LABORATORY STUDY REPORT

Enhancing Natural Attenuation Through Bioaugmentation with Aerobic Bacteria that Degrade Cis-1,2-Dichloroethene

ESTCP Project ER-0516

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EXECUTIVE SUMMARY

Monitored natural attenuation (MNA) and enhanced in situ bioremediation (EISB) remedies hold the promise of reducing the costs associated with cleanup of Department of Defense (DoD) sites impacted by chlorinated solvents. However, there are many DoD sites where tetrachloroethene (PCE) and trichloroethene (TCE) are only undergoing partial dechlorination to cis-1,2-dichloroethene (cDCE), even when sufficient electron donor is present or added. As a result, there are a significant number of plumes at DoD and related sites where PCE and TCE have been dechlorinated to cDCE, but where the cDCE persists and migrates uncontrolled in groundwater rather than undergoing further dechlorination to ethene (the desired end product).

Through a project funded by the Strategic Environmental Research and Development Program (SERDP; project ER-1168) a novel aerobic bacterium (JS666) was isolated and described that is able to use cDCE as a sole carbon and energy source under aerobic conditions, converting it to carbon dioxide and water without the addition of exotic co-factors (Coleman et al., 2002a,b).

Subsequently, the Environmental Security Technology Certification Program (ESTCP) funded additional work under project ER-0516 to optimize the growth of JS666, to further characterize its ability to degrade solvent mixtures, and to assess whether JS666 can transfer its ability to degrade cDCE to other in situ microorganisms. These studies were conducted in preparation for a field scale demonstration to evaluate the effectiveness of JS666 as an in situ bioaugmentation agent to degrade cDCE and other chlorinated solvents in groundwaters that are or will become aerobic.

This laboratory study report presents the results of the Growth, Kinetic, and Optimization Assessment conducted under Task 1 of ESTCP project ER-0516. Laboratory experiments were conducted by Georgia Institute of Technology and Cornell University to establish factors that will allow optimal cell growth for production purposes and to collect data required to evaluate the application of JS666 under various field conditions.

Results of these experiments indicate that the JS666 culture can be effectively grown for field application. Moreover, cells that have been stored or stockpiled over a short period of time can rapidly recover the ability to degrade cDCE, lending promise to the culture's in-situ activity following transport to and subsequent injection at a suitable field site. Kinetic assays have also allowed for estimation of biodegradation rates (e.g., cDCE degrades at 120 ug/L/day at 23°C using a 4 x 10^4 cells/mL inoculum). Because JS666 can co-metabolize trans-1,2-dichloroethene (tDCE), TCE, vinyl chloride (VC), 1,2-dichloroethane (1,2-DCA), or ethene while growing on cDCE, its application can be expanded to sites containing not only cDCE but mixtures of cDCE and these other co-contaminants. Furthermore, microcosms studies conducted with a range of aquifer materials suggest that JS666 will survive and remain active in subsurface environments. Experiments with various inoculum levels suggest that 10^4 cells/mL will be an effective dose of JS666. Unfortunately, no evidence was found to suggest that the ability to degrade cDCE can be transferred from JS666 to indigenous bacteria. As such, when assessing bioaugmentation with

JS666 as a remedial alternative it is necessary to ensure that the conditions at the site are suitable for JS666 to thrive. Ideal conditions for JS666 include pH conditions above 6.5, dissolved oxygen levels from >0.01 to <8 mg/L, and low conductivity (<15 mS/cm).

In the following sections, the results of the laboratory study are summarized in more detail.

Cultivation of JS666

Investigation of the cultural conditions necessary for reliable production of a JS666 inoculum for bioaugmentation revealed that pH must be kept above 6.5 for sustained cDCE-degradation; and previous studies (Coleman et al., 2002a) indicated that temperature must be kept under 30ºC (and preferably below 25 ºC). The need for pH-neutrality may present challenges at some cDCE-contaminated sites and suggests that buffer (current recommendation is 40 mM phosphate) may have to be introduced along with this bioaugmentation agent. On the other hand, it may be possible to adapt JS666 to lower pH through selection of low-pH-tolerant variants [Note: since JS666 was originally enriched and isolated at neutral pH, it is not surprising that our current variety prefers neutral pH.] We are presently investigating adaptation through pH "challenges" to the culture.

Two different cultural behaviors were observed: 1) "Bad Behavior" with high-density cultures (> 0.3 OD_{600}) recently exposed to co-substrates such as glycerol, succinate or ethanol, in which cDCE-degradation rates steadily decline, cDCE degradation is not sustainable, and behavior resembles that of co metabolism; and 2) "Good Behavior" observed in the original studies of Coleman et al. (2002a), with low-density (< 0.05 OD_{600}) cultures frequently transferred to fresh medium and which have not seen any substrate but cDCE for many generations. We successfully demonstrated production of a stable, sustainable inoculum source exhibiting "good behavior" through frequent transfer/dilution into fresh media.

Tests were conducted to define growth requirements and tolerances of JS666 to obtain optimum production of a high-population-density inoculum source as well as to insure *in situ* activity following bioaugmentation. Results suggested that minimization of ionic strength while maintaining neutral pH can increase the growth of JS666 on cDCE. Best growth in serum bottles was obtained in ½-strength MSB (40 mM phosphate buffer) at pH 7.2 under atmospheric air. No effects on cDCE degradation were found from vitamin addition; lowered O_2 concentrations; sulfite, thiosulfate, CO_2, CO_2 plus H_2, or CO addition. No density dependent phenomena were observed.

Studies investigating the oxygen-tolerance of JS666 showed that cultivation at elevated (above normal, ambient atmospheric) levels of oxygen negatively impacted culture-health. This was manifested by less cumulative cDCE degraded (before degradation ability ceased) with increasing oxygen partial pressures from 105 mmHg to 600 mmHg. The likely cause is damage from production of reactive oxygen species (ROS) produced during aerobic oxidation of cDCE.

In bioreactor studies, growth on cDCE was substantially better in low ionic strength media (10 mM phosphate) than in reactors with 40 mM phosphate. Growth in the bioreactor is eventually limited by the accumulation of a water-soluble factor, most likely NaCl as a result of chloride release from cDCE and neutralization by NaOH. Soluble metabolites were eliminated by medium exchange with cell retention by addition of a loop to a transverse filtration apparatus. Review of the longest bioreactor run shows that onset of slow growth occurred when total cDCE pumped into the reactor since the last medium exchange reached 1.3 – 1.5 ml L^{-1}, a 4-5 fold increase in total cDCE degradation over that obtained in serum bottles.

The objective of 100 L of cells at OD_{600} = 1 for bioaugmentation dictates that cDCE-grown cells of JS666 be stored or stockpiled. Tests showed that cells stored as pellets at -80°C without cryopreservatives, recovered activity more rapidly than cells stored under any other condition. A cell pellet frozen at -80° C for 35 days was thawed at room temperature, suspended in ½ MSB, then used to inoculate a 1-L reactor. Growth on cDCE, as indicated by sustained increases in OD_{600} began 3 days after inoculation. The results show that after moderate term storage at -80°, pellets of cDCE-grown cells can rapidly recover the ability to degrade cDCE and can be stockpiled for bioaugmentation.

Degradation of Mixtures of Chloroethenes

Previous studies (Coleman et al., 2002a) reported that JS666 can degrade chloroethenes and chloroethanes besides cDCE, without being able to grow on them. Because cDCE might be present in mixtures of chloroethenes and chloroethanes at contaminated sites for which bioaugmentation with JS666 would be considered, its response to such contaminant mixtures is of interest. In this phase of study, we investigated the relative kinetics and mutual effects of binary mixtures of cDCE at ~ 2 ppm in the presence of lesser concentrations (50 to 450 ppb) of VC, TCE, or 1,2-DCA. The good news, so far as bioremediation is concerned, is that although the co-presence of VC, TCE, or 1,2-DCA reduces the maximum degradation rate of cDCE, the rate remains substantial and cDCE can be completely degraded, as can the co-substrates.

The patterns of VC or TCE degradation in presence of cDCE suggest (but alone do not prove) some sort of parallel, co-metabolic degradation systems for cDCE and these co-substrates, with possibly some inhibitory/damaging byproducts of VC or TCE transformation. Co-presence of VC or TCE caused cDCE degradation rates to be halved, but the effect was not proportional to concentrations of VC or TCE. On the other hand, degradation of the co-substrate was either improved (VC) or unaffected (TCE) by the presence of cDCE.

This is in stark contrast to the patterns of 1,2-DCA degradation in presence of cDCE, which showed clearer signs of true competition: cDCE degradation was modestly inhibited by 1,2-DCA (but competitive inhibition would be expected to be modest, since molar concentrations of 1,2-DCA were far lower than those of cDCE) in a roughly linear decline with increasing 1,2-DCA concentration; and 1,2-DCA degradation was markedly inhibited by the very-much higher,

cDCE concentration. These results are consistent with our observation that JS666 can grow on 1,2-DCA, but not on VC or TCE. Different pathways are likely at work.

Assess Ability to Sustain Induction of cDCE-oxidizing Enzymes When Using Nonchlorinated Co-subtrates.

Succinate, acetate and ethanol were tested to determine their ability to boost cell growth without inhibition of the cDCE degradation pathway in order to facilitate production of the culture. The presence of ethanol yielded the most biomass and the fastest cDCE consumption per bottle; however, no-ethanol controls had the highest specific activity for cDCE degradation. Higher specific activities resulted from growth of JS666 on cDCE plus acetonitrile (MeCN). The result indicates that MeCN can be added as a co-substrate to get a large initial cell mass without long-term loss of the ability to grow on cDCE. Because the ability to grow on MeCN is rarer than the ability to grow on ethanol, it should be easier to maintain purity in cultures grown with MeCN than in cultures grown with ethanol.

The JS666 genome suggests that JS666 has the pathway to degrade *cis*-1,2-dichloroethane (DCA). Experiments showed that JS666 grows on DCA, but at much lower concentrations of DCA than of DCE. Estimates of the dimensionless Henry's constant for DCA at 22°C is much lower than for cDCE. This suggests that DCA is more toxic than DCE at comparable total concentrations because much more DCA is dissolved in the aqueous phase.

Assessment of the sustenance of cDCE oxidation activity in mixed-culture, subsurface environments; the effect of co-presence of degradable organics on sustenance of cDCE oxidation activity in mixed-culture, subsurface material; and the sustenance of cDCE oxidation activity in a rich, mixed-culture environment containing a wide variety of potential competitors.

The intention of the experiments described herein, was to assess the survivability of JS666 in subsurface materials and to determine under what conditions this culture could be applied for successful bioaugmentation. Microco sm experiments were performed under ideal conditions, and then systematically challenged with inhospitable conditions and other potential barriers. cDCE degradation was monitored, and because this organism would later be used in field tests of bioaugmentation, a molecular probe was applied to track growth or die-off of JS666 within some microcosms to test the probe's efficacy. Microcosms were constructed with subsurface materials from five sites: Savannah River Site (SRS), Hill Air Force Base (AFB), Robins AFB, Ft. Lewis, and Aerojet. Additionally, microcosms were constructed using two dilutions of primary sewage effluent: unautoclaved (contributing both complex organic substrates as well as competing and/or predatory microbes) and autoclaved (contributing only complex organics).

In neutral-pH-buffered microcosms constructed from all five site-materials, high concentration (~ 60 mg/L) of cDCE was completely degraded within 10 to 15 days when inoculated with "good-behaving" transfer culture at 4 x 10^5 cells/ml. Without inoculation of JS666, no

significant cDCE degradation was observed. Studies were undertaken to determine effective inoculum size, using three levels –- 1X, 0.1X, and 0.01X (where 1X corresponds to the 4 x 10^5 cell/ml concentration) – with SRS soil. In microcosms constructed of SRS soil + minimal salts medium (MSM), cDCE was depleted in about 20 days at 1X and was about 50% depleted in 60 days at both 0.1X and 0.01X inoculum levels. With a more realistic initial cDCE concentration (0.6 mg/L), complete degradation was observed in about 5 days at 1X and 0.1X, and in about 20 days at the 0.01X inoculum level. Therefore, we believe 10^4 cells/ml is a reasonable suggested inoculum level for field application.

As a rigorous test of both microbial competition/predation, and of the co-presence of alternative substrates, we undertook studies in which municipal primary sewage effluent was added to SRS-soil microcosms along with JS666. Both raw and autoclaved sewage effluent were employed (the former representing a source of competing/predatory microbes plus alternative substrates; the latter representing a source of alternative substrates only). Each was employed at two levels – 1% and 10% v/v. Inoculum levels of 1X and 0.1X were used. Without JS666 addition, no significant degradation of cDCE occurred.

All JS666-inoculated microcosms prepared with either 1% or 10% autoclaved primary effluent were able to degrade 60 mg/L cDCE, regardless of the initial inoculation level – with the lower inoculum level requiring more time to degrade the cDCE. This demonstrates that even in the presence of a mixture of alternative — and most likely preferable — carbon sources and competing/predatory microbes, JS666 is able to degrade large amounts of cDCE.

Additional Microbial Ecology Work

A key microbial ecology question is whether the ability to degrade cDCE is transmissible to indigenous microbes. In spite of the presence of at least two possible means of gene horizontal transfer, the two megaplasmids and the Mu-like bacteriophage, the ability to degrade cDCE is not readily transmissible between bacteria under laboratory conditions. The two megaplasmids were not demonstrated to be transmissible. We were unable to cure the megaplasmids from JS666 by growth on nonselective media, or by growth in presence of the DNA damaging agents acridine orange, ethidium bromide, or mitomycin C. As we were unable to cure wild-type JS666 of its plasmids, we were therefore unable to demonstrate conjugation between wild type JS666 and a plasmid-cured derivative of JS666. Experiments were performed to transfer the cDCE degradation ability from JS666 to *P. naphthalenivorans* CJ2 by conjugation on solid surface of rich media. No evidence for transfer of cDCE degradation genes to *P. naphthalenivorans* CJ2 was observed. The presence of a Mu-like phage was tested. The apparent transfer of nalidixic acid resistance to nonresistant JS666 by culture supernatant indicates active phage.

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LIST OF ABBREVIATIONS

AFB	Air Force Base
AO	acridine orange
ºC	degrees Celcius
cDCE	cis-1,2-dichloroethene
CHCA	cyclohexanecarboxylic acid
DCA	1,2-Dichloroethane
DDI	distilled – deionized water
DNA	deoxyribonucleic acid
DoD	Department of Defense
EISB	enhanced in situ bioremediation
ESTCP	Environmental Security Technology Certification Program
EtBr	ethidium bromide
FID	flame ionization detector
g	grams
gc	gas chromatograph
HCl	hydrochloric acid
L	liter
MeCN	acetonitrile
mg/L	milligrams per liter
ml	milliliter
mM	millimolar
MMC	mytomicin C
mmHg	millimeters of mercury
MNA	monitored natural attenuation
MSM	minimal salts medium
NaCl	sodium chloride
NaoH	sodium hydroxide
OD_{600}	optical density at 600 nm; measurement of cell density
pA	peak area
PFGE	Pulsed Field Gel Electrophoresis
PLE	tetrachloroethene
ppb	parts per billion
ppm	parts per million
qPCR	quantitative polymerase chain reaction
RfLP	restriction fragment length polymorphism
ROS	reactive oxygen species
RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
SERDP	Strategic Environmental Research and Development Program
SRS	Savannah River Site

TBE	Tris-Borate-EDTA
TCD	thermal conductivity detector
TCE	trichloroethene
TMS	trace metals solution
VC	vinyl chloride
v/v	volume per volume

1. SUBTASK 1.1: OPTIMIZING CULTURE CONDITIONS FOR PRODUCTION OF JS666 FOR INOCULATION IN THE FIELD.

1.1 Materials and Methods

1.1.1 Chemicals and Media

cis-1,2-Dichloroethene (cDCE) (97%) was obtained from Sigma-Aldrich. All other chemicals were reagent grade. Strain JS666 was maintained on a minimal salts medium (MSM) modified from Hartmans *et al*. (1992), which contained per liter of deionized water: 0.95 g KH_2PO_4, 2.27 g K_2HPO_4, and 0.67 g $(NH_4)2SO_4$. Filter-sterilized trace metal solution (TMS, 2 ml/L) was added after the above solution was autoclaved and cooled to room temperature. The TMS contained per liter of deionized water: 60 g $MgSO_4{\cdot}7H_2O$, 6.37 g EDTA, 1.0 g $ZnSO_4{\cdot}7H_2O$, 1.0 g $CaCl_2{\cdot}2H_2O$, 1.0 g $FeSO_4{\cdot}7H_2O$, 1.0 g $NaMoO_4$ $\cdot 2H_2O$, 1.0 g $CoCl_2{\cdot}6H_2O$, and 1.0 g $MnSO_4{\cdot}H_2O$. The pH of the MSM was 7.2 unless otherwise indicated. MSM at pH values other than 7.2 was prepared by adjusting the ratio of KH_2PO_4/K_2HPO_4 in the medium. Nutrient agar and/or trypticase soy agar (Becton Dickinson) at one-quarter strength with 15 g/L agar (Fisher Scientific) were used as non-selective media. Cultures used for inoculation were streaked on a non-selective agar plate to check for abnormal colony morphologies indicative of contamination.

1.1.2 Experimental Cultures

Frozen glycerol stocks of JS666 (15% glycerol in MSM) were used to inoculate one liter of MSM that contained 10 mM succinate and 40 µL of cDCE (0.53 mmoles) in order to quickly generate a large amount of biomass. Cultures were re-spiked with an additional 40 µL of cDCE before all the cDCE had been consumed. At the onset of the degradation of the second spike of cDCE, the culture was centrifuged at 7000 revolutions per minute (RPM) for 10 minutes, and the supernatant decanted. Cells were resuspended in approximately 30 ml of MSM by vortexing and centrifuged again at 10,000 RPM for 4 minutes. Cell pellets were resuspended in the appropriate volume of MSM to yield the desired OD_{600}. Finally, 0.5 ml of this concentrated culture was used to inoculate experimental serum vials that contained MSM with cDCE as the sole carbon source. Experiments were carried out in 160-ml serum vials (Wheaton) with 100 ml of liquid and 60 ml of headspace (air plus excess oxygen). Teflon-coated butyl rubber stoppers were crimp-sealed to the serum vials. The initial inoculum of JS666, neat cDCE, and additional oxygen were injected via a syringe. Cultures were inverted at an angle and shaken on a horizontal shaker at 160 RPM at 22° C in the dark.

1.1.3 Analytical Methods

The concentration of cDCE was measured using a Perkin Elmer gas chromatograph (GC) fitted with a 1% SP-1000 Carbopack column (Supelco) and flame-ionization detector (FID). Oxygen consumption and carbon dioxide production were monitored using a thermal-conductivity detector (TCD). The total moles of cDCE per bottle were determined by comparing the peak areas from a 100-µL headspace sample to cDCE standards prepared in water with the same liquid-to-headspace ratio. An Eppendorf Biophotometer was used to measure the optical density at 600 nm (OD_{600}) and estimate the biomass concentration. The pH measurements were taken using an Accumet micro electrode with a calomel reference.

