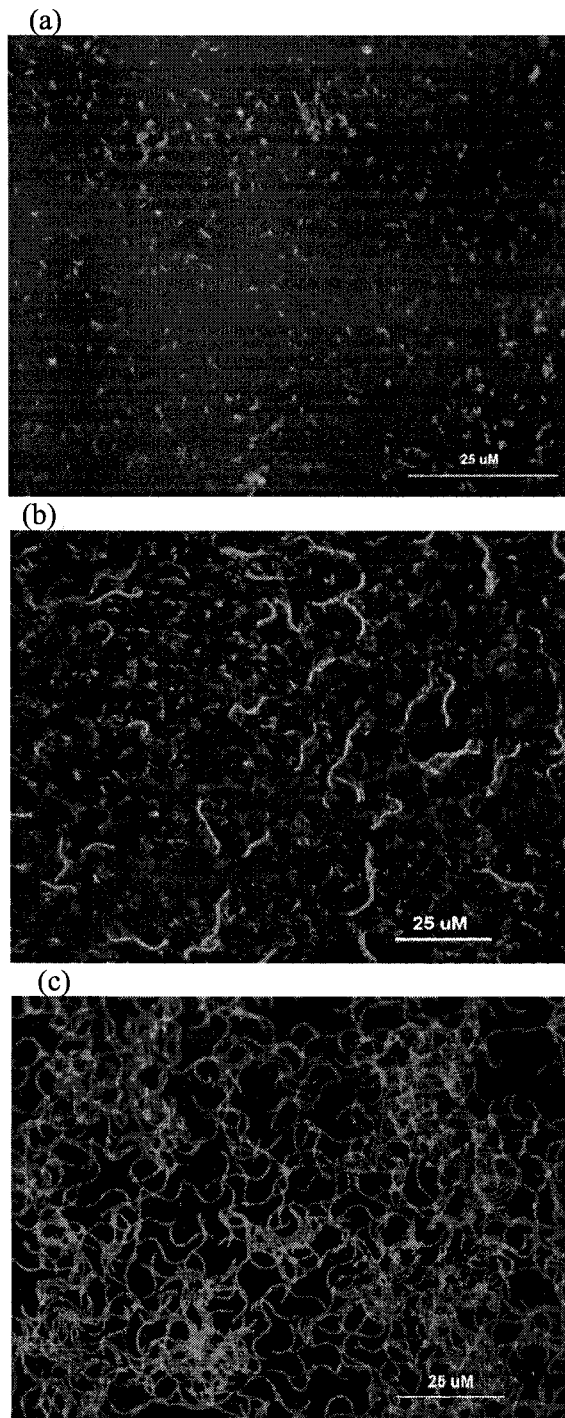
Figure 4.4 Bacterial Morphology as a Function of Protistan Abundance and TOC Concentration vs Time

Figure 4.5: Morphology of Bacteria Stained with Acridine Orange with Protists Present, Absent and KB-1 Amended
  a) **Protists Absent, 0% elongated, TOC Amended**
  b) **Protists Present, 75% elongated, TOC Amended**
  c) **TOC and KB-1 + Protists Present, 100% elongated**

(organic carbon, electron donor) were present to initiate reductive dechlorination (TCE → cDCE), however, something prevented further biodegradation.

The goal of organic carbon amendments is two fold in most bioremediation scenarios: to generate redox conditions conducive to reductive dechlorination (Bradley, 2003; Norris et al., 1994; US EPA, 2000), and stimulate fermenters to produce the electron donors (acetate or H_2), so that dehalorespiring bacteria will reductively dechlorinate TCE to ethene (Norris et al., 1994; Gerritse et al., 1999, US EPA, 2000). Clearly, the addition of sodium lactate partially accomplished this goal as TCE was degraded to cDCE. The stall at cDCE suggested the microbial community was unable to degrade cDCE because of: unfavorable redox conditions, resource limitation (electron donor, nutrient availability), or a paucity of bacteria capable of using cDCE as an electron acceptor [N.B., Because protists were not present during this period, predation was not a factor.]

Redox was not measured during the first experiment (protists removed) and therefore we could not determine whether the sulfate reducing or methanogenic conditions typically required for reductive dechlorination of cDCE were occurring (Bradley, 2003). If less reducing conditions existed in the reactors, dechlorination of cDCE could have been energetically unfavorable (Bradley, 2003; Heimann and Jakobsen, 2006; Chapelle, 2001).

Energetics may also have played a role in the stall at cDCE. When numerous electron acceptors are present, the reactions that yield(s) the most energy will usually be favored. Numerous potential electron acceptors exist at Site 32, including O_2, Fe^{3+}, SO_4^{-2}, TCE, and cDCE (Eighmy et al., 2007). The Gibbs free energies of reaction for sulfate reduction and cDCE dechlorination (using H_2 as an electron donor) are similar at standard conditions (-151 kJ/mol of electron donor (ED) for $SO_4 \rightarrow HS^-$ and -121.1 kJ/mol of ED for cDCE $\rightarrow$ VC; calculated from values given in Benjamin, 2002). In theory, at standard conditions, sulfate, present in high concentrations (100 – 130 mg SO_4^{-2}/L) at Site 32, would need to be significantly depleted before cDCE dechlorination would predominate (Cord-Ruwisch et al., 1998; Heimann and Jakobsen, 2006). However, it should be noted that the calculation of Gibbs free energy of reaction is dependant on numerous factors including: pH, temperature, partial pressure, and electron donor and acceptor concentrations. Hence, while under one set of conditions sulfate reduction may be more energetically favored, under slightly different conditions cDCE may be the preferred electron acceptor. While it is beyond the scope of this paper to fully evaluate the energetic pathways present at Site 32, it is possible that the high sulfate concentrations made its use as an electron acceptor more energetically favorable than cDCE dechlorination, resulting in a stall at cDCE.

It is also possible that *Dehalococcoides ethenogenes* was not present in the reactors. Previous studies (Naser, 2003) have shown that although *Dehalococcoides ethenogenes* was reported at Site 32, it was often undetectable or in very low abundance.

Within five days of protists returning to the reactors (Day 83), a dramatic

morphological shift was observed in the bacterial community (Figures 4.4a and 4.5a);

greater than 90% of the bacteria had shifted from coccoidal to an elongated form, with a

L : W ratio of ~ 10:1. A TCE spike was injected on Day 85, and was rapidly converted to

cDCE, and once again stalled there. Apparently, the morphological change had no

immediate influence on bacterial community's ability to biodegrade cDCE. Instead, the

change in morphology appeared to be a direct response to the presence of protists; a

likely defense mechanism to avoid predation (Corno and Jurgens, 2006; Flynn et al.,

2000; Jurgens et al., 1998). Most nanoflagellates graze by phagocytosis (Kinner et al.,

1998; Fenchel, 1987); elongation makes the bacteria too large for predators to consume

them. Often, bacteria that are unable to elongate become selectively preyed upon by

protists (Jurgens and Gude, 1994; Corno and Jurgens, 2006).

4.4.2 <u>Protists Present</u>

The protists present experiment was repeated twice: Run 1 lasted 51 days before it

had to be terminated due to extremely cold temperatures at the BBC site (i.e., it was no

longer possible to pump groundwater from BBC6). Run 2 lasted 100 days once

groundwater could be obtained again.

Run 1 (data not shown) lasted for 19 days prior to the injection of organic carbon

(~ 120 mg TOC/L). Neither TCE degradation nor progeny (cDCE, VC) generation

occurred. Within five days of organic carbon injection, 90% of the bacteria were

elongated (L:W ≈ 10:1). This morphology remained predominant until the termination of

the experiment at Day 51. There was no statistically significant increase in bacterial or protistan abundance when lactate was added (bacteria = $2.3 \times 10^6 \pm 8 \times 10^5$ cells/L to $7 \times 10^6 \pm 1.7 \times 10^6$ cells/L ($p = 0.05$), protists = 91.7 ± 85.2 cells/L to 163 ± 134.4 cells/L ($p = 0.05$)).

The first 55 days of Run 2 were at ambient TOC (< 10 mg C/L), while the remainder (45 days) had elevated lactate concentrations (110 – 120 mg TOC/L). There were problems controlling the TCE concentration in the reactors, with the measured concentration ranging from 2.2 – 7.2 μM, exceeding the desired concentration of 4.8 μM. This was initially addressed by reducing the amount of TCE in the refill water in an effort to lower the TCE concentration in the reactors. In spite of these problems, relevant data was obtained. The TCE concentration increased from ~ 2.7 μm to ~7 μm, while the cDCE and VC remained stable (Figure 4.1b). The bacterial abundance remained stable at $4.6 \times 10^6 \pm 8.5 \times 10^5$ cells/L; 100% of the bacteria were coccoidal. Protistan abundance remained stable at a mean of 60 ± 48.5 cells/L. The lack of cDCE or VC production again corroborated earlier findings that Site 32 is electron donor limited.

Days 55 to 100 modeled an organic carbon amended system (~ 120 mg TOC/L). The TCE concentration spiked to ~6.8 μM on Day 55, and rapidly declined after the amount of TCE in the refill water was again reduced (Figure 4.1b). The TCE concentration reached the target range (4 – 5 μM) on Day 80, however, rapidly increased to ~7 μM and then ~10 μM on Day 90. The increase in TCE was traced back to a leaking syringe and inadequate mixing time of the refill water. This was fixed by replacing the syringe, and modifying the refill water procedure to include a stir bar and allowing it to

mix at low speed for ~15 min prior to use. The cDCE and VC concentrations remained

stable, suggesting that no reductive dechlorination of TCE was occurring. The mean

bacterial abundance was ~5.5 x10^6 ±1.9x10^6 cells/L, and was not significantly different

(p = 0.05) from the bacterial abundance prior to organic carbon amendment. Five days

after adding the organic carbon amendment, > 90% of the bacteria were elongated (L:W

~ 10:1); a morphological change that continued for the remainder of the experiment

(Figure 4.4b). The mean protistan abundance was 152 ± 151.3 cells/L, however, due to

the variability and high standard deviation, this was not significantly different from the

abundance during the ambient TOC conditions (< 10 mg TOC/L). Within seven days of

organic carbon injection, the measured Eh was approximately -212 mV, indicating sulfate

reducing conditions predominated.

In contrast to the protists absent experiment where reductive dechlorination of

TCE occurred, but stalled at cDCE, no reductive dechlorination was observed when

protists were present. Because the same reactors, groundwater and environmental

conditions existed, the data suggested that the presence of protists prevented significant

dechlorination of TCE. This corroborates research by Lewis (2005) and computer

modeling by Travis and Rosenberg (1997), which suggested that protists inhibit TCE

biodegradation by direct grazing of cells.

Morphological changes in response to predation, similar to those we observed

(Figure 4.4 and 4.5), have been reported by Simek et al. (1997), Shikano et al. (1990),

and Jurgens et al. (1999) (though none of these studies involved TCE). It is unclear if the

morphological change observed in the bacterial community resulted from a change in the existing species, or if it was a newly dominant species. It is possible that the bacteria responsible for TCE biodegradation to cDCE elongated to avoid predation. However, with the exception of the first few days when protists were allowed to return during the protists absent experiment, elongated bacteria were not associated with TCE biodegradation. This suggested that the elongated cells observed were a newly dominant species and not an elongated form capable of dechlorination.

In balanced systems, (i.e., mature natural systems) predation and resource availability control prey composition and abundance (Power, 1992). It seems that the bacteria capable of TCE dechlorination were grazed significantly when protists were present, even when organic carbon was added, while other bacteria were able to maintain their population(s) by elongating.

It is interesting to note that TCE degradation did occur in the protists removed experiment when protists were allowed to return (Day 83, Figure 4.3), while no degradation occurred when protists were present throughout the entire experiment. This is likely because in the protists absent experiment, the presence of organic carbon, suitable electron donor (TCE), and no predation pressure created ideal conditions for dechlorinating species to become dominant and acclimated. The increased predation pressure caused by the introduction of protists likely selected for bacteria able to elongate. The total chlorinated ethene data suggest these bacteria were not able to dechlorinate TCE even though higher concentrations of organic carbon were present.

Conversely, the high initial abundance of dechlorinators before protists were introduced likely resulted in sufficient members being present so that they were able to completely degrade the TCE spike on Day 85 before being removed from the system by predation. We hypothesize that the bacterial community for Days 90 – 100 in the protists removed was similar to that in the protists present experiment, for if the protists absent experiment had been allowed to run longer and another TCE spike occurred, we hypothesize that results similar to the protists present experiment (i.e., no TCE dechlorination) would have been obtained.

4.4.3 KB-1 Amended and Protists Present

For Days 0 to 30, the organic carbon concentration was ambient (< 10 mg TOC/L) and indigenous bacteria were the only ones present along with protists. No significant changes in TCE, cDCE or VC were observed. The mean bacterial abundance was $2.5 \times 10^6 \pm 1.8 \times 10^6$ cell/L (Table 4.2), with 100% coccoidal bacteria. The mean protistan abundance was 355 ± 166 cells/L. Once again, the reactor was electron donor limited (i.e., Site 32 *in situ* conditions prevailed).

KB-1 and sodium lactate were added on Day 30; the resulting organic carbon concentration was ~120 mg TOC/L (Figure 4.1c). Within three days of injecting the amendments, TCE concentrations declined significantly and approached zero, while cDCE concentrations increased from ~ 2 μM to a peak of 7 μM. The concentration of cDCE remained constant from Days 35 to Day 50, and then began to steadily decrease,

approaching the detection limit (2 μg/L) on Day 80. An increase in VC from ~0.5 μM to ~2 μM began on Day 58, and corresponded with the decrease in cDCE concentration. The VC concentration reached a peak of ~2.2 μM on Day 70, and decreased to the detection limit (2 μg/L) on Day 82. Total chlorinated ethenes remained constant at 8.6 ± 1.08 μM until Day 71 when they rapidly decreased. The data suggested that TCE was being sequentially degraded to VC and ethene through reductive dechlorination and likely mineralized to CO_2. The cDCE biodegradation rate was calculated (Table 4.1) using the period when DCE was actively being degraded. ~10 days elapsed between the peak cDCE concentration and the start of cDCE degradation. Although VC reduction occurred, we were unable to calculate the reaction rate constant because degradation was so rapid that insufficient data was collected. Once degradation was first observed (~ Day 31), half-lives for TCE, cDCE and VC were ~ 4 days, ~25 days, and ~15 days, respectively. These values fall within the range of reported half-lives of dehalogenation of chlorinated ethenes using KB-1 (Table 4.3).

A mean Eh of -220 mV was obtained within seven days of organic carbon injection and remained there until the end of the experiment, indicating that sulfate reducing conditions predominated through the remainder of the experiment.

The mean bacterial abundance during this period was 11.5 x10^6 ± 1.9 x 10^6 cells/L, significantly greater (p = 0.05) than at any other point in this study (Figure 4.2c). This was most likely because the KB-1 culture provided a large initial increase in total

numbers. Within three days of the organic carbon and KB-1 amendments, 100% of the
bacteria had shifted to an elongated morphology (L:W ~ 10:1) (Figures 4.4c and 4.5c).

Protistan abundance increased significantly in response to the increased bacterial
numbers (734.5 ± 211.6 cells/L, $p = 0.05$). However, on Day 60, the protistan abundance
began to decrease (< 20 cells/L by Day 65), and the bacteria reverted to the coccoidal
form. The decrease in protistan abundance in the reactors coincided with an increase in
VC concentration. VC is toxic to many eukaryotic species, and it is possible that protistan
abundance was reduced as the VC concentration increased (Euro Chlor, 1999).

Table 4.3: Summary of Anaerobic Biodegradation Half-Lives in the Literature (days)

Reference	Matrix	TCE	cDCE	VC
GeoSyntec Whitepaper on KB-1 Cox et al, (2002)	Groundwater with KB-1	~25	~35	~5
Yager et al. (1997)	Bottlecosm with Petroliferous Dolomite	27 – 67	NA	NA
Byl and Williams (2000)	Bottlecosm with Karst (TN)	4	NA	NA
Castellanos et al. (2003)	Microcosm Sterile Control	> 500	> 500	> 500
Castellanos et al. (2003)	Microcosm Electron Donor Amended	> 500	203	> 500
Castellanos et al. (2003)	Microcosm KB-1 Amended	4	15	39
Castellanos et al. (2003)	Groundwater	331	> 500	> 500

It is clear that the amendment of KB-1 resulted in a significantly faster conversion of TCE to cDCE than was observed with the indigenous bacteria in the absence of protists (Table 4.3). This was likely due in part to the large abundance of dechlorinators present in the KB-1. Detectable conversion of TCE to cDCE and beyond occurred when the bacteria were in the coccoidal form, further supporting the argument that the elongated bacteria were not capable of significant biodegradation of chlorinated ethenes. The transformation of cDCE to VC slowed once the bacterial morphology shifted to the elongated form. Only when the coccoidal morphology predominated again ~15 days later was VC fully degraded. It is also possible that the rate of chlorinated ethene biodegradation by the elongated bacteria was too low to be detected in this experiment.

PCR analysis of 4 reactor samples was conducted by the United State Geological Survey (USGS) (Voytek, 2007). It indicated that two days after the injection of KB-1, the bacterial community consisted primarily of *Geobacter spp.* and *Dehalococcoides ethenogenes*. By five days (i.e., during the cDCE stall and after the bacterial elongation), the composition had shifted primarily to species in the gamma-*Proteobacterium* genus, with little *Geobacter* and no detectable *Dehalococcoides ethenogenes*. Gamma-*Proteobacterium* has been previously detected in BBC6 groundwater (Naser, 2005), and a literature review yielded no published instances where they were found to be capable of reductive dechlorination. In addition, gamma-Proteobacterium was also reported as elongating under heavy predation in Simek et al. (1997). *Geobacter* spp. has been identified as only capable of degrading TCE to cDCE, while *Dehalococcoides*

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ethenogenes has been shown to fully dechlorinate TCE to ethene (Lu and Kampbell, 2006). We hypothesize that protistan grazing probably placed a strong top-down predation force on the bacterial community, and species that were unable to elongate (i.e., *Geobacter spp., Dehalococcoides ethenogenes*), were grazed to extremely low levels, thus severely limiting biodegradation of TCE and its progeny in the reactors. gamma-*Proteobacterium*, present in the groundwater being injected into the reactors, likely became dominant simply because they have a adequate method to avoid predation. The samples collected for analysis likely contained only unattached bacteria, and thus probably did not include any attached bacteria that existed in the reactors. While it appears that *Dehalococcoides ethenogenes* and *Geobacter spp.* were removed by grazing, it is possible that they attached to the reactor surface to avoid predation or improve mass transfer kinetics. Little research has been done on the ability of dechlorinators to attach to surfaces, and it is unclear if they have this ability. This needs to be investigated.

It is unlikely that protistan predation was able to totally eliminate *Dehalococcoides ethenogenes* from the reactors because protists do not actively "hunt" for bacteria, but rather graze (Corno and Jurgens, 2006; Jurgens et al, 1998). It is also possible that *Dehalococcoides ethenogenes* has the ability to attach to surfaces, significantly decreasing its susceptibility to grazing. This ensures that when the predation pressure is reduced, and if conditions are favorable, *Dehalococcoides ethenogenes* could increase in abundance and potentially degrade cDCE or VC, as was observed in this experiment. The rapid increase in VC concentration on Day 70 and decrease in protistan abundance coincided with the change in morphology from elongated to coccoidal

bacteria. This suggests that once predation pressure was reduced, *Dehalococcoides ethenogenes* was able to out-compete gamma-Proteobacterium and become dominant again, while rapidly converting cDCE to VC, VC to ethene, and likely ethene to CO_2.

4.4.4 Impact of Protists on TCE Biodegradation

When the indigenous bacterial community was present, even with adequate electron donor, protists appeared to completely inhibit dechlorination of TCE to cDCE. When protists were removed TCE was degraded to cDCE, but stalled there. When KB-1 and adequate electron donor were present, TCE dechlorination to VC was possible. This dichotomy was probably due to differences in the initial bacterial species distribution and abundance: the KB-1 amendment had a very high abundance of *Geobacter* spp. and *Dehalococcoides ethenogenes*, while the indigenous community likely had only a few, if any (Naser, 2003). In both cases, the protists likely grazed the bacteria, selecting for species able to elongate. The KB-1 amendment appeared to accomplish full conversion of TCE to cDCE prior to the attenuation of the dechlorinators. Complete dechlorination of TCE appeared to only occur under certain circumstances; high TOC, protists absent, and dechlorinating bacteria present (Figure 4.6).

Visual inspection of the bacteria using epifluorescence microscopy indicated that the elongated form of bacteria were identical in all three experiments. Protistan predation probably acted as a strong top-down force which selected against dechlorinating bacteria either through direct grazing, or through grazing of other species that are required for

reductive dechlorination to occur (e.g., fermenters). Protists select prey based upon numerous factors including: cell size, surface charge, morphology, and motility (Chrzanowski and Simek, 1990; Griebler et al., 2002; Pfandl et al., 2004; Young, 2006). Bacteria from Site 32 range in size from 0.2 to 0.8 µm, and are within the preferred size range for nanoflagellates (2 – 4 µm) (Kinner et. al., 1998; Lewis, 2005). By elongating to 1 – 10 µm, the bacteria were too large to be consumed by the protists, and thus avoided predation. Species that were unable to elongate (e.g., dechlorinators), were probably heavily grazed. It is interesting to note that bacterial elongation only occurred when protists were present and organic carbon was amended. Elongation is energetically unfavorable, and requires additional energy that may not be available under *in situ* organic carbon concentrations. It is possible that the organic carbon amendment allowed the bacteria to produce enough energy so that elongation became a viable option to avoid predation. If predation pressure had been present for a long enough time in the reactors, the composition and abundance of the bacterial community in all three experiments might have been similar, with dechlorinators excluded in favor of species with adequate predation defense mechanisms. Normally, as the bacterial community shifts to elongated bacteria, the protistan community responds with an increased abundance of larger species or cells that are able to graze the elongated bacteria (Jürgens et al., 1998; Geradi, 1990). However, in the subsurface, pore size restrictions and colloidal filtration limit the maximum protistan cell size to 2 – 4 µm; thus elongated bacteria would not be grazed by larger protists (Eighmy et al., 2007; Becker, 2003). Hence, the elongated bacteria were essentially un-regulated by top-down forces.

Duhamel and Edwards (2007) stated that a change in bacterial species is required for complete degradation of TCE to ethene, while Becker (2006) concluded that competition between bacteria for resources (i.e, electron donors) dictates the final community composition. Our results suggested that when *Dehalococcoides ethenogenes* is part of the initial bacterial community composition at Site 32 and organic carbon amendment is injected, complete reductive dechlorination may occur if TCE degraders have an effective method to combat predation. Otherwise, they will probably be grazed from the system, rendering energetics and intraspecific competition moot.

Little is known about the attachment capabilities of dechlorinators, specifically *Dehalococcoides ethenogenes*. Attachment to fracture surfaces offers many potential benefits, including increased electron donor/acceptor flux, as well as significantly reduced predation. The microbiological techniques used in this study focused on suspended bacteria, and therefore the conclusions reached probably do not apply to bacteria that are able to attach. If dechlorinators could be made to attach, the potential exists for significantly higher biodegradation rates, in part because dechlorinators could exist with little predation pressure. Further research is required to fully evaluate the attachment abilities of dechlorinators, and how predation influences the success of such systems.

Protists Absent

Days 0 - 26
Low TOC
No TCE - cDCE Conversion
No cDCE - VC conversion
No VC - ethene conversion
Protists Absent
No Elongation

Days 26 - 60
High TOC
TCE being converted to cDCE
No cDCE - VC conversion
No VC - ethene conversion
Protists Absent
No elongation

Days 60 - 83
High TOC
TCE converted to cDCE
No cDCE - VC Conversion
No VC - Ethene Conversion
Protists Absent
No elongation

Days 83 - 90
High TOC
TCE Spike converted to cDCE
No cDCE - VC conversion
no VC - ethene conversion
Protists Increasing
% of elongated Bacteria Increasing

Days 90 - 100
High TOC
No Detectable TCE Conversion
No cDCE - VC Conversion
No VC - Ethene Conversion
Protists Present
100% Elongated Bacteria

Partial biodegradation
TCE -> cDCE

Protists Present

Days 0 - 55
Low TOC
No TCE - cDCE Conversion
No cDCE - VC Conversion
No VC - ethene conversion
Protists Present
No Elongation

Days 55 - 60
High TOC
No TCE - cDCE Conversion
No cDCE - VC Conversion
No VC - Ethene Conversion
Protists Present
% of Elongated Bacteria Increasing

Days 60 - 100
High TOC
No TCE - cDCE Conversion
No cDCE - VC Conversion
No VC - Ethene Conversion
Protists Present
100% Elongated Bacteria

No Biodegradation
of TCE

KB-1 + Protists Present

Days 0 - 30
Low TOC
No TCE - cDCE conversion
No cDCE - VC conversion
No VC - ethene conversion
Redox: + 20 mV
Low Abundance of Dehalococcoides
Low Abundance of Geobacter
Low Abundance of Gamma Proteobacterium
Protists Present
No Elongation

Days 30 - 35
High TOC
TCE converted to cDCE
No cDCE - VC conversion
No VC - ethene conversion
Redox: 272 mV
KB-1 Amended
High Abundance of Dehalococcoides
High Abundance of Geobacter
Low Abundance of Gamma Proteobacterium
Protists Present
< 20% Elongation

Days 35 - 50
High TOC
All TCE converted to cDCE
Small amount of cDCE conversion
No VC - ethene conversion
Redox: 212 mV
Low Abundance of Geobacter
Dehalococcoides Undetectable
High Abundance of Gamma Proteobacterium
Protists Present
100% elongation

days 50 - 65
High TOC
All TCE converted to cDCE
Small amount of cDCE conversion
VC concentration increasing
Redox: 212 mV
Protistan Abundance Decreasing
% of Elongated Bacteria Decreasing

Days 65 - 100
High TOC
All TCE converted to cDCE
All cDCE converted to VC
All VC converted to ethene
Redox: 212 mV
Low Abundance of Geobacter
High Abundance of Dehalococcoides
Low Abundance of Gamma Proteobacterium
Protists Absent
0% Elongation

Complete Mineralization
TCE => CO2

Figure 4.6: Summary of Conditions Present and Results of Protists Absent, Present, and KB-1 + Protists Experiments

Although it is assumed that the bulk of the degradation observed in this experiment was due to dehalorespiration, it is possible that co-metabolism was responsible for some of the reduction in TCE concentration. Although specific species, such as *Dehalococcoides ethenogenes,* would become less important to the degradation of chlorinated ethenes under a co-metabolism dominated system, the basic conclusions in this experiment would remain unchanged as predation could influence dehalorespiration and co-metabolism in a similar manner.

It is likely that the success of microbially-mediated reductive dechlorination as a treatment method in fractured bedrock and other aquifers where protists are present is highly dependant on the composition of the microbial community, how it is regulated (i.e., resource controlled or predation controlled), and at what threshold the bacterial community reacts to protistan grazing. In fractured bedrock systems, the depth, size of fractures, and presence of toxic compounds (e.g., VC) may all play a role in controlling protistan predation on bacteria, and may partially explain the successes and failures of bioremediation in these systems. Our results clearly show that the presence of protists can affect microbially-mediated reductive dechlorination, and that protistan predation as a top-down force may prevent the success of bioaugmentation with amendments such as KB-1. It is likely that when protists are present and bioaugmentation is employed, the bulk of the degradation occurs within the first few days, prior to the dechlorinators being grazed to very low levels. In order to achieve complete dechlorination, it may be necessary to continually introduce *Dehalococcoides ethenogenes* to replace cells lost by grazing, or to limit grazing altogether. It is possible, for example, that the transitory

presence of VC at modest concentrations may inhibit or eliminate protists. Research

needs to be done to not only identify what regulates the protistan community, but whether

predation can be controlled or eliminated. It appears that if predation could be

significantly reduced, the addition of a dechlorinating culture such as KB-1 in

combination with a suitable electron donor, has the potential to very rapidly dechlorinate

TCE without stalling. Further inquiry is needed in order to determine how the presence of

protists initiates a morphological shift in the bacterial community, and how the presence

and relative abundance of dechlorinators, including *Dehalococcoides ethenogenes,*

changes with a top-down predation.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

At the beginning of this research, four questions were posed that can now be answered:

1) How is the biodegradation rate of TCE influenced by the presence of protists under ambient and organic carbon amended systems?

The presence of protists clearly inhibits biodegradation of TCE under both ambient and amended organic carbon conditions. This is probably due to protistan grazing on dechlorinating bacteria.