1.1.4 General Methods for Culture Development.

All serum bottle studies were performed in 160 ml serum bottles containing 50 ml of culture medium. Bottles were capped with aluminum seals with Teflon-faced silicone septa. cDCE (Aldrich catalog #D62004) was added as a neat solution using a 5 µl syringe with the plunger in the needle (Hamilton). Before cDCE addition, the syringe needle was passed through a gas flame to sterilize the end. The cDCE was drawn into the syringe, the septum on the serum bottle was passed through the gas flame, and cDCE was added to the bottle. The serum bottle was tilted to rinse the drop of cDCE from the needle tip. The needle was passed through the flame again after being withdrawn from the bottle. The same needle hole was used for all cDCE additions and headspace sample withdrawals from the serum bottles.

All serum bottle experiments were initiated with cDCE-grown cultures that were harvested by centrifugation. Cell pellets were suspended in a small volume (1-2 ml) of fresh medium and 100 – 200 µl of such suspensions were used to inoculate experimental bottles. Serum bottles were incubated at 150 rpm on a gyrotary (New Brunswick) shaker at room temperature (20-24°C) under ambient room light.

cDCE was analyzed with a gas chromatograph (HP 6890) equipped with a flame ionization detector and a 1.83 m x 2.1 mm packed column containing 1% SP-1000 on 60/80 Carbopack B (Supelco). The initial oven temperature was 100°C and ramped to 150°C at 20°/min, then to 175°C at 10°/min. Detector temperature was 275°C. cDCE eluted at 3.88 min and tDCE at 4.0 min. 100 µl headspace samples were used for all cDCE analyses.

1.2 Results and Discussion

1.2.1 Effect of pH on cDCE Degradation by Strain JS666

Duplicate JS666 cultures with an initial OD_{600} of 0.42 were established in MSM. The cultures were supplied cDCE as the sole carbon source. The concentration of cDCE, pH, and OD_{600} were monitored over time. Rates of cDCE degradation were initially rapid, but began to slow after six

spikes of cDCE (4 µL neat cDCE each) had been degraded. In one culture, after 14 days, seven spikes of cDCE had been degraded (Figure 1.1). After the degradation of the seventh spike of cDCE, the pH had dropped to 6.5, and degradation rates decreased dramatically. A duplicate culture behaved similarly. At this point, the pH was adjusted in one of the cultures to 7.2 by adding NaOH. Degradation of cDCE in this culture was restored and could be maintained for five more spikes of cDCE before the rates of degradation slowed appreciably. Degradation did not resume in the culture that did not receive a pH adjustment (data not shown).

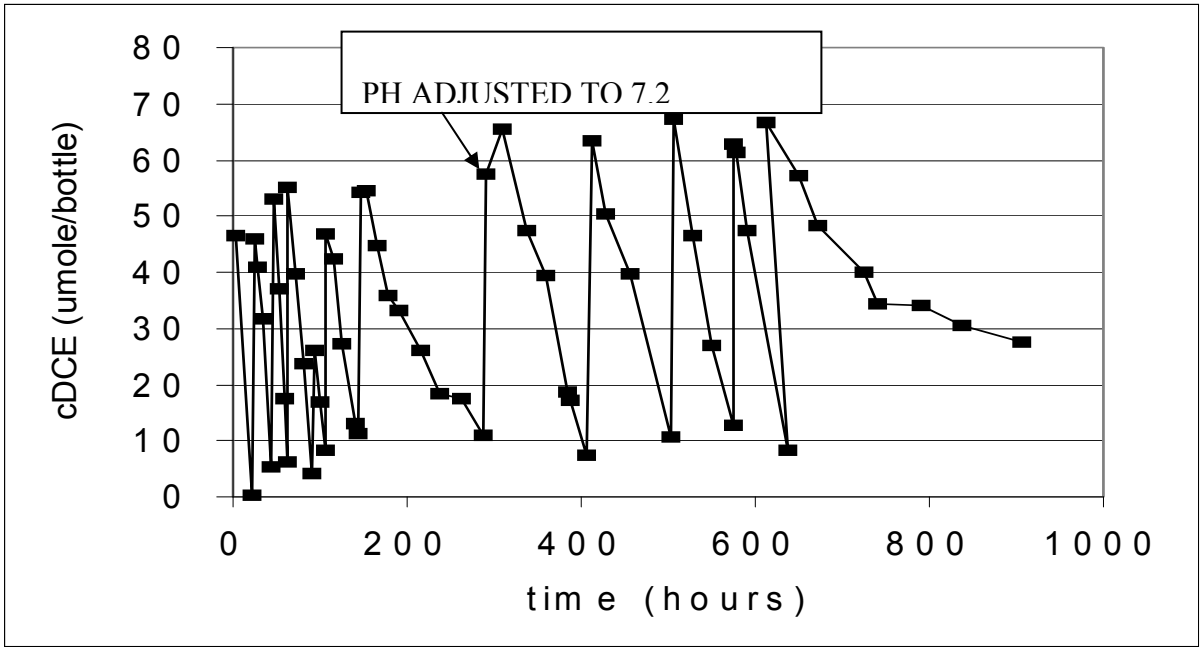


Figure 1.1 Degradation of cDCE by JS666 Culture (starting OD_{600} = 0.42) **in MSM. After 14 Days, the pH Was Adjusted to 7.2 by Adding NaOH, and the cDCE Activity Resumed.**

The oxidation of cDCE by strain JS666 causes the release of HCl, thereby lowering the pH of the cultures by an amount proportional to the amount of cDCE degraded. Since cDCE degradation activity could be restored by adjusting the pH, it seemed likely that the degradation of cDCE by JS666 might be inhibited at low pH. To test the sensitivity of JS666 cDCE degradation at low pH, duplicate JS666 cultures were set up in MSM with different initial pH values of 6.7, 7.0, and 7.5. Cultures were supplied cDCE as the sole carbon source. A comparison of the cDCE concentration over time in the cultures revealed that the culture with the lowest starting pH of 6.7 (Figure 1.2a) degraded fewer spikes of cDCE before degradation activity began to slow than cultures with higher starting pH (Figure 1.2b and 1.2c). Figure 1.3 shows the decrease in pH of these cultures over time. It is evident that the pH decreased and then began to level-off, indicating that degradation activity has ceased in all cultures. However, the terminal pH values of the cultures were not the same. For the cultures with lower starting pH values (pH = 6.7 and 7.0), the pH leveled off around 6.3-6.4. In these cultures, it is plausible that the decrease in degradation activity was due to low pH. Thus, it appears that degradation of cDCE may be inhibited at pH values below 6.3-6.4. However, in the culture with the highest starting pH (7.5), the pH leveled off near 7.0 suggesting that degradation of cDCE slowed for some reason other than low pH.

A closer look at the cDCE concentration over time shows that the degradation rate was initially fast, but got progressively slower with each additional spike of cDCE degraded. This degradation pattern resembles that of a co-metabolic process, even though cDCE degradation was previously shown to be coupled to growth in strain JS666 (Coleman et al., 2002a).

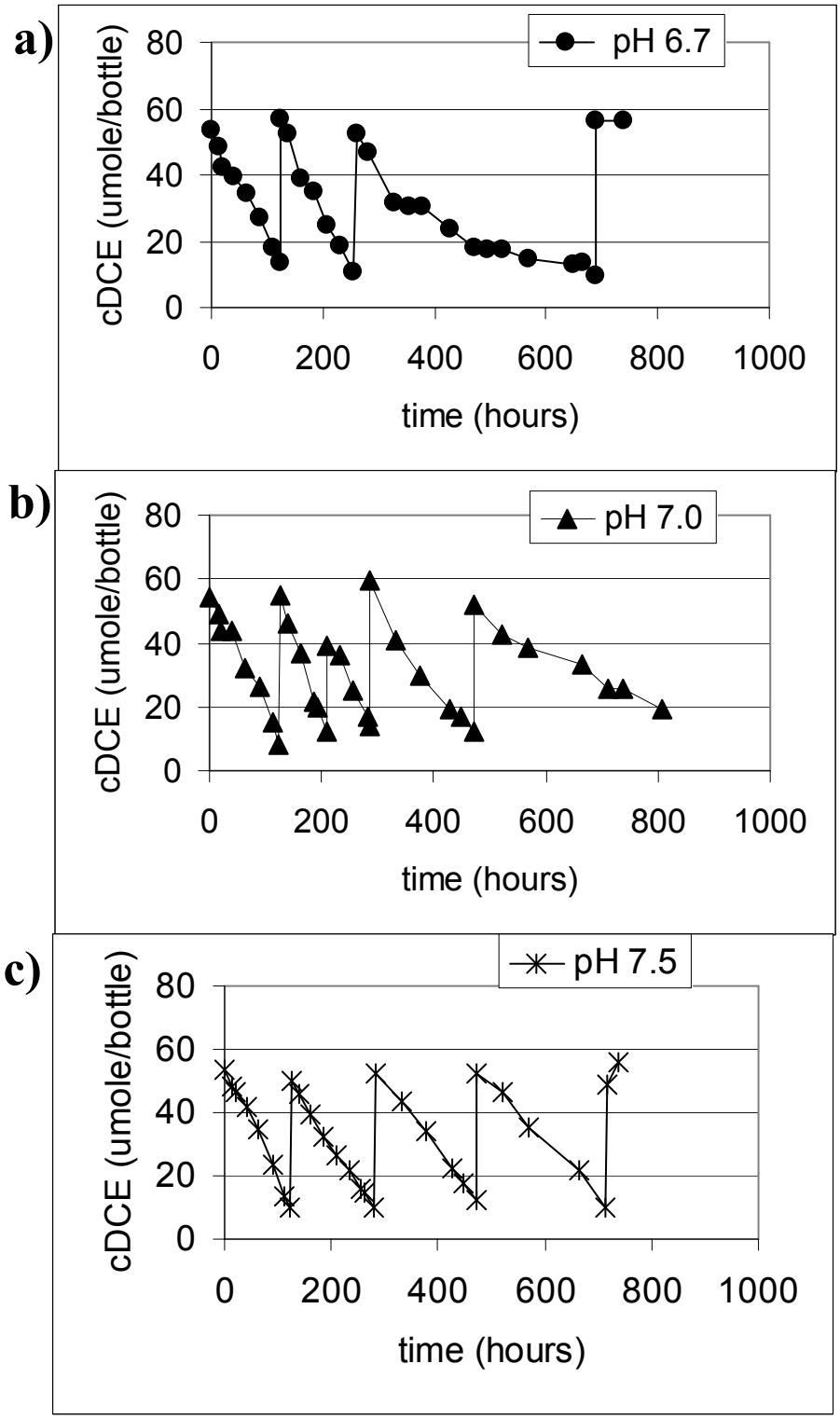


Figure 1.2 Concentration of cDCE Over Time for JS666 Cultures (OD_{600} = 0.45) **in MSM with an Initial pH of a) 6.7, b) 7.0, and c) 7.5. cDCE was supplied as the sole carbon source. Single Replicates are Shown for Clarity, but Duplicates Behaved Similarly.**

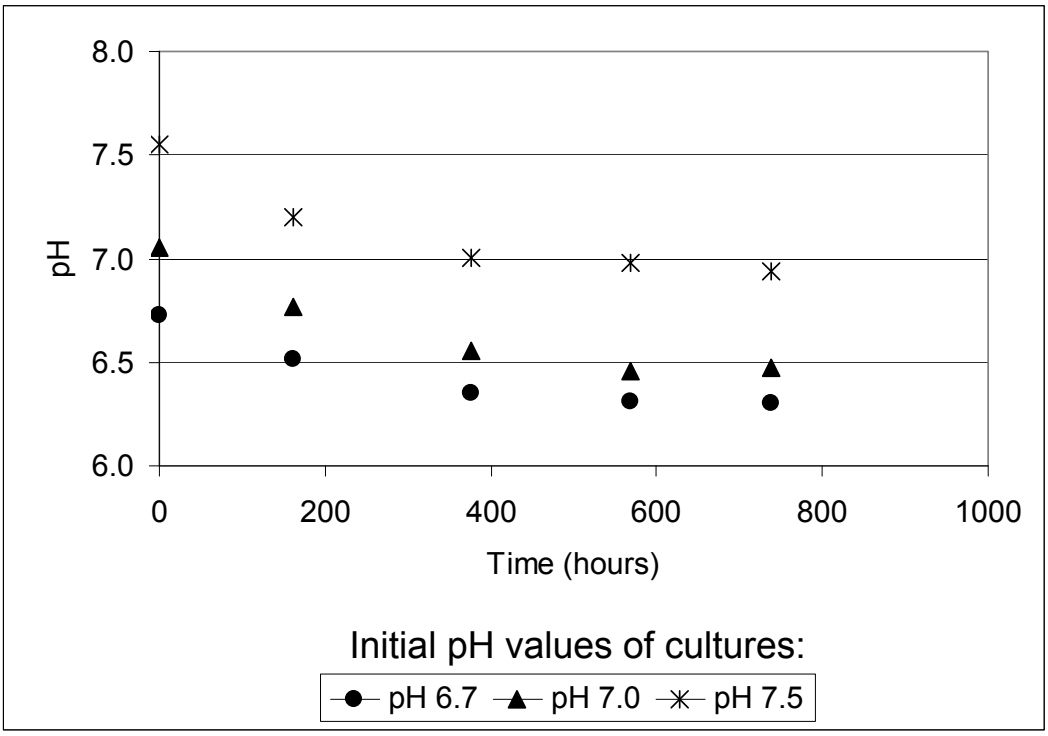


Figure 1.3 Decrease in pH Over Time in JS666 Cultures in MSM at Different Initial pH Values. cDCE was Provided as the Sole Carbon Source. Values Shown are the Average of Duplicate Measurements.

1.2.2 Effect of Initial Biomass Concentration on cDCE Degradation

JS666 cultures in MSM with cDCE were setup with different initial OD_{600} values to investigate the effect of biomass concentration on cDCE degradation. Cultures were prepared in duplicate at OD_{600} values of 0.13, 0.27, and 0.67. One would expect that degradation of cDCE by cultures with lower OD_{600} values would be slower compared to higher OD_{600} cultures. However, the total amount of cDCE degraded by all cultures should be the same (before inhibited by low-pH values) when degraded by a strain like JS666 whose growth is coupled to cDCE degradation. In this experiment, degradation by cultures with a starting OD_{600} of 0.13 (Figure 1.4a) was about half as fast as degradation by cultures with an initial OD_{600} of 0.27 (Figure 1.4b), as expected. However, fewer spikes of cDCE were degraded by the cultures with a lower starting OD_{600}. Furthermore, the decline in cDCE degradation activity could not be attributed to low pH, which was still above 6.7 in these cultures. The cultures with the highest initial OD_{600} (0.67, Figure 1.4c) degraded the most spikes of cDCE. However, the rate of degradation was initially rapid and decreased with each additional spike of cDCE added. After degradation had slowed in the highest OD_{600} culture, one of the duplicates was resuspended in fresh MSM while the pH in the other culture was adjusted to 7.2 with NaOH. It was hypothesized that resuspension would (1) remove potentially toxic byproducts or intermediates and/or (2) replenish necessary nutrients in the cultures. However, neither the resuspension in fresh media nor the pH adjustment restored

degradation activity. The organism in this experiment appeared to be unable to sustain growth on cDCE and degradation appeared co-metabolic. This degradation activity was very different from the activity seen in the initial pH experiment (Figure 1.1).

The difference in degradation pattern seen in the cultures could be due to development of a strain of JS666 that can degrade cDCE but has lost the ability to grow on it. Prolonged exposure of JS666 to a preferred substrate like succinate may have suppressed the expression of genes responsible for growth on cDCE. Alternatively, the exposure of JS666 to succinate may have selected for a strain that has been cured of a plasmid required for growth on cDCE. It is known that JS666 contains two large plasmids. A strain of JS666 unburdened with one or more of these plasmids would be able to reproduce more quickly in the presence of succinate and cDCE, and thus, out-compete strains that maintained both plasmids. The use of succinate as a co-substrate can be advantageous to achieve higher cell yields, but only as long as cDCE degradation activity is sustainable in the presence of the co-substrate.

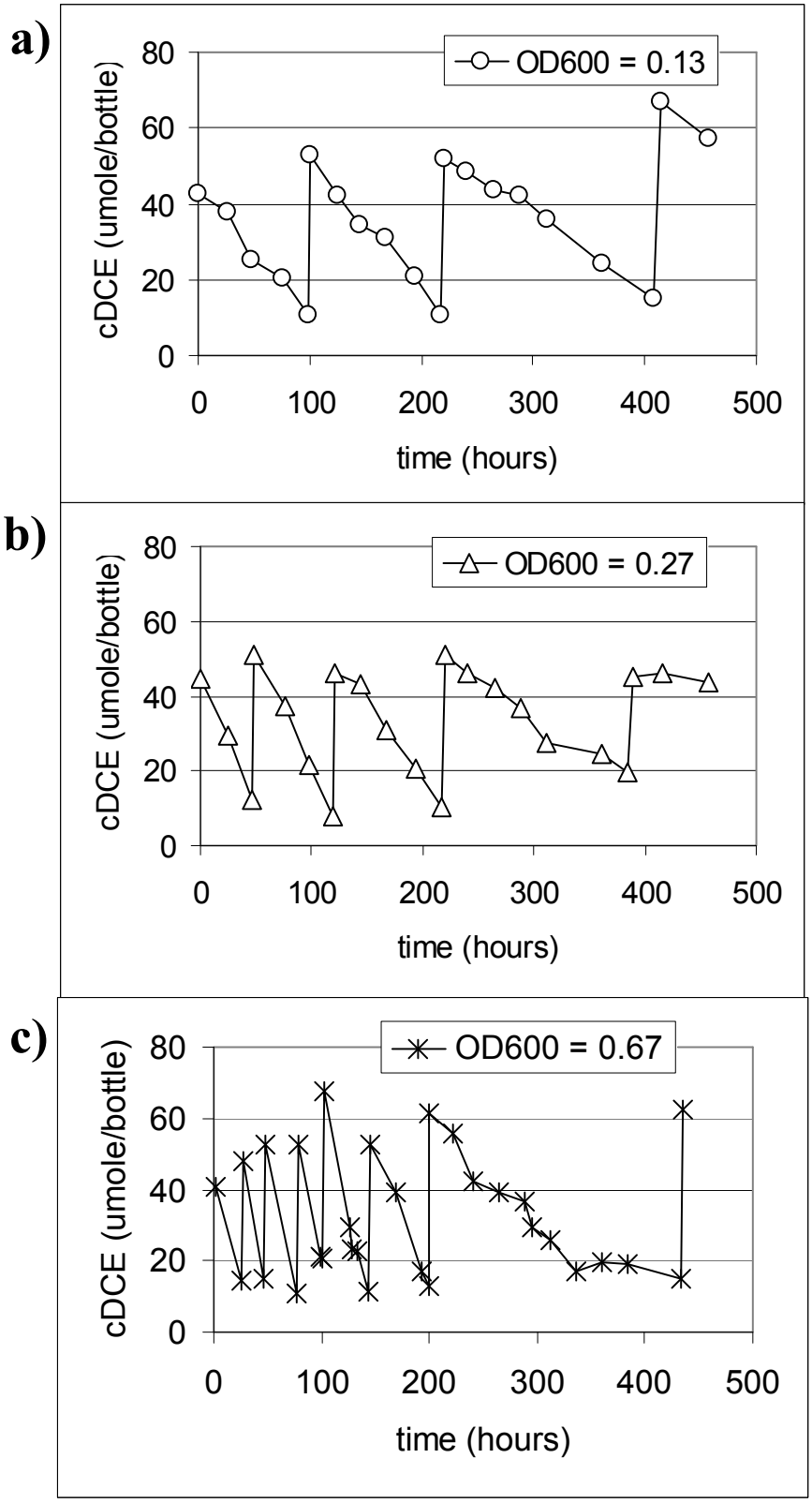


Figure 1.4 Degradation of cDCE by JS666 Cultures in MSM with cDCE at Different Starting Biomass Concentrations. Single Replicates are Shown for Clarity, but Duplicates Behaved Similarly.

Although the differences seen in degradation patterns could be due to the presence of a strain that has lost the ability to grow on cDCE, it is also possible that cultures that appeared to have lost the ability to grow on cDCE were actually contaminated. Because of this possibility, cultures were screened for contamination in three ways: 1) cultures were streaked on agar plates to observe colony morphologies, 2) cell morphologies were investigated using microscopy, and 3) RFLP was performed on the 16S rRNA of select cultures using two restriction enzymes (Msp1 and Hha1). None of the above techniques revealed evidence of contamination in the cultures used in the experimental studies reported herein.

1.2.3 Achieving "Good Behavior" – Studies with Low-Density Transfer Cultures

In essence, we have observed two types of behavior with JS666: 1) "Bad Behavior" with high-density cultures (> 0.3 OD_{600}) recently exposed to co-substrates such as succinate or ethanol, in which cDCE-degradation rates steadily decline and behavior resembles that of cometabolism; and 2) "Good Behavior" observed in the original studies of Coleman et al. (2002a), with low-density (< 0.05 OD_{600}) cultures frequently transferred to fresh medium and which have not seen any substrate but cDCE for many generations.

We set out to create an inoculum source of "good-behaving" JS666 using the transfer-culture technique (in which 5% v/v transfers were made to fresh medium after only a few cDCE spikes were degraded). Results are shown below in Figures 1.5 and 1.6.

These transfer cultures were established with cDCE as the sole carbon source by inoculating 1 ml of frozen culture into one liter of MSM with 40 µl of cDCE. The frozen cultures used for both culturing techniques described were functionally equivalent. After the degradation of 2-5 spikes of cDCE, 5% transfers were routinely made into 160-ml serum bottles containing 95 ml of MSM with cDCE. It is worth noting that 1-ml frozen cultures contained a final concentration of 15% glycerol, and JS666 is able grow on glycerol. The glycerol was likely rapidly consumed in the initial culture. If trace amounts remained, they would be diluted out upon the first transfer, leaving cDCE as the sole, remaining carbon source.

Figure 1.5 shows the first four transfers to fresh media with cDCE as the sole carbon source. A single lineage of transfer cultures is shown, but a duplicate lineage behaved similarly. Degradation of cDCE was sustained using this method for more than nine transfers to date, over a period of six months. The transfer cultures were able to degrade a significantly greater total quantity of cDCE compared to the typical 5-6 spikes of cDCE degraded in cultures reported previously (neglecting cultures in which degradation declined prematurely because of pH limitations). The initial lag phase prior to the onset of degradation after the first transfer was on the order of 40 days. With each successive transfer, the lag phase was reduced. Eventually, the OD_{600} of the transfer cultures routinely increased from below detection to 0.05-0.07, and the lag was reduced to less than one day.

Growth of JS666 in one of the transfer cultures (Figure 1.6) was confirmed with heterotrophic plate counts. Viable cell counts increased from 1.5 x 10^7 CFU/ml to 1.5 x 10^9 CFU/ml during the degradation of 5-6 spikes of cDCE (Figure 1.6c, note the log scale). More interesting was that the rate of cDCE degradation rapidly increased and then rapidly decreased (Figure 1.6b). The initial increase in degradation rate likely reflects the growth of the organism. However, the degradation rate began to decline when the viable cell counts were still exponentially increasing. This suggests that the cells were still viable, but that perhaps the cDCE degradation enzymes were damaged or no longer being expressed.

It is unclear how the culture was able to support exponential growth while the rates of disappearance of the only available carbon source were declining. It is possible that the organism could have been using an accumulated intermediate or dead cell remains for growth. If only a small fraction of the biomass were viable, the dead cells could have supplied sufficient carbon and energy source for the active cells. To investigate this, the contributions of viable and total biomass to protein concentration were estimated. The terminal OD_{600} was approximately 0.05. Using the correlation described earlier, this could account for a protein concentration of 0.01 mg/ml. Viable counts indicated that the terminal concentration of cells was approximately 10^9 CFU/ml. Assuming that the mass of protein per cell is 1.55 x 10^{-13} g, then viable cells could account for a protein concentration of 0.16 mg/ml (Neidhardt et al., 1990). This crude calculation suggests that the contribution of dead cells to the overall protein concentration in the culture was small. There is a significant amount of uncertainty in this calculation, but there is no data to support the fact that the cells were growing on dead remains of other cells.

In summary, the resuspending of cultures with succinate-grown inoculum in fresh medium does not appear to allow sustenance of cDCE degradation. Rather, degradation rates are rapid at first, but progressively decline with each additional spike of cDCE. OD_{600} values also tend to decrease in such cultures. On the other hand, 5% transfers of actively degrading culture to fresh media allow for sustained cDCE degradation and a decreased lag phase with each transfer. In these transfer cultures, an increase in the degradation rates is seen as degradation proceeds, and growth is evident from OD_{600} measurements and viable plate counts.

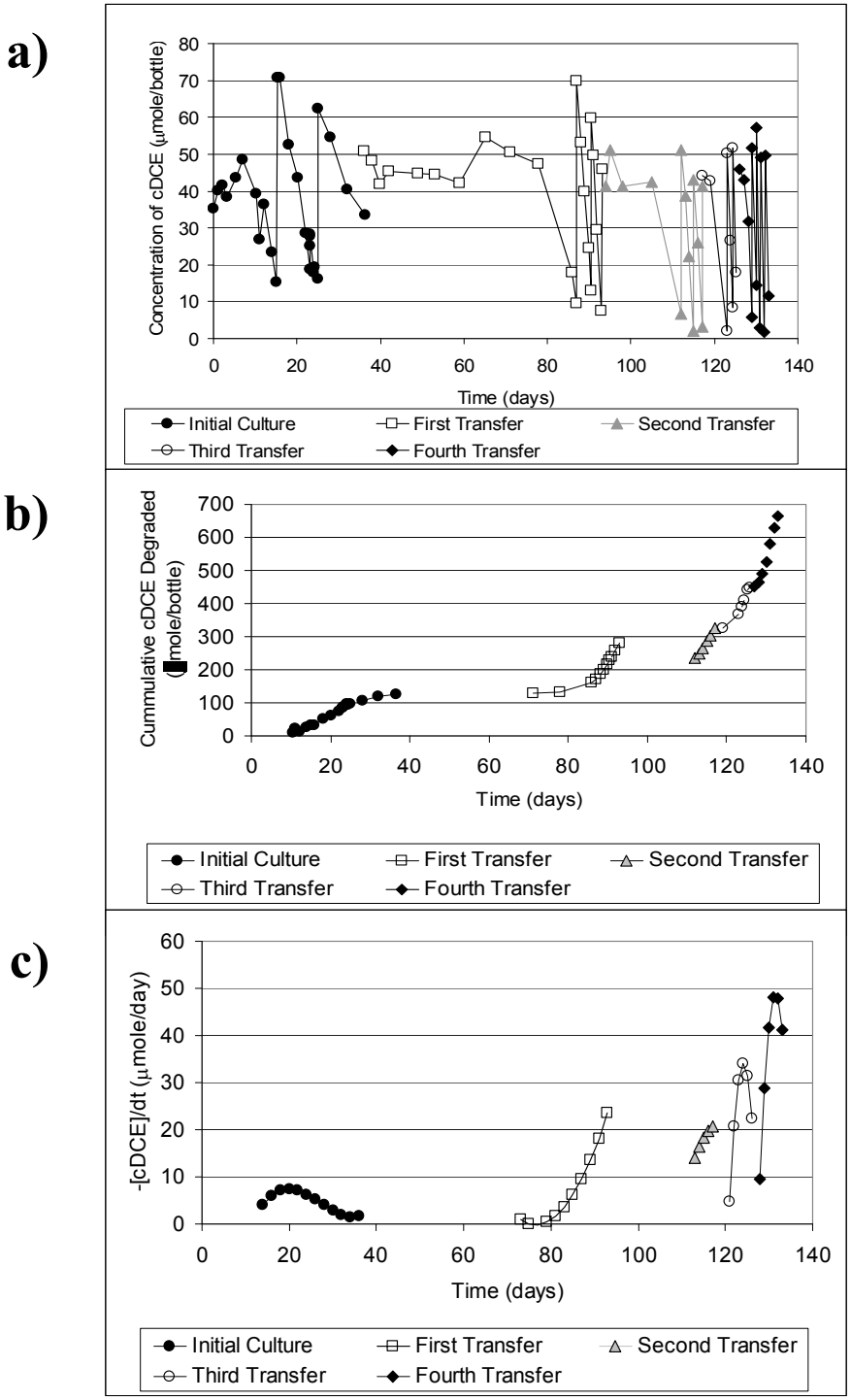


Figure 1.5 First Four Transfers of JS666 Culture to Fresh Medium (MSM with cDCE as the sole carbon source)**. Initial Culture was Inoculated with 1 ml of Frozen Culture that Contained 15% Glycerol. a) cDCE degradation over time, b) cumulative cDCE degradation over time, and c) rate of disappearance of cDCE over time.**

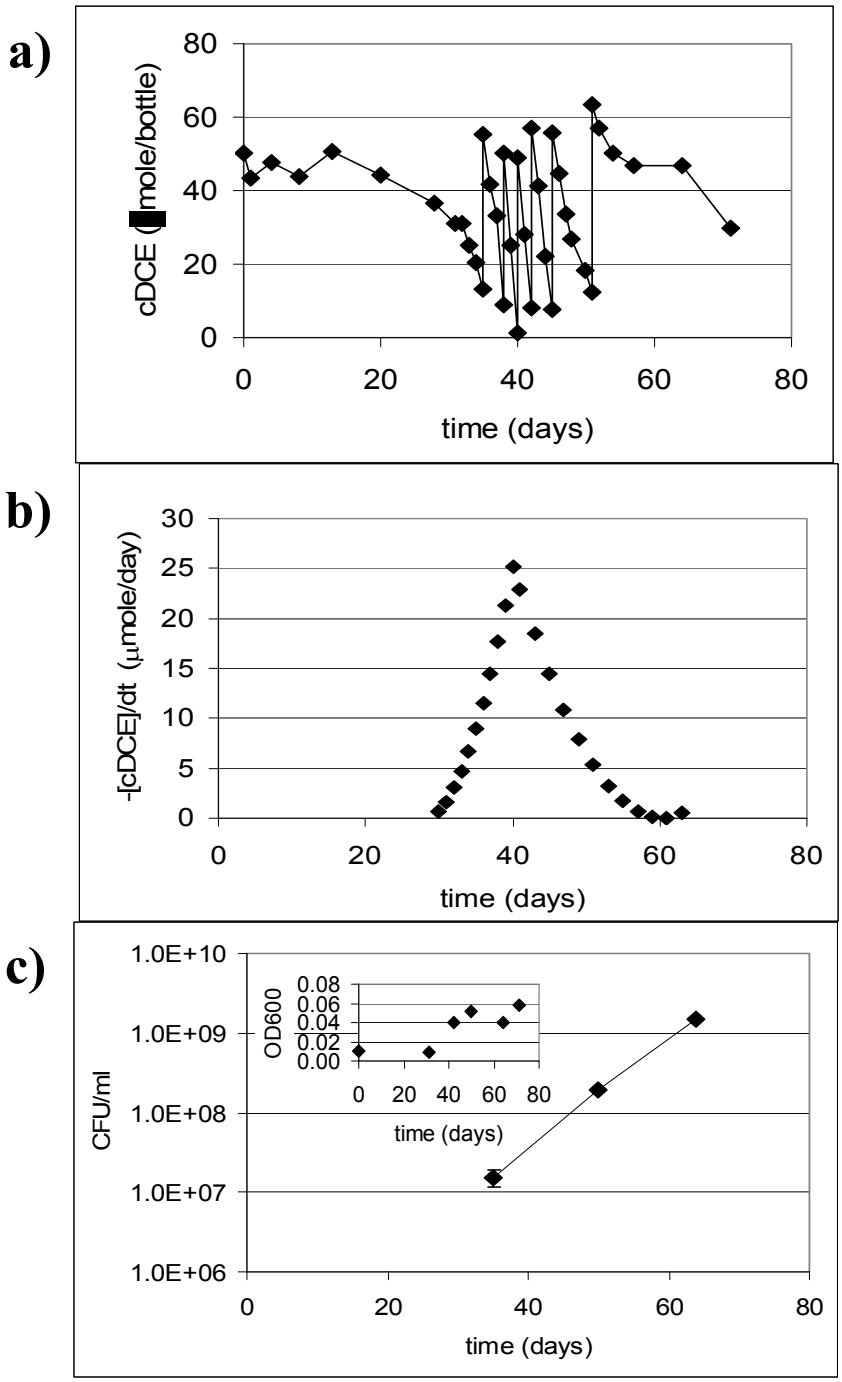


Figure 1.6 Degradation of cDCE a), Rate of Disappearance of cDCE b), and Growth Measured by Heterotrophic Plate Counts c) and OD_{600} (inset) **in a MSM and cDCE culture seeded with a 5% transfer of an actively degrading culture. For Heterotrophic Plate Counts, Error Bars Represent the Standard Deviation of Triplicate Samples.**

Conclusions — Culture Sustenance

A strain of JS666 that has lost the ability to grow on cDCE (i.e., a "bad-behaving" culture) would not necessarily be precluded from use as a bioaugmentation agent. Concentrations of cDCE in the subsurface are likely to be low. Under such field conditions, growth of JS666 on cDCE is unlikely (and probably unnecessary). Even with a potentially altered strain of JS666, it is probable that the cDCE would be completely degraded in the field before any decrease in degradation activity would be noticeable. However, it is apparent that our source of JS666 remains capable of "good behavior"—equivalent to that observed and reported by Coleman et al. (2002a)—but frequent transfers on cDCE-only are required for maintenance. Though tedious, this method is advocated for maintenance of the inoculum source in most of the subsequent microcosm studies described later in this report. As is presented later, we did undertake one microcosm study with a "bad-behaving" inoculum, amended with ethanol as co-substrate, to test the hypothesis that bioaugmentation need not depend on use of a "good-behaving" inoculum.

1.2.4 Effect of Oxygen on cDCE Degradation by JS666

We became suspicious that the elevated oxygen levels we had been employing were perhaps detrimental to JS666, albeit in subtle ways. We therefore conducted a study to examine this possibility.

We grew up a frozen plug on succinate and ambient O_2. Once the culture was actively degrading, it was centrifuged, washed, and resuspended into serum vials with cDCE as the sole carbon source. After 1.5 spikes of cDCE had been degraded (t=2.4 days) in the serum vials, the cultures were challenged with different levels of oxygen (1ab-ambient, 2ab-165 mmHg, 3ab-260 mmHg, 4ab-600 mmHg).