2) Does the presence of protists influence the occurrence of stalls and their duration?

Protistan grazing on dechlorinators (e.g., *Dehalococcoides ethenogenes, Geobacter spp.*) appeared to directly cause stalls. Such selective grazing could prevent further dechlorination of TCE and its progeny because bacteria capable of dechlorination of cDCE-VC and VC-ethene were not sufficiently abundant. Protistan grazing on fermenters could also cause stalls by limiting the amount of preferred electron donor (H_2) reaching the dechlorinating bacteria.

3) How does the presence of protists influence the abundance and composition of the bacterial community?

Protists did not appear to influence the abundance of the bacterial community in *in situ* or organic carbon amended conditions. However, the

presence of protists did appear to impact the bacterial community composition, and grazing-resistant species became dominant, seemingly be elongation.

4) How does the presence of protists influence the TCE degradation rate, bacterial abundance and community structure in a bioaugmented system?

The presence of protists did not appear to significantly impact the bacterial abundance in a bioaugmented system, however, they seemed to significantly impact the bacterial community composition, favoring a grazing-resistant consortium that had a lower TCE biodegradation rate.

The results of this study indicate that the presence of protists in an organic carbon amended fractured bedrock system can inhibit reductive dechlorination of TCE. The presence of protists resulted in elongation of the bacteria. While in the elongated form, no significant reduction of TCE, or production of cDCE was observed, suggesting reductive dechlorination was not occurring. Similar results were observed when a high-density *Dehalococcoides ethenogenes* dominated culture was injected; reductive dechlorination and mineralization only occurred at low protistan abundance. The results of this study suggest that TCE-contaminated systems undergoing enhanced bioremediation may stall at cDCE or VC despite the initial presence of *Dehalococcides ethenogenes*. Although *Dehalococcides ethenogenes* were present prior to organic carbon injection, after organic carbon injection their abundance was reduced to non-detectable levels, while other species were able to thrive. This suggests that a community shift occurred and *Dehalococcides ethenogenes* was competitively excluded. The likely cause was predation, suggesting that to produce an environment where *Dehalococcides ethenogenes*

is able to reach levels capable of fully mineralizing TCE's progeny to ethene and CO_2, it may be necessary to inhibit protistan predation. This could be accomplished through removal of protists from the system, or forcing a chemical or physical change in the dechlorinating bacteria that make them unappealing to protists. It is unclear if this is possible, or feasible, and more research is required. Little is known about the attachment capabilities of dechlorinators, specifically *Dehalococcoides ethenogenes*. Attachment offers many benefits, including increased electron donor/acceptor flux, as well as significantly reduced predation. If dechlorinators could be made to attach, the potential exists for significantly higher biodegradation rates. Further research is required in this area.

Further research is needed to fully examine the role of protistan predation in bioremediation. The results of this study suggest that predation has an effect, however, it does not identify the threshold at which predation becomes limiting, nor does it identify what role predation has in controlling bacterial species distribution. We hypothesize that protistan predation, either directly or indirectly, leads to a change in the composition of the bacteria community, with *Dehalococcides ethenogenes* and other species capable of reductive dechlorination diminished in significance via grazing pressure. To test this hypothesis, it would be useful to repeat this experiment and use molecular biology techniques to identify what bacterial and protistan species are present and their relative abundance, and include a analysis of the total bacterial community (i.e., including attached cells).

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APPENDICES

APPENDIX A: DATA GRAPHS

TCE, cDCE, and Vinyl Chloride Concentrations vs Time with Protists Removed in Reactor 1
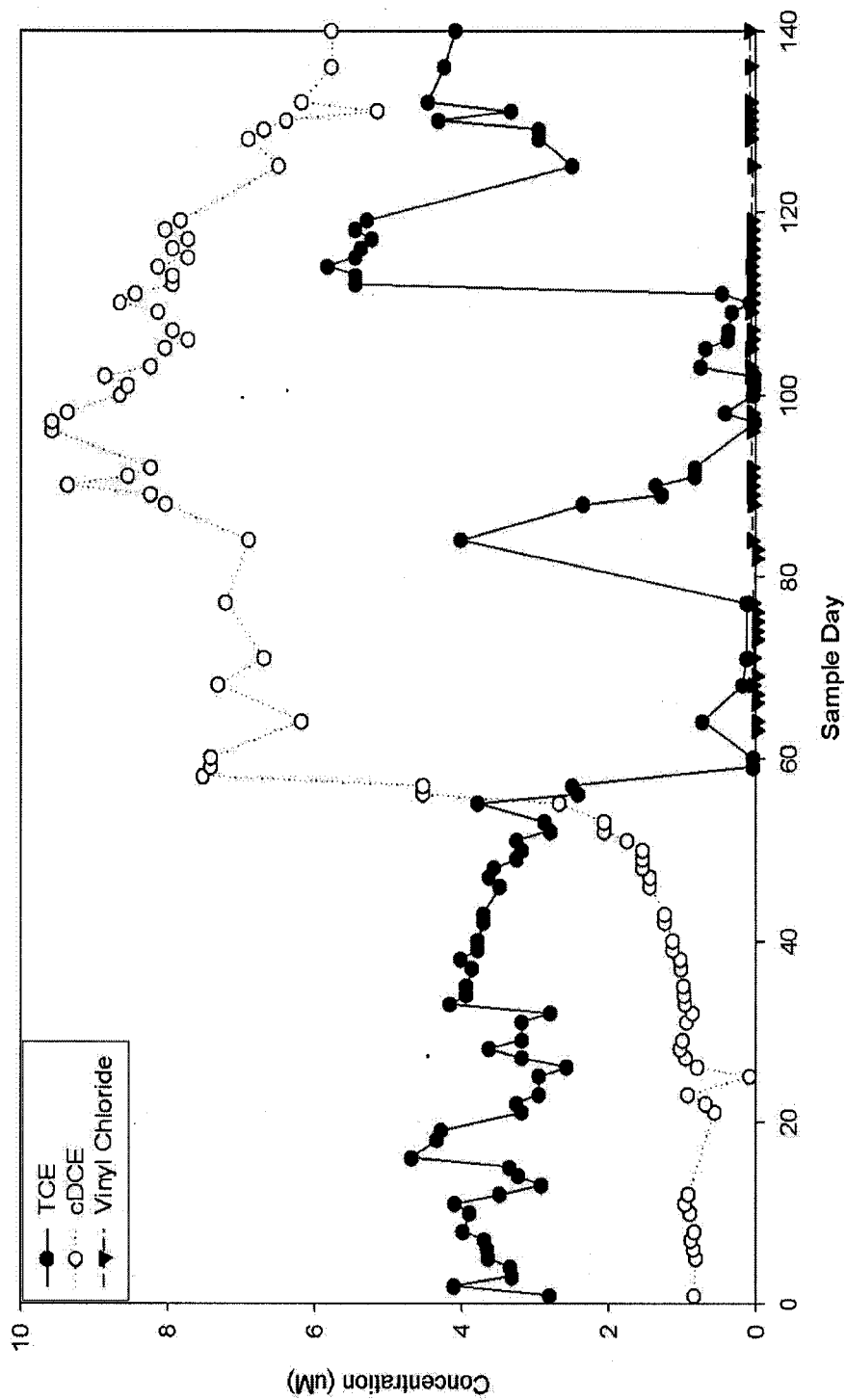
104

TCE, cDCE, and Vinyl Chloride Concentrations vs Time with Protists Removed in Reactor 2

105
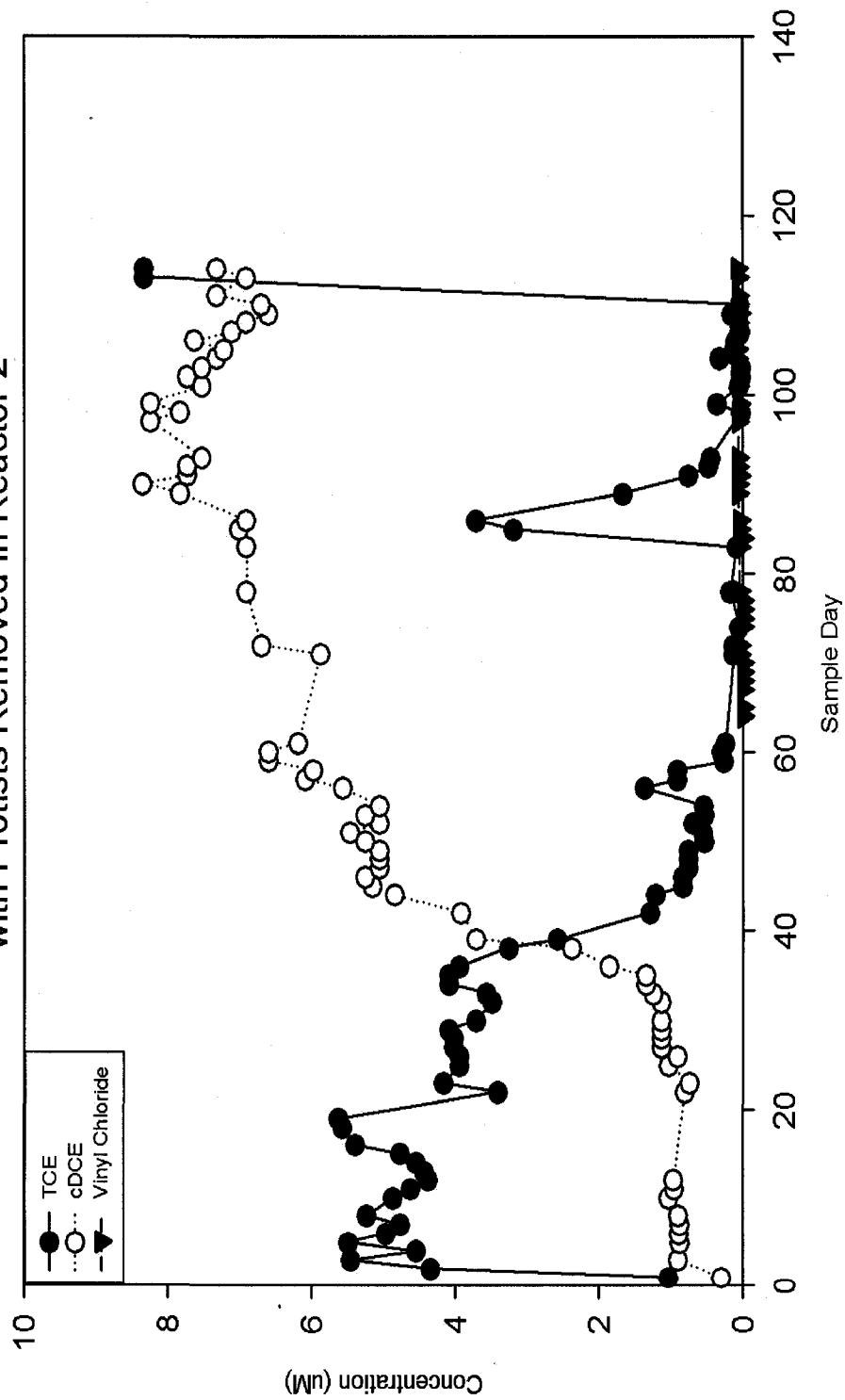
Bacteria Abundance vs Time with Protists Removed, Reactor 1
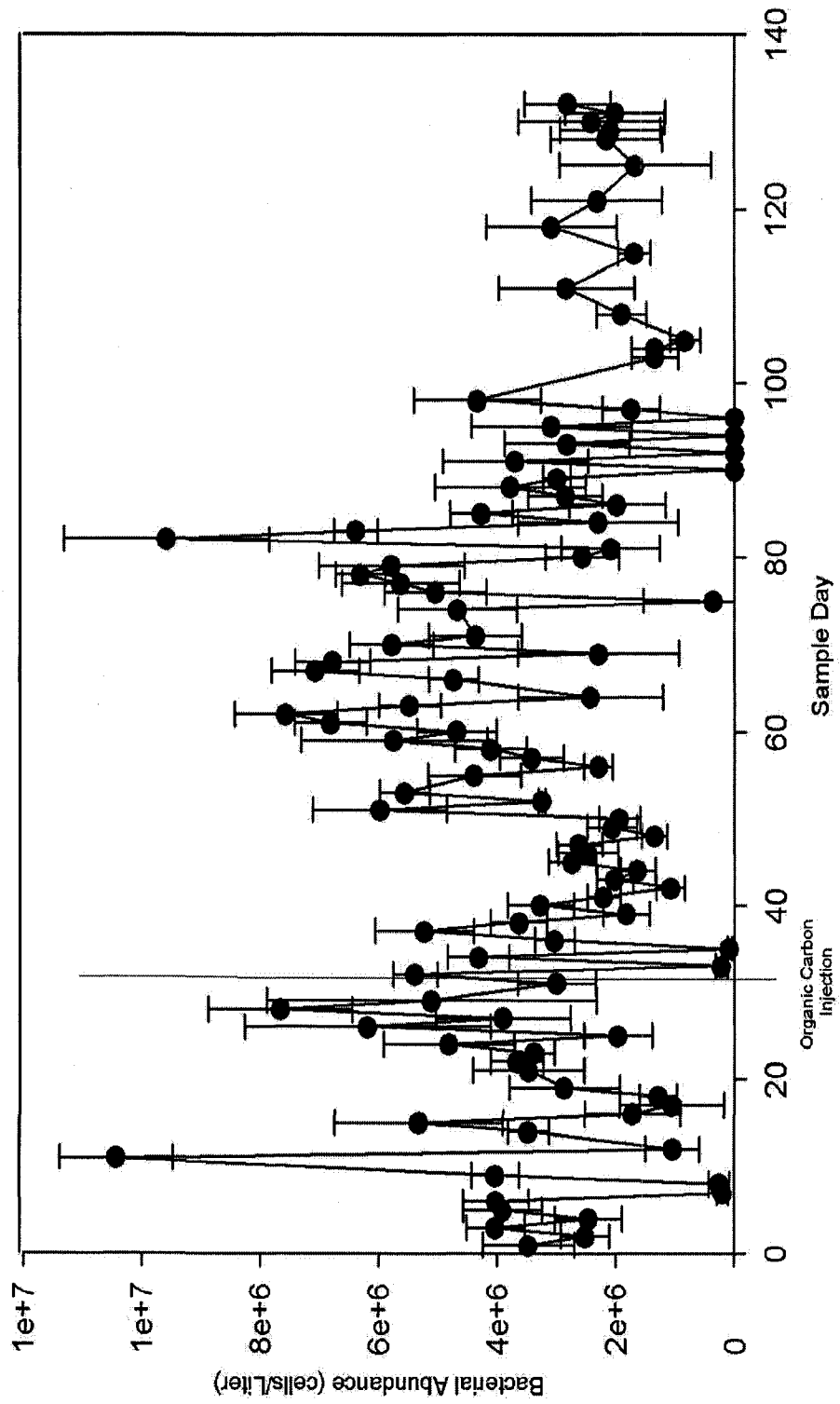
Bacterial Abundance vs Time with Protists removed in Reactor 2
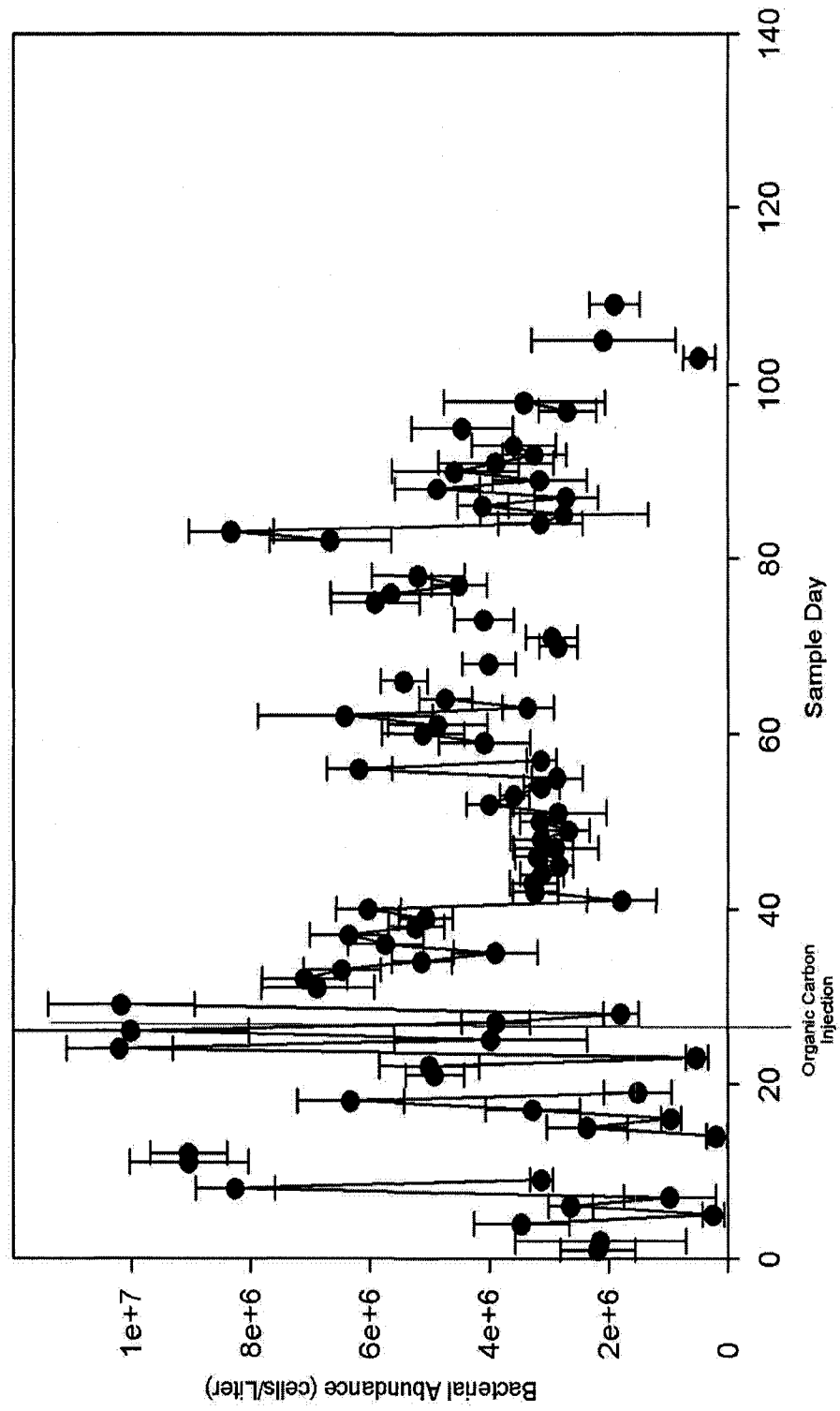
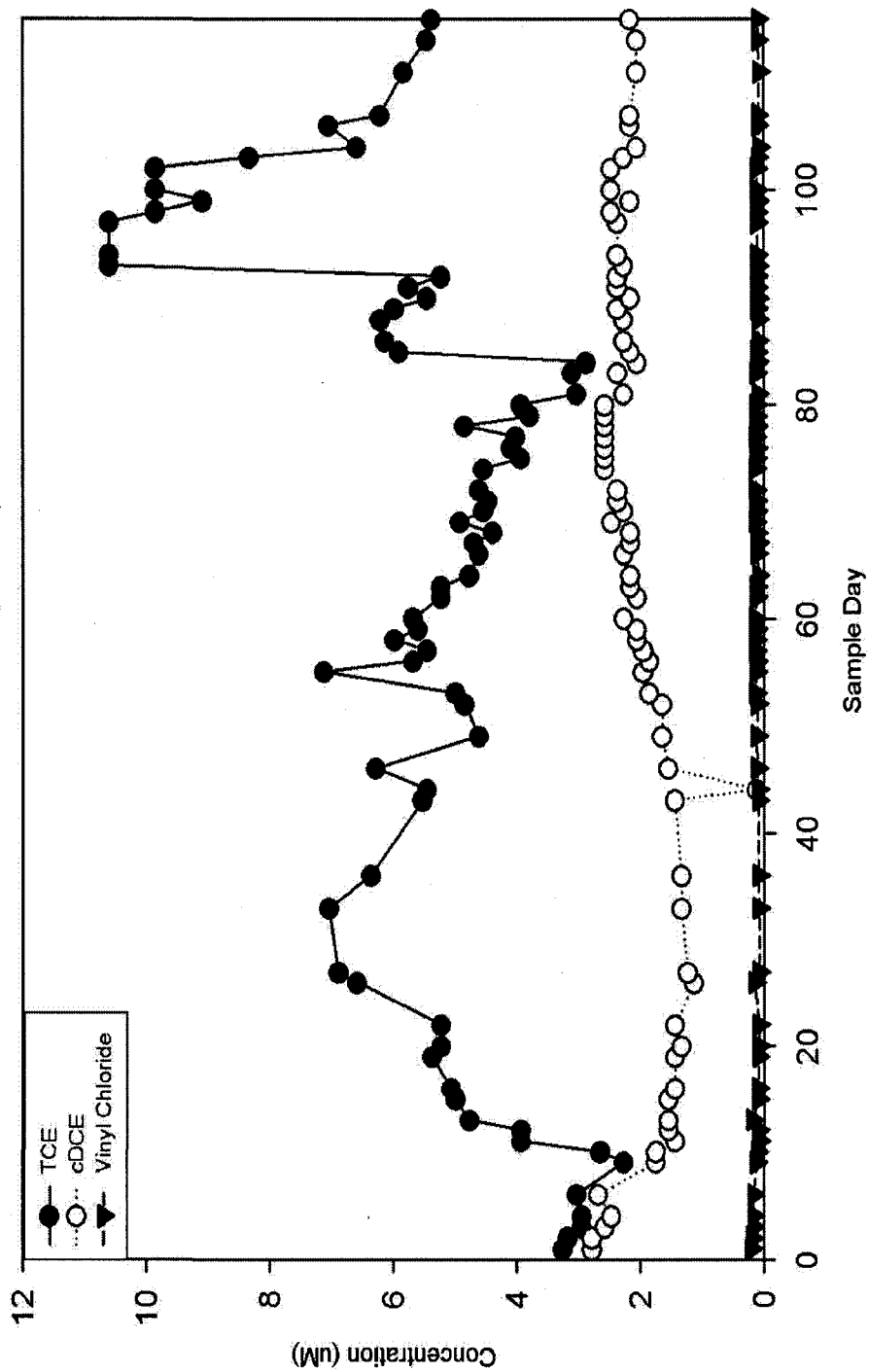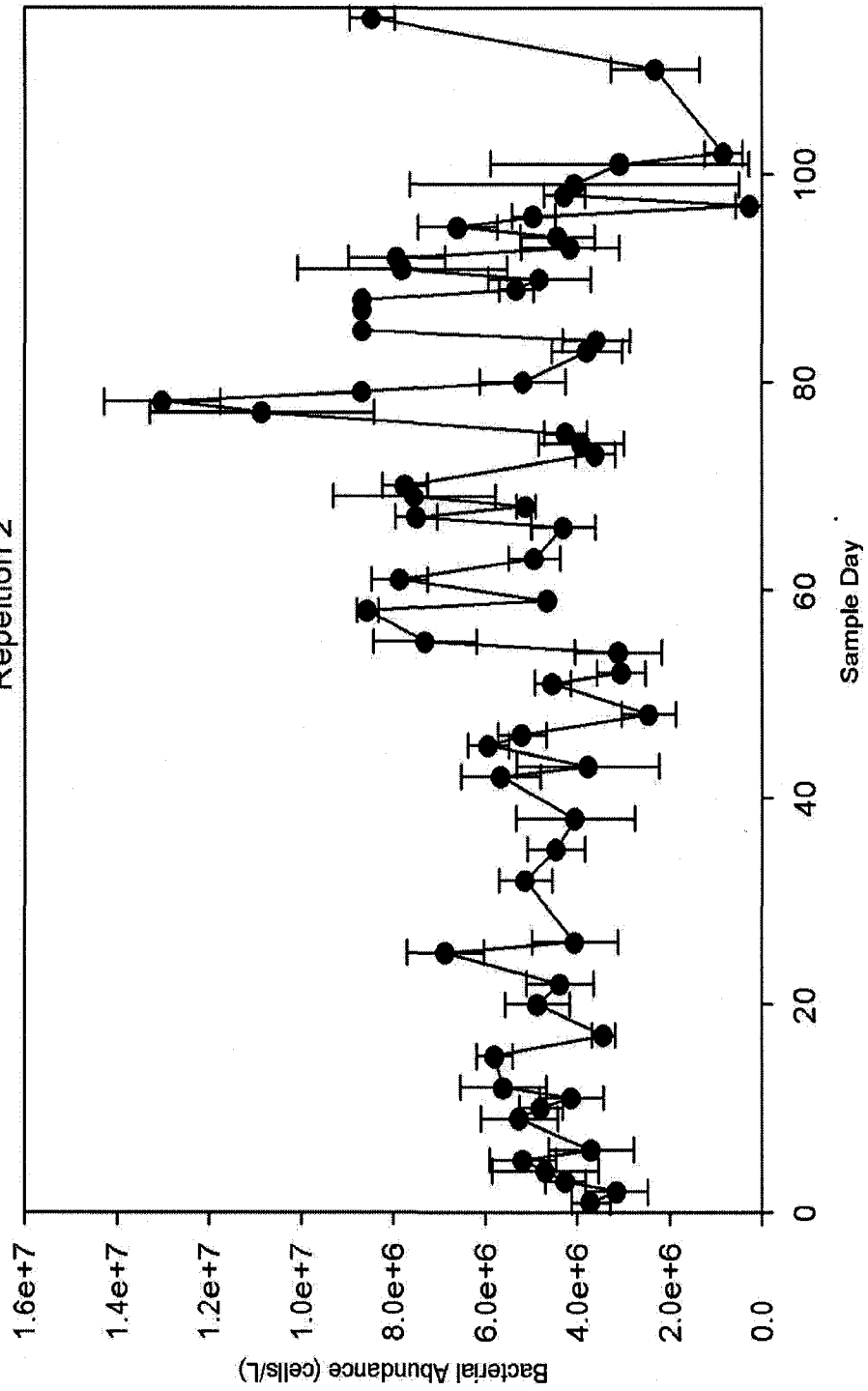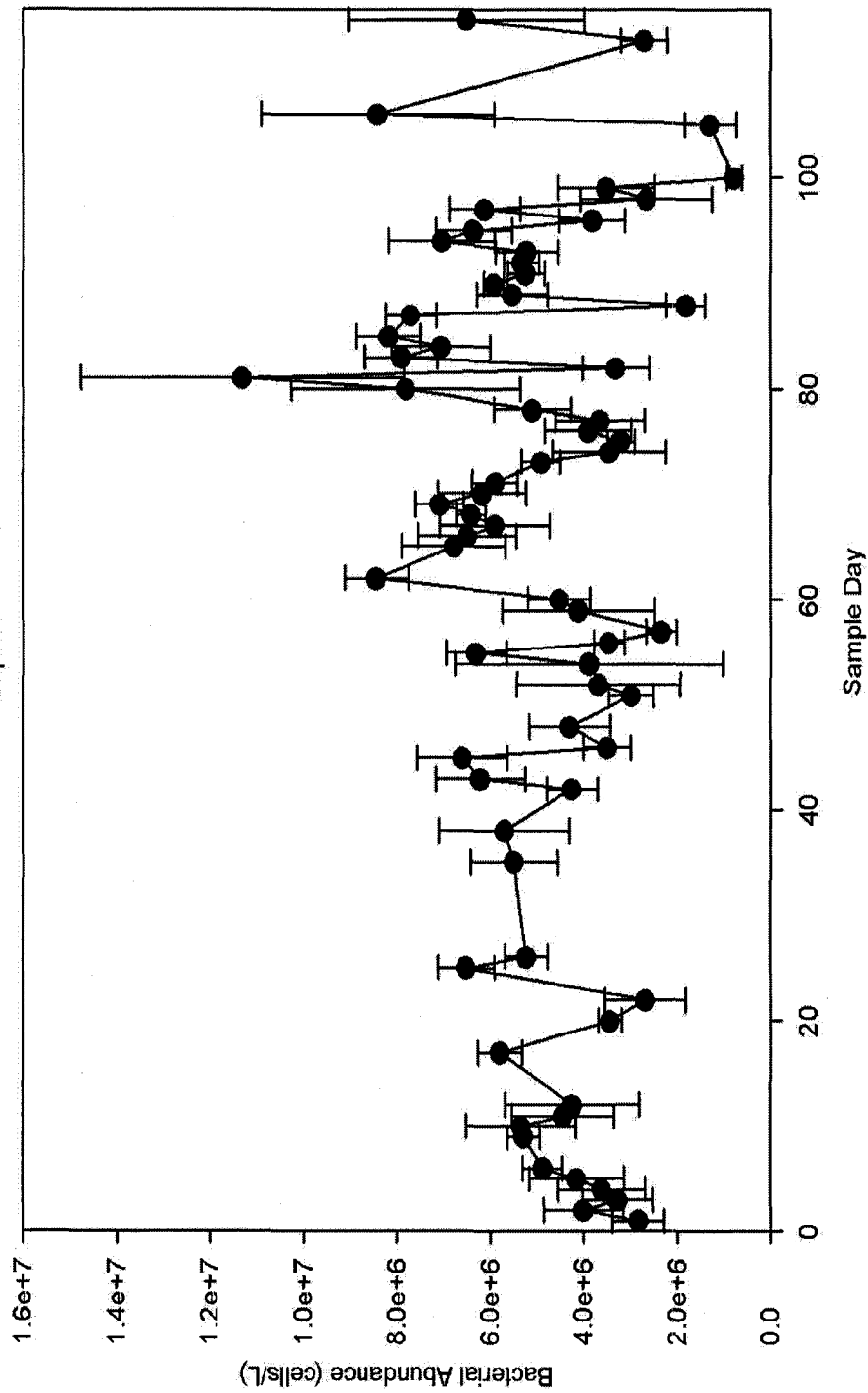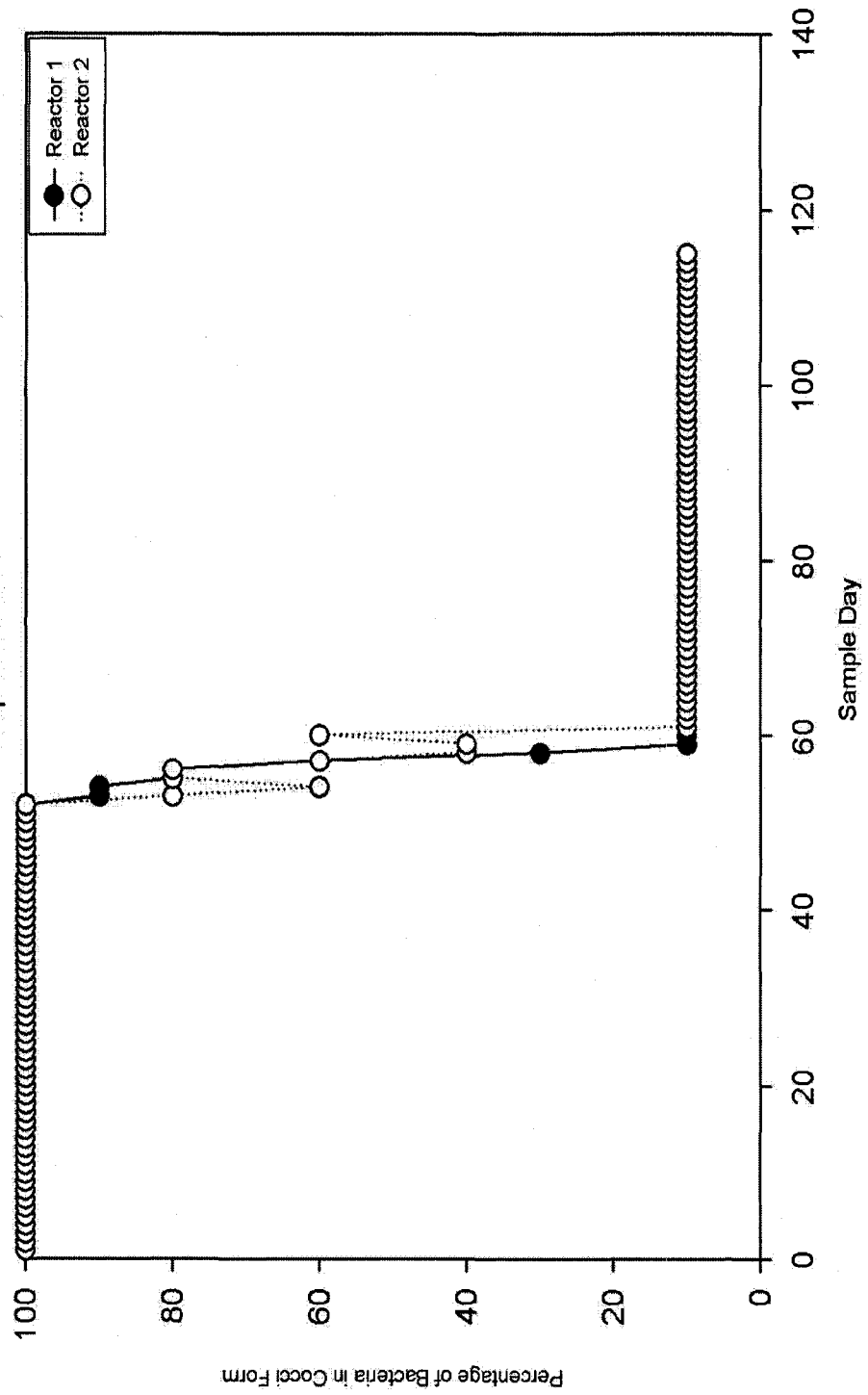Protistian Abundance vs Time with Protists Removed in Reactor 1
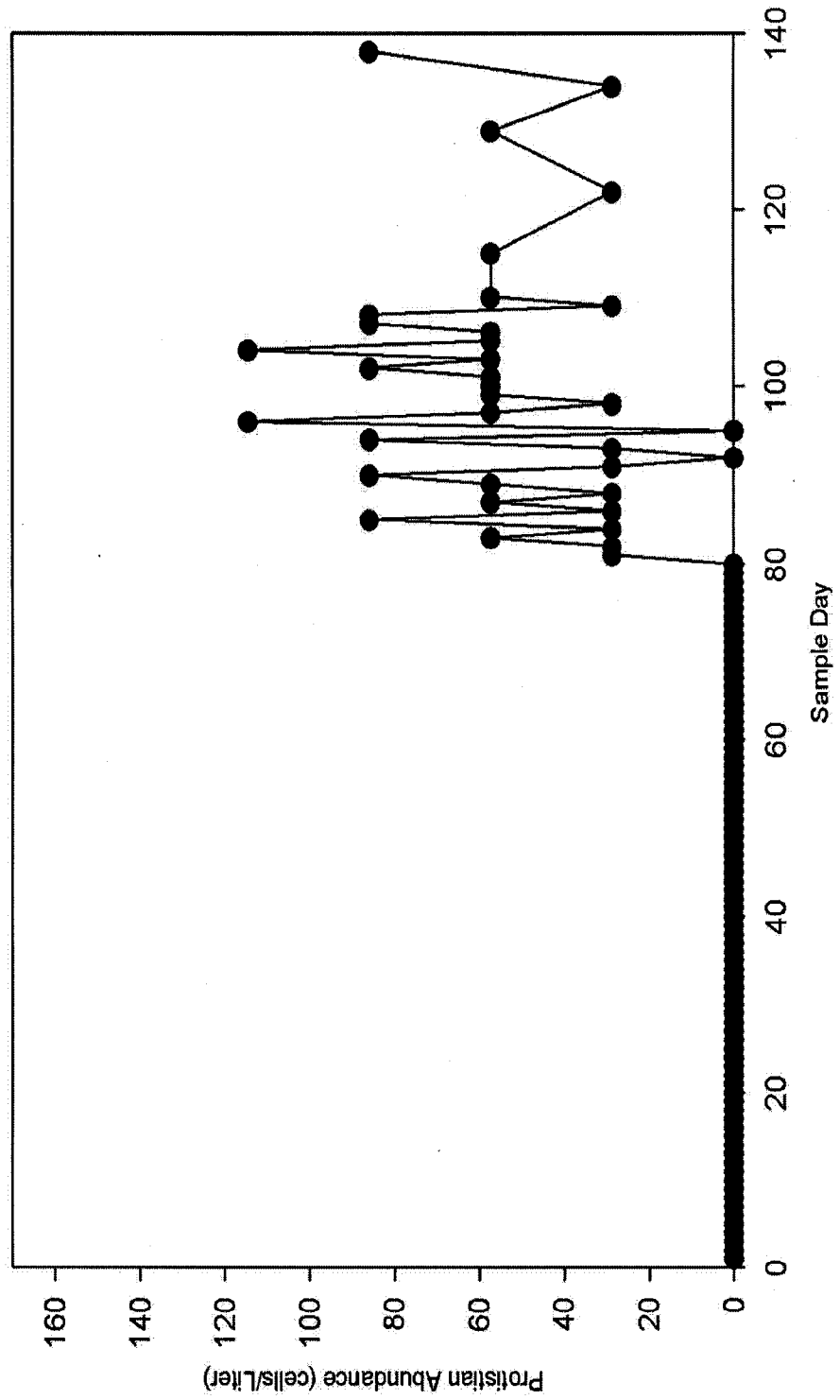
Protistian Abundance vs Time with Protists removed in Reactor 2