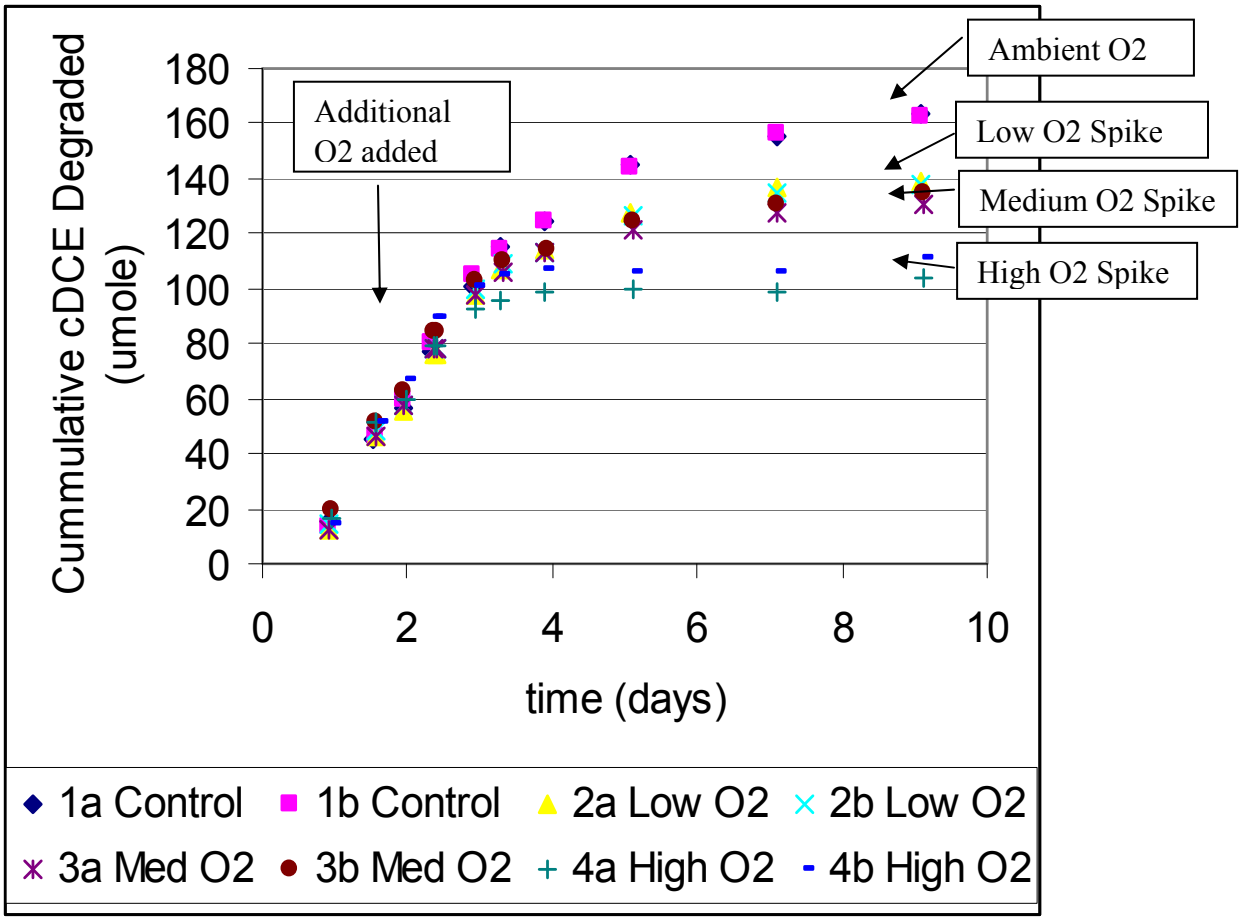
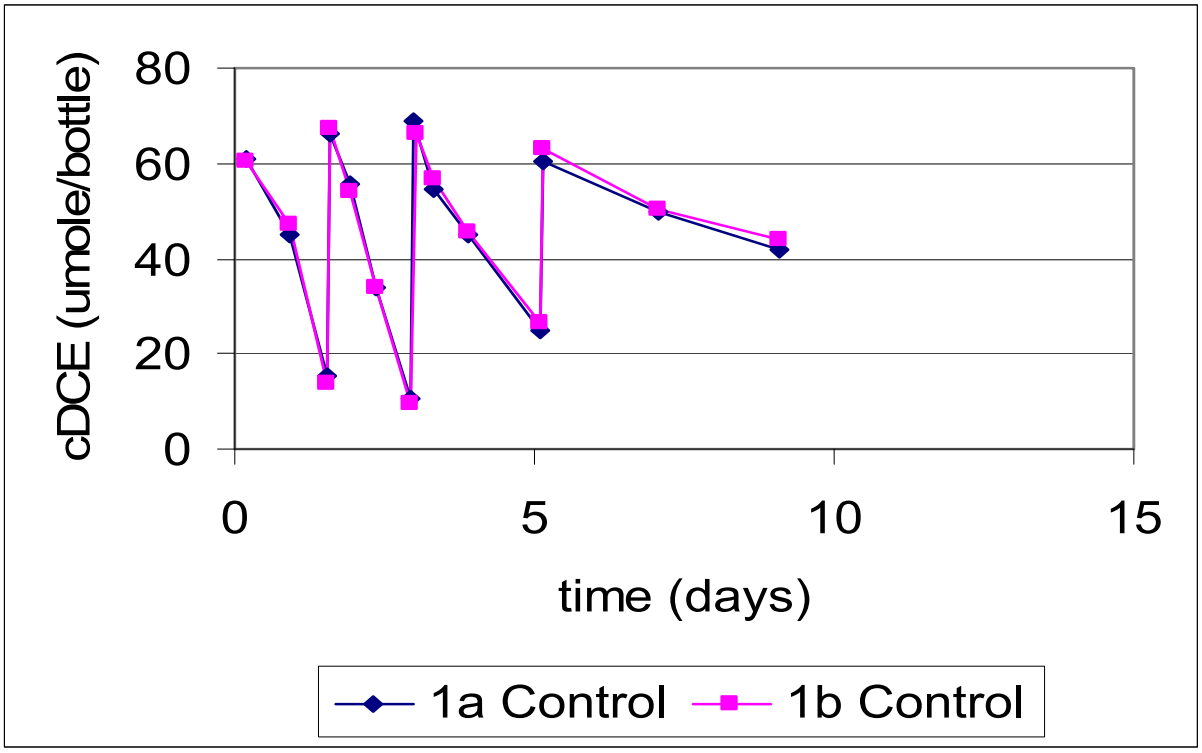
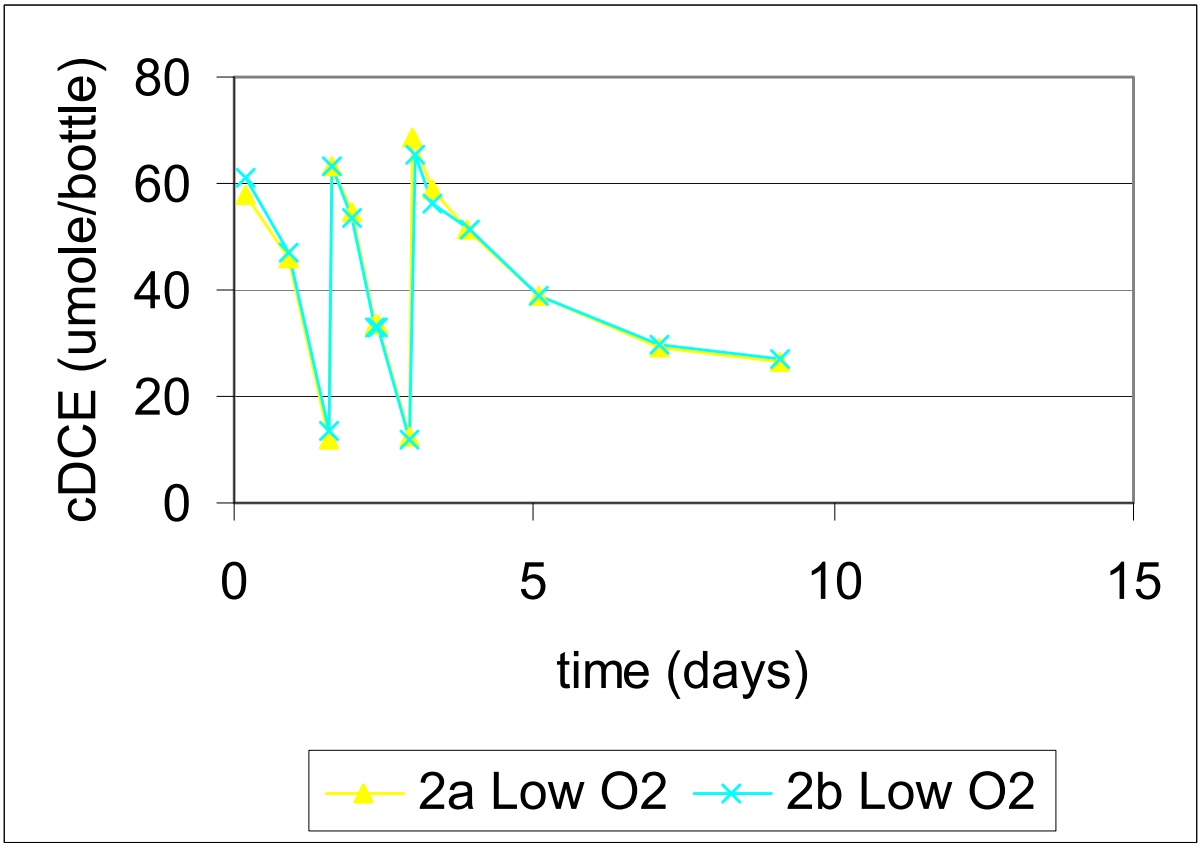


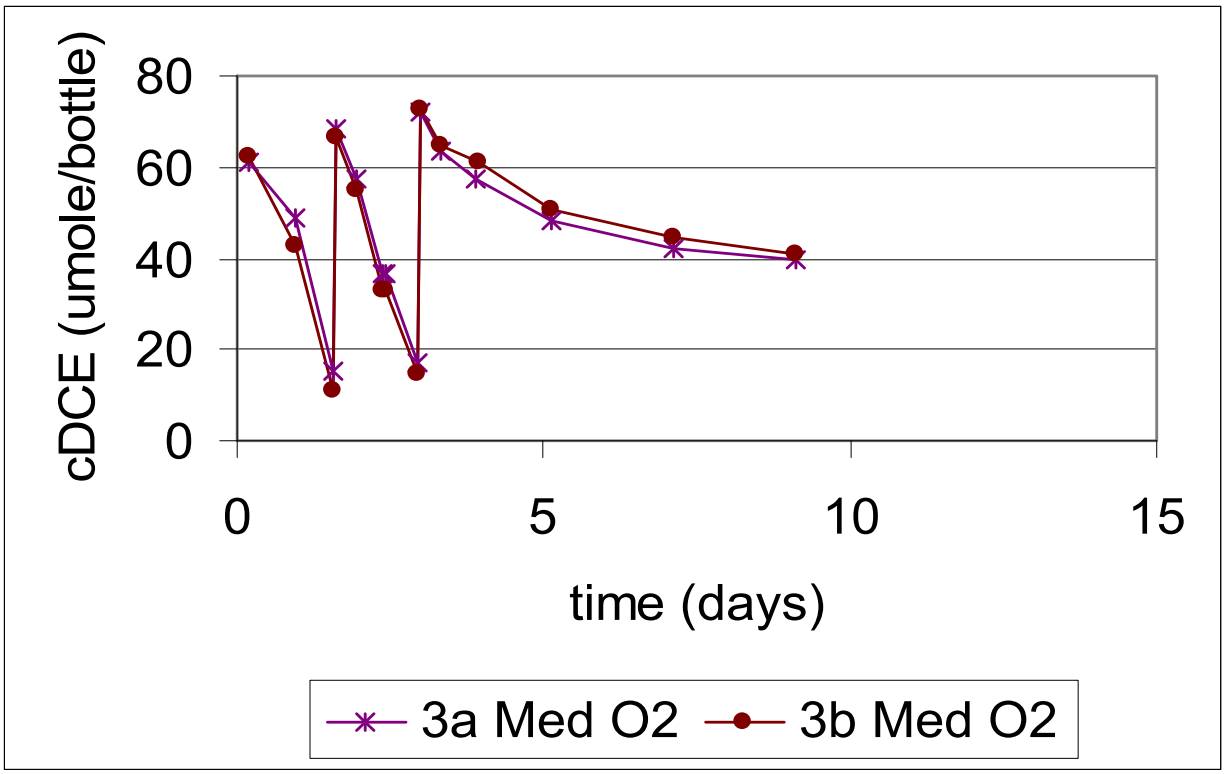
Figure 1.7. Effect of Oxygen on Cumulative cDCE Degradation by JS666.

Cultures that were challenged with higher concentrations of O2 degraded less cumulative cDCE than those with lower levels (Figure 1.7). The effect of the additional oxygen was not immediate, but rather became evident a few days after the O2 spike was administered.

Rates of degradation declined in all cultures (Figures 1.8 and 1.9). Therefore, although high oxygen concentrations appear to inhibit cDCE degradation, this by itself does not seem to explain why rates are declining in succinate-grown cells but not in transfer cultures. Perhaps this difference in degradation between these two culturing techniques is due to the biomass concentration.


cDCE (umole/bottle)
80
60
40
20
0
0
5
10
15
time (days)
1a Control
1b Control


cDCE (umole/bottle)
80
60
40
20
0
0
5
10
15
time (days)
2a Low O2
2b Low O2



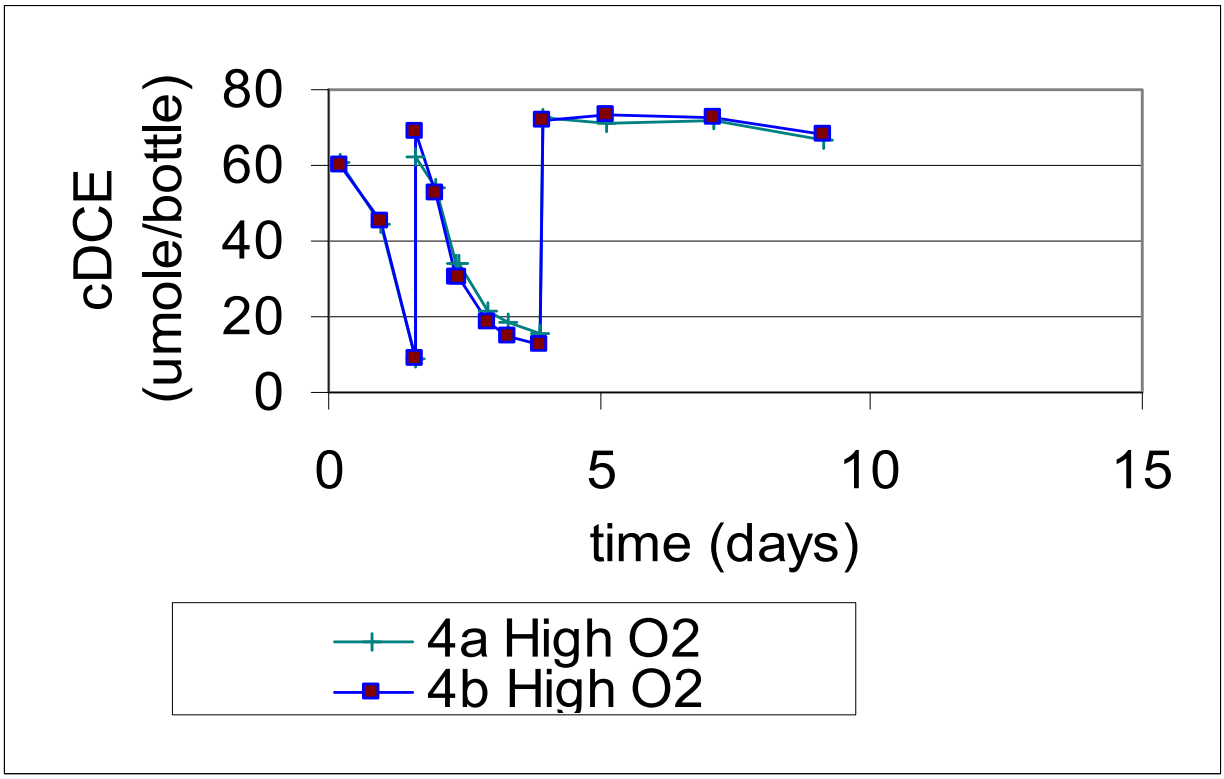


Figure 1.8. cDCE Degradation Patterns in Bottles Challenged with Different Oxygen Levels.

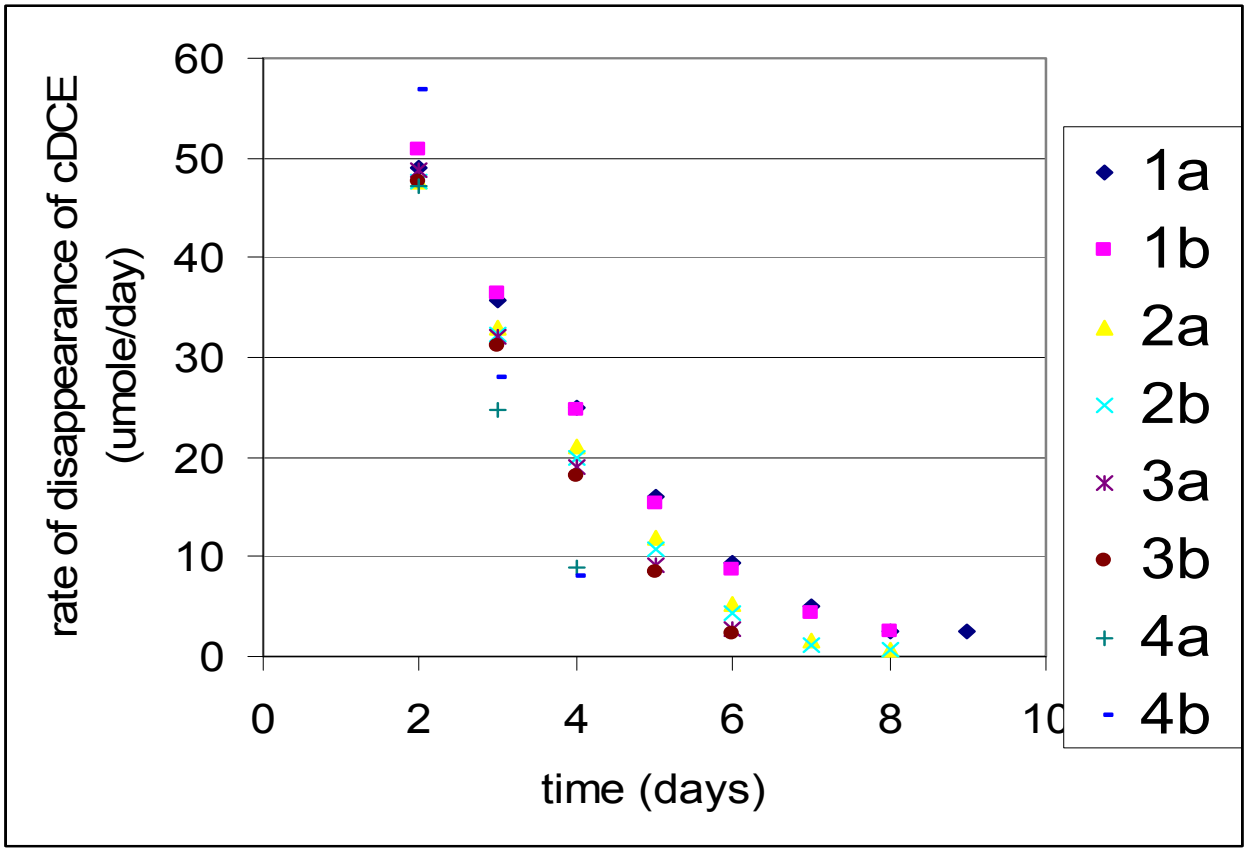


Figure 1.9 Rates of cDCE Degradation in Bottles Challenged with Different Oxygen Levels.

In light of these results, we conducted all future microcosm studies at (or below) ambient oxygen levels. We also reduced the agitation level to <100 rpm, and we reduced the inoculum level, since we suspected that JS666 does not work as effectively at high inoculum levels.

We also tested whether lowered levels of O_2 affected cDCE degradation. Half of the air in the headspace in serum bottles was replaced with N_2. Bottles were inoculated with cDCE-grown cells and cDCE was added and analyzed at 0, 6 and 23 h. No difference in cDCE degradation was seen between the test bottles and controls.

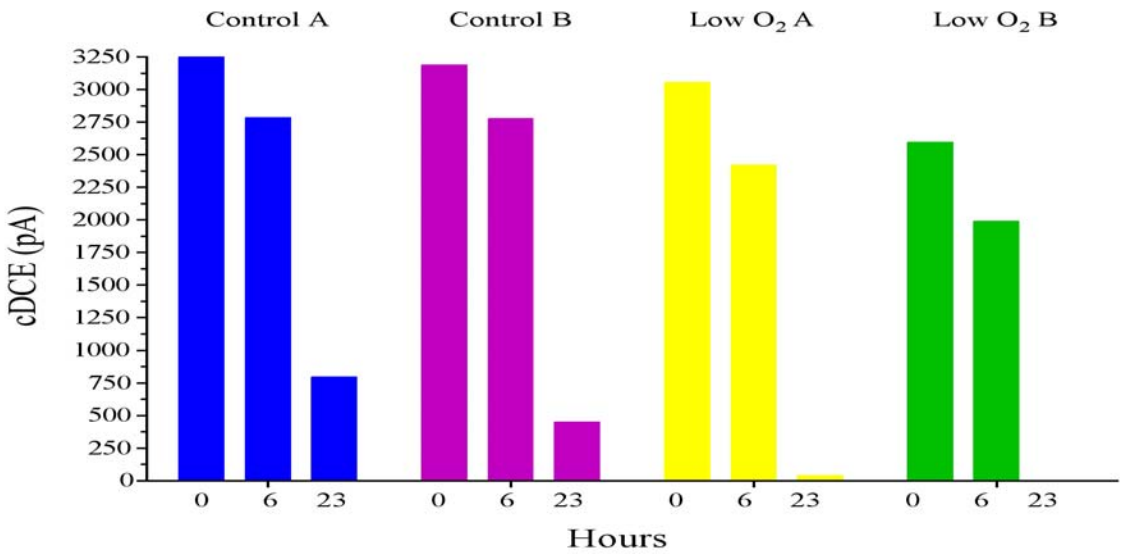


Figure 1.10 Effect of Oxygen Concentration on Degradation of cDCE.

1.2.5 Growth Medium Studies.

The growth medium (Coleman, Mattes et al. 2002) used in the initial studies of *Polaromonas* sp. JS666 was based on a MSM developed for mycobacteria that degrade vinyl chloride. *Polaromonas* belongs to the family Comamonadaceae of the order Burkholderiales, many of whose members are normally grown in media with higher concentrations of the macro- and micronutrient components. One of the initial goals of the sub-task was to find a growth medium that provided better buffering against pH changes caused by chloride release during growth on cDCE. Because the recently isolated *P. naphthalenivorans* strain CJ2 (Jeon, Park et al. 2004) is routinely grown on a mineral medium (MSB) originally developed for pseudomonads (Stanier, Palleroni et al. 1966), growth of JS666 in the two media was compared.

Table 1.1. Components of MSB and MSM culture media.

		Concentration in final solution (mM)	
Solution A	Component	MSB	MSM
	Na_2HPO_4	40	
	KH_2PO_4	40	6.99
	K_2HPO_4		13.05
Solution B	(Hutner's Base – vitamin free)		
	Nitrilotriacetic acid	0.85	
	$MgSO_4$	2.4	0.00049
	$CaCl_2 \cdot 2H_2O$	0.45	0.0000068
	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.00015	
	$NaMoO_4 \cdot 2H_2O$		0.0000008
	$FeSO_4 \cdot 7H_2O$	0.007	0.000018
Metals 44	EDTA	0.0067	0.000034
	$ZnSO_4 \cdot 7H_2O$	0.038	0.000007
	$MnSO_4 \cdot H_2O$	0.009	0.0000062
	$FeSO_4 \cdot 7H_2O$	0.018	
	$CuSO_4 \cdot 5H_2O$	0.0016	0.0000008
	$Co(NO_3)_2 \cdot 6H_2O$	0.00085	
	$CoCl_2 \cdot 6H_2O$		0.0000017
	$Na_2B_4O_7 \cdot 10H_2O$	0.00046	
Solution C	$(NH_4)_2SO_4$	7.6	5.08

Phosphate Buffer

MSM was varied by changing the proportions of the phosphate buffer component (Part A) and the micronutrients (Part B). When the concentration of phosphate buffer equaled or exceeded 60 mM, growth of JS666 was inhibited, but when Part A was doubled to bring the phosphate buffer to 40 mM, growth was better than in the original MSM. When Part A was doubled, and Part B was varied between 1X and 10X the concentration in MSM, slightly better growth was seen at the higher micronutrient concentrations. MSB normally contains 80 mM phosphate buffer, and essentially the same micronutrients as MSM, but at up to 4 orders of magnitude higher concentrations. It was diluted to one-half strength (½ MSB) to bring the phosphate buffer to an acceptable concentration for JS666 and compared to MSM with double Part A. No difference in growth was seen between the two media; thereafter ½ MSB was adopted as the standard medium for JS666.

pH Optimum

pH was varied in ½ MSB. The most rapid initial use (at 4 h) of cDCE and most extensive final use (at 20 h) took place at pH 7.2. Periodic adjustment of the pH with ammonium hydroxide allowed the addition of up to 18 spikes of cDCE before growth finally stopped. Additional oxygen was required after 12 cDCE spikes.

Table 1.2 Effect of pH on cDCE Consumption.

Initial pH	6.8	7.0	7.2	7.4
cDCE remaining	pA			
4h	1496	1500	1190	1252
8h	1291	1468	868	1279
20h	1199	1129	591	846
Final pH	6.7	6.8	6.9	7.1

Vitamins

Balch's vitamins (Gerhardt, Murray et al. 1994) were tested to determine if vitamin addition would enhance cDCE degradation. Cultures with vitamins initially degraded cDCE much more rapidly than cultures without vitamins, but degradation stopped after the initial activity and cultures without vitamins went on to complete degradation of the initial spike of cDCE before the cultures with vitamins. After a lag period, the cultures with vitamins resumed cDCE degradation. There was no overall benefit to vitamin addition, and vitamins were omitted from all subsequent media formulations.

Table 1.3 Vitamin Concentration in Medium.

Vitamin	Concentration in medium (µg/L)
p-Aminobenzoic acid	50
Folic acid	20
Biotin	20
Nicotinic acid	50
Calcium pantothenate	50
Riboflavin	50
Thiamine HCl	50
Pyridoxine HCl	100
Cyanocobalamin	1
Thioctic acid	50

Chemolithotrophy

Recent work has shown that chemolithotrophic organisms closely related to JS666 are responsible for half the bacterial growth in bottled natural mineral waters (Loy, Beisker et al. 2005). The composition of the bottled waters suggested that thiosulfate or sulfur might be the energy source for the chemolithotrophic growth.

The JS666 genome (http://genome.ornl.gov/microbial/bpro_js666/) contains several genes that might encode sulfite oxidase as well as other enzymes for sulfur metabolism. If JS666 could derive energy from sulfur compounds during growth on cDCE, one might expect a higher total growth yield from cDCE plus sulfur compounds. JS666 also appears to have genes that encode enzymes of the Calvin-Benson cycle, specifically for the key enzyme ribulose 1,5-bisphosphate carboxylase (RubisCO) which might allow the organism to fix CO_2 and again result in a higher growth yield from cDCE plus CO_2 than from cDCE alone.

Sulfite, thiosulfate, CO_2, CO_2 plus H_2, and CO were added to serum bottles with cDCE. No difference was seen between controls with cDCE only and experimental bottles in the rate of cDCE degradation, and there was no difference in final growth as measured by increased optical density when the cDCE was completely utilized. Closer examination of the gene sequence annotated as RubisCO revealed that is more closely related to RubisCO-like (Hanson and Tabita 2001) proteins than to RubisCO and is likely not involved in the Calvin-Benson cycle.

Ionic Strength

A recent paper (Müller, Walter et al. 2006) explored the relationship between ammonium concentration, ionic strength, and toxicity to bacteria. The authors found that at molar concentrations, $(NH_4)_2SO_4$ was toxic to the bacteria that they tested due to increased ionic strength of the medium, and not due to the ammonium. Along similar lines, the slowdown in growth observed when JS666 is cultured at high phosphate buffer strengths or after multiple additions of cDCE may be due to the increased ionic strength of the medium caused by both Cl^- release and pH adjustment. We used an experimental design similar to that of Muller *et al.* (Müller, Walter et al. 2006) to test whether high ionic strength inhibits cDCE degradation by JS666. Because our previous experiments found inhibition of cDCE degradation at phosphate concentrations of 60 mM, our ionic strength experiments were carried out at a maximum concentration of 100 mM sulfate. Serum bottles contained ½-MSB and varying combinations of $(NH_4)_2SO_4$ and Na_2SO_4. After inoculation with JS666, the bottles were sealed and cDCE was added to each bottle. cDCE was monitored by GC, and cDCE was added daily to bring the amount of cDCE in the bottle back to the starting concentration. The experiment was terminated at 6 days. Final salinity was measured with a refractometer. The initial salinity of ½-MSB is 4.5 and the ionic strength is 0.099. Ionic strength was calculated as $I = ½ \sum c_i z_i^2$, where I is ionic strength, c_i is the concentration of the ith ion present in solution, and z_i is the charge (Borkowski 2005).

Table 1.4 Effect of Final Salinity and Final Ionic Strength on cDCE Degradation.

Bottle	mM $(NH_4)_2SO_4$	mM Na_2SO_4	Final OD_{600}	Final Salinity ‰	Final Ionic Strength	Total µl cDCE
A	25	0	.0835	8.0	0.182	15.5
B	50	0	.0688	10.5	0.257	14.5
C	100	0	.0194	15.0	0.403	7.5
D	25	25	.0600	10.5	0.257	15.0
E	25	75	.0368	15.5	0.402	6.0
F	0	0	.0941	5.0	0.108	17.0

The results suggest that total ionic strength affects cDCE consumption. A deleterious effect was seen at 25 mM SO_4^{-2}, and became progressively more pronounced with increasing ionic strength irrespective of the ionic species.

Additional cultures were grown in serum bottles prepared with ½ and ¼ strength MSB, with varying amounts of NaCl. NaCl is the predominant model ionic species in literature on the effects of ionic strength on bacterial growth. The striking result is that JS666 appears to be particularly sensitive to NaCl. The ionic strength of ¼ MSB with 50 mM NaCl is the same as ½ MSB with no additional NaCl, yet degradation of cDCE slowed dramatically in the NaCl supplemented bottles after only a few additions of cDCE while degradation continued in the ½ MSB bottles. The results also indicate that the greater buffering capacity of ½ MSB over ¼ MSB outweighs the effects of higher ionic strength. Overall, the results suggest that minimization of ionic strength while maintaining neutral pH can increase the growth of JS666 on cDCE.

Figure 1.11 Effect of NaCl on cDCE Degradation.

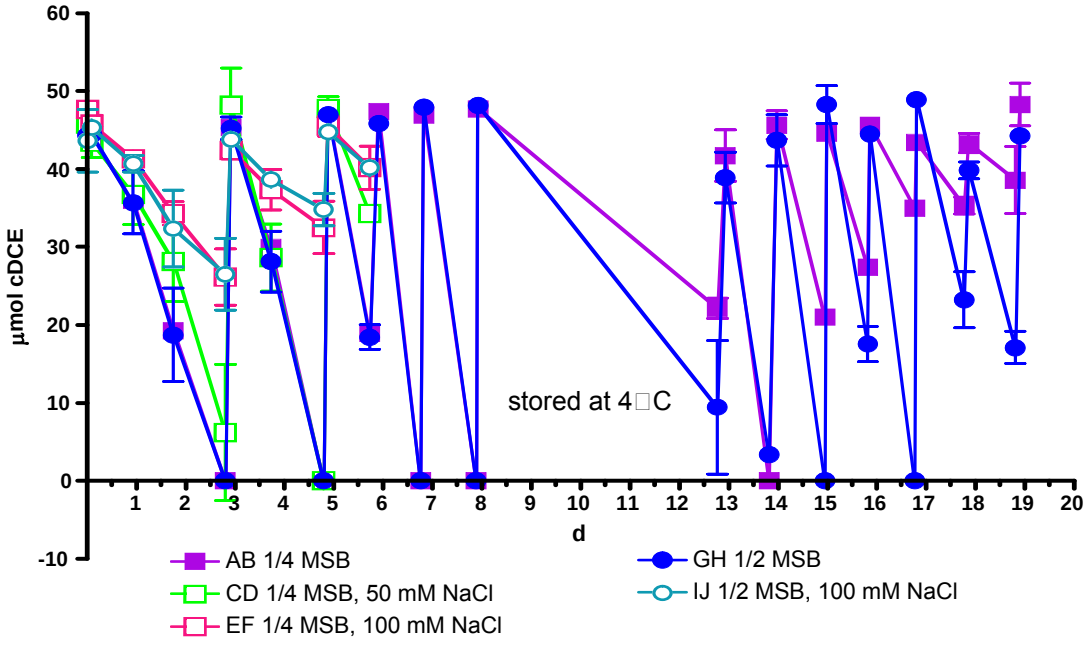


Table 1.5 Effect of Initial Ionic Strength on cDCE Degradation.

Bottle	Medium	mM NaCl	Final OD_{600}	Initial ionic strength	Final pH	Total µl cDCE
A	¼ MSB	0	.1068	.05	5.62	28.1
B	¼ MSB	0	.1034	.05	5.68	27.5
C	¼ MSB	50		.10		9
D	¼ MSB	50		.10		9.0
E	¼ MSB	100		.15		5.0
F	¼ MSB	100		.15		5.7
G	½ MSB	0	.1565	.10	6.12	33.5*
H	½ MSB	0	.1589	.10	6.13	36.1*
I	½ MSB	100		.20		5.0
J	½ MSB	100		.20		5.3

***cDCE still being degraded but experiment terminated when ¼ MSB bottles stopped degrading cDCE.**

Cation Effect

The strong inhibitory effect of NaCl on cDCE degradation raised the question of whether there is an effect of different cations in combination with chloride. Duplicate bottles were constructed with ¼-MSB supplemented with NaCl, KCl, NH_4Cl, and $MgCl_2$ with the added chloride kept constant at 100 mM. cDCE in all chloride-supplemented bottles except for the NH_4Cl-supplemented bottle, as well as in the ½-MSB control bottles was initially degraded more slowly than in the ¼-MSB bottles. However, after several cDCE additions, all cultures adapted to the chloride supplements except for the NH_4Cl-supplemented cultures in which cDCE-degradation slowed markedly from the initial rates.

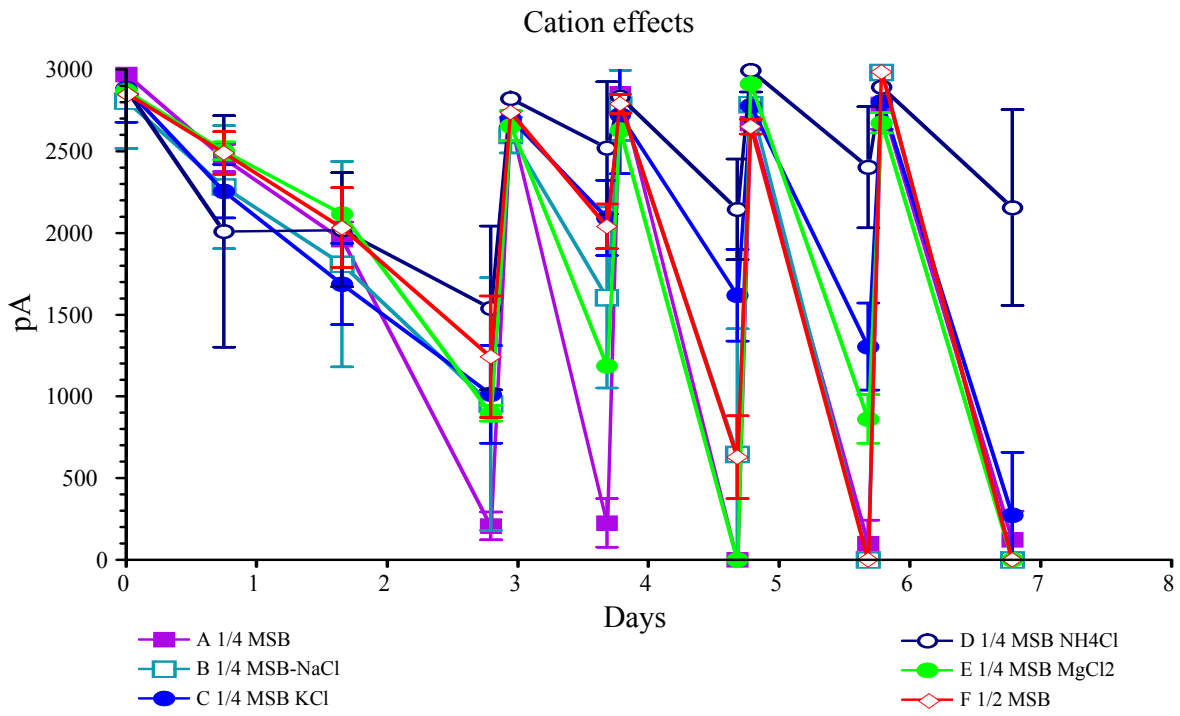


Figure 1.12 Effect of Cations on cDCE Degradation.

Nitrogen Source

The effect of alternate nitrogen sources on cDCE degradation was examined in serum bottles. Duplicate bottles of ¼-MSB made without nitrogen were provided 2 mM nitrogen as $(NH_4)_2SO_4$, $NaNO_2$, $NaNO_3$, or urea. Ammonium sulfate is the normal nitrogen source in MSB. Additional bottles were provided with no nitrogen; a trace amount of ammonium (40 nM) is in ¼-MSB when no additional nitrogen is supplied. Only ammonium and nitrate served as nitrogen sources during cDCE degradation. Urea was expected to be a good nitrogen source because the genome of JS666 contains genes for all 3 subunits of urease with 60 to 83% identity to known ureases. However, growth with urea was indistinguishable from no nitrogen or nitrite supplementation.

No genes for assimilatory nitrite or nitrate reductases are annotated in the JS666 genome, but the bacterium clearly grows when nitrate is provided as the sole nitrogen source.

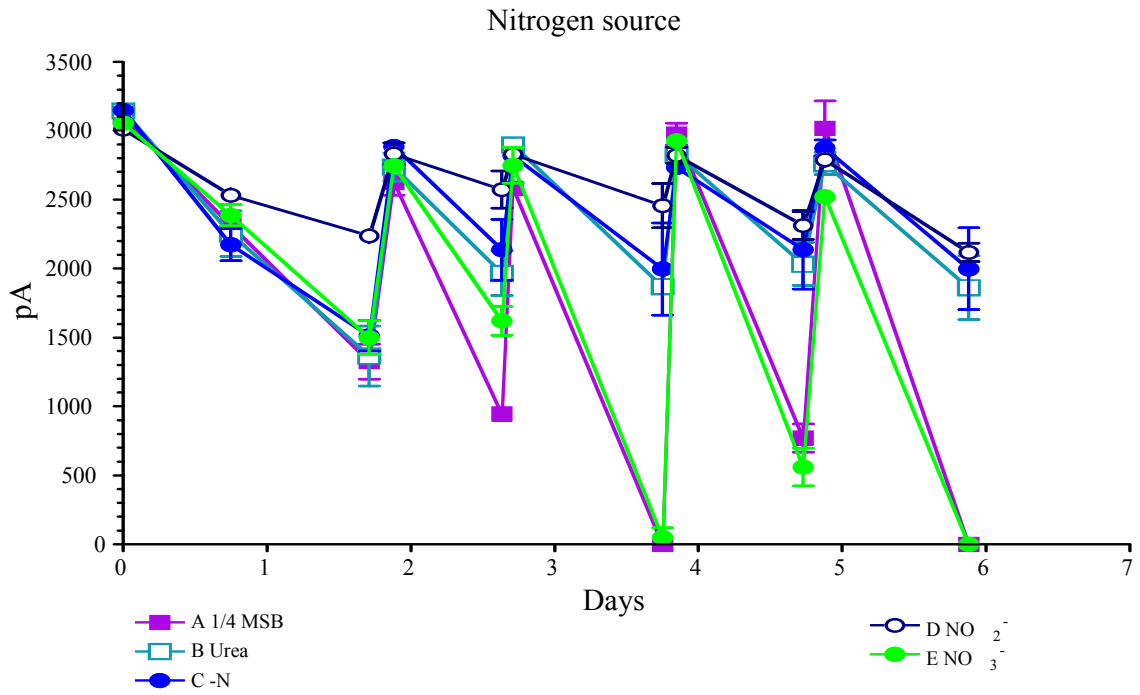


Figure 1.13 Effect of Nitrogen Source on cDCE Degradation.