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Percentage of Bacteria in the Cocci Form vs Time with Protists removed in Reactor 1

Protists Allowed to Return

Sample Day
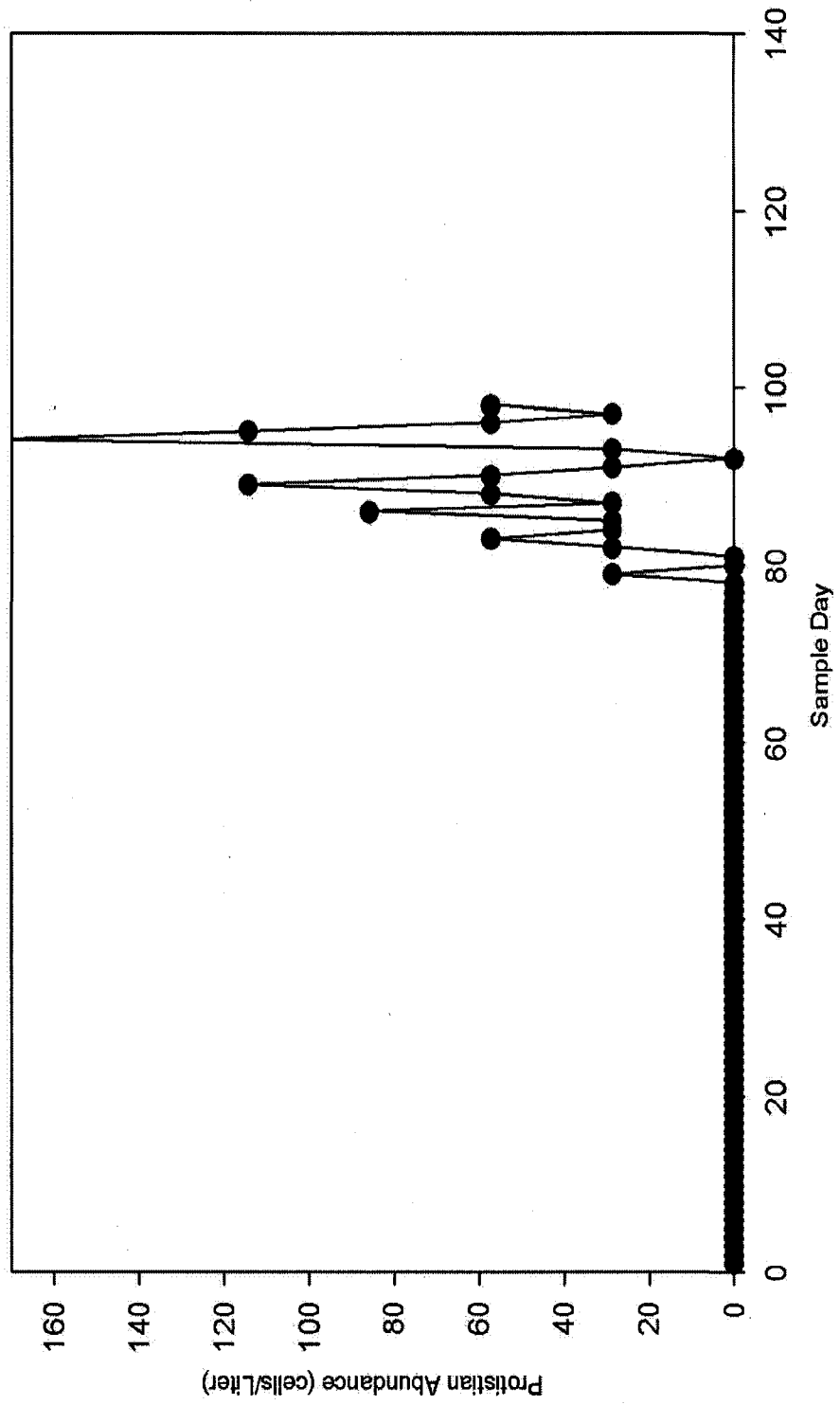
Percentage of Bacteria in the Cocci Form
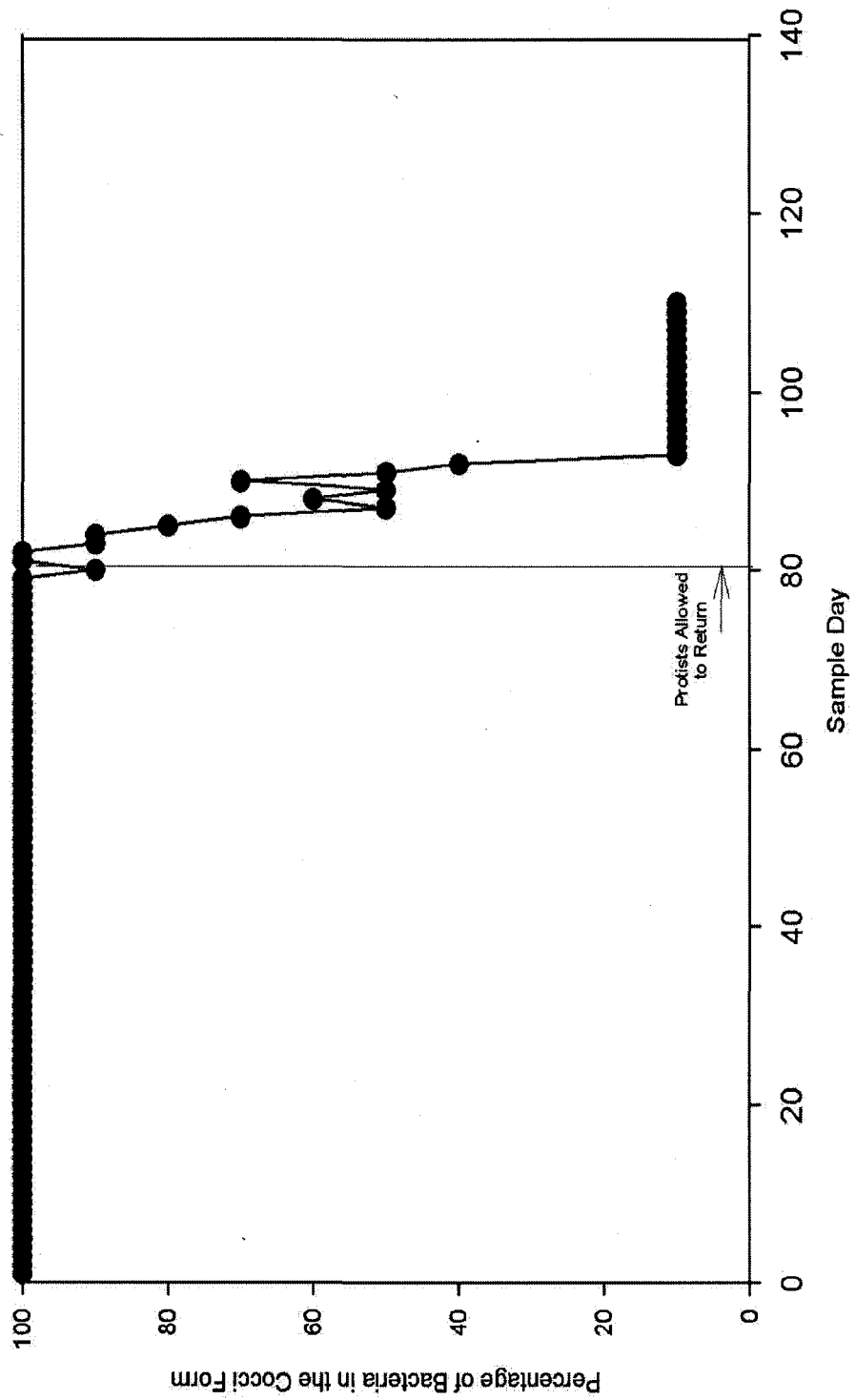
Percentage of Bacteria in the Cocci Form vs Time with Protists Removed in Reactor 2

Total Organic Carbon Concentration vs Time with Protists Removed
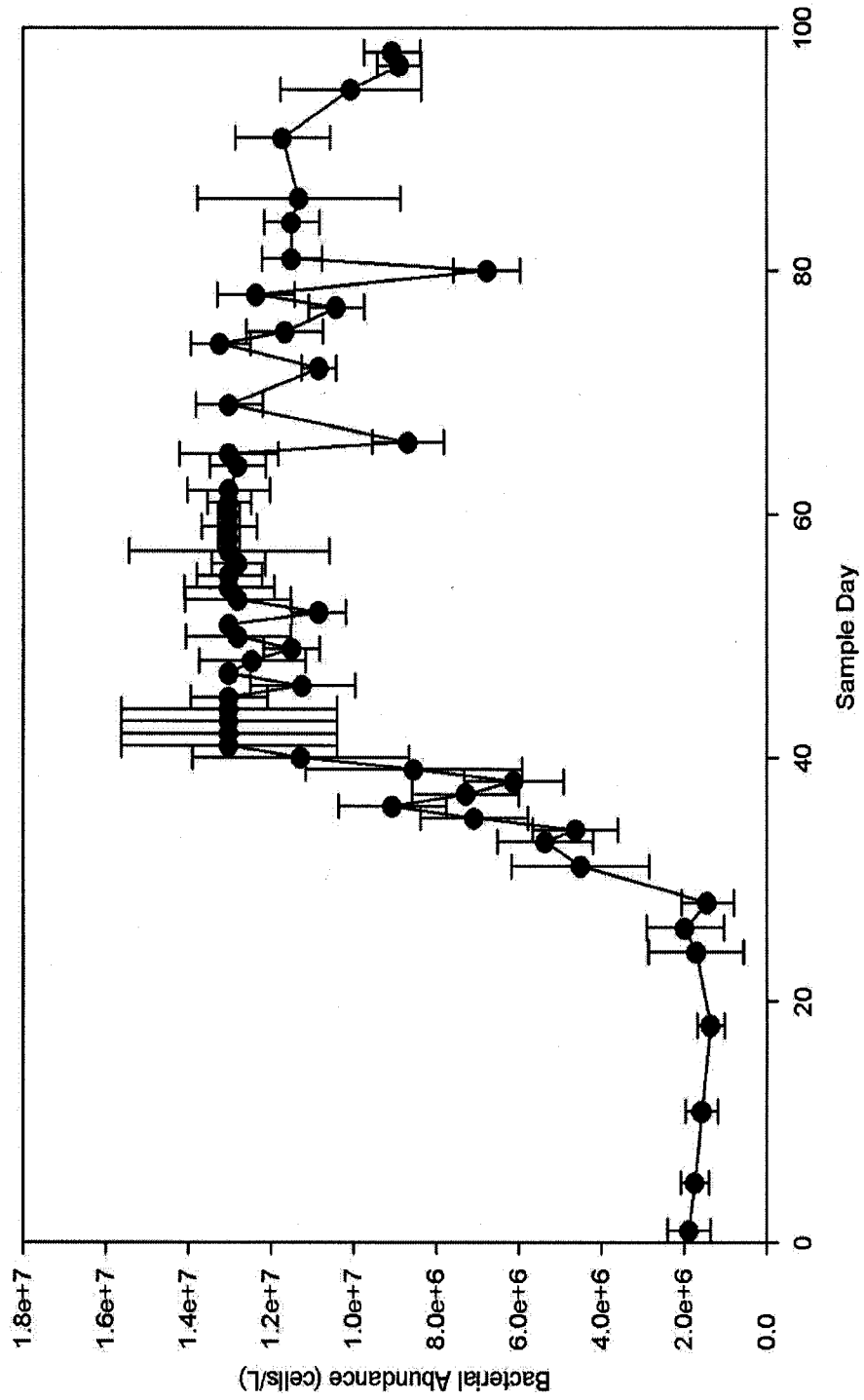
112

TCE, cDCE, and Vinyl Chloride Concentrations vs Time with Protists Present
Reactor 1, Repetition 1

113
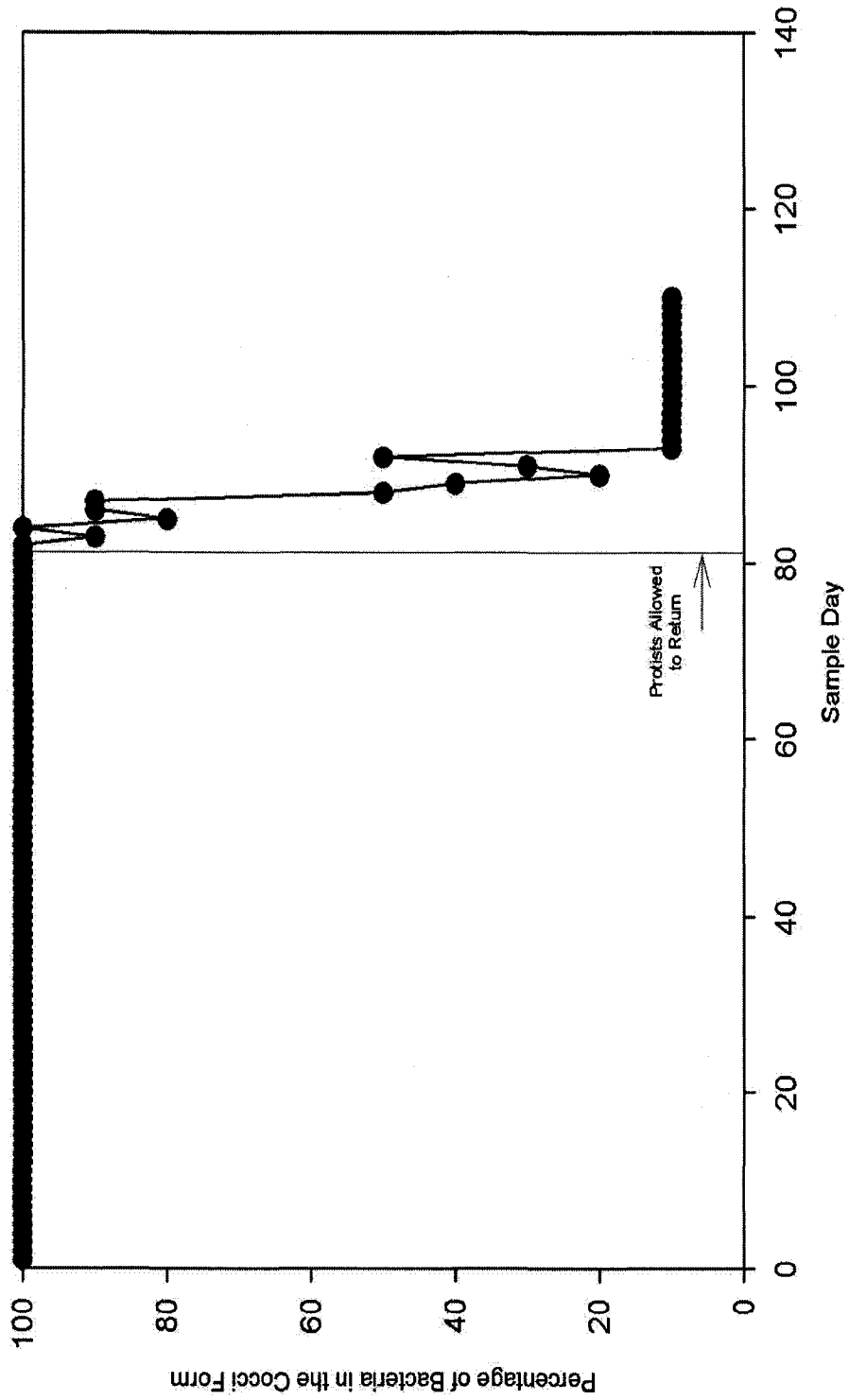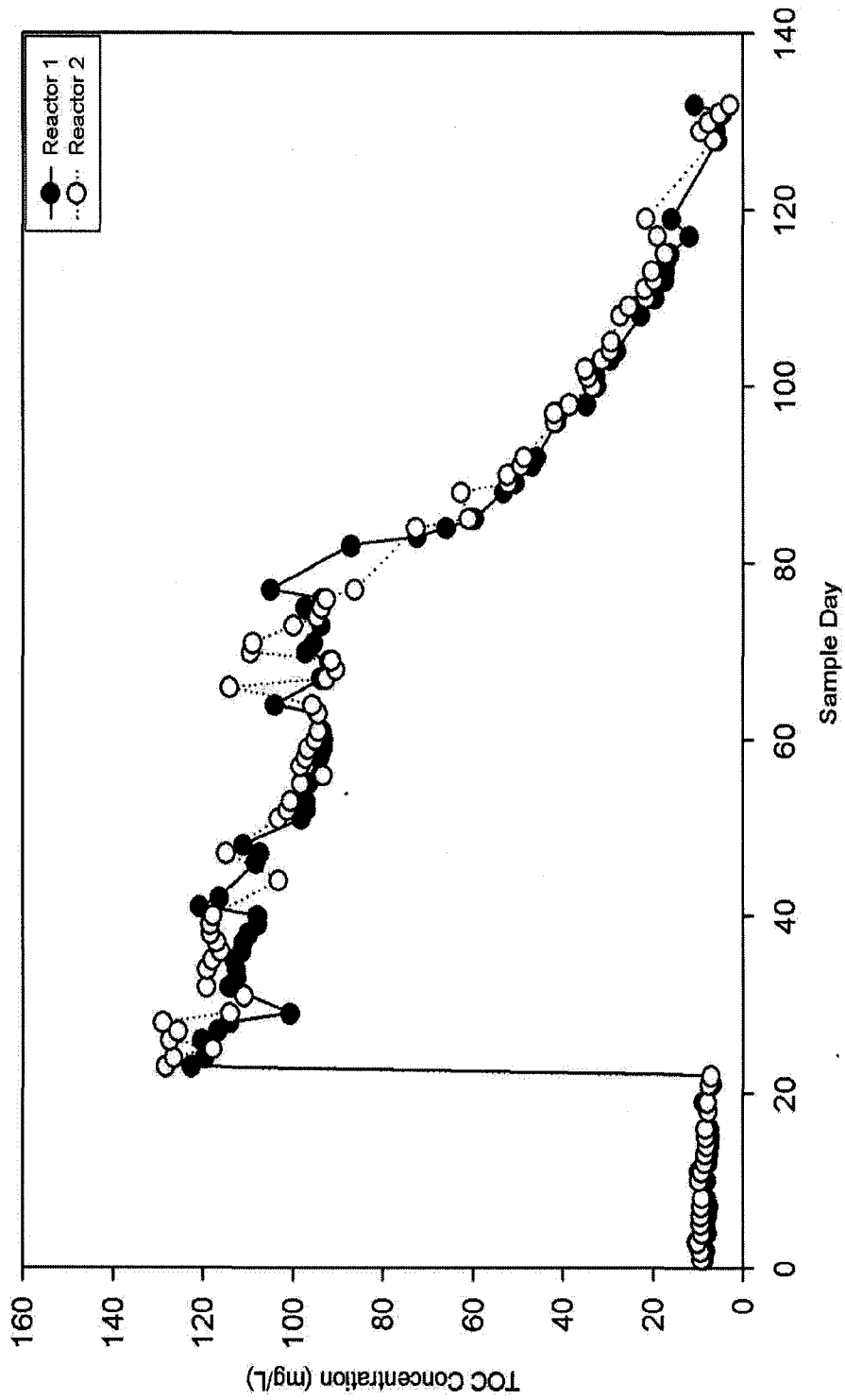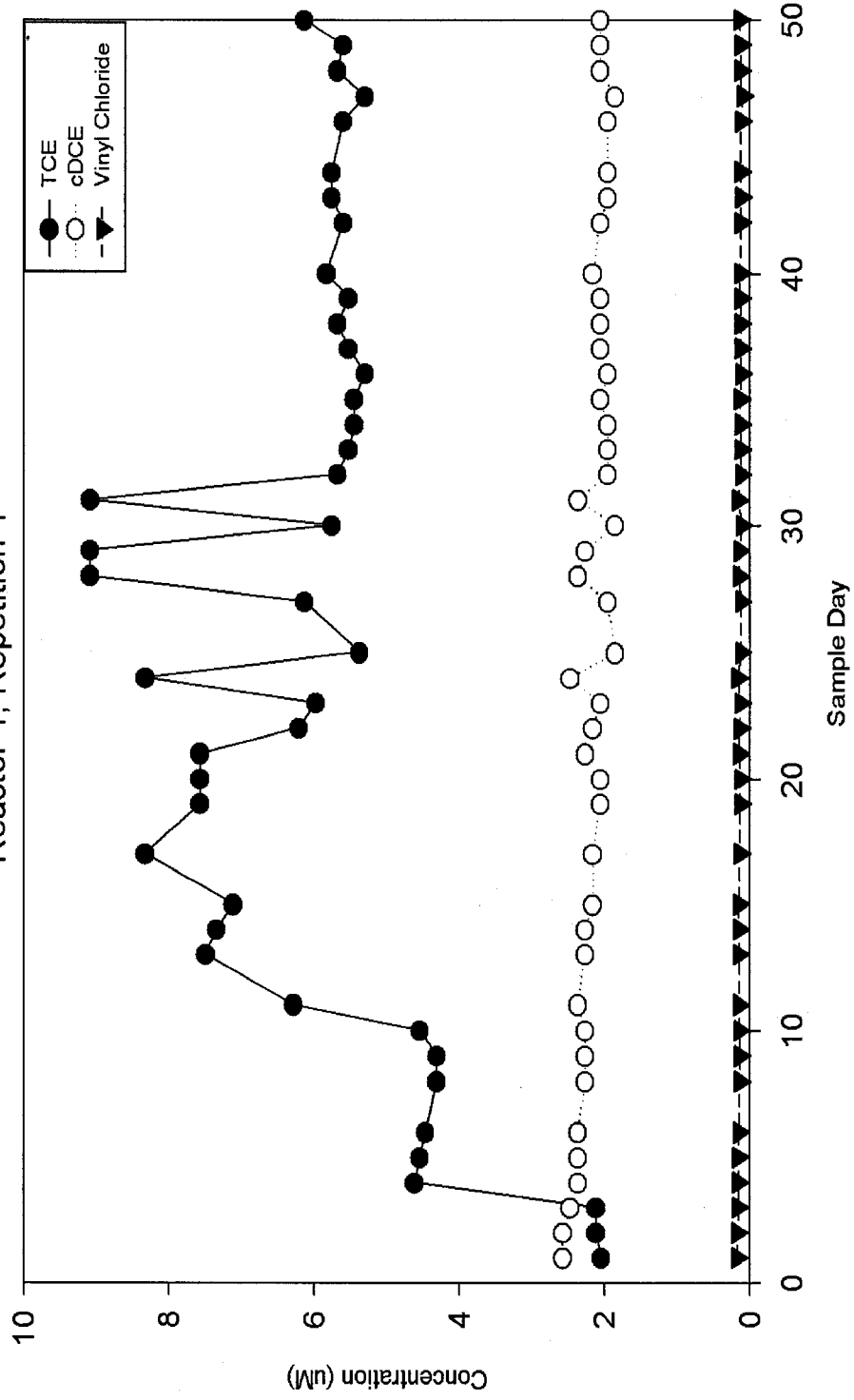
TCE, cDCE, and Vinyl Chloride Concentrations vs Time with Protists Present
Reactor 2, Repetition 1