Summary of Medium Investigations

At the start of the investigation *Polaromonas* sp. JS666 could be grown on cDCE to 0.2 OD_{600} in small serum bottles. cDCE at a nominal concentration of 800 µM (3 µl) could be added to serum bottle cultures 4-5 times before cDCE degradation slowed (Coleman, Mattes et al. 2002). Studies conducted under this effort demonstrated that acid pH inhibits cDCE degradation and that pH adjustment back to 7.2 maintains degradation of cDCE for up to 18 additions before growth stops. Phosphate concentrations of 60 mM or above inhibit cDCE degradation, but the need to buffer against pH changes must be balanced against the deleterious effects of high phosphate concentrations. When a medium buffered with 40 mM phosphate (½-MSB) is used, 35 µl of cDCE per 50 ml of medium can be routinely supplied to a culture without the need to adjust pH. No additional amendments are needed for cell growth.

1.2.6 Bioreacter Studies

Bioreactor studies were initiated to apply the lessons learned in the serum bottle studies to growth of large batch cultures of JS666. The ultimate goal is to obtain a culture for use in the field bioaugmentation study, the maximum requirements for which are 100 L of culture with OD_{600} = 1.0, actively growing on cDCE. The bioreactor experiments were conducted simultaneously with the serum bottle studies and were modified as the serum bottle studies supplied new insights.

Initial Conditions

JS666 was grown in batch in a 1L fermenter with ½-MSB and pH controlled at 7.2 by automatic addition of 5 N ammonium hydroxide. No additional air was supplied and the volume of medium in the reactor (400 ml) was based on maintaining a similar headspace to aqueous volume as in the serum bottles. The reactor was vented to the outside atmosphere via a single ⅛" I.D. tube fitted with a sterile air filter. Neat cDCE was added in pulses via syringe pump. Temperature was maintained at 20°C. Stirring was accomplished with a Rushton impeller at 500 rpm. The stirring speed was experimentally determined to give maximum mixing without causing splashing and formation of vortices. Under the initial conditions, growth in the reactor mimicked the growth in the serum bottles. That is, when no additional carbon source was supplied, growth reached a plateau at 0.2 OD_{600} after the equivalent of 18-20 additions of cDCE. Addition of O_2, which was found to be limiting in serum bottles after 12 cDCE additions, did not stimulate cDCE degradation. Addition of micronutrients and sulfate also failed to stimulate cDCE degradation. When the non-growing cells were transferred from either the serum bottle or the reactor into fresh serum bottles, growth on cDCE resumed.

Ionic Strength

The salinity in the fermenter was 8% when cDCE degradation reached a plateau. The ionic strength would have increased over the course of the run due to Cl^- release from cDCE and from NH_4OH added initially to raise the starting pH to 7.2, and during the run to maintain pH at 7.2. To test whether total ionic strength inhibited cDCE degradation in the fermenter, the medium in the reactor was modified so that the phosphate buffer concentration was reduced to 10 mM while keeping all the other nutrients the same as in ½ MSB, thereby reducing the ionic strength to 0.04. Growth on cDCE was substantially better in the low ionic strength reactor than in reactors with 40 mM phosphate. The OD_{600} reached 0.27 before growth slowed (Panel A, Figure 1.12). Previous best growth in the reactor on cDCE alone was 0.18. The doubling time was approximately 76 hours, roughly the doubling time that Coleman (Coleman, Mattes et al. 2002) reported in serum bottles. After growth stopped, the culture was aseptically removed from the reactor, centrifuged and the pellet was returned to the reactor vessel which had been filled with fresh media and autoclaved. After a short lag period, cDCE degradation resumed. The OD_{600}

reached 0.4 before growth slowed (Panel B, Figure 1.12). During the reactor runs, pH in the reactor was adjusted with ammonium hydroxide. Chloride release from cDCE degradation would produce ammonium chloride in combination with the ammonium hydroxide. The serum bottle studies, which were being carried out at the same time, indicated that ammonium chloride in the 50-100 mM range is inhibitory to cDCE degradation. Rough calculations of the cDCE degraded indicated that as much as 40 mM ammonium chloride was in the reactors when growth slowed.

The culture was aseptically removed from the reactor a second time, cells pelleted and returned to fresh media in the reactor. NaOH was substituted for NH_4OH for pH adjustment based on serum bottle studies that indicated NH_4Cl is inhibitory to cDCE degradation. The culture grew with a substantially shortened lag period and reached an OD_{600} of 0.6 before growth slowed (Panel C, Figure 1.12 for purposes of comparison the axis ranges are the same in all panels). Good growth following transfer to fresh culture media could indicate one of two things: growth is inhibited by the build up of metabolites; or growth is limited by the lack of nutrients. The sensitivity of JS666 to high concentrations of various ionic species suggests that it is toxic metabolites, possibly chloride salts that ultimately limits growth on cDCE.

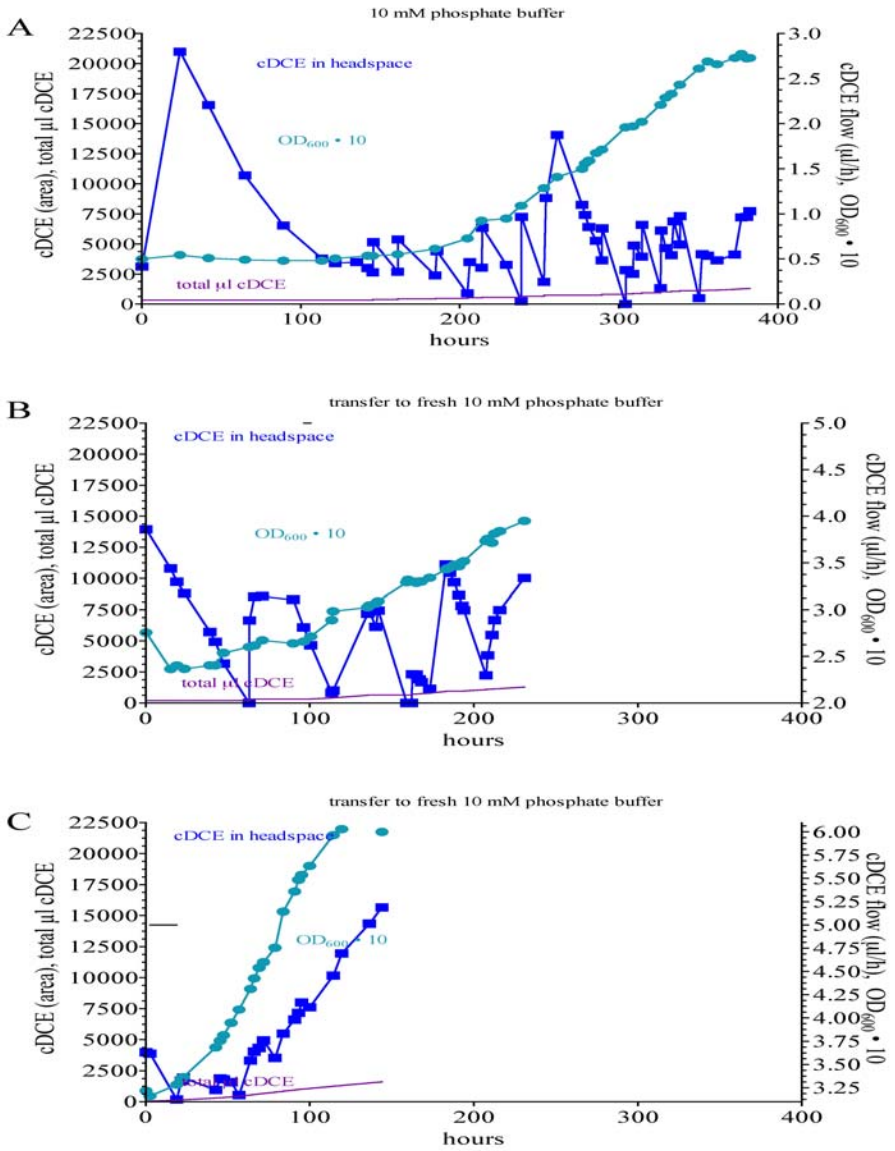


Figure 1.14 Growth of JS666.

Inhibition Investigation

To distinguish between inhibition due to metabolite accumulation and inhibition due to nutrient limitation, half of the culture (350 ml) was removed. The cells were separated from the culture medium by centrifugation and returned to the reactor with 350 ml of deionized water. The effect was dilution of medium in the reactor thus diluting accumulated metabolites as well as the nutrient concentration. If nutrient concentration was limiting before dilution, there should be no growth following further dilution. If metabolite accumulation was limiting before dilution, there would be additional growth following dilution provided that dilution did not now limit nutrients. The result was additional growth of the culture following dilution. The OD_{600} increased by 0.1

on 500 µl of cDCE. The final OD_{600} was 0.89. The results indicate that nutrients are not limiting in the reactor when growth slows, but that the accumulation of some water soluble component, that can be eliminated by removal and replacement of the culture fluid, is inhibitory to cDCE degradation.

Table 1.6 Results of Inhibition Investigation.

Medium exchange no.	µl cDCE added	OD_{600} increase	
0	1300	0.2	initial
1	1300	0.15	
2	1300	0.27	
3	1500	0.3	
4	500	0.1	1:1 dilution w/deionized water

Accumulation of Metabolites

The above experiment did not distinguish between accumulation of ionic species such as chloride salts that accumulate as pH is adjusted and the accumulation of organic metabolic intermediates of cDCE. We examined the gas chromatograms for accumulation of possible organic metabolic intermediates. We found a pattern of very small peaks that were not integrated by the automatic integration protocols, and that accumulated in both the bioreactor and in serum bottles after multiple additions of cDCE. Unknown peak 1 elutes at the time that authentic DCA elutes (4.4 min). The peak is well separated from the cDCE peak (3.8 min). Unknown peak 1 is not detected when a new culture is initially provided with cDCE. The size of the peak fluctuates within a small range (7 to 20 pA units) that is not correlated with the number of injections of cDCE. When authentic DCA is provided to the cultures at the same time as cDCE, the size of the peak that remains at 4.4 min after multiple injections of cDCE is in the same range as when no authentic DCA is provided.

Unknown peak 2 elutes at 3.9 min and is not differentiated from the cDCE peak in the initial injection of cDCE. Unknown 2 either is not present or is masked by the large cDCE peak. Unknown peak 2 first appears as a shoulder on the tailing side of the cDCE peak as cDCE is consumed. As more cDCE is consumed the shoulder is resolved as a separate peak. The size of unknown peak 2 becomes larger as additional cDCE is provided. The peak detected after one injection cDCE is in the 60 to 80 pA units range, after multiple cDCE injections, the peak size increases to 200 to 300 pA units.

Because unknown peak 1 eluted at the same time as DCA, an experiment was done in serum bottles to test whether DCA was degraded at the same time as cDCE. Bottles were provided with DCE and DCA at 10:1 and 50:1 ratios (5 µl cDCE and 0.5 µl DCA, or 5 µl cDCE and 0.1 µl DCA). cDCE degradation rates were no different in control bottles that received only cDCE (5 µl) and in the 50:1 ratio bottles. DCA in the 50:1 bottles was just detectable (7 pA units) at the

time of inoculation. Bottles that received the 10:1 ratio of cDCE to DCA were inhibited until DCA reached a level of approximately 30 pA units.

Unknown peaks were analyzed by GC-MS. Unknown peak 1 has the same retention time as authentic DCA on the GC used for standard cDCE analysis. On the GC-MS unknown peak 1 had a retention time of 11.15 min vs. 11.78 min for DCA and the fragments detected were at m/z 44, 48, 49, 50, and 51. The same cluster of small fragments in the same ratios appear in the mass spectra for *cis*- and *trans*-DCE, but not in spectra for DCA, 1,1-DCE, chloroacetaldehyde, or 2-chloroethanol.

Unknown peak 2 has a retention time of 3.9 min vs. 3.8 min for cDCE in the standard cDCE analysis. On the GC-MS the retention time of 7.42 min for unknown peak 2 matched that for tDCE (7.41 min) and was well separated from cDCE (8.85 min). The mass spectra of the *cis* and *trans* isomers are indistinguishable. The cDCE stock contains tDCE as an impurity. We interpret the results as follows: when cultures are fed cDCE, the tDCE remains after the cDCE is consumed. An experiment was done in serum bottles to determine whether tDCE inhibits cDCE degradation at the concentrations observed to accumulate in serum bottles after multiple injections of cDCE. Duplicate serum bottle cultures were fed with cDCE (5 µl per bottle) or cDCE plus tDCE (0.5 or 0.1 µl per bottle). cDCE disappeared from all bottles at the same rate and the tDCE remained in the bottles after the cDCE was consumed. The results indicate that tDCE accumulation does not inhibit cDCE degradation at the concentrations seen under culture conditions. The results also suggest that unknown peak 1 is a metabolite of cDCE and not of tDCE.

Chloride Removal

Some soluble factor, that is removed when the culture medium is replaced, is responsible for the slowdown in growth of JS666 after multiple additions of cDCE, even after pH adjustment. tDCE, a contaminant of the cDCE used to feed the JS666 cultures accumulates in the culture medium, but experiments with tDCE addition demonstrated that tDCE does not inhibit cDCE degradation at the concentrations at which tDCE accumulates in cultures. Bottle experiments indicated that ionic strength and chloride ions (apart from their role in lowering culture fluid pH) significantly slowed growth on cDCE. We attempted to remove chloride from serum bottle cultures using Amberlite IRN-78, a strong basic anion exchange resin, previously reported to remove chloride in industrial applications (Yun and Buchanan 2001). Resin was soaked in ½-MSB in serum bottles for 10 min before the ½-MSB was decanted and replaced with fresh medium. The initial soak was to counter the rise in pH (to about 12) caused by the unsoaked resin. After soaking, pH in the medium rose to 8.3, a second soak did not further affect pH. Bottles with resin were autoclaved and cooled before addition of cells and cDCE. In two separate experiments, cultures incubated with resin, initially degraded cDCE more slowly than control cultures without resin. Control cultures removed cDCE rapidly until approximately 37 µl of cDCE had been added to

the bottles (approximately 10 mM). The first experiment was terminated after 59 µl of cDCE had been added to the culture incubated resin, but before cDCE degradation had slowed.
In a subsequent experiment, a range of resin amounts (10 to 40 g/L) were added to duplicate bottles. More cDCE was consumed in bottles with 10 or 20 g/L resin than in control bottles without resin. Much less cDCE was consumed in bottles with 40 g/L resin than in the control bottles. Bottles with 40 g/L resin also developed a white precipitate, presumably due to disruption of the balance of ionic species in the medium by the action of the anionic exchange resin.

Table 1.7 Effect of Resin Concentration on cDCE Degradation.

g/L Resin	0	10	20	40
Bottle 1 µl cDCE consumed	47.0	61.4	89.9	12.0
Bottle 2 µl cDCE consumed	47.6	63.0	80.8	14.1

JS666 was grown in a 1-L bioreactor, using modified ½-MSB as the growth medium, with 10 mM rather than 40 mM phosphate buffer. A small column filled with approximately 5 g of Amberlite IRN-78 resin was substituted for the base addition bottle. When the pH of the culture medium dropped below the pH 7.2 set point, culture medium was pumped from the reactor vessel, through the column and back into the reactor. The column was exchanged for a fresh column when the resin was no longer able to restore the pH to the set point. All other operating conditions were as described for previous bioreactors. Before the run was terminated by a break in the recycle line (and loss of the culture), OD_{600} tripled, and approximately 2800 µl of cDCE was consumed, and growth had not slowed. The flattening of growth around 300 h was due to the inability of air addition to maintain 40% O_2 saturation in the reactor. Automatic continuous aeration stripped cDCE from the reactor and growth stopped. When O_2 was substituted for air, there was a 10 h lag period before growth resumed. Growth in previous bioreactors slowed or stopped after the addition of approximately 1300 µl of cDCE and one doubling. The results indicated that the removal of excess chloride rather than the addition of base to neutralize chloride yields better growth of JS666.

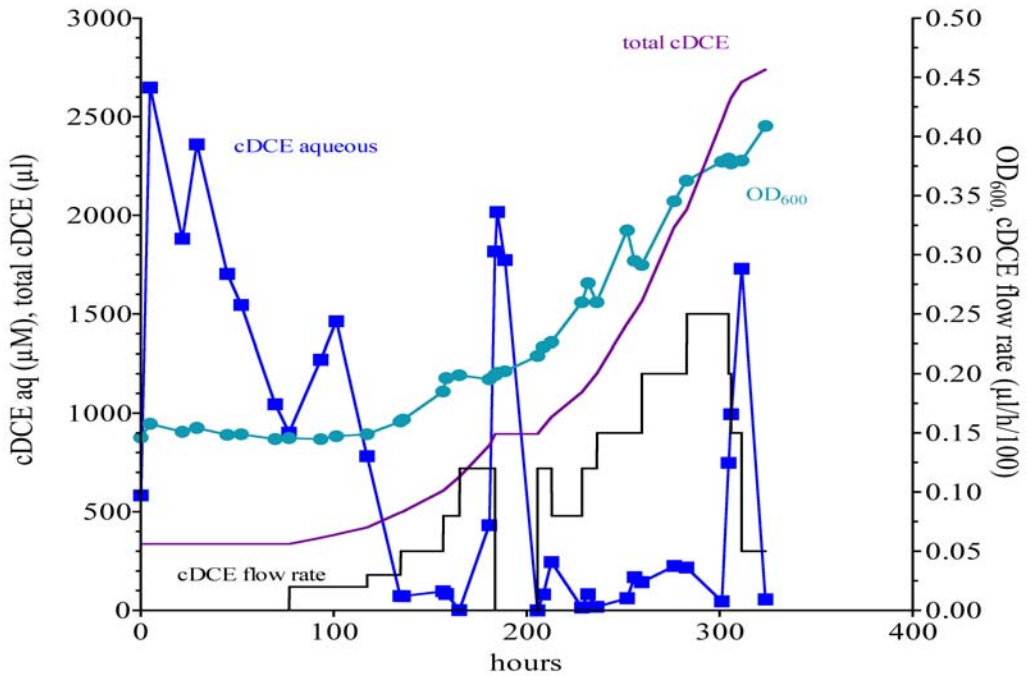


Figure 1.15 Growth of JS666 in 1-L Bioreactor.

Resin Regeneration

The bioreactor was restarted with a larger resin column and an additional loop to regenerate the Amberlite IRN-78 resin with NaOH when the resin's capacity to remove chloride was exhausted. The goal was to determine the maximum amount of growth that could be achieved before a medium exchange was required. The attempt was unsuccessful, as the cells failed to grow after resin regeneration, possibly due to removal of essential nutrients along with excess chloride. The failure of extended chloride removal by anion exchange resin caused us to focus on cell retention during medium exchange to remove inhibiting metabolites.