114
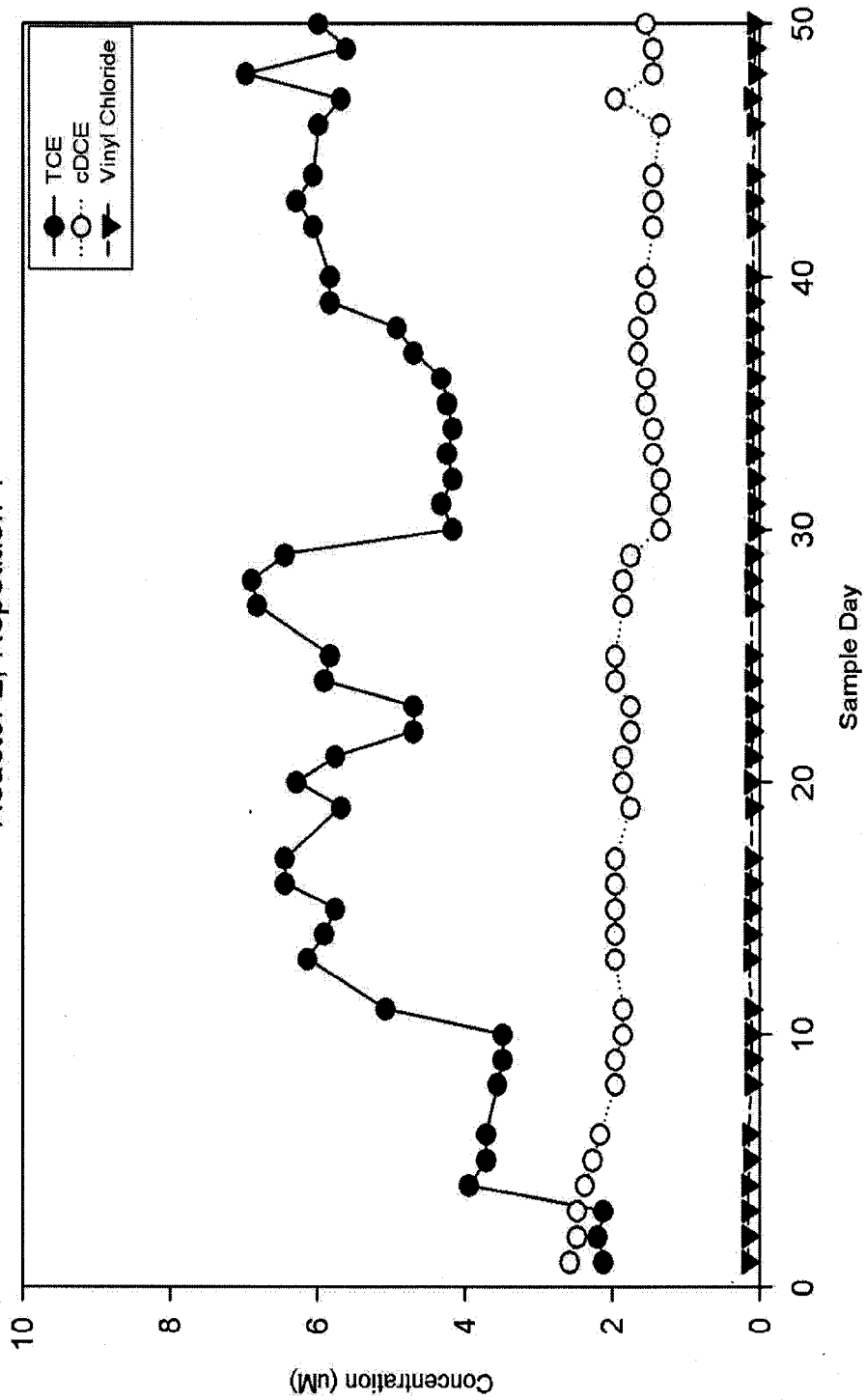
Bacteria Concentration vs Time with Protists Present in Reactor 1
Repetition 1
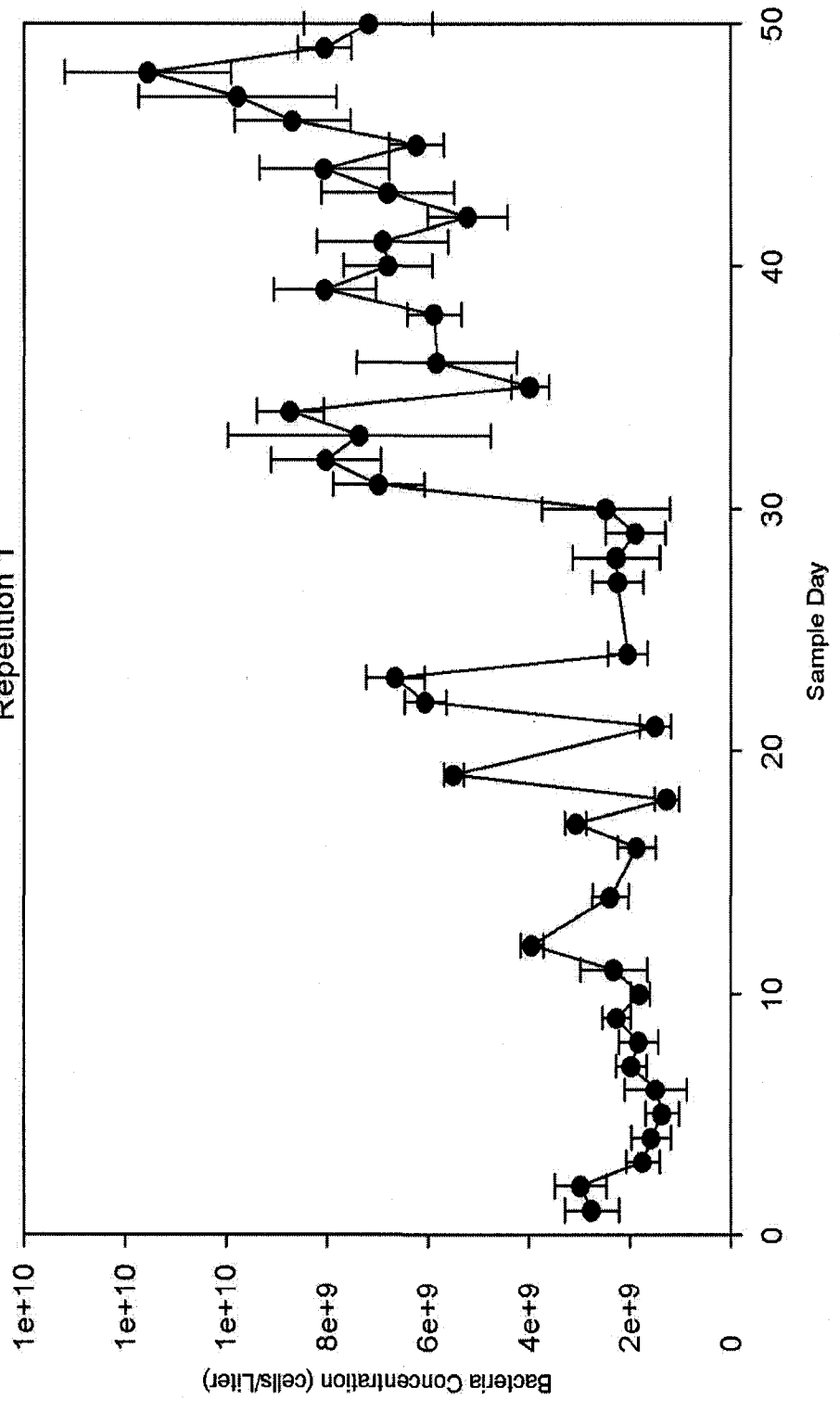
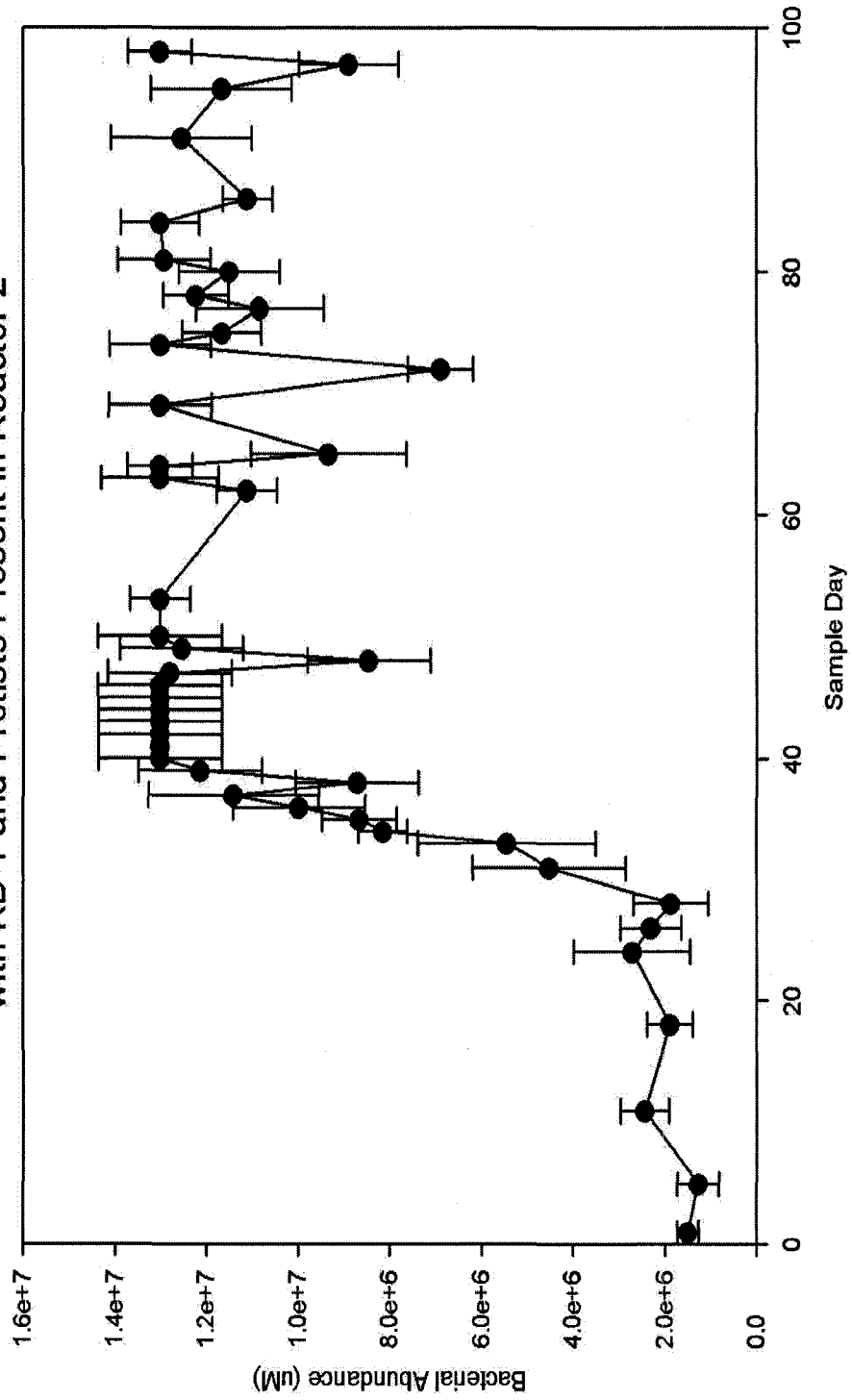Mean TCE, cDCE and Vinyl Chloride Concentrations with KB-1 and Protists Present

116
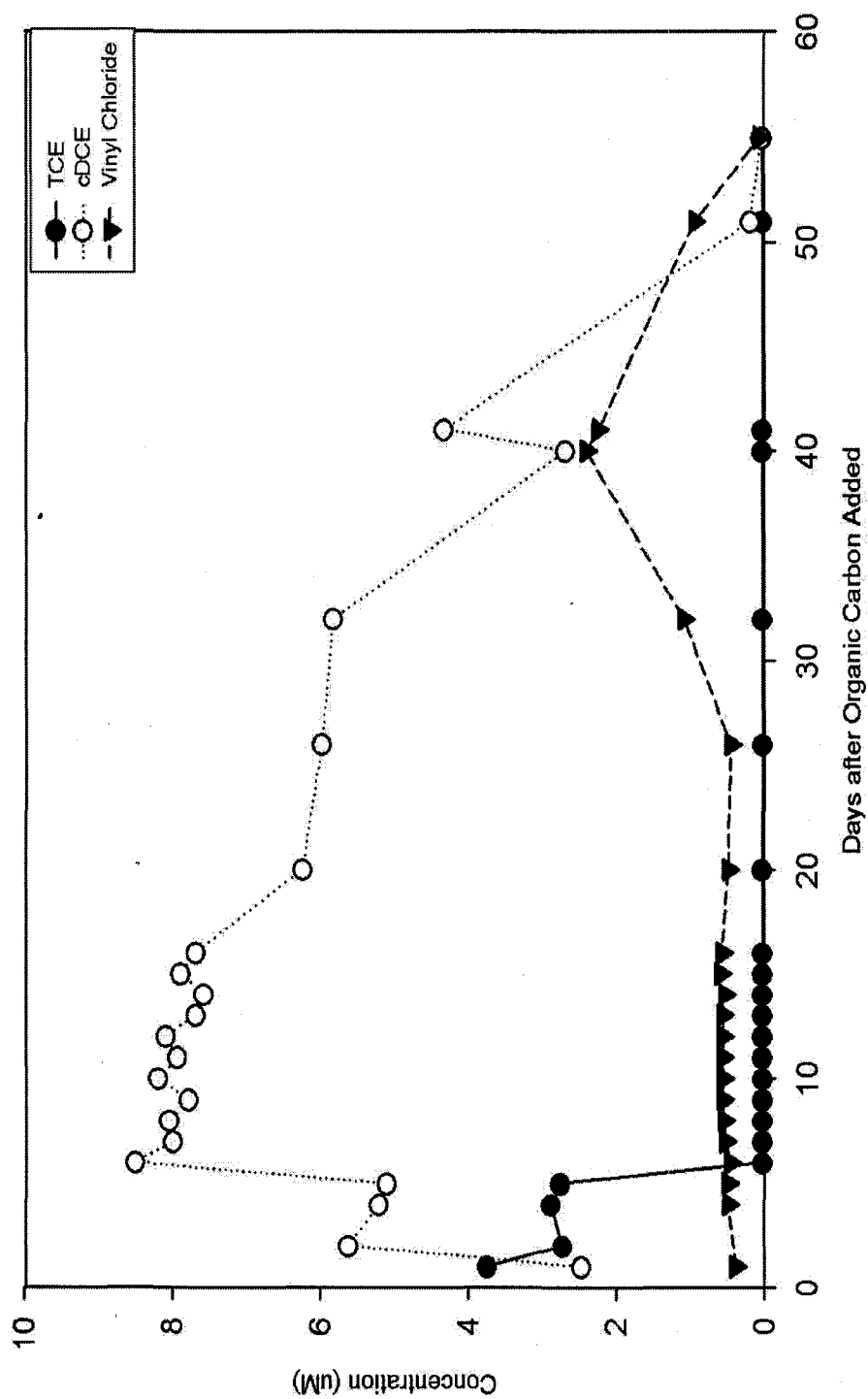
Bacterial Abundance vs Time with Protists Present in Reactor 2
Repetition 1

Protistian Abundance vs. Time with Protists Present in Reactor 1
Repitition 1

118
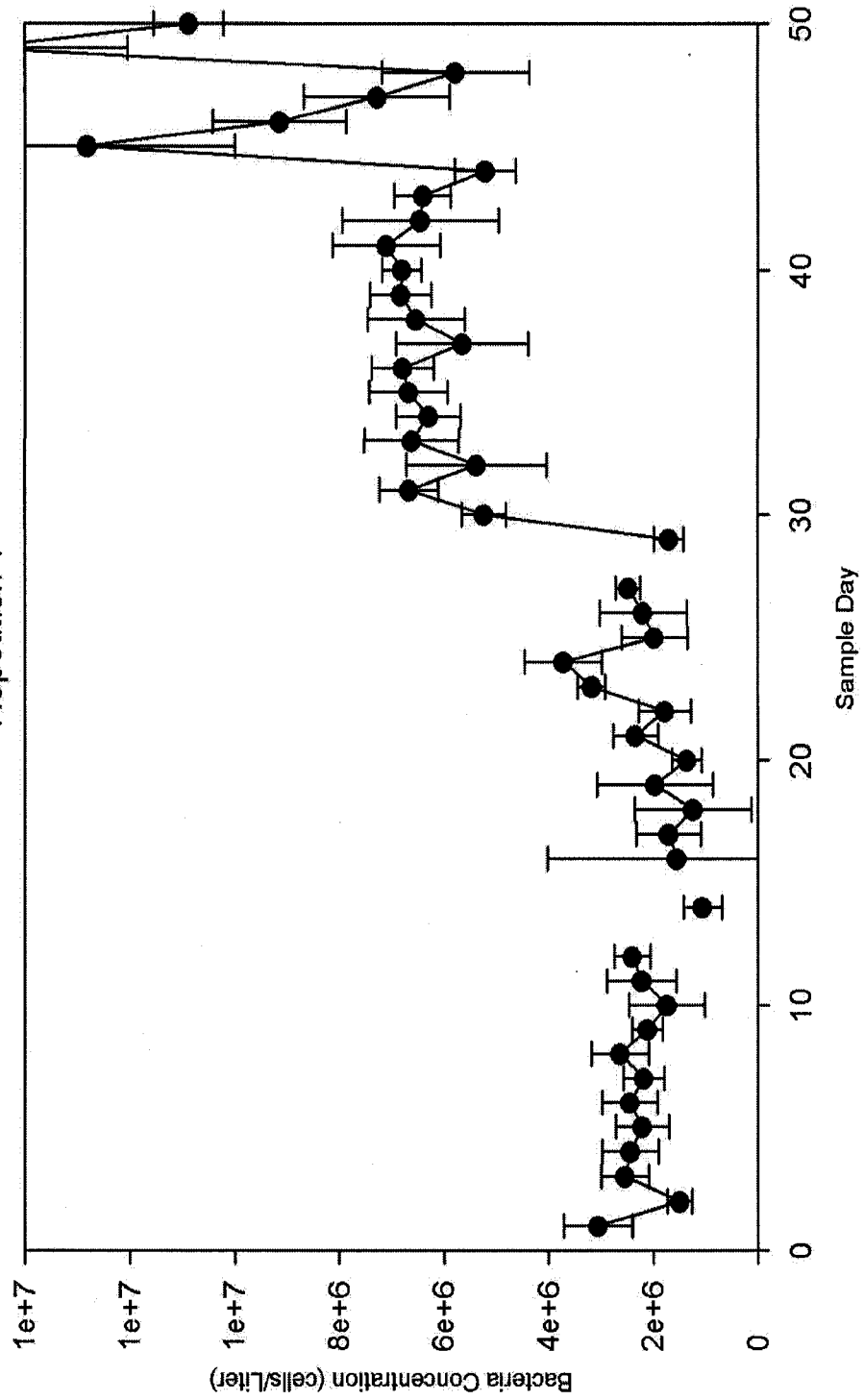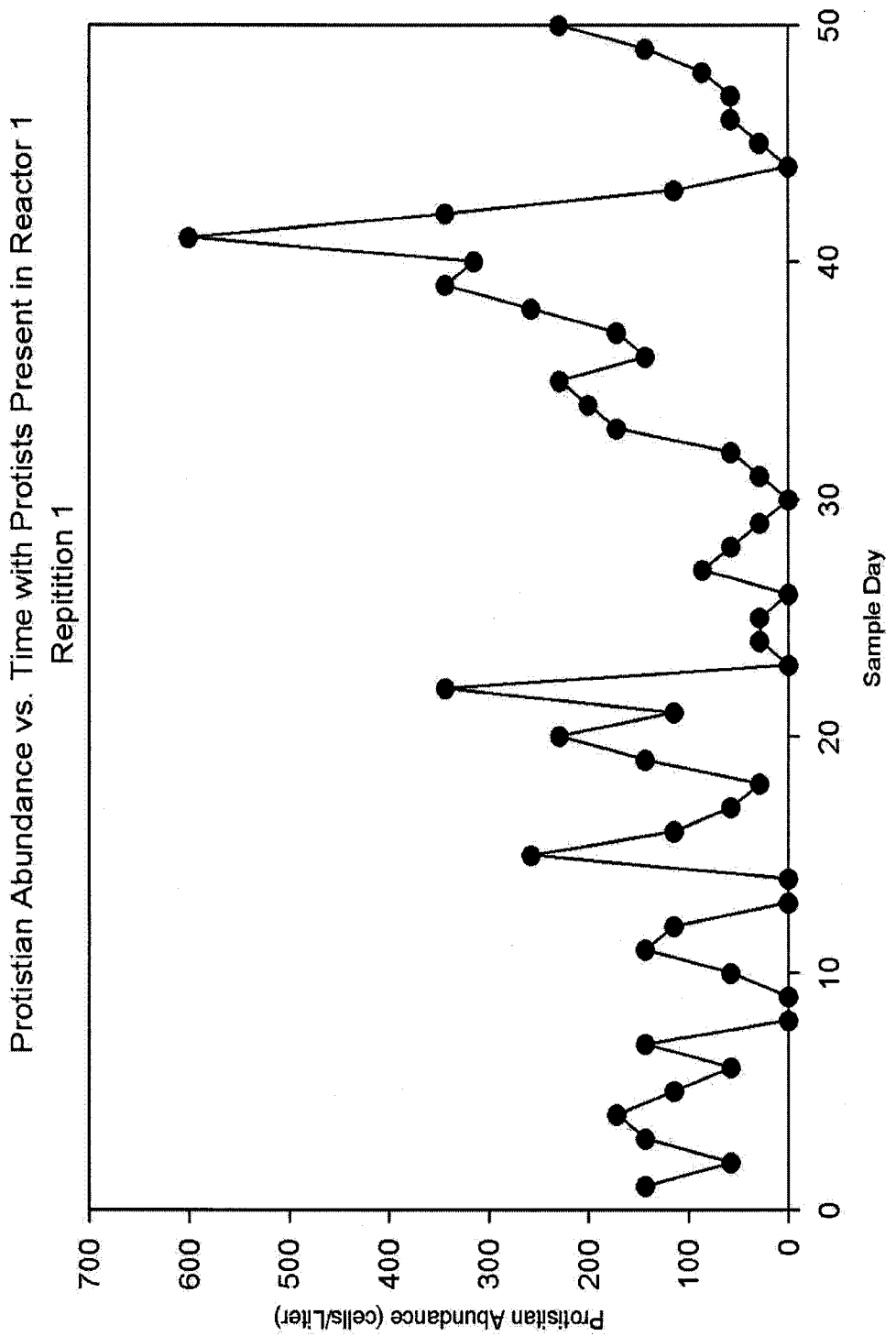
Protistian Abundance vs Time with Protists Present in Reactor 2
Repitition 1
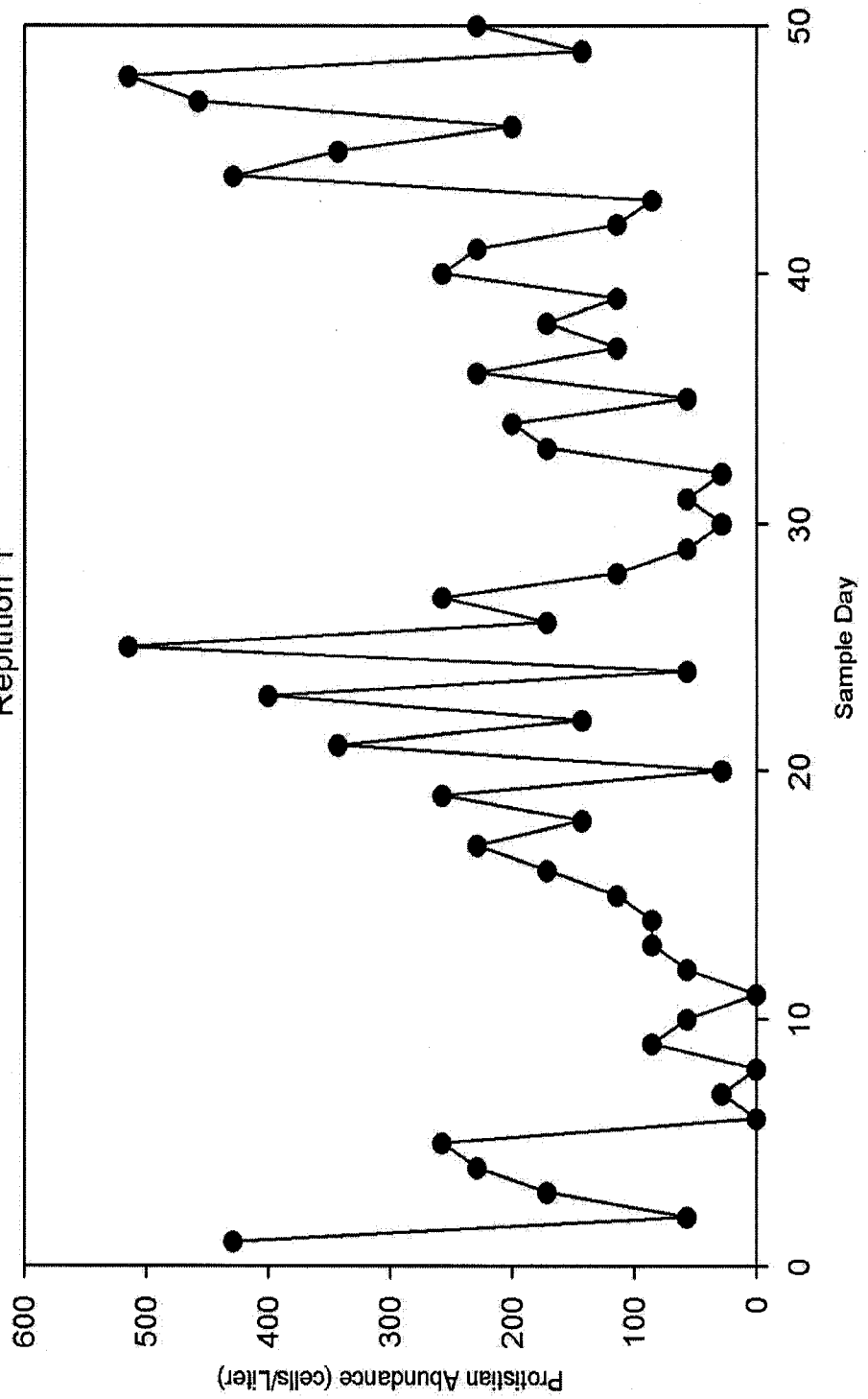
Total Organic Carbon Concentration vs Time with Protists Present
Repetition 1
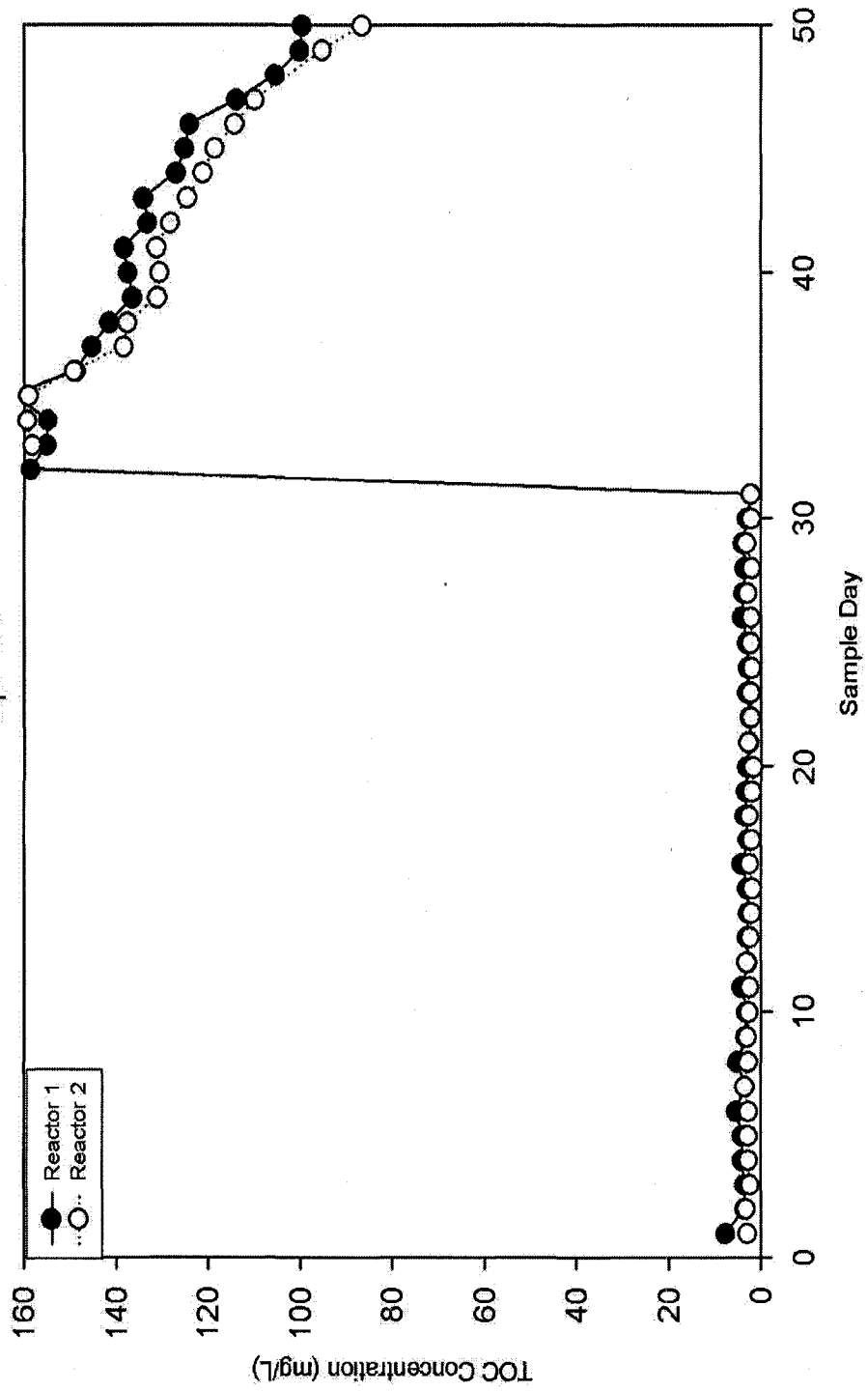
120

TCE, cDCE and Vinyl Chloride Concentations vs Time with Protists Present
Reactor 1, Repetition 2

121

TCE, cDCE and Vinyl Chloride Concentrations vs Time with protists present in Reactor 2, Repetition 2

122
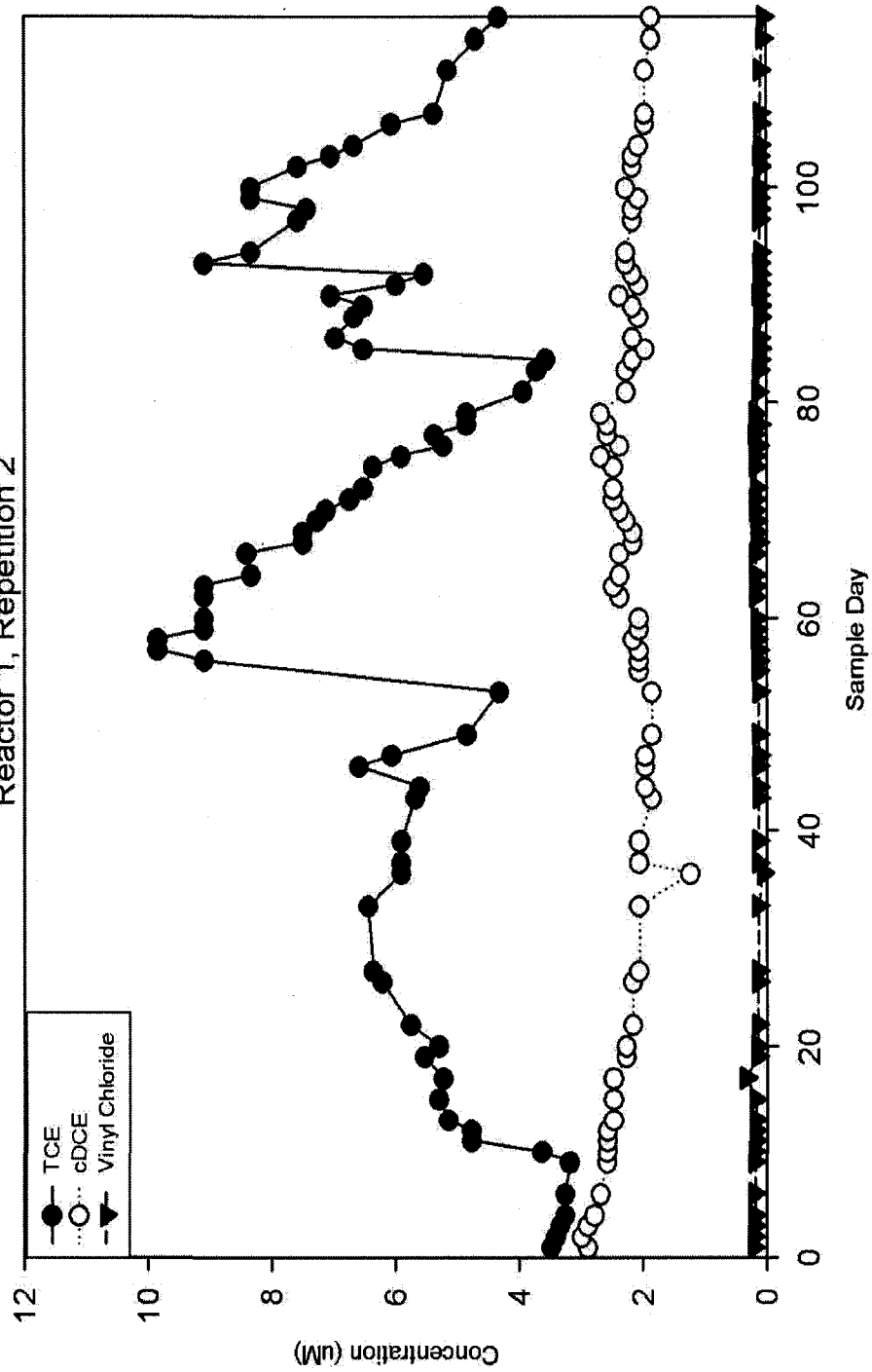
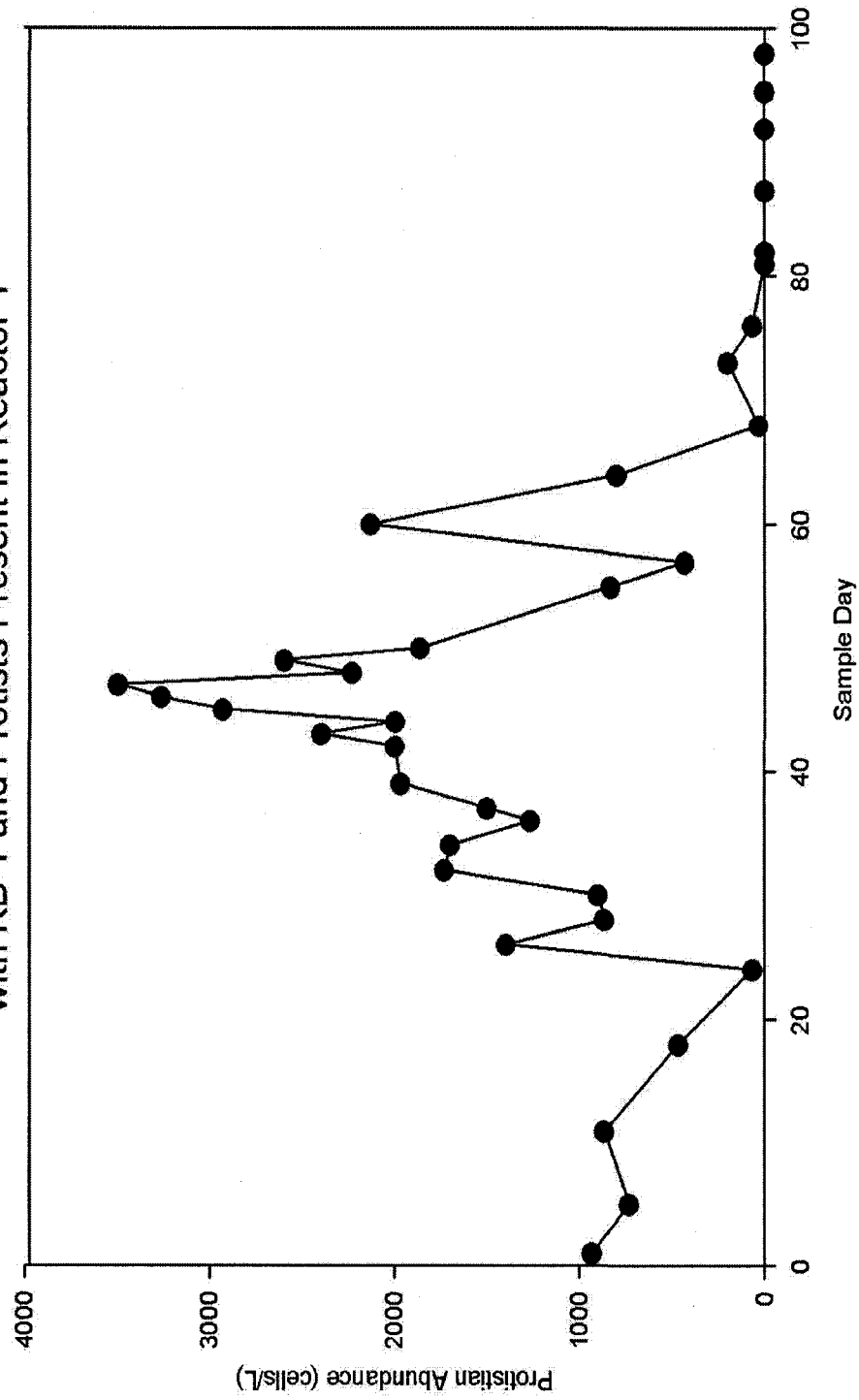Bacterial Abundance vs Time with Protists Present in Reactor 1
Repetition 2

Bacterial Abundance vs Time with Protists Present in Reactor 2
Repetition 2

124

Percentage of Bacteria in the Cocci Form with Protists Present
Repetition 2

Protistian Abundance vs Time with Protists Present in Reactor 1
Repetition 2
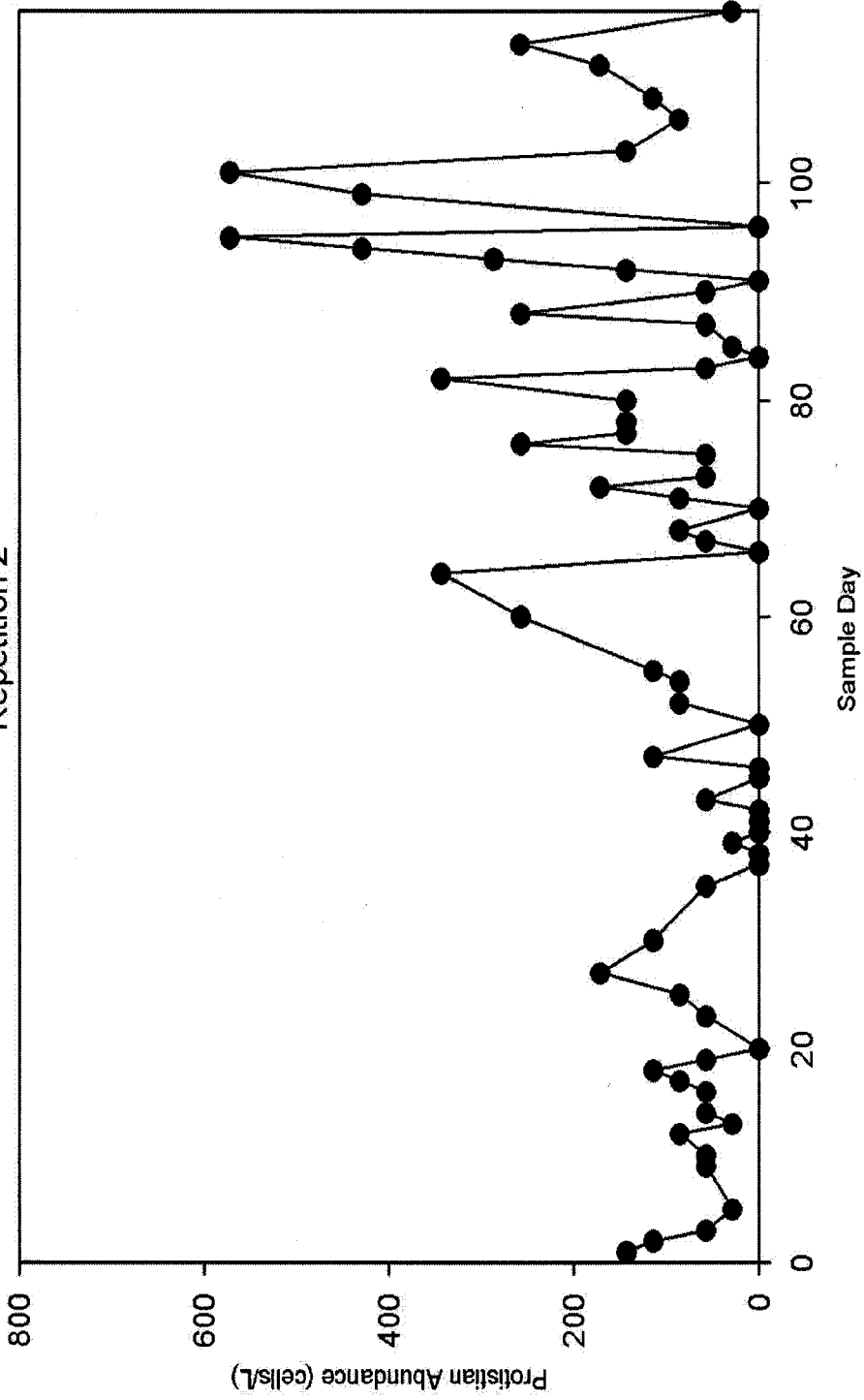
Protistian Abundane vs Time with Protists Present in Reactor 2
Repetition 2
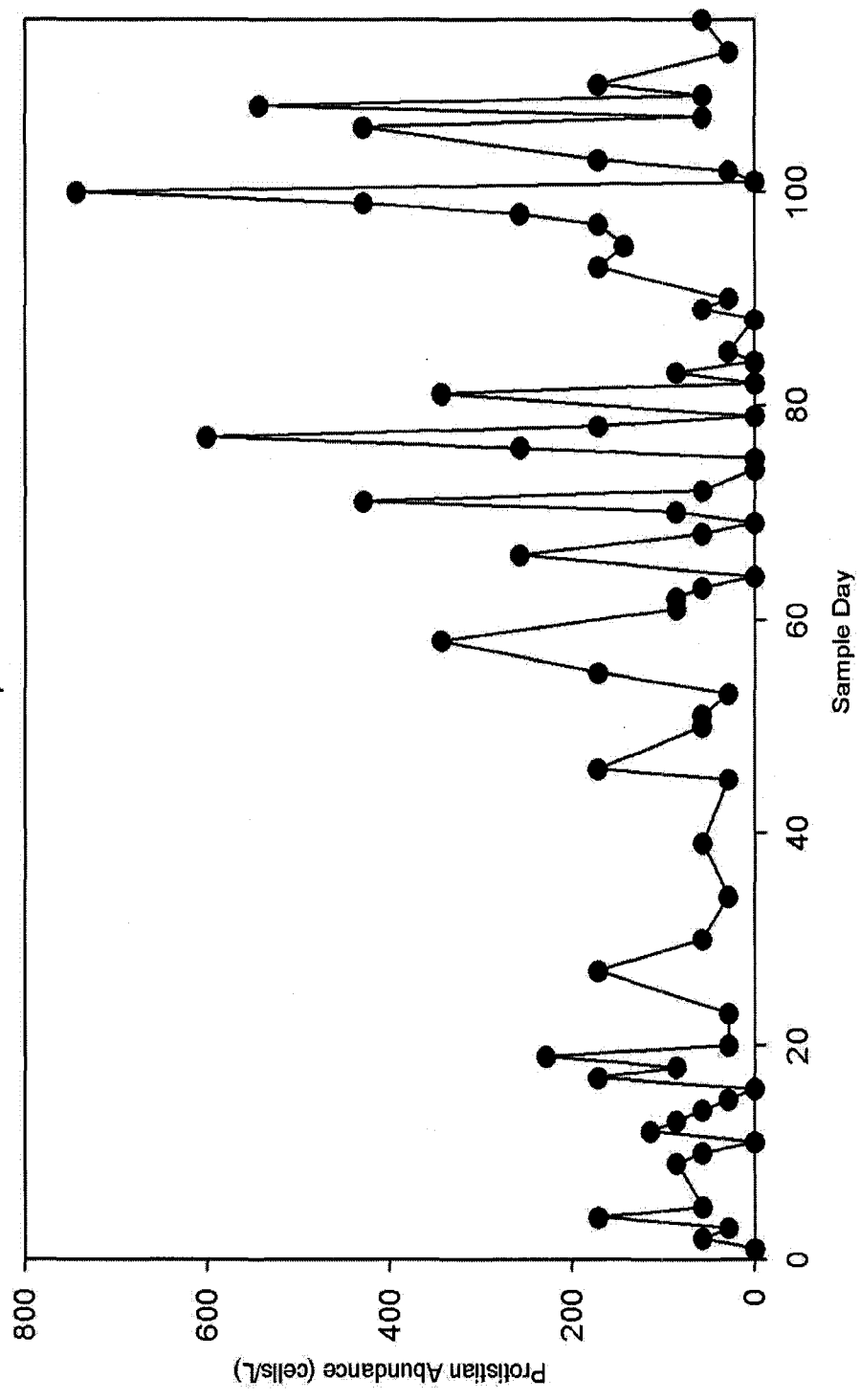
Total Organic Carbon Concentration vs Time with Protists Present Repetition 2
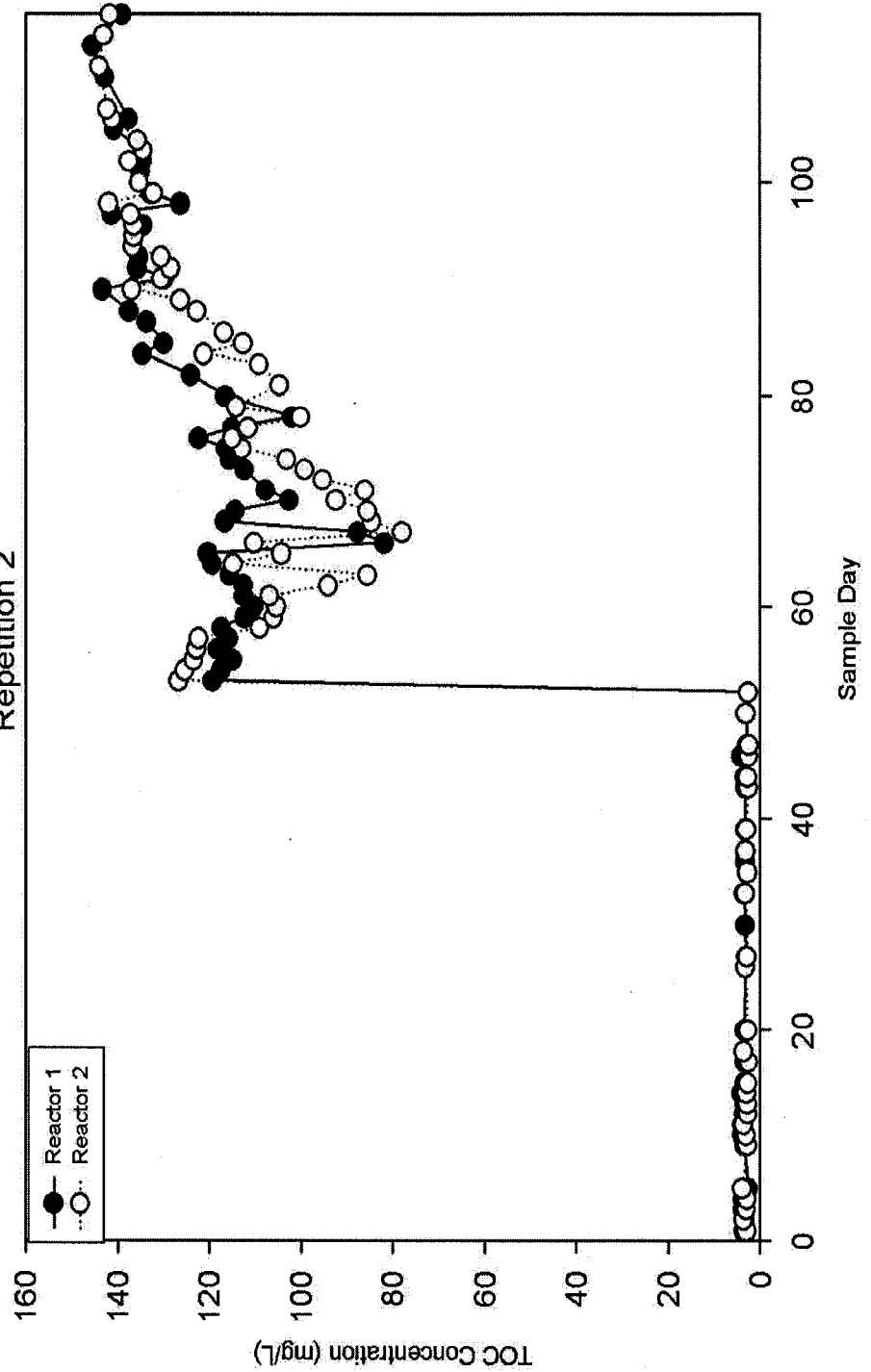
ORP vs Time with Protists Present
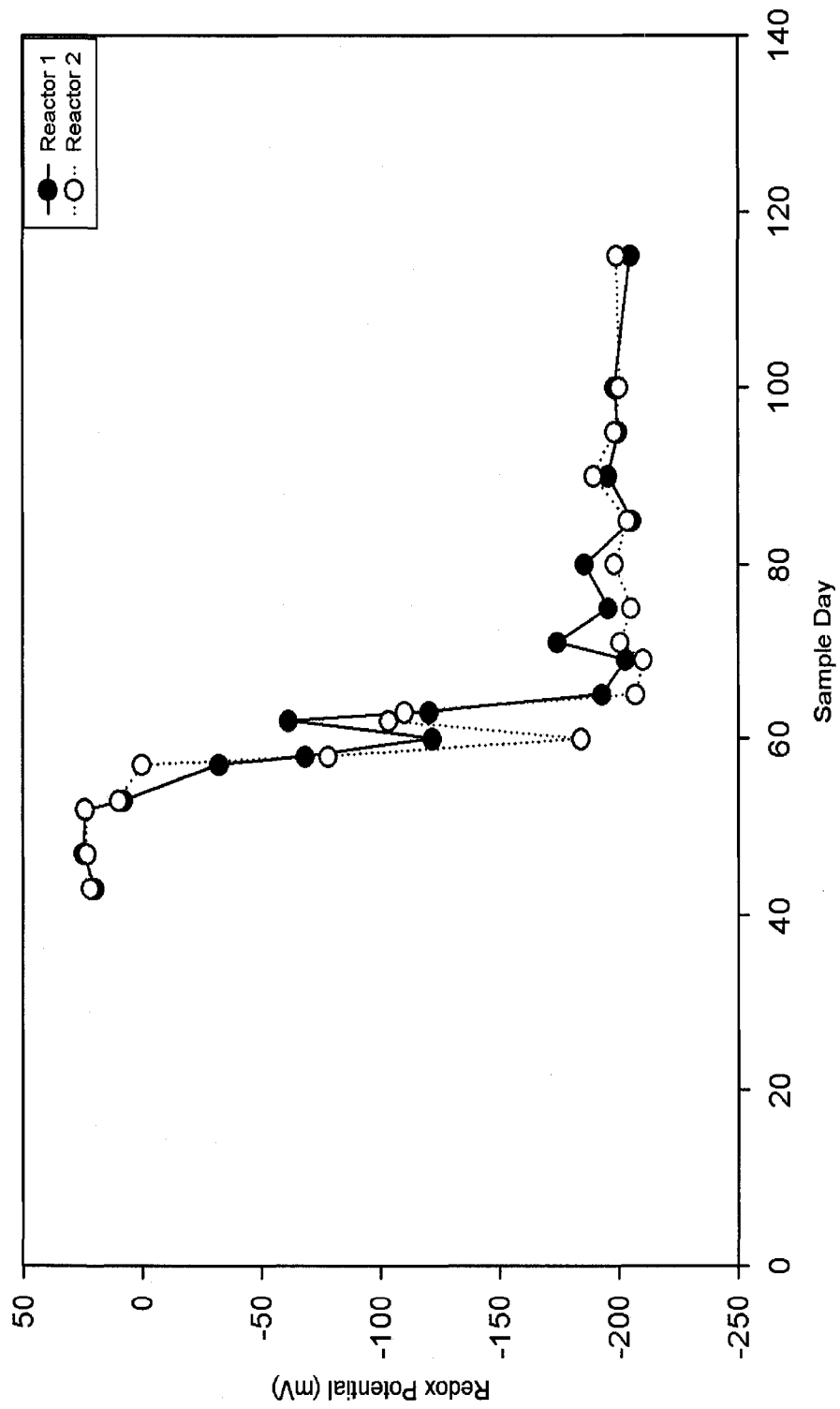
TCE, cDCE, and Vinyl Chloride Concentrations vs Time with Protists and KB-1 Present in Reactor 1
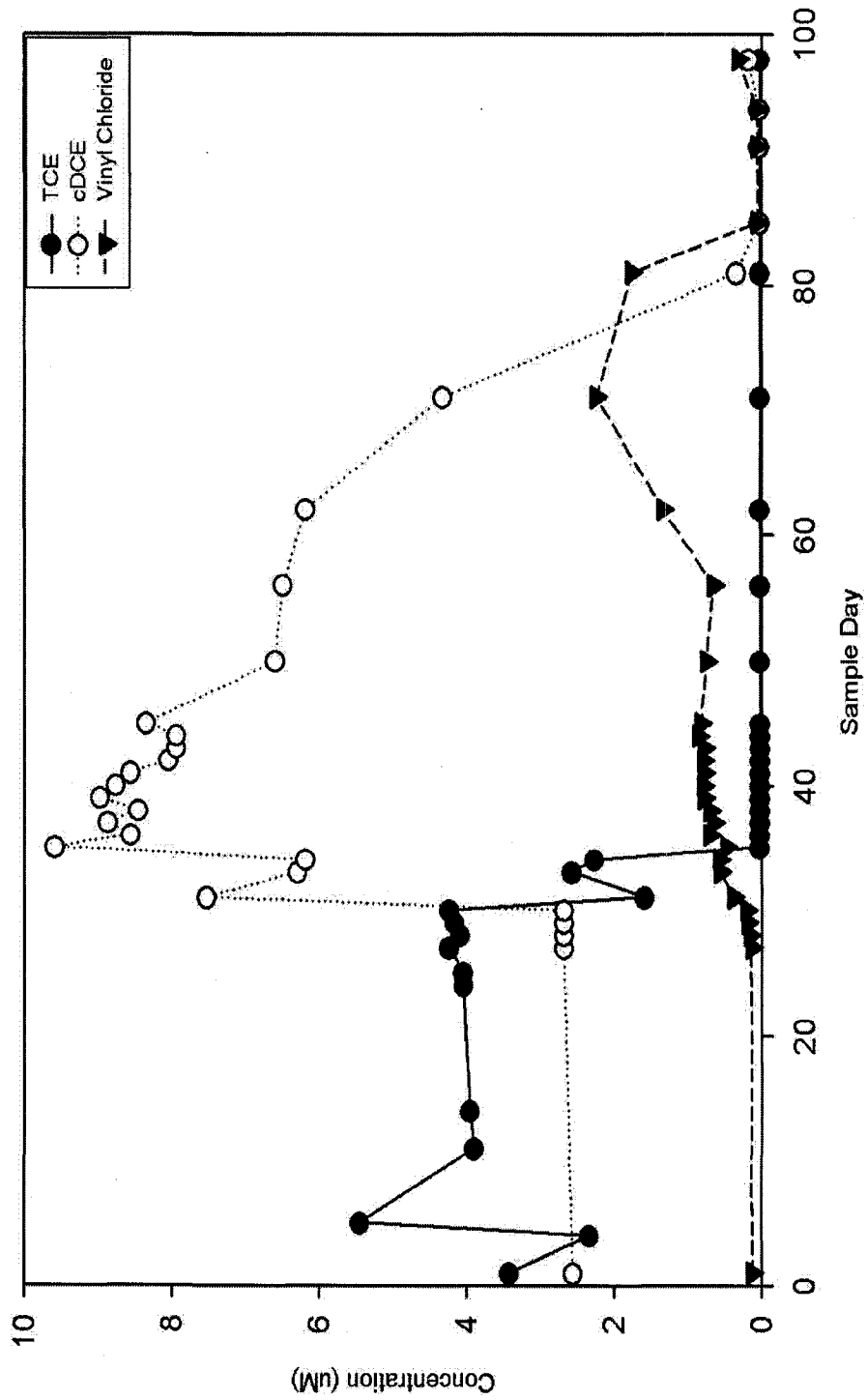
130