Medium Exchange With Cell Retention

The culture medium in the 1-L bioreactor vessel could be exchanged by aseptic removal of the culture, harvesting by centrifugation, and transferring the cells to another sterile vessel containing fresh medium. The handling-intense procedure provided many opportunities for contamination of the culture and also resulted in an extended lag period after the exchange was made. Even more handling would be required for a 4-L bioreactor. In order to minimize such handling and the lag period, other bioreactor designs were considered. Reactors for immobilized cells such as fluidized bed reactors could not be used because JS666 does not form biofilms. Hollow-fiber reactors were considered but none were readily available that were made of cDCE-resistant materials, and had sufficient culture capacity at a cost-effective price. The configuration that we adopted added a loop to a transverse filtration apparatus (Millipore Pellicon) containing a

filter cassette with 0.4 µm pore size (Durapore membrane). To exchange culture medium, a peristaltic pump sends the culture medium to the filter cassette at 4 L min^{-1}. During transverse filtration a proportion of the medium is forced through the filter to waste (the filtrate) while the cells are retained on the filter surface and recirculated back to the bioreactor vessel in the medium that did not go through the filter (the retentate). The cells become concentrated in the medium as the filtrate is removed. When only a small volume of concentrated cells remain in the bioreactor, the waste line is closed and lines to a sterile medium reservoir are opened. The tubing lines in the peristaltic pump are exchanged so that sterile medium back-flushes cells off of the filter surface and into the bioreactor vessel. The process takes about 15 min to change 4 L of culture medium.

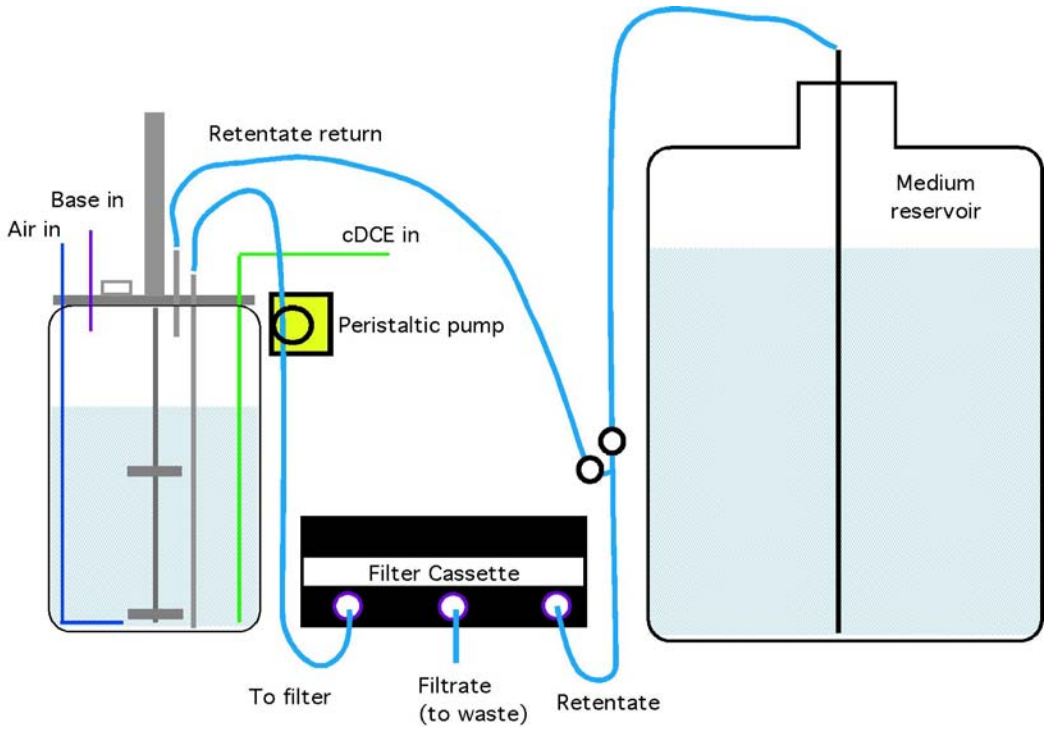


Figure 1.16 Schematic of Bioreactor.

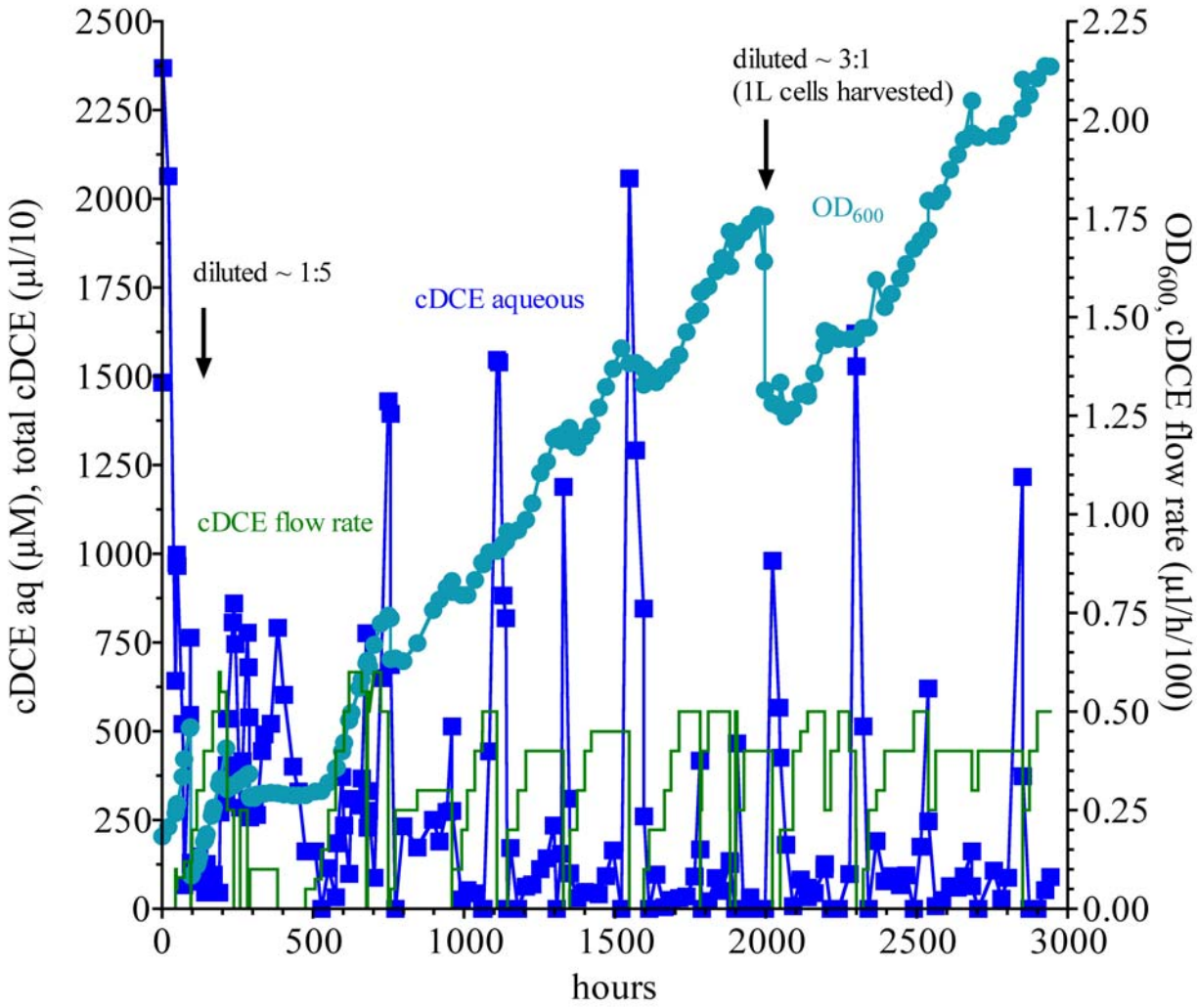


Figure 1.17 Growth of JS666 in 4-L Bioreactor.

The contents of a 1-L bioreactor vessel were transferred to a bioreactor with a 4-L working volume. Modified ½ MSB was added to bring the volume to 4 L. pH was controlled at 7.0 by addition of NaOH. cDCE was initially pulsed into the culture with a syringe pump, but when degradation began, cDCE was pumped in continuously at rates from 3.2 to 50 µl h^{-1}. The medium was exchanged whenever growth began to slow based on OD_{600} and the increase in cDCE concentration in the reactor. Review of the run shows that onset of slow growth occurred when total cDCE pumped into the reactor since the last medium exchange reached 1.3 – 1.5 ml L^{-1}, a 4-5 fold increase in total cDCE degradation over that obtained under the original serum bottle conditions.

Culture Storage

Limited bioreactor capacity dictates that cDCE-grown cells of JS666 be stored or stockpiled. Cells from an actively growing culture were stored at 4°, -20°, and -80°C, with and without culture media and/or cryoprotectant (10% glycerol). Periodically, cultures were removed from storage conditions and inoculated into fresh media to test how stable the cDCE-degrading phenotype is maintained under the various storage regimes.

Results indicate that in the very short term (< 4 days) 4°C > -80° > -20°. For the frozen temperature treatments, cells that were stored as pellets, recovered activity more rapidly than did cells that were stored in the original culture medium. The addition of glycerol as a cryopreservative did not enhance recovery, nor did the addition of dilute yeast extract. Removal of the culture fluid did not affect recovery of cells stored at 4°C. When storage time exceeded 1 week, the -80°C cell pellet recovered cDCE degradation activity much more rapidly than cells stored under any other condition.

A portion of the culture from the 4-L bioreactor was centrifuged to remove the culture medium and the cell pellet was frozen at -80°C for 35 days. The pellet was removed from the freezer, allowed to thaw at room temperature, suspended in ½ MSB, and then used to inoculate a 1-L reactor. Growth on cDCE, as indicated by sustained increases in OD_{600} began 3 days after inoculation. The results show that after moderate term storage at -80°C, pellets of cDCE-grown cells can rapidly recover the ability to degrade cDCE.

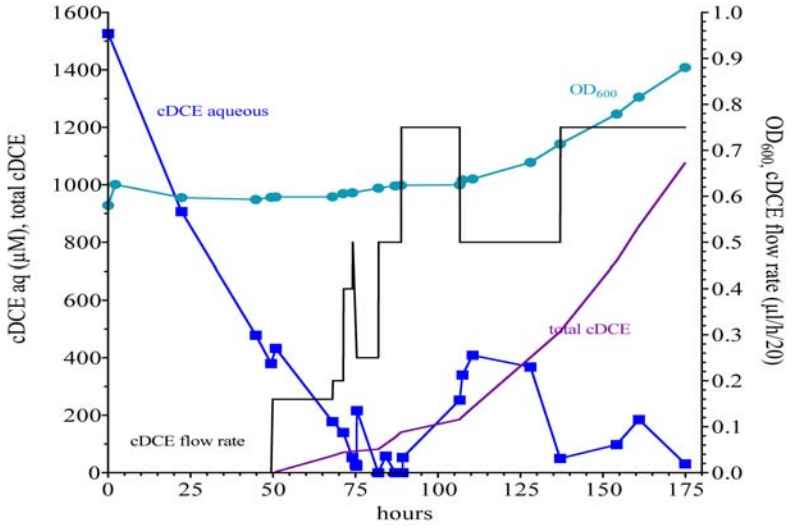


Figure1.18 Growth of JS666 as Measured by OD_{600}.

1.2.7 Density Dependence

Two studies were done to test for density-dependent degradation of cDCE. There are bacterial behaviors that depend on the absolute density of the microorganisms in cultures such as the ability to form biofilms or pathogenicity. In some instances, culture density affects whether and how rapidly a growth substrate is catabolized (Nishino and Spain 1993). To test whether cell density of JS666 affects cDCE degradation, a series of different volume serum bottles (160, 73, and 39 ml bottles) were inoculated with the same volume of washed JS666 cells (300 µl). The bottles contained proportionally the same amount of ½-MSB (50, 23, and 12 ml, respectively), and were spiked proportionally with cDCE (3, 1.37, and 0.73 µl, respectively). If density-dependent phenomena were involved, one would expect that the lag period before degradation of cDCE begins would be shorter in the dense culture. No differences were observed in the lag periods. In the second experiment the same size serum bottles (160 ml) were inoculated with JS666 over a 3 order of magnitude volume range. Again no differences were observed in the lag periods, although the degradation rates varied with inoculum size. We can conclude that cDCE degradation in JS666 is not density dependent.

1.3 Summary and Conclusions

Investigation of the cultural conditions necessary for reliable production of a JS666 inoculum for bioaugmentation revealed that pH must be kept above 6.5 for sustained cDCE-degradation; and previous studies (Coleman et al., 2002a) indicated that temperature must be kept under 30ºC (and preferably below 25ºC). The need for pH-neutrality will present challenges at some cDCE-contaminated sites, and suggests that buffer (current recommendation is 40 mM phosphate) may have to be introduced along with this bioaugmentation agent for successful implementation. On the other hand, it may be possible to adapt JS666 to lower pH through selection of low-pH-tolerant variants [Note: since JS666 was originally enriched and isolated at neutral pH, it is not surprising that our current variety prefers neutral pH.] We are presently investigating adaptation through pH "challenges" to the culture.

Two different cultural behaviors were observed: 1) "Bad Behavior" with high-density cultures (> 0.3 OD_{600}) recently exposed to co-substrates such as glycerol, succinate or ethanol, in which cDCE-degradation rates steadily decline, cDCE degradation is not sustainable, and behavior resembles that of cometabolism; and 2) "Good Behavior" observed in the original studies of Coleman et al. (2002a), with low-density (< 0.05 OD_{600}) cultures frequently transferred to fresh medium and which have not seen any substrate but cDCE for many generations. We successfully demonstrated production of a stable, sustainable inoculum source exhibiting "good behavior" through frequent transfer/dilution into fresh media.

2. SUBTASK 1.2: EVALUATING THE EFFECTS OF MIXTURES OF CHLOROETHENES ON cDCE AND OTHER CHLOROETHENE TRANSFORMATIONS.

2.1 Introduction

Previous studies (Coleman et al., 2002a) reported that JS666 can degrade chloroethenes and chloroethanes besides cDCE, without being able to grow on them. Because cDCE might be present in mixtures of chloroethenes and chloroethanes at contaminated sites for which bioaugmentation with JS666 would be considered, its response to such contaminant mixtures is of interest. In this phase of study, we investigated the relative kinetics and mutual effects of binary mixtures of cDCE at ~ 2 ppm in the presence of lesser concentrations of VC, TCE, and 1,2-DCA.

2.2 Materials and Methods

Studies were conducted in 100-ml liquid volumes in 160-ml glass serum bottles fitted with Teflon-lined, butyl-rubber stoppers and aluminum crimp-caps as described elsewhere in this report. The inoculum was a "good-behaving" cDCE transfer culture (23˚C; agitated) in MSM, fed spikes of 59 mg/l (nominal concentration) cDCE as sole carbon substrate. The inoculum culture was aseptically operated as described in a previous section of this report — 5% v/v transfers were made to fresh MSM, usually during mid-degradation of the second spike of cDCE. In preparation for conducting a kinetic experiment, the entire 100-ml culture was added to 1-liter of MSM in a larger serum bottle – essentially creating a 10x scale-up of the usual system. Midway through the second, nominal-59-mg/l spike of cDCE, this one-liter culture was distributed to several centrifuge tubes and spun-down in a centrifuge to harvest the cells. The concentrated cells from centrifugation were composited back into a 160-ml serum bottle and purged with air to remove any residual cDCE. Care was taken to avoid temperatures in excess of 25˚C through this entire process, but aseptic conditions were not maintained – the rationale being that the culture was soon to be used in a one-time, short-term (ca. 12- to 40-h) experiment at low substrate levels in which growth (of anything) would be insignificant. The concentrated cells were distributed to a series of identical, 160-ml serum bottles with final liquid volume (by MSM addition) of 100 ml.

The series of experimental bottles were capped as usual, and dosed with desired chlorinated substrates from aqueous stock solutions, to avoid experiencing any kinetic delays in dissolution from otherwise adding neat, chlorinated-organic substrates. cDCE was dosed at 2 µmol per bottle (ca. 1.8 mg/l actual aqueous concentration when partitioning to the headspace is taken into account). The potential, competing chloroethene or chlorethane was dosed at 0.2, 0.4, or 0.6 µmol per bottle. Note that all of these levels were considerably lower than used in most of the other studies reported herein. Our intention was to use concentrations more typical of what one

might encounter at contaminated sites. All bottle-types were run in duplicate. Bottles were agitated (160 rpm) in an inverted position on an orbital shaker in a constant-temperature (23˚C) room. At both beginning and end of an experimental run, samples were taken for DNA analysis (FluoroSkan) as a measure of relative biomass concentration (ca. 2 µg DNA/ml).

2.3 Results

In the following, only single examples of individual bottle-types are shown. In all cases, replicates behaved substantially the same.

Vinyl Chloride

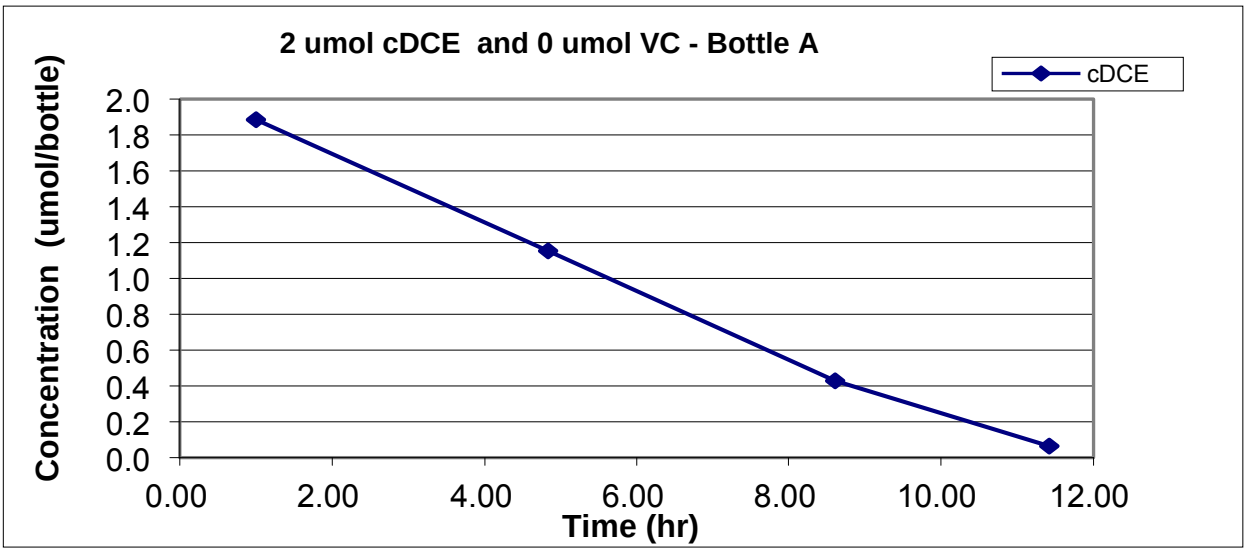


Figure 2.1 Degradation of cDCE Alone.