TCE, cDCE and Vinyl Chloride Concentrations vs Time
with KB-1 and Protists Present in Reactor 2

131

Bacterial Abundance vs Time with KB-1 and Protists Present in Reactor 1

132

TCE, cDCE, Vinyl Chloride Concentrations vs Time with KB-1 and Protists Present in Reactor 2

Percentage of Bacteria in the Cocci form with KB-1 and Protists Present
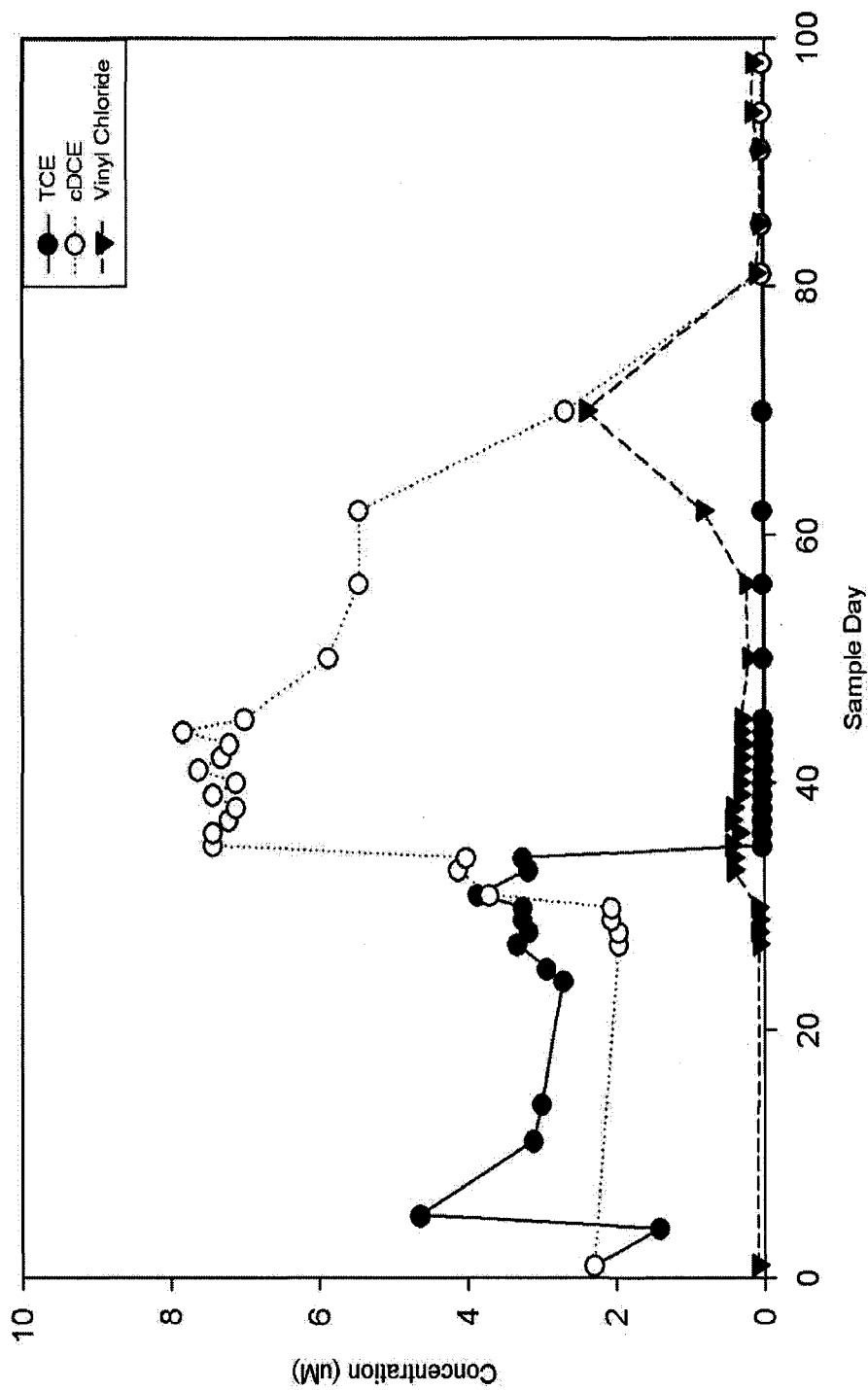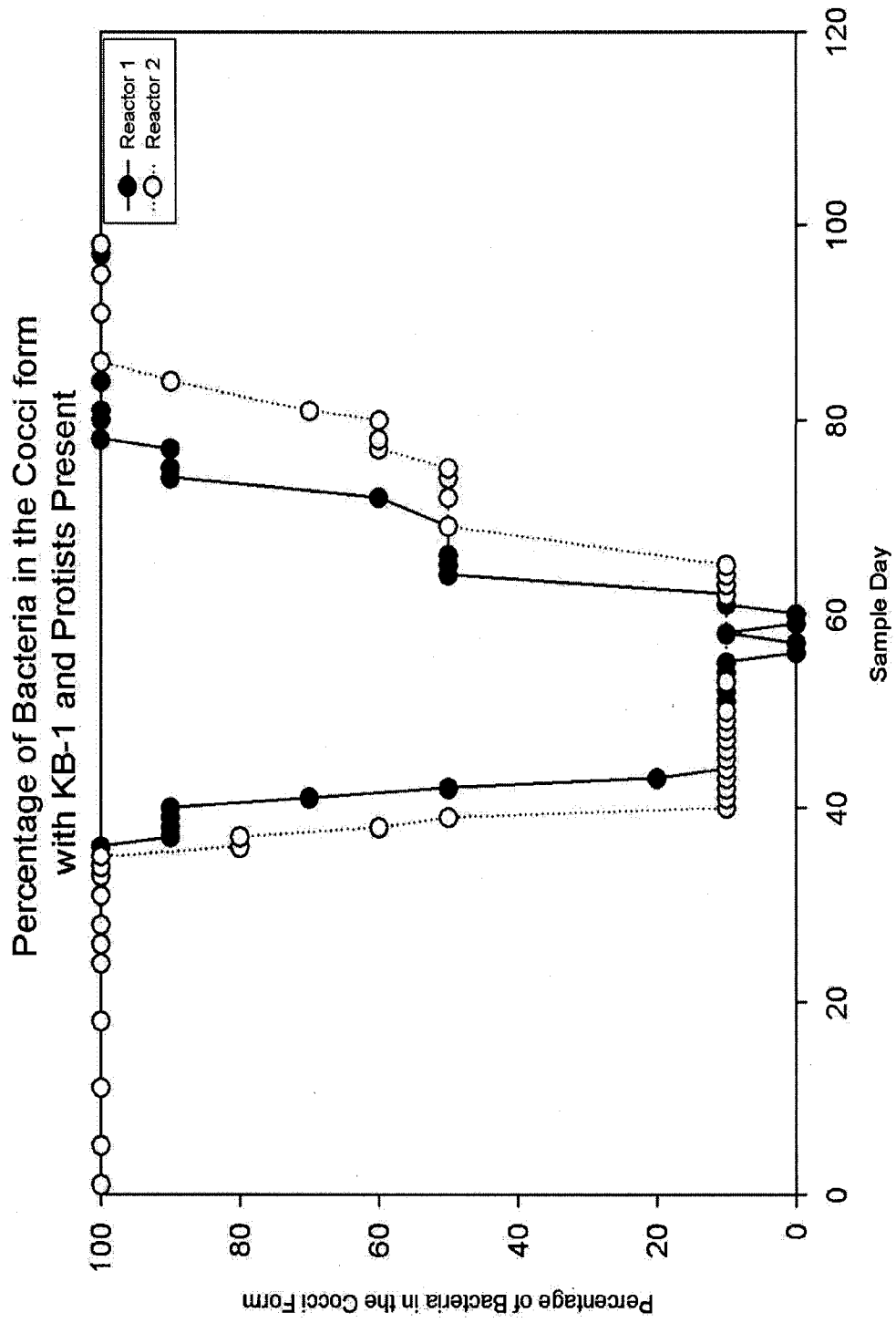
Protistian Abundance vs Time
with KB-1 and Protists Present in Reactor 1

Protistian Abundance vs Time
with KB-1 and Protists Present in Reactor 2

Total Organic Carbon Concentration vs Time with Protists and KB-1 Present

137
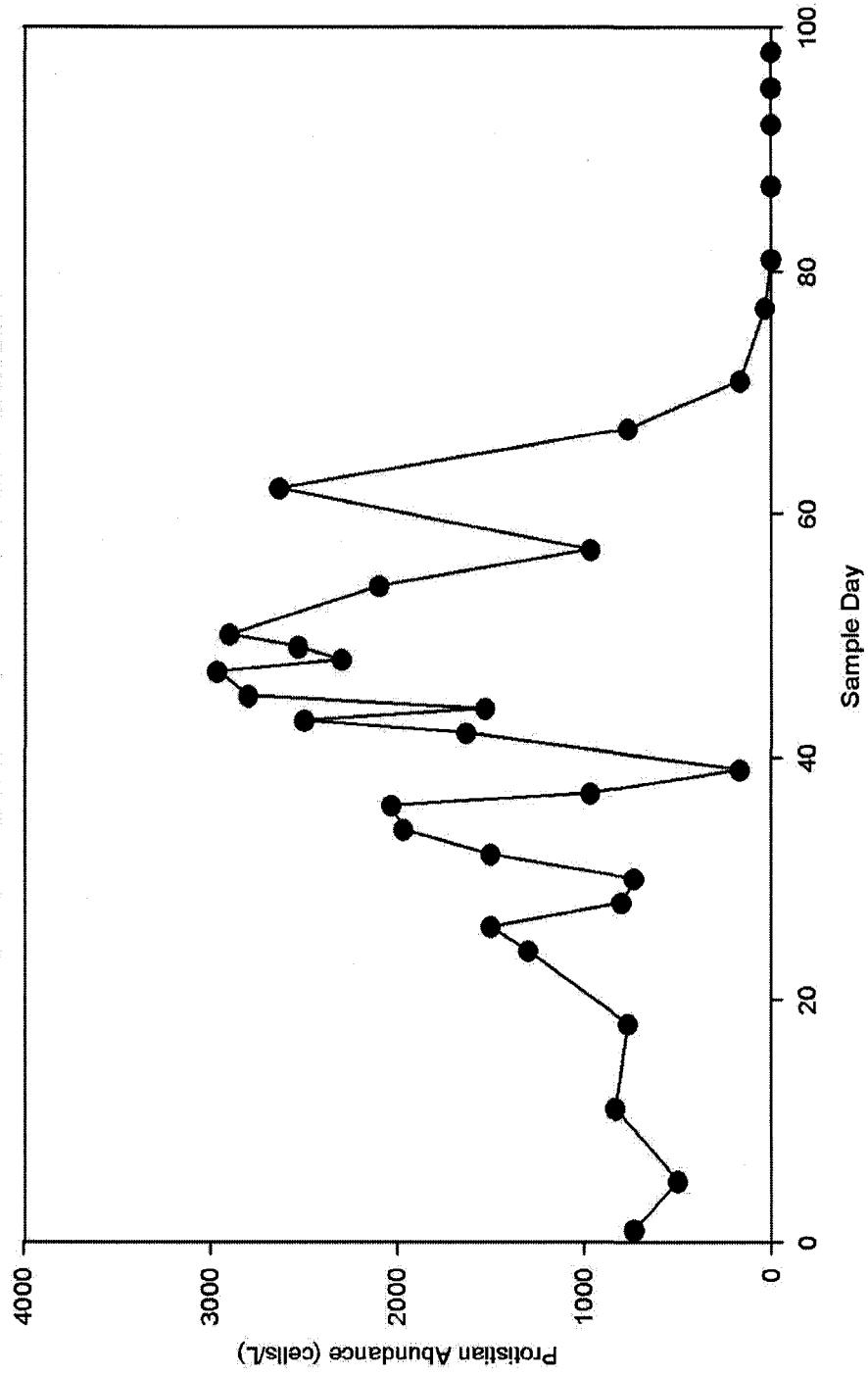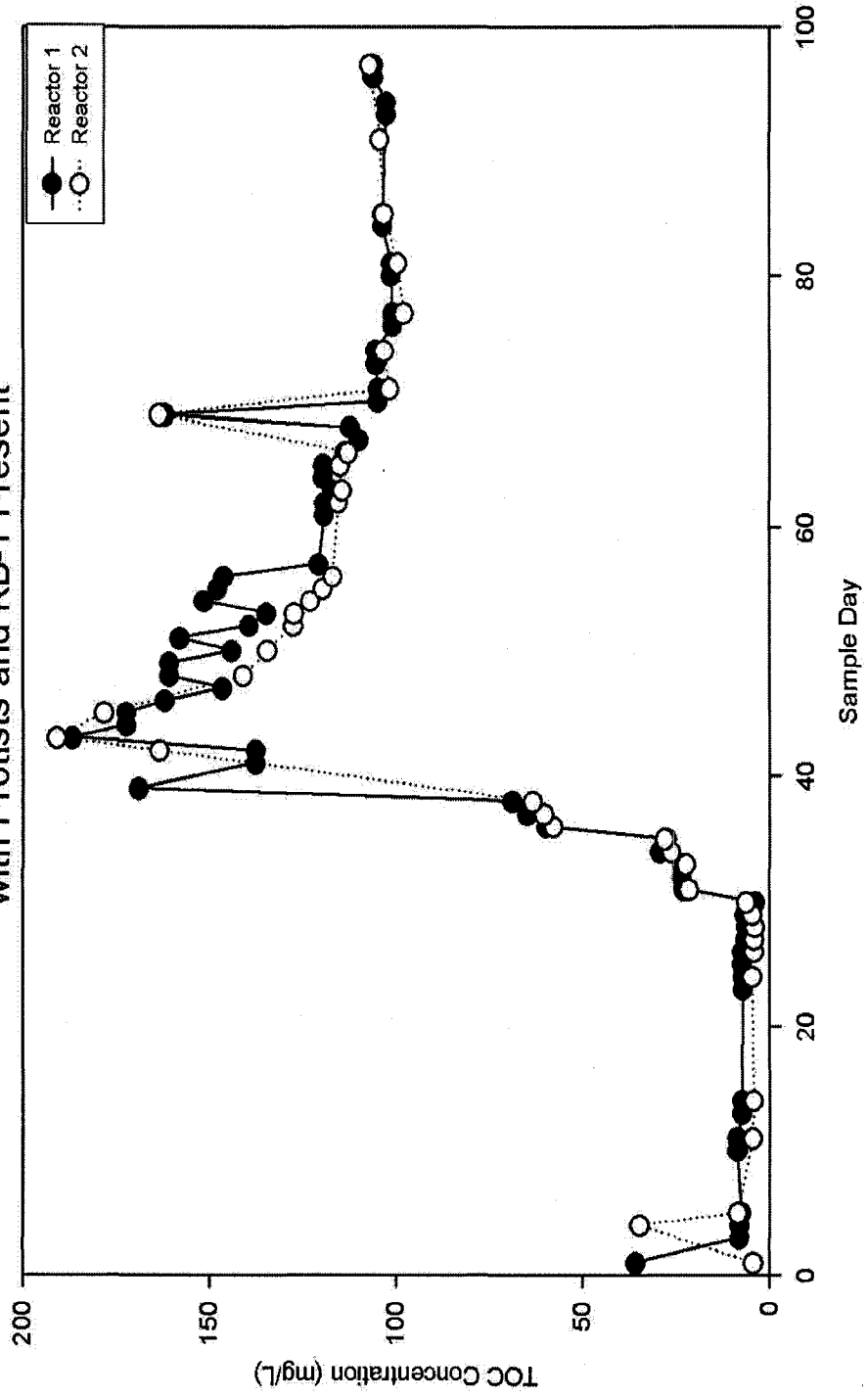
ORP vs Time with KB-1 and Protists Present
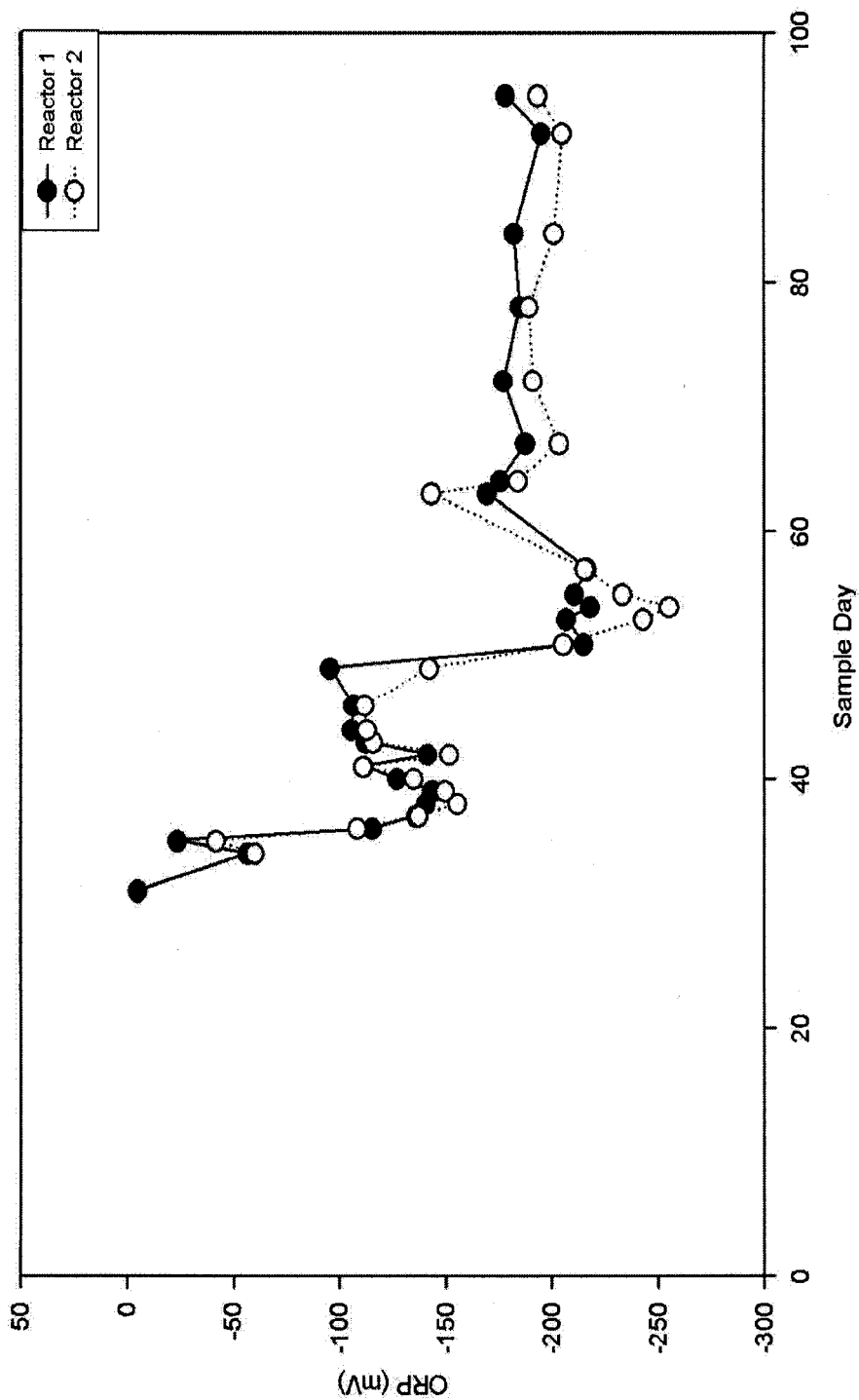
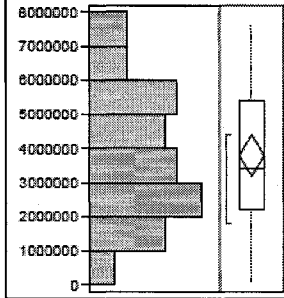Mean TCE, cDCE and Vinyl Chloride Concentrations vs Time with Protists Removed

139
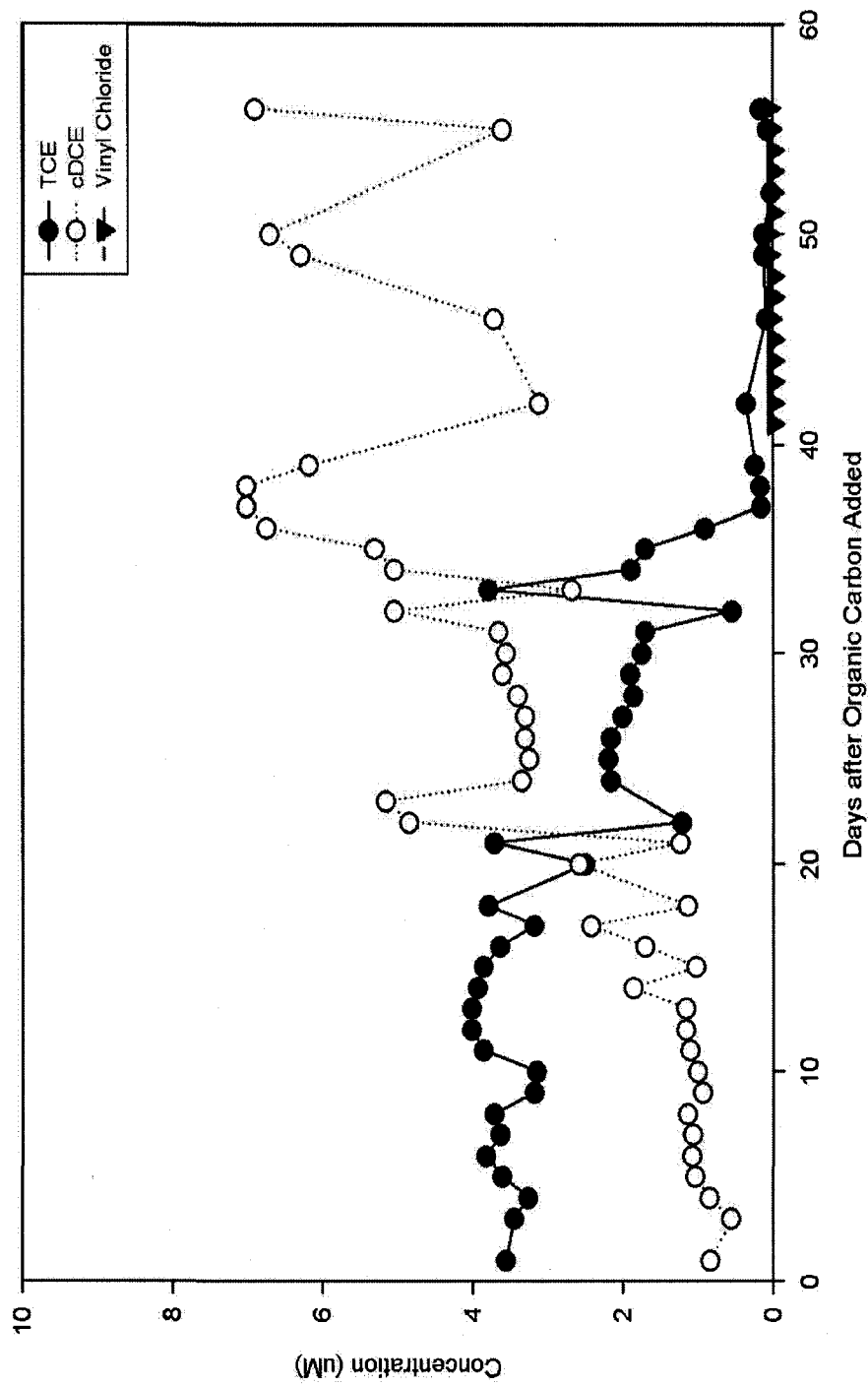
APPENDIX B: STATISTICAL ANALYSIS (JMP OUTPUT)

Bacteria Comparison- Distribution

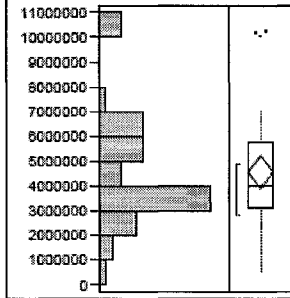## Phase=1

Distributions

Bacteria R1

Quantiles

100.0%	maximum	7669310
99.5%		7669310
97.5%		7660629.4
90.0%		6809930.8
75.0%	quartile	5394993
50.0%	median	3424497
25.0%	quartile	2204872
10.0%		1464417
2.5%		100260.9
0.5%		86806
0.0%	minimum	86806

Moments

Mean	3806039.4
Std Dev	1969834.5
Std Err Mean	300366.44
upper 95% Mean	4412203.4
lower 95% Mean	3199875.4
N	43

Bacteria R2

Quantiles

100.0%	maximum	10195365
99.5%		10195365
97.5%		10192761
90.0%		7023516.9
75.0%	quartile	5802981
50.0%	median	3984395.5
25.0%	quartile	3125016
10.0%		2719198
2.5%		619252.02
0.5%		525176
0.0%	minimum	525176

Moments

Mean	4554762.5
Std Dev	2152427.2
Std Err Mean	332126.73
upper 95% Mean	5225506
lower 95% Mean	3884019
N	42

Phase=2

Distributions

Bacteria R1
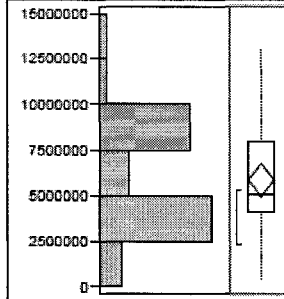
Quantiles

100.0%	maximum	13020900
99.5%		13020900
97.5%		13020900
90.0%		8680600
75.0%	quartile	7917792
50.0%	median	5041256.5
25.0%	quartile	4095072.8
10.0%		2857219.8
2.5%		272571
0.5%		272571
0.0%	minimum	272571

Moments

Mean	5851905.9
Std Dev	2723553.3
Std Err Mean	453928.55
upper 95% Mean	6773423.7
lower 95% Mean	4930388
N	36

Bacteria R2
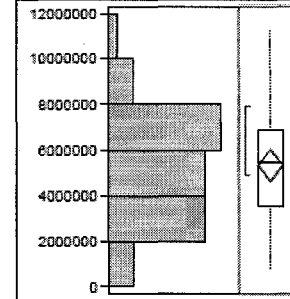
Quantiles

100.0%	maximum	11310822
99.5%		11310822
97.5%		11095326
90.0%		8096395.8
75.0%	quartile	6846993.5
50.0%	median	5431885.5
25.0%	quartile	3504792.3
10.0%		2438814.6
2.5%		823029.13
0.5%		785594
0.0%	minimum	785594

Moments

Mean	5321104.5
Std Dev	2210756.6
Std Err Mean	341127.15
upper 95% Mean	6010024.7
lower 95% Mean	4632184.2
N	42
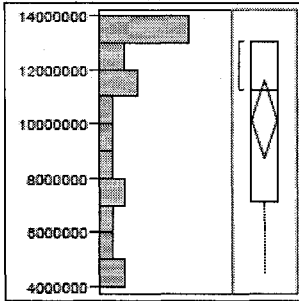
Distributions

Bacteria R1

Bacteria R2

Quantiles (Bacteria R1)

100.0%	maximum	13020900
99.5%		13020900
97.5%		13020900
90.0%		13020900
75.0%	quartile	13020900
50.0%	median	11284780
25.0%	quartile	7189707
10.0%		4777802.2
2.5%		4513912
0.5%		4513912
0.0%	minimum	4513912

Moments (Bacteria R1)

Mean	10186477
Std Dev	3142161.2
Std Err Mean	685675.79
upper 95% Mean	11616772
lower 95% Mean	8756182.8
N	21
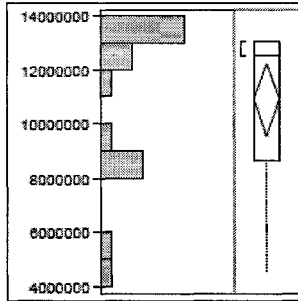
Quantiles (Bacteria R2)

100.0%	maximum	13020900
99.5%		13020900
97.5%		13020900
90.0%		13020900
75.0%	quartile	13020900
50.0%	median	12543467
25.0%	quartile	8667579
10.0%		5447077
2.5%		4513912
0.5%		4513912
0.0%	minimum	4513912

Moments (Bacteria R2)

Mean	10895981
Std Dev	2776519.4
Std Err Mean	636977.25
upper 95% Mean	12234220
lower 95% Mean	9557741
N	19

Bacteria Comparison- Fit Y by X

Oneway Analysis of Bacteria R1 By Phase
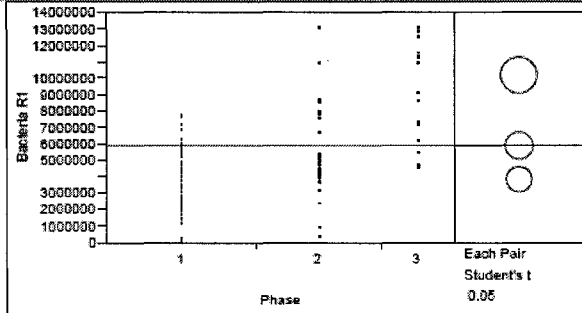
Missing Rows   29

Means Comparisons

Comparisons for each pair using Student's t

t	Alpha
1.98472	0.05

Abs(Dif)-LSD

	3	2	1
3	-1548542	2956746	5044570
2	2956746	-1182718	912304
1	5044570	912304	-1092177

Positive values show pairs of means that are significantly different.

Level		Mean
3	A	10186477
2	B	5851906
1	C	3806039

Levels not connected by same letter are significantly different

Level	- Level	Difference	Lower CL	Upper CL	p-Value	
3	1	6380438	5044570	7716306	1.784e-15	
3	2	4334572	2956746	5712397	1.1283e-8	
2	1	2045867	912304	3179429	0.0005351	

Oneway Analysis of Bacteria R2 By Phase

Missing Rows   26

Means Comparisons

Comparisons for each pair using Student's t

t	Alpha
1.96397	0.05

Abs(Dif)-LSD

	3	2	1
3	-1480615	4313143	5079485
2	4313143	-995851	-229509
1	5079485	-229509	-995851

Positive values show pairs of means that are significantly different.

Level		Mean
3	A	10895981
2	B	5321104
1	B	4554763

Levels not connected by same letter are significantly different

Level	- Level	Difference	Lower CL	Upper CL	p-Value	
3	1	6341218	5079485	7602951	1.146e-16	
3	2	5574878	4313143	6836609	4.978e-14	
2	1	766342	-229509	1762193	0.1298836	

Oneway Analysis of Bacteria R1 By Phase

Missing Rows   27

Means Comparisons

Comparisons for each pair using Student's t

t	Alpha
1.98498	0.05

Abs(Dif)-LSD

	3	2	1
3	-1627341	2695394	4814560
2	2695394	-1166151	960514
1	4814560	960514	-1081736

Positive values show pairs of means that are significantly different.

Level		Mean
3	A	10002336
2	B	5891289
1	C	3806039

Levels not connected by same letter are significantly different

Level	- Level	Difference	Lower CL	Upper CL	p-Value
3	1	6196296	4814560	7578033	3.402e-14
3	2	4111047	2695394	5526700	9.9196e-8
2	1	2085249	960514	3209985	0.0003949

Oneway Analysis of Bacteria R2 By Phase
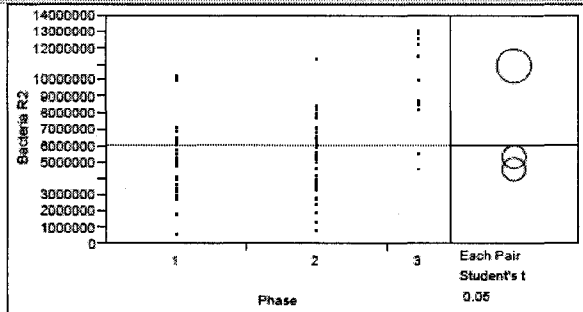
Missing Rows   23

Means Comparisons

Comparisons for each pair using Student's t

t	Alpha
1.98397	0.05

Abs(Dif)-LSD

	3	2	1
3	-1480615	4313143	5079485
2	4313143	-995851	-229509
1	5079485	-229509	-995851

Positive values show pairs of means that are significantly different.

Level		Mean
3	A	10895981
2	B	5321104
1	B	4554763

Levels not connected by same letter are significantly different

Level	- Level	Difference	Lower CL	Upper CL	p-Value
3	1	6341218	5079485	7602951	1.146e-16
3	2	5574876	4313143	6836609	4.978e-14
2	1	766342	-229509	1762193	0.1299936

144

Protist Comparsion- Fit Y by X

Oneway Analysis of Protists R1 By Phase
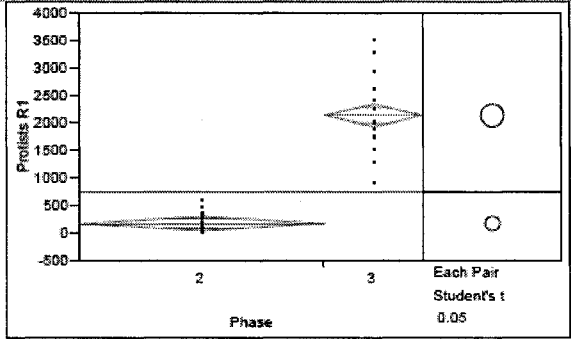
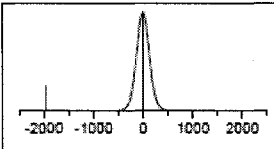Missing Rows   32

Oneway Anova

Summary of Fit

Rsquare	0.835144
Adj Rsquare	0.831847
Root Mean Square Error	402.3474
Mean of Response	727.8731
Observations (or Sum Wgts)	52

t Test

2-3

Assuming equal variances

Difference	-1960.1	t Ratio	-15.9153
Std Err Dif	123.2	DF	50
Upper CL Dif	-1712.7	Prob > |t|	0.0000
Lower CL Dif	-2207.4	Prob > t	1.0000
Confidence	0.95	Prob < t	0.0000

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Phase	1	41004331	41004331	253.2954	<.0001
Error	50	8094171	161883.41		
C. Total	51	49098501			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
2	37	162.27	66.15	29.4	295.1
3	15	2122.33	103.99	1913.7	2331.0

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for each pair using Student's t

t	Alpha
2.00856	0.05

Abs(Dif)-LSD

	3	2
3	-295.1	1712.7
2	1712.7	-187.9

Positive values show pairs of means that are significantly different.

Protist Comparsion- Fit Y by X

Oneway Analysis of Protists R2 By Phase
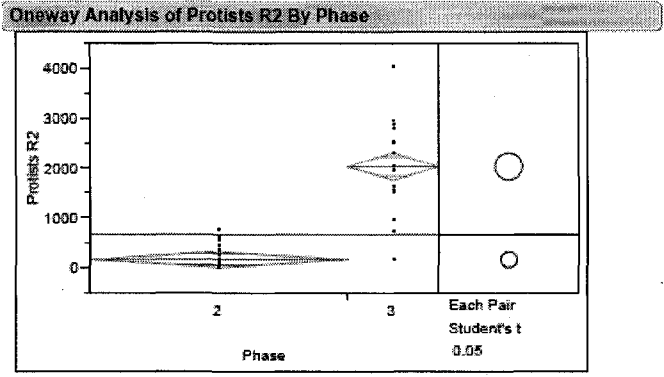
Missing Rows   27

Oneway Anova

Summary of Fit

Rsquare	0.720382
Adj Rsquare	0.715298
Root Mean Square Error	524.2854
Mean of Response	652.7193
Observations (or Sum Wgts)	57

t Test

2-3

Assuming equal variances

Difference	-1877.2	t Ratio	-11.9036
Std Err Dif	157.7	DF	55
Upper CL Dif	-1561.2	Prob > |t|	0.0000
Lower CL Dif	-2193.3	Prob > t	1.0000
Confidence	0.95	Prob < t	0.0000
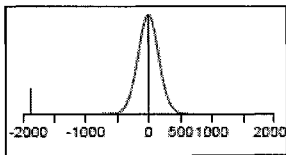
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Phase	1	38948938	38948938	141.6968	<.0001
Error	55	15118134	274875.15		
C. Total	56	54067070			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
2	42	158.71	80.90	-3.4109	320.8
3	15	2035.93	135.37	1765	2307.2

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for each pair using Student's t

	t	Alpha
	2.00404	0.05

Abs(Dif)-LSD

	3	2
3	-383.7	1561.2
2	1561.2	-229.3

Positive values show pairs of means that are significantly different.

Protist-TCE comparison (Phase comparison)- Fit Y by X

Oneway Analysis of Protists R1 By Phase
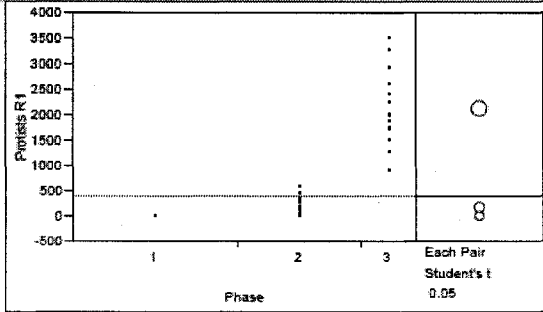
Missing Rows   30

Means Comparisons

Comparisons for each pair using Student's t

t	Alpha
1.98580	0.05

Abs(Dif)-LSD

	3	2	1
3	-213.5	1772.8	1948.5
2	1772.8	-139.8	37.9
1	1948.5	37.9	-121.9

Positive values show pairs of means that are significantly different.

Level		Mean
3	A	2122.3333
2	B	169.0857
1	C	0.0000

Levels not connected by same letter are significantly different

Level	- Level	Difference	Lower CL	Upper CL	p-Value	
3	1	2122.333	1948.472	2296.194	5.652e-42	
3	2	1953.248	1772.793	2133.702	7.584e-38	
2	1	169.086	37.929	300.243	0.0120768	

Oneway Analysis of Protists R2 By Phase
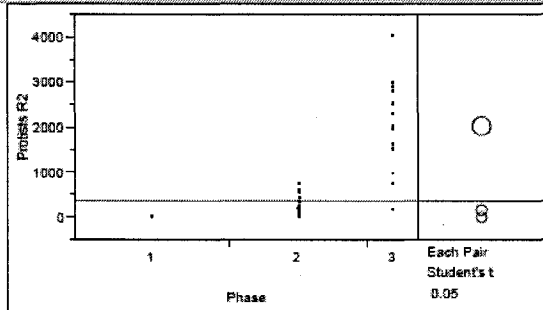
Missing Rows   24

Means Comparisons

Comparisons for each pair using Student's t

t	Alpha
1.98422	0.05

Abs(Dif)-LSD

	3	2	1
3	-283.0	1640.8	1805.5
2	1640.8	-171.2	-5.3
1	1805.5	-5.3	-161.6

Positive values show pairs of means that are significantly different.

Level		Mean
3	A	2035.9333
2	B	161.1951
1	B	0.0000

Levels not connected by same letter are significantly different

Level	- Level	Difference	Lower CL	Upper CL	p-Value	
3	1	2035.933	1805.47	2266.400	4.084e-32	
3	2	1874.738	1640.84	2108.635	5.094e-29	
2	1	161.195	-5.28	327.673	0.0575766	

Oneway Analysis of TCE Phase I By Reactor
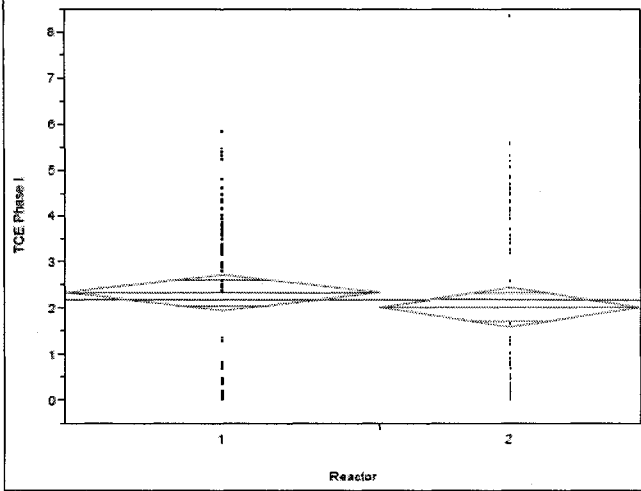
Missing Rows   77

Oneway Anova

Summary of Fit

Rsquare	0.00605318
Adj Rsquare	0.00092974
Root Mean Square Error	2.02231101
Mean of Response	2.16433055
Observations (or Sum Wgts)	196

t Test

1-2

Assuming equal variances

Difference	0.31536	t Ratio	1.086954
Std Err Dif	0.29013	DF	194
Upper CL Dif	0.88756	Prob > |t|	0.2784
Lower CL Dif	-0.25685	Prob > t	0.1392
Confidence	0.95	Prob < t	0.8608
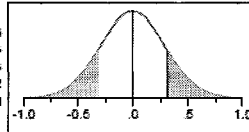
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Reactor	1	4.83190	4.83190	1.1815	0.2784
Error	194	793.40992	4.08974		
C. Total	195	798.24182			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	107	2.32753	0.19550	1.9419	2.7131
2	89	2.01217	0.21436	1.5894	2.4350

Std Error uses a pooled estimate of error variance

148

Protist Experiment Phase I (no protists) reactor comparison- Fit Y by X

Oneway Analysis of DCE Phase I By Reactor
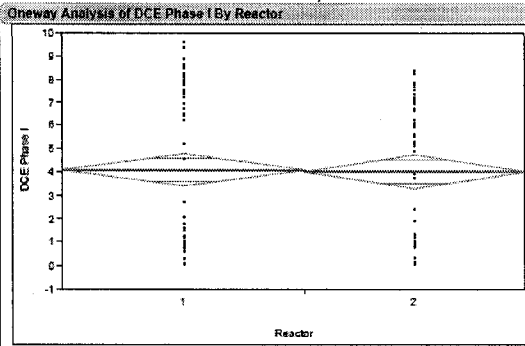
Missing Rows 103

Oneway Anova

Summary of Fit

Rsquare	0.000144
Adj Rsquare	-0.00581
Root Mean Square Error	3.293287
Mean of Response	4.04436
Observations (or Sum Wgts)	170

t Test

1-2

Assuming equal variances

Difference	0.0797	t Ratio	0.155486
Std Err Dif	0.5058	DF	168
Upper CL Dif	1.0773	Prob > |t|	0.8766
Lower CL Dif	-0.9200	Prob > t	0.4383
Confidence	0.95	Prob < t	0.5617
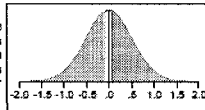
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Reactor	1	0.2624	0.2624	0.0242	0.8766
Error	168	1822.8583	10.8503		
C. Total	169	1823.1207			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	89	4.08184	0.34916	3.3926	4.7711
2	81	4.00318	0.36600	3.2806	4.7257

Std Error uses a pooled estimate of error variance

Oneway Analysis of VC Phase I By Reactor
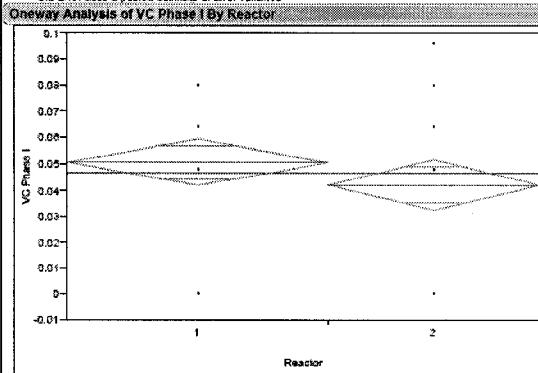
Missing Rows 188

Oneway Anova

Summary of Fit

Rsquare	0.020214
Adj Rsquare	0.008409
Root Mean Square Error	0.030189
Mean of Response	0.046871
Observations (or Sum Wgts)	85

t Test

1-2

Assuming equal variances

Difference	0.00862	t Ratio	1.309679
Std Err Dif	0.00659	DF	83
Upper CL Dif	0.02172	Prob > |t|	0.1943
Lower CL Dif	-0.00448	Prob > t	0.0971
Confidence	0.95	Prob < t	0.9029
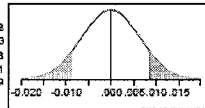
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Reactor	1	0.00156059	0.001561	1.7124	0.1943
Error	83	0.07564298	0.000911		
C. Total	84	0.07720356			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	47	0.050723	0.00440	0.04197	0.05948
2	38	0.042105	0.00490	0.03236	0.05185

Std Error uses a pooled estimate of error variance

Protist Experiment Phase I (no protists) reactor comparison- Fit Y by X

Oneway Analysis of Bacteria Phase I By Reactor
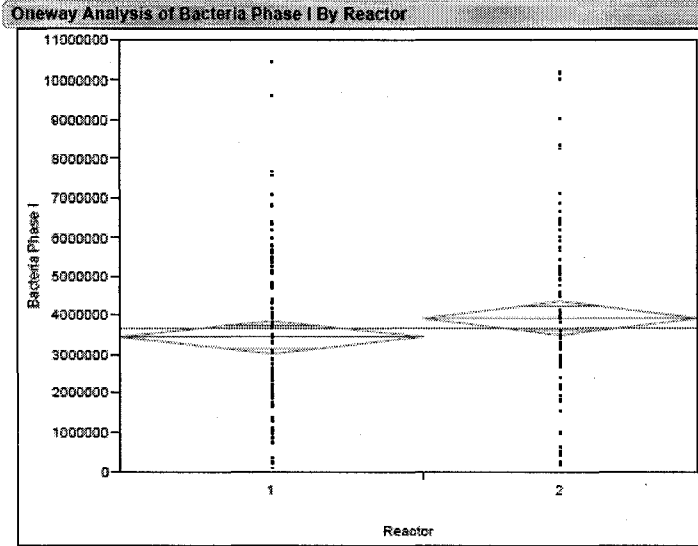
Missing Rows   75

Oneway Anova

Summary of Fit

Rsquare	0.012327
Adj Rsquare	0.007288
Root Mean Square Error	2141580
Mean of Response	3646592
Observations (or Sum Wgts)	198

t Test

1-2

Assuming equal variances

Difference	-476696	t Ratio	-1.56407
Std Err Dif	304780	DF	196
Upper CL Dif	124373	Prob > |t|	0.1194
Lower CL Dif	-1077766	Prob > t	0.9403
Confidence	0.95	Prob < t	0.0597

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Reactor	1	1.12197e13	1.122e+13	2.4463	0.1194
Error	196	8.98928e14	4.5864e12		
C. Total	197	9.10147e14			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	104	3420282	209999	3006133.5	3834429.7
2	94	3896978	220887	3461357.5	4332598.7

Std Error uses a pooled estimate of error variance

Protist Experiment Phase I (no protists) reactor comparison- Fit Y by X

Oneway Analysis of TOC Phase I By Reactor
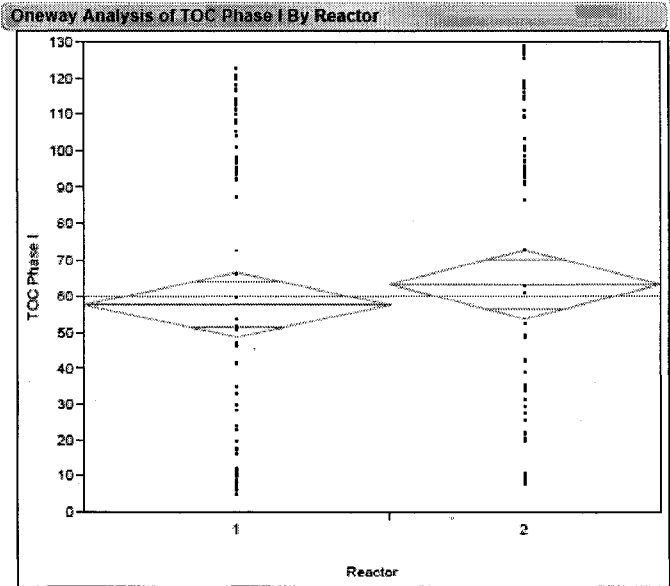
Missing Rows   90

Oneway Anova

Summary of Fit

Rsquare	0.003945
Adj Rsquare	-0.00156
Root Mean Square Error	44.42713
Mean of Response	60.25027
Observations (or Sum Wgts)	183

t Test

1-2

Assuming equal variances

Difference	-5.573	t Ratio	-0.84698
Std Err Dif	6.580	DF	181
Upper CL Dif	7.410	Prob > |t|	0.3981
Lower CL Dif	-18.567	Prob > t	0.8009
Confidence	0.95	Prob < t	0.1991

-20 -15 -10 -5 0 5 10 15 20
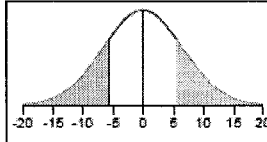
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Reactor	1	1415.93	1415.93	0.7174	0.3981
Error	181	357252.42	1973.77		
C. Total	182	358668.36			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	97	57.6311	4.5109	48.730	66.532
2	86	63.2044	4.7807	53.752	72.657

Std Error uses a pooled estimate of error variance

Protist Experiment Phase II (protists) reactor comparison- Fit Y by X

Oneway Analysis of TCE Phase II By Reactor 2

Reactor 2
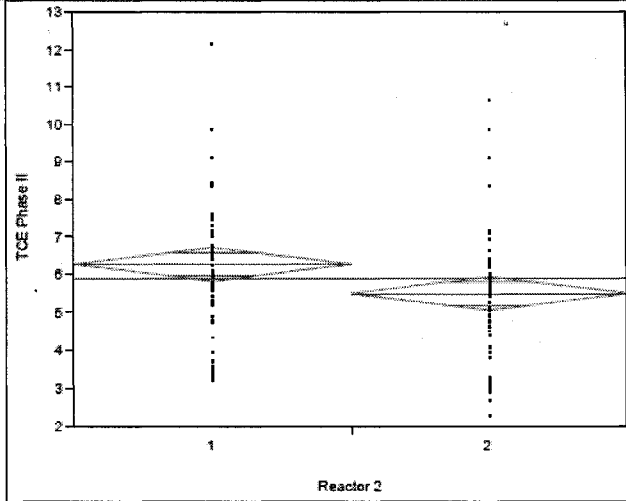
Missing Rows   87

Oneway Anova

Summary of Fit

Rsquare	0.040632
Adj Rsquare	0.033923
Root Mean Square Error	1.900779
Mean of Response	5.873103
Observations (or Sum Wgts)	145

t Test

1-2

Assuming equal variances

Difference	0.77696	t Ratio	2.460994
Std Err Dif	0.31571	DF	143
Upper CL Dif	1.40102	Prob > |t|	0.0150
Lower CL Dif	0.15290	Prob > t	0.0075
Confidence	0.95	Prob < t	0.9925
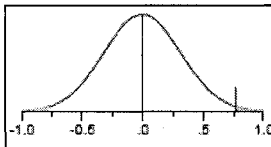
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Reactor 2	1	21.88186	21.8819	6.0565	0.0150
Error	143	516.65344	3.6130		
C. Total	144	538.53530			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	73	6.25890	0.22247	5.8192	6.6987
2	72	5.48194	0.22401	5.0391	5.9247

Std Error uses a pooled estimate of error variance

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Protist Experiment Phase II (protists) reactor comparison- Fit Y by X

Oneway Analysis of DCE Phase II By Reactor 2
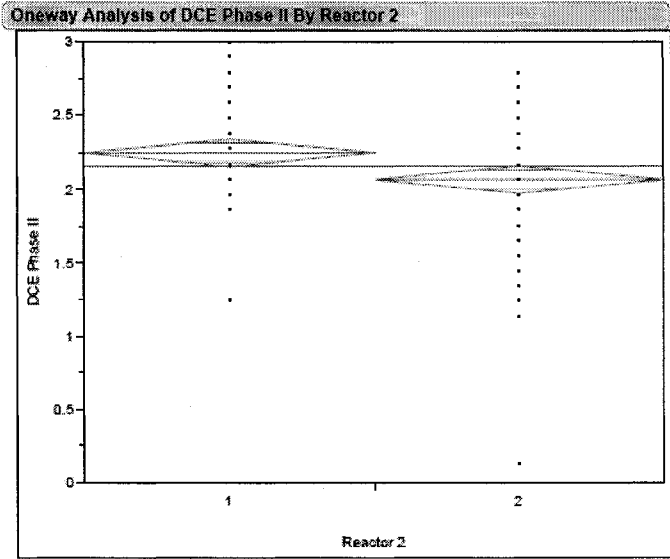
Missing Rows   87

Oneway Anova

Summary of Fit

Rsquare	0.049521
Adj Rsquare	0.042874
Root Mean Square Error	0.39505
Mean of Response	2.155724
Observations (or Sum Wgts)	145

t Test

1-2
Assuming equal variances

Difference	0.179102	t Ratio	2.729558
Std Err Dif	0.065616	DF	143
Upper CL Dif	0.308804	Prob > |t|	0.0071
Lower CL Dif	0.049400	Prob > t	0.0036
Confidence	0.95	Prob < t	0.9964
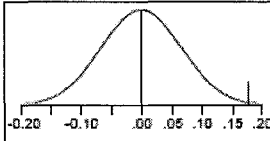
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Reactor 2	1	1.162755	1.16276	7.4505	0.0071
Error	143	22.317194	0.15606		
C. Total	144	23.479949			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	73	2.24466	0.04624	2.1533	2.3361
2	72	2.06556	0.04656	1.9735	2.1576

Std Error uses a pooled estimate of error variance

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Oneway Analysis of VC Phase II By Reactor 2
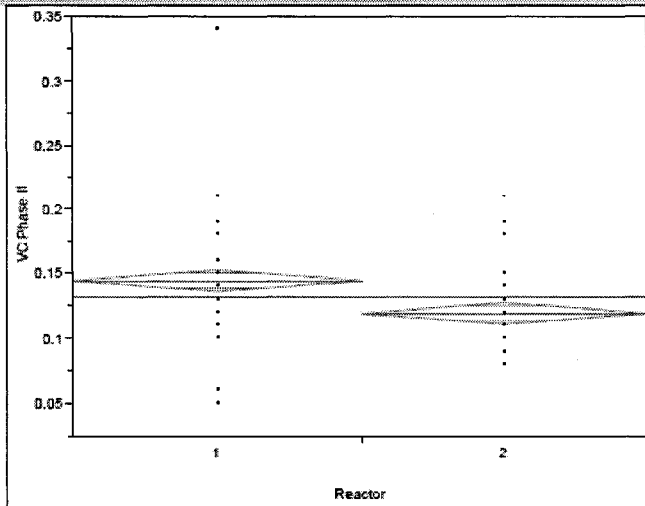
Missing Rows   87

Oneway Anova

Summary of Fit

Rsquare	0.108869
Adj Rsquare	0.102637
Root Mean Square Error	0.036134
Mean of Response	0.131379
Observations (or Sum Wgts)	145

t Test

1-2

Assuming equal variances

Difference	0.025086	t Ratio	4.179733
Std Err Dif	0.006002	DF	143
Upper CL Dif	0.036949	Prob > |t|	<.0001
Lower CL Dif	0.013222	Prob > t	<.0001
Confidence	0.95	Prob < t	1.0000
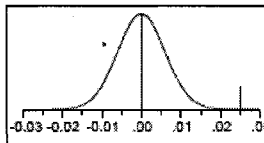
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Reactor 2	1	0.02281061	0.022811	17.4702	<.0001
Error	143	0.18671353	0.001306		
C. Total	144	0.20952414			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	73	0.143838	0.00423	0.13548	0.15220
2	72	0.118750	0.00426	0.11033	0.12717

Std Error uses a pooled estimate of error variance

Protist Experiment Phase II (protists) reactor comparison- Fit Y by X

Oneway Analysis of Bacteria Phase II By Reactor 2
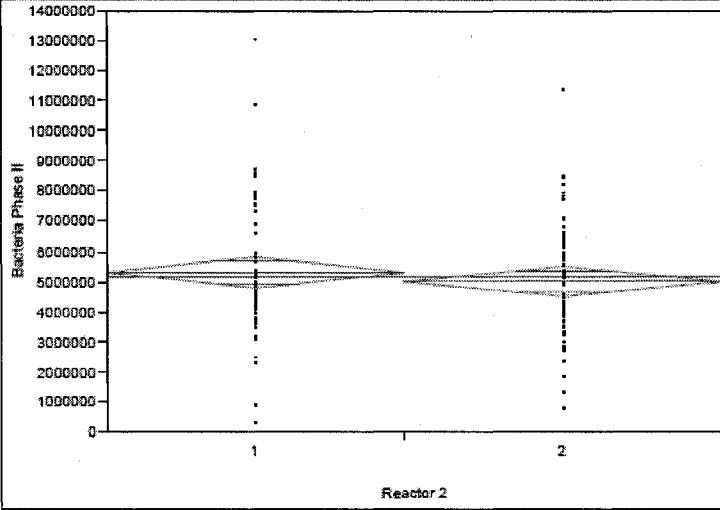
Missing Rows   99

Oneway Anova

Summary of Fit

Rsquare	0.005367
Adj Rsquare	-0.00223
Root Mean Square Error	2084699
Mean of Response	5146142
Observations (or Sum Wgts)	133

t Test

1-2

Assuming equal variances

Difference	304194	t Ratio	0.840778
Std Err Dif	361798	DF	131
Upper CL Dif	1019898	Prob > |t|	0.4020
Lower CL Dif	-411520	Prob > t	0.2010
Confidence	0.95	Prob < t	0.7990
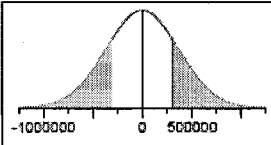
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Reactor 2	1	3.0722e+12	3.0722e12	0.7069	0.4020
Error	131	5.69322e14	4.346e+12		
C. Total	132	5.72394e14			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	64	5302951	260587	4787447.4	5818455.4
2	69	4998768	250968	4502292.5	5495242.7

Std Error uses a pooled estimate of error variance

Protist Experiment Phase II (protists) reactor comparison- Fit Y by X

Oneway Analysis of Protists Phase II By Reactor 2

Reactor 2
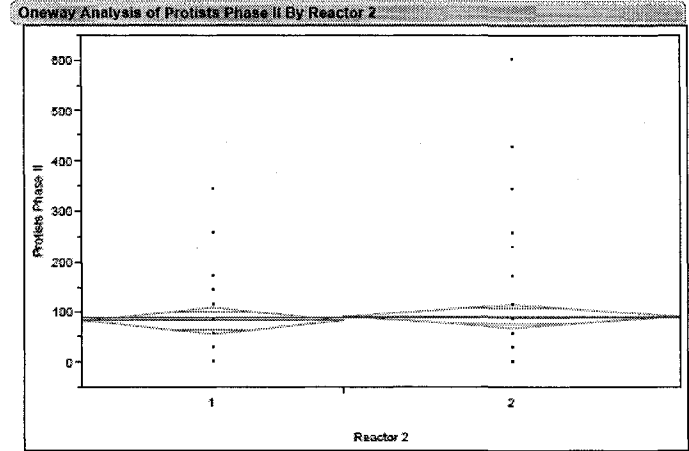
Missing Rows   106

Oneway Anova

Summary of Fit

Rsquare	0.001902
Adj Rsquare	-0.00615
Root Mean Square Error	98.58679
Mean of Response	86.96032
Observations (or Sum Wgts)	126

t Test

1-2

Assuming equal variances

Difference	-8.609	t Ratio	-0.48615
Std Err Dif	17.709	DF	124
Upper CL Dif	26.442	Prob > |t|	0.6277
Lower CL Dif	-43.660	Prob > t	0.6861
Confidence	0.95	Prob < t	0.3139

-60 -40 -20   0 10 20 30 40 50 60
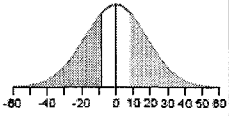
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Reactor 2	1	2297.1	2297.09	0.2363	0.6277
Error	124	1205199.7	9719.35		
C. Total	125	1207496.8			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	55	82.1091	13.293	55.798	108.42
2	71	90.7183	11.700	67.561	113.88

Std Error uses a pooled estimate of error variance

)

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Protist Experiment Phase II (protists) reactor comparison- Fit Y by X

Oneway Analysis of TOC Phase II By Reactor 2
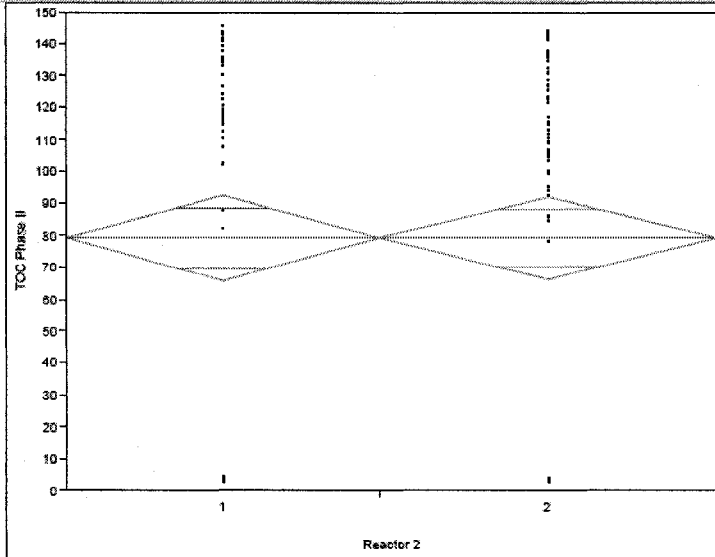
Missing Rows   78

Oneway Anova

Summary of Fit

Rsquare	4.825e-8
Adj Rsquare	-0.00658
Root Mean Square Error	57.75488
Mean of Response	79.18041
Observations (or Sum Wgts)	154

t Test

1-2
Assuming equal variances

Difference	0.025	t Ratio	0.002708
Std Err Dif	9.315	DF	152
Upper CL Dif	18.429	Prob > |t|	0.9978
Lower CL Dif	-18.379	Prob > t	0.4989
Confidence	0.95	Prob < t	0.5011

-30 -20 -10   0   10   20   30
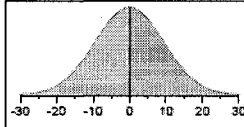
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Reactor 2	1	0.02446235	0.024462	0.0000	0.9978
Error	152	507011.62	3335.60		
C. Total	153	507011.65			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	74	79.1935	6.7138	65.929	92.458
2	80	79.1683	6.4572	66.411	91.926

Std Error uses a pooled estimate of error variance

Oneway Analysis of Bacteria Phase III By Reactor
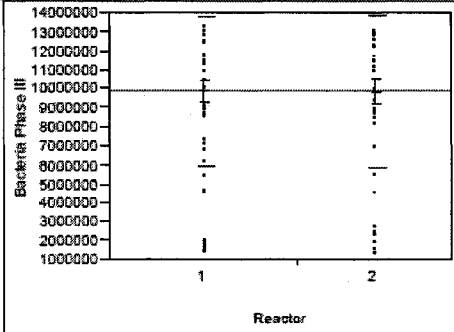
Missing Rows   99

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
1	55	9860688	3946274	532115	8793860.9	10927515
2	45	9843222	4013594	598311	8637404.7	11049039

t Test

1-2

Assuming unequal variances

Difference	17466	t Ratio	0.021814
Std Err Dif	800702	DF	93.4796
Upper CL Dif	1607394	Prob > |t|	0.9826
Lower CL Dif	-1572461	Prob > t	0.4913
Confidence	0.95	Prob < t	0.5087
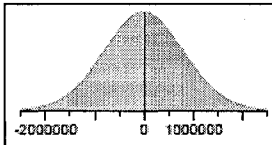
Practical Equivalence between 2 and 1

Specified Practical Difference Threshold	100
Actual Difference in Means	-17466.4
Std Error of Difference	799334.7

Test	t Ratio	p-Value
Upper Threshold	-0.02198	0.4913
Lower Threshold	-0.02173	0.5086
Max over both		0.5086
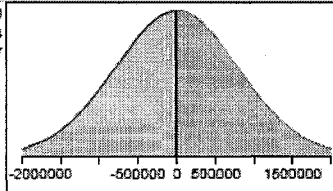
Oneway Analysis of TCE Phase III By Reactor
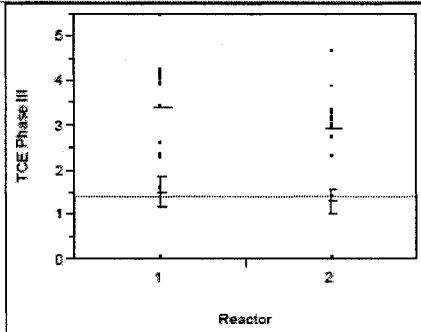
Missing Rows   131

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
1	34	1.49898	1.89630	0.32521	0.83733	2.1606
2	34	1.29570	1.60085	0.27454	0.73713	1.8543

t Test

1-2

Assuming unequal variances

Difference	0.2033	t Ratio	0.477633
Std Err Dif	0.4256	DF	64.19318
Upper CL Dif	1.0535	Prob > |t|	0.6345
Lower CL Dif	-0.6469	Prob > t	0.3173
Confidence	0.95	Prob < t	0.6827

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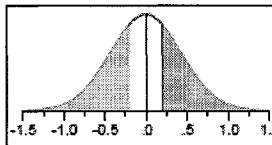
Oneway Analysis of DCE Phase III By Reactor
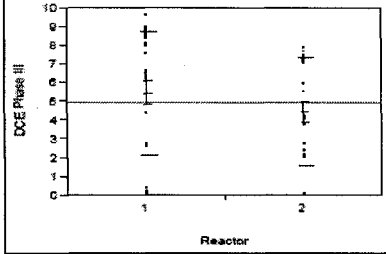
Missing Rows   143

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
1	28	5.41249	3.32461	0.62829	4.1233	6.7016
2	26	4.37890	2.86254	0.54664	3.2573	6.5005

t Test

1-2

Assuming unequal variances

Difference	1.0336	t Ratio	1.241089
Std Err Dif	0.8328	DF	52.98613
Upper CL Dif	2.7040	Prob > |t|	0.2200
Lower CL Dif	-0.6368	Prob > t	0.1100
Confidence	0.95	Prob < t	0.8900

Oneway Analysis of VC Phase III By Reactor
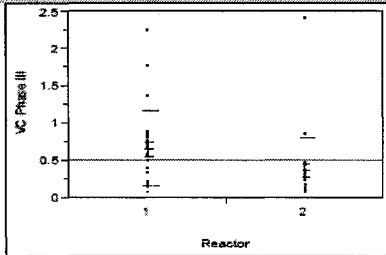
Missing Rows   143

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
1	28	0.636000	0.503929	0.09523	0.44080	0.83140
2	28	0.351429	0.435976	0.08239	0.18237	0.52046

t Test

1-2

Assuming unequal variances

Difference	0.284571	t Ratio	2.269795
Std Err Dif	0.125928	DF	52.90532
Upper CL Dif	0.537161	Prob > |t|	0.0280
Lower CL Dif	0.031981	Prob > t	0.0140
Confidence	0.95	Prob < t	0.9860

Oneway Analysis of Protists Phase III By Reactor
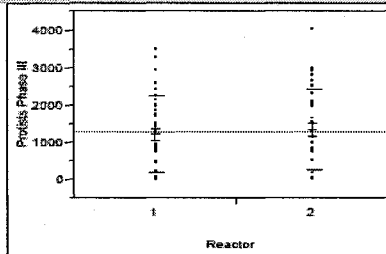
Missing Rows   131

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
1	35	1190.29	1034.68	174.89	834.85	1545.7
2	33	1322.09	1085.91	189.03	937.54	1707.1

t Test

1-2

Assuming unequal variances

Difference	-131.81	t Ratio	-0.51181
Std Err Dif	257.53	DF	65.2398
Upper CL Dif	382.48	Prob > |t|	0.6105
Lower CL Dif	-646.09	Prob > t	0.6947
Confidence	0.95	Prob < t	0.3053

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APPENDIX C: USGS GENETIC ANALYSIS

Summary of work for Joe Cunningham UNH

Assessment of bioreactor sample-change in microbial community over time

1) **real time PCR of Dehalococcoides and Geobacter in all samples**

    Geobacter and Dehaloccoides abundances were much higher in KB1 sample than all others, with Geobacter dominating. JC4 (10/27 R1) sample had approximately an order of magnitude fewer Geobacter and Dehalococcoides, and JC2 and JC3 (11/10 and 11/15) had detectable but low Geobacter abundances and no detectable Dehalococcoides. See slide 2.

2) **TRFLP (DNA fingerprint) analysis of bacterial 16SrRNA gene PCR products from all samples digested with MnlI (primers used were 46f-FAM and 519r)**

    Different peak sizes on the TRFLP electropherogram show that the bacterial community has shifted over time (and JC2 and JC3 share the same dominant peaks, but they are in different proportions). See slide 3.

3) **Clone library analysis of initial KB1 culture (JC1) and reactor samples after morphological change(JC 2 and 3 combined)**

    Geobacter, Pelobacter, and Dehalococcoides dominate the clone library from JC1, but the clone library from JC2 and 3 is dominated by other organisms such as one closely related to a gamma proteobacterium and one closely related to an epsilon proteobacterium found in dechlorinating consortia or communities. It appears that the community has dramatically shifted, but still is populated by many organisms found in other dechlorinating consortia or communities. See slides 4 and 5. The tables show how many different types of organisms we obtained by RFLP (restriction fragment length polymorphism) analysis of the clones in the library, what proportion of the clone library they represented, and once the clones' DNA were sequenced, the closest cultured and uncultured sequences found in the NCBI blast data base (http://www.ncbi.nlm.nih.gov/BLAST/).

Real-time PCR assessment of Dehalococcoides and Geobacter

Sample	Description	DHC/ng DNA	Geobact/ng DNA
JC1	KB1 3/15/07	2610	37680
JC2	11/10 R2	BLD	80
JC3	11/15 R1	BLD	720
JC4	10/27 R1	148	2209

DNA fingerprint (TRFLP) showing changes in bioreactor over time

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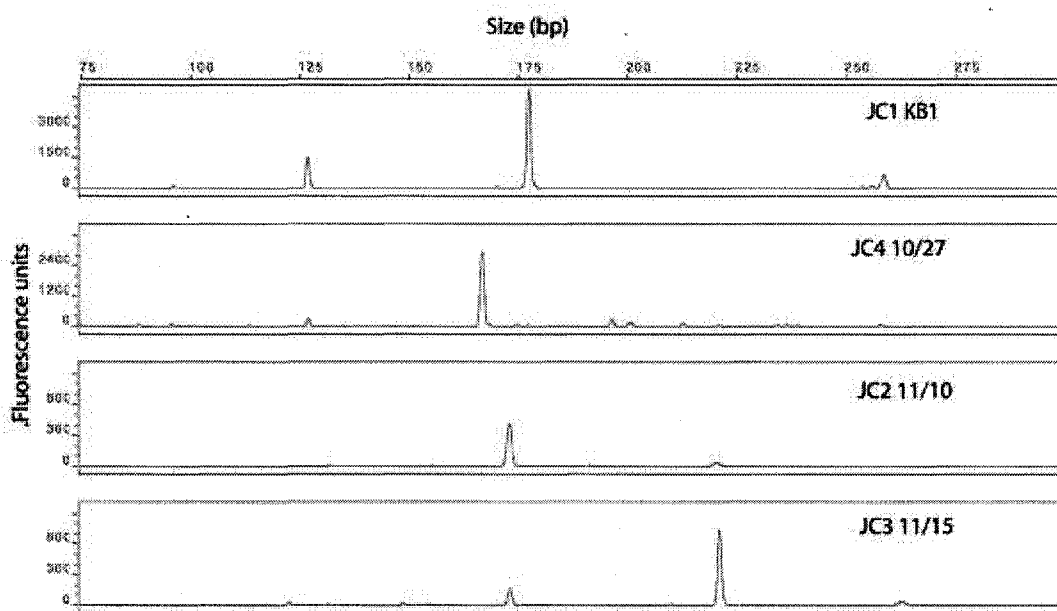
Joe Cunningham bioreactor samples_clone library analyses

#1 KB1 3/15/07 (description from JC- very high concentration dechlorinating culture sample)

restriction analysis of 46f/519r library digested with MspI and HinpI and subsequent sequencing of representative patterns

RFLP pattern	# of clones	% of total library	Blast seq closest relatives uncultured	Blast seq closest relatives cultured
1	6	14	DQ191700 Uncultured bacterium clone AME E30 "Microbial community dynamics of psychrophilic anaerobic bioreactors treating a solvent-containing pharmaceutical wastewater" 457/470 (97%)	CP000482 Pelobacter propionicus DSM 2379 "Complete sequence of chromosome of Pelobacter propionicus DSM 2379" 452/470 (96%)
2	2	5	DQ191700 uncultured bacterium clone AME E30 "Microbial community dynamics of psychrophilic anaerobic bioreactors treating a solvent-containing pharmaceutical wastewater" 457/471 (97% sim)	CP000482 Pelobacter propionicus DSM 2379 "Complete sequence of chromosome of Pelobacter propionicus DSM 2379" 452/471 (95% sim)
3	25	58	AY780563 Uncultured Geobacter sp. clone KB-1 "Microbial composition of chlorinated ethene-degrading cultures dominated by Dehalococcoides" (100% sim 465/465)	AY914177 Geobacter lovleyi "Geobacter lovleyi sp. nov. Strain SZ, a Novel Metal-Reducing and Tetrachloroethene-Dechlorinating Bacterium" (99% sim. 464/465)
4	3	7	DQ833298 Uncultured Dehalococcoides sp. clone PMVC7 "populations Implicated in the Anaerobic Reductive Dechlorination of Vinyl Chloride in Highly Enriched Communities from Pere Marquette River Sediment Material" 426/426 (100%)	EF059530 Dehalococcoides sp. JN18_V108_B "The Dehalococcoides Population in Sediment-Free Mixed Cultures Metabolically Dechlorinates the Commercial Polychlorinated Biphenyl Mixture Aroclor 1260" 426/426 (100%)
5	5	12	AY780563 Uncultured Geobacter sp. clone KB-1 "Microbial composition of chlorinated ethene-degrading cultures dominated by Dehalococcoides" 471/471 (100%)	AY914177 Geobacter lovleyi "Geobacter lovleyi sp. nov. Strain SZ, a Novel Metal-Reducing and Tetrachloroethene-Dechlorinating Bacterium" 470/471 (99%)
6	1	2	AY780563 Uncultured Geobacter sp. clone KB-1 "Microbial composition of chlorinated ethene-degrading cultures dominated by Dehalococcoides" 469/472 (99%)	AY914177 Geobacter lovleyi "Geobacter lovleyi sp. nov. Strain SZ, a Novel Metal-Reducing and Tetrachloroethene-Dechlorinating Bacterium" 468/472 (99%)
7	1	2	DQ401522 Uncultured bacterium clone R3ENDE/ "Microbial community structure of anaerobic granular sludge during low-temperature anaerobic digestion of toluene contaminated wastewater" 431/453 (95%)	AY737507 Geobacter hephaestius "Geobacter hephaestius sp. nov., a sulfur- and iron-reducing bacterium isolated from anoxic rice paddy soil" 422/455 (92%)
total #	43	100		

library #2 combination of 11/10 R2 and 11/15 R1
(description-reactor sample; low-medium density elongated bacteria)
restriction analysis of 46f/519r library digested with MspI and HinpI and subsequent sequencing of representative patterns

RFLP Pattern	# of clones	%	Blast seq closest relatives uncultured	Blast seq closest relatives cultured
8	26	55	DQ830638 Uncultured bacterium clone PAS3_E07 "Comparison of Bacterial Communities and Diversity of Soil from Three Sites at an Experimental Rice Station" 450/470 (95%)	DQ767881 Acidaminococcaceae bacterium FCF9A "New strains of fermentative bacteria from Lake Fryxell, McMurdo Dry Valleys, Antarctica" 447/470 (95%)
9	14	30	EF393413 Uncultured bacterium clone ORSFC2 "Phylogenetic Characterization of Polychlorinated Biphenyl Dechlorinating Consortia Under Different Anaerobic Treatments of Ohio River Sediments" 387/400 (96%)	none in top 100 blast hits
10	4	9	AY667251 "Uncultured bacterium clone TANB6 Molecular characterization of a dechlorinating community resulting from in situ biostimulation in a trichloroethene-contaminated deep, fractured basalt aquifer and comparison on to a derivative laboratory culture" 43	AF218076 Sulfurospirillum halorespirans strain PCE-M2 "Description of Sulfurospirillum halorespirans sp. nov., an anaerobic, tetrachloroethene-respiring bacterium, and transfer of Dehalospirillum multivorans to the genus Sulfurospirillum as Sulfurospirillu
11	2	4	EF491568 Uncultured gamma proteobacterium clone PC-10 "Historical volcanic activities on bacterial diversity and vertical distribution in deep-sea sediments of the tropical West Philippine Basin" 456/457 (99%)	DQ501957 Serratia marcescens "Molecular detection of the biowarfare simulant Serratia marcescens from a 50-year-old munition buried at Fort Detrick, Maryland" 456/457 (99%)
12	1	2	EF393413 Uncultured bacterium clone ORSFC2 "Phylogenetic Characterization of Polychlorinated Biphenyl Dechlorinating Consortia Under Different Anaerobic Treatments of Ohio River Sediments" 383/397 (96%)	none in top 100 blast hits
total #	47	100		

APPENDIX D: SITE 32 LAYOUT

CONCEPTUAL MODEL OF DISSOLVED CONTAMINANT
MIGRATION PATHWAYS IN GROUND WATER AT SITE 32

Site 32 Cross Section

North

Upper Sand

Marine Clay

Lower Sand

Bedrock

Groundwater Flow

Sheet Pile/Pump & Treat

Site 32 Source Area

ca. 40m

TCE (pink) 200–80,000 ppb
DCE (blue) 5–5,000 ppb
VC   1–20 ppb
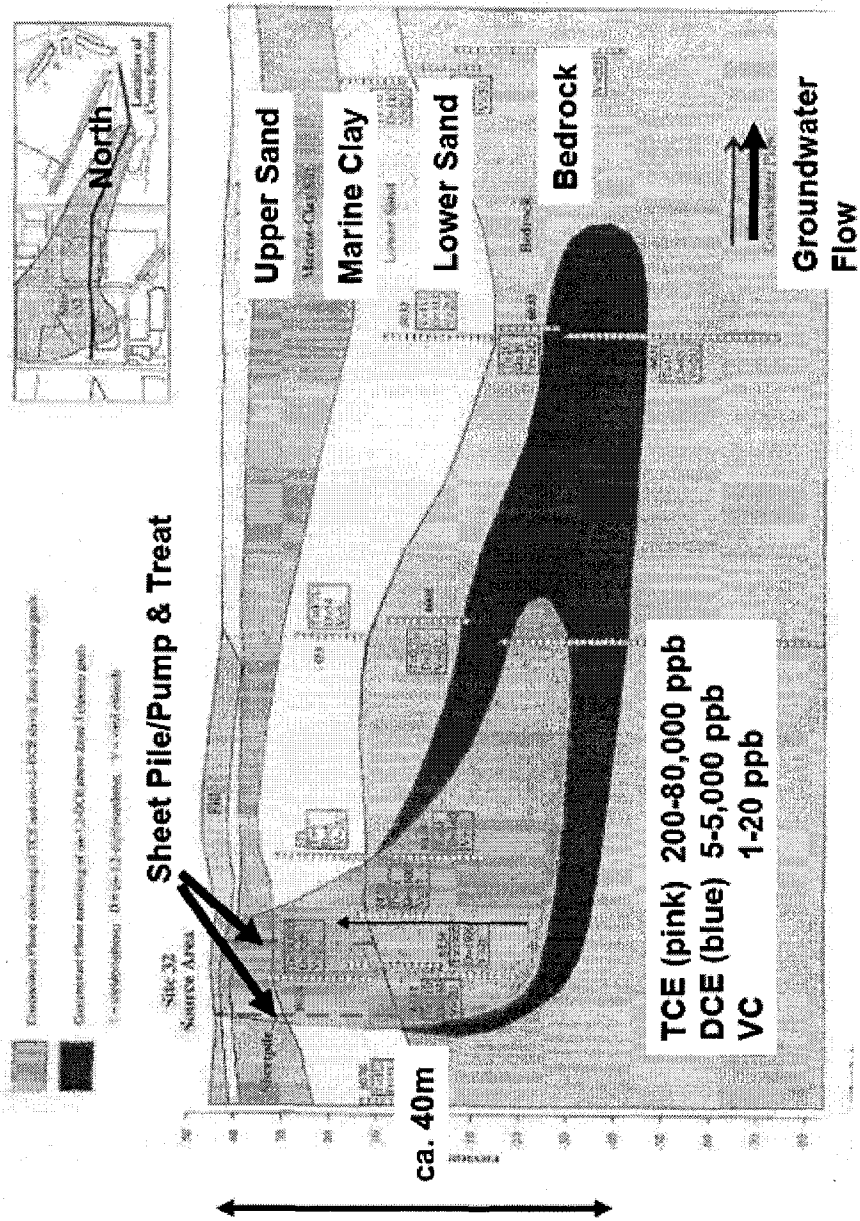
Bedrock Bioremediation Center,
UNH. 2003

Pease International Tradeport, Site 32.
Extent of VOC contaminant plume in 1992, 1998 and 2002.