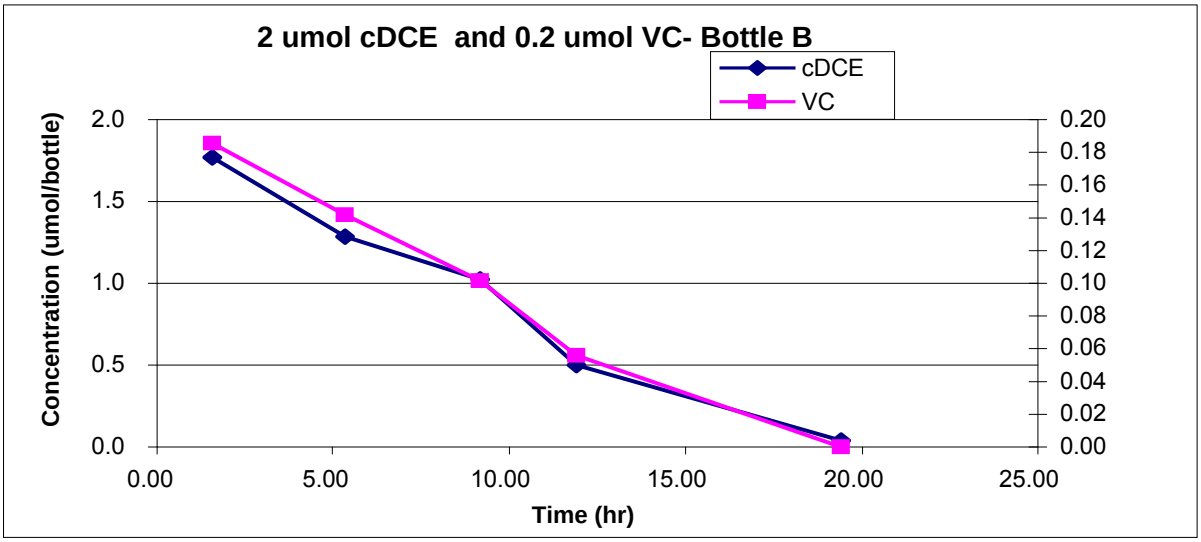


Figure 2.2 Degradation of cDCE and VC at Lowest VC Level.

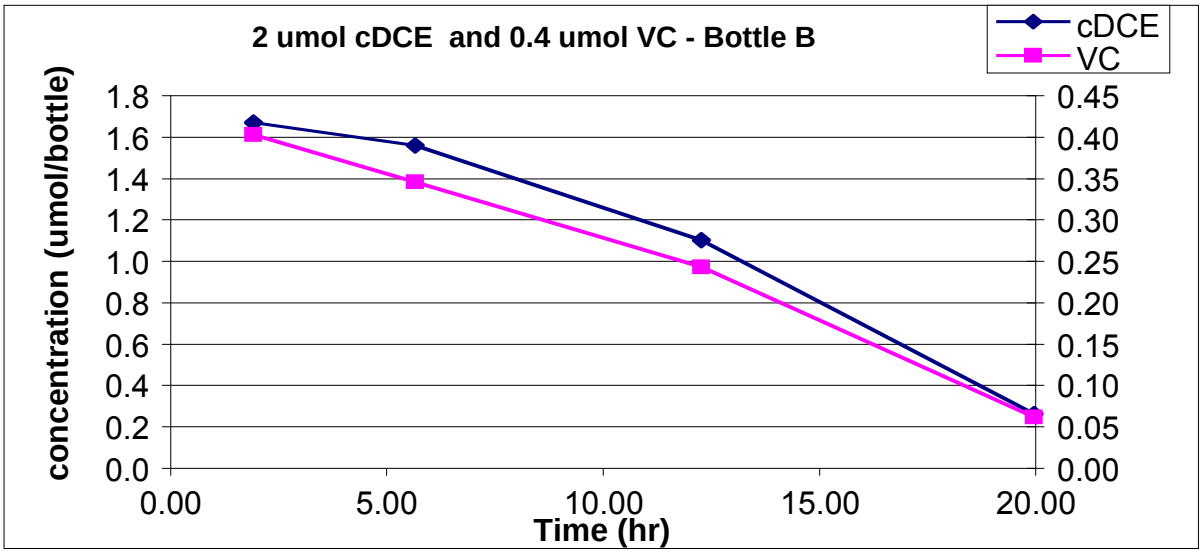


Figure 2.3 Degradation of cDCE and VC at Medium VC Level.

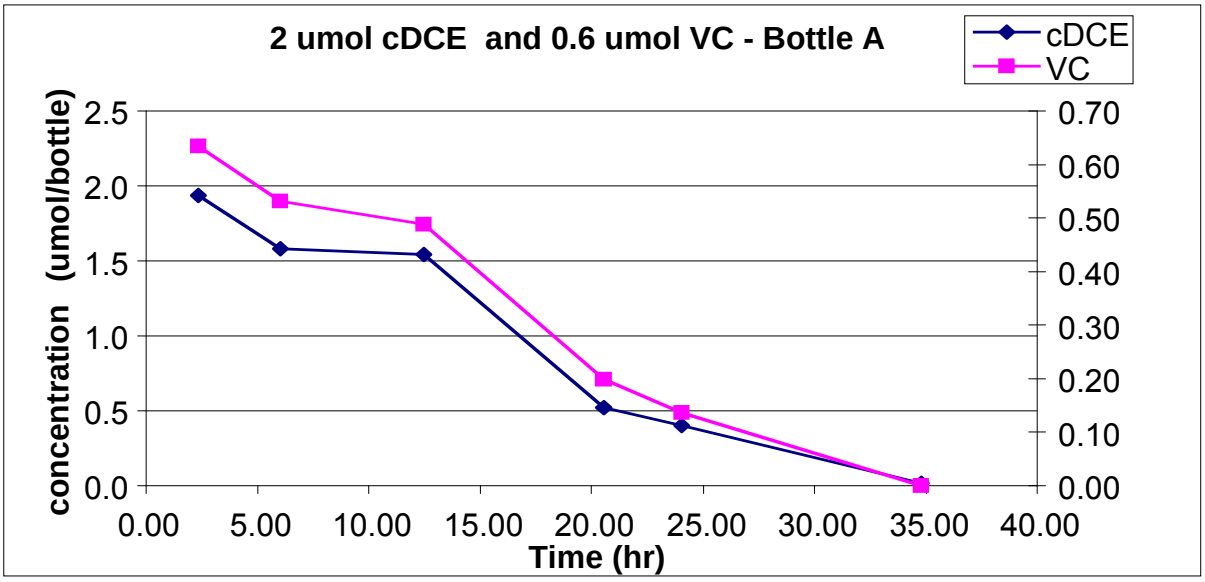


Figure 2.4 Degradation of cDCE and VC at Highest VC Level.

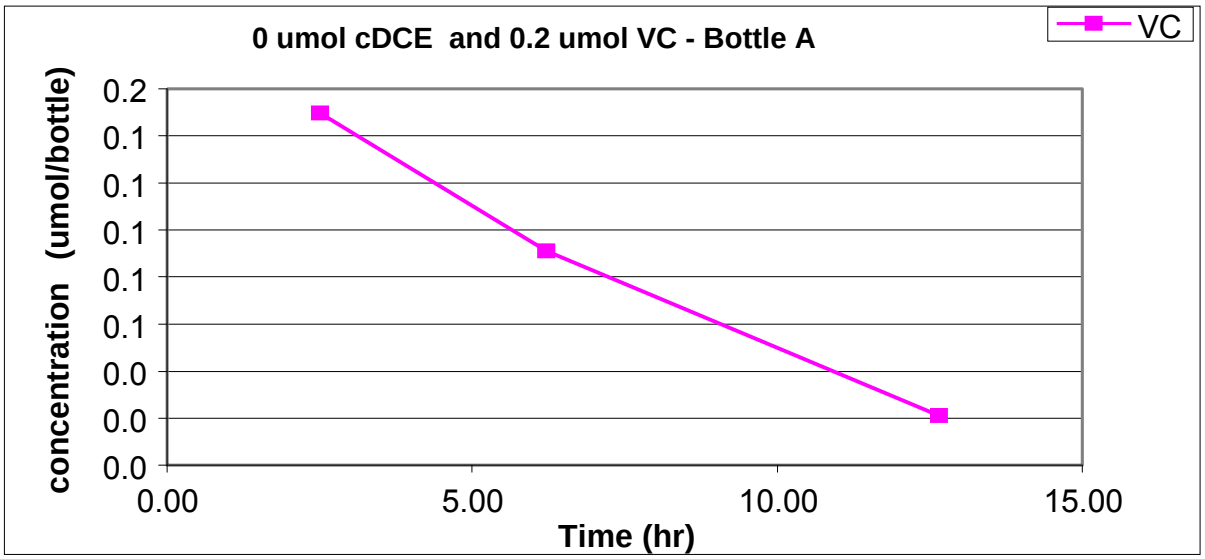


Figure 2.5 Degradation of VC Alone at Lowest VC Level.

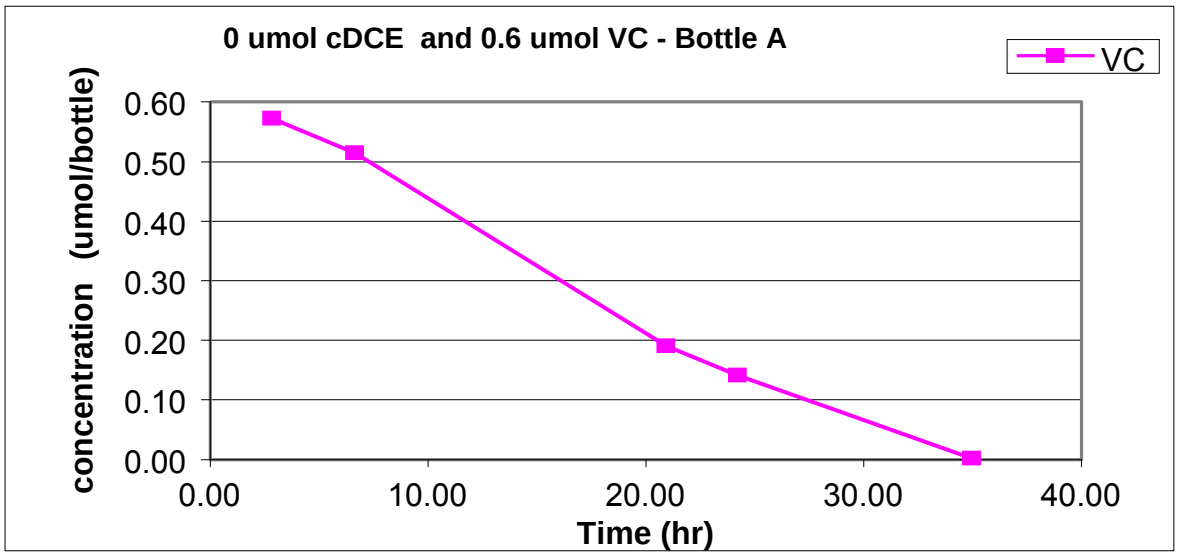


Figure 2.6 Degradation of VC Alone at Highest VC Level.

The way in which degradations of cDCE and VC track each other in binary mixture is interesting i.e., when the vertical scales are adjusted so that each starts out at the same position, their degradation curves nearly overlap so as to be identical. It is not a situation where one compound is degraded first or preferentially. Their "fraction remaining" values are virtually the same at any time.

In Figure 2.7 below, the results are presented in another way: The maximum rates of cDCE degradation are shown as function of initial, aqueous VC concentration (taking into account partitioning to headspace). These actual, aqueous VC concentrations are given in units of mg/l, since these units likely are more meaningful to practitioners than units of µM. It is apparent that VC concentrations above about 100 ppb cause a 50% decline in cDCE degradation rate (which did not worsen at still higher VC concentrations) – yet it is also apparent from all of the foregoing data, that cDCE degradation nonetheless proceeds to completion. That's the good news, from a bioremediation standpoint.

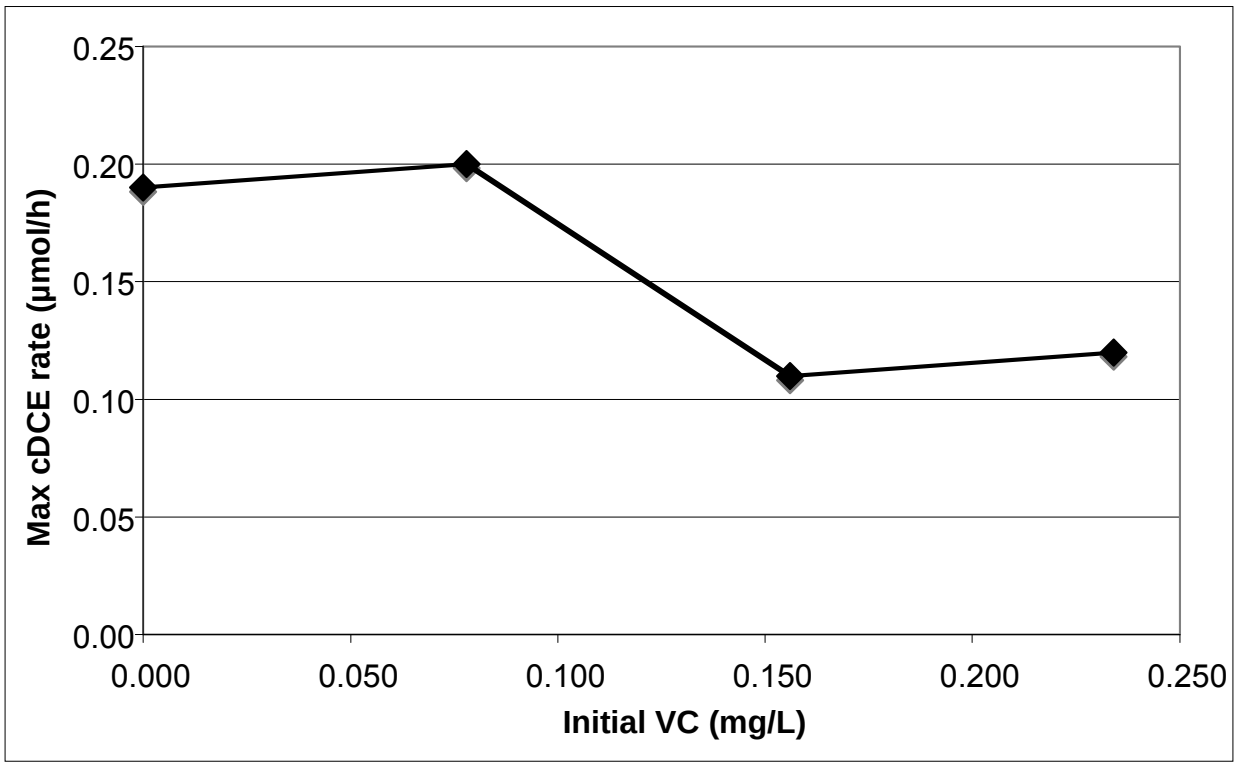


Figure 2.7 Maximum cDCE Degradation Rate as Function of Initial, Aqueous VC Concentration.

In Figure 2.8 below, the maximum rate of VC degradation is shown as function of initial, aqueous VC concentration–with and without presence of cDCE. It is apparent that, over the range of VC concentrations investigated, the VC degradation rate was virtually first-order with respect to VC concentration (and perhaps *enhanced by* the co-presence of its growth substrate, cDCE).

It should be pointed out, however, that the mutual effects of cDCE and VC upon each other's degradation are more complex than these simple examinations of maximum rates suggest. It is apparent that the shape of the cDCE degradation curves–in particular, the time when maximum degradation rates occurred–was influenced by co-presence of VC. In the absence of VC, maximum rate of cDCE degradation tended to occur at initial time-points, whereas in the presence of VC, maximum rate of cDCE degradation was delayed until 10 to 20 hours after initiation.

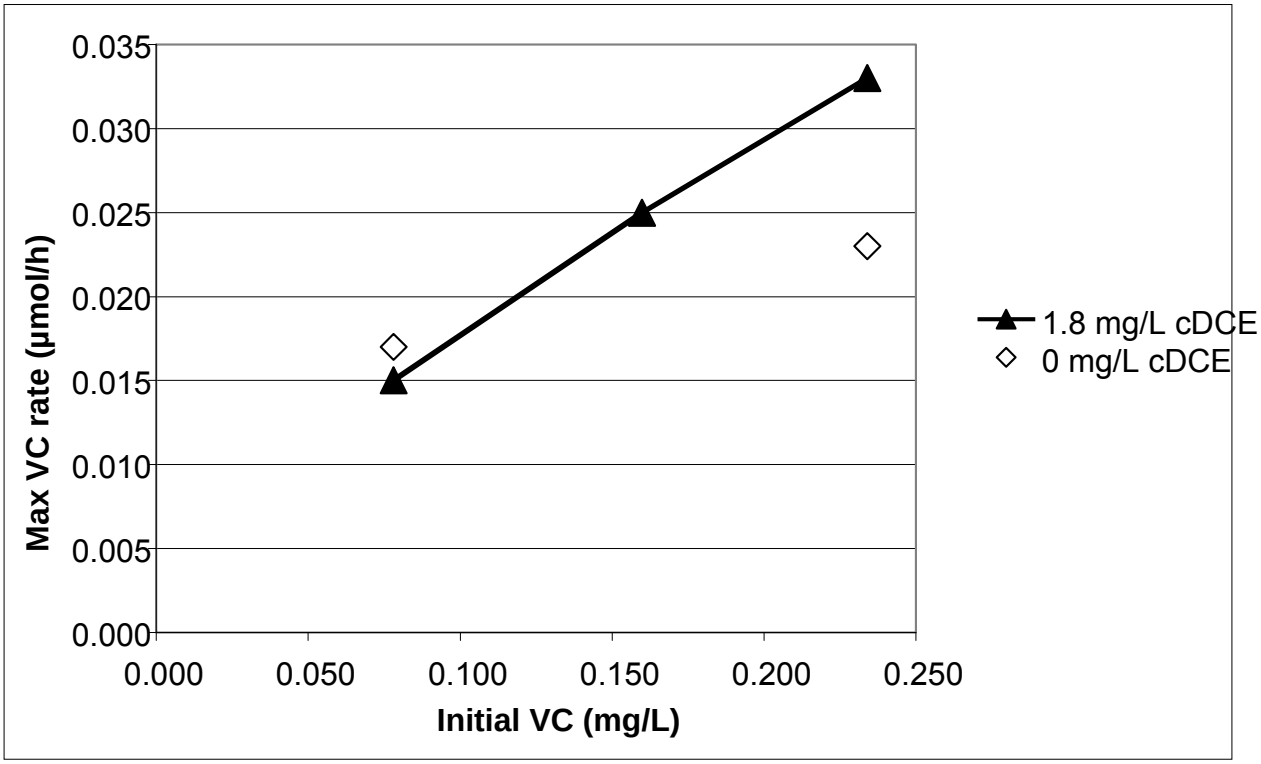


Figure 2.8 Maximum VC Degradation Rate Versus Initial, Aqueous VC Concentration — in Presence or Absence of cDCE.

Trichloroethene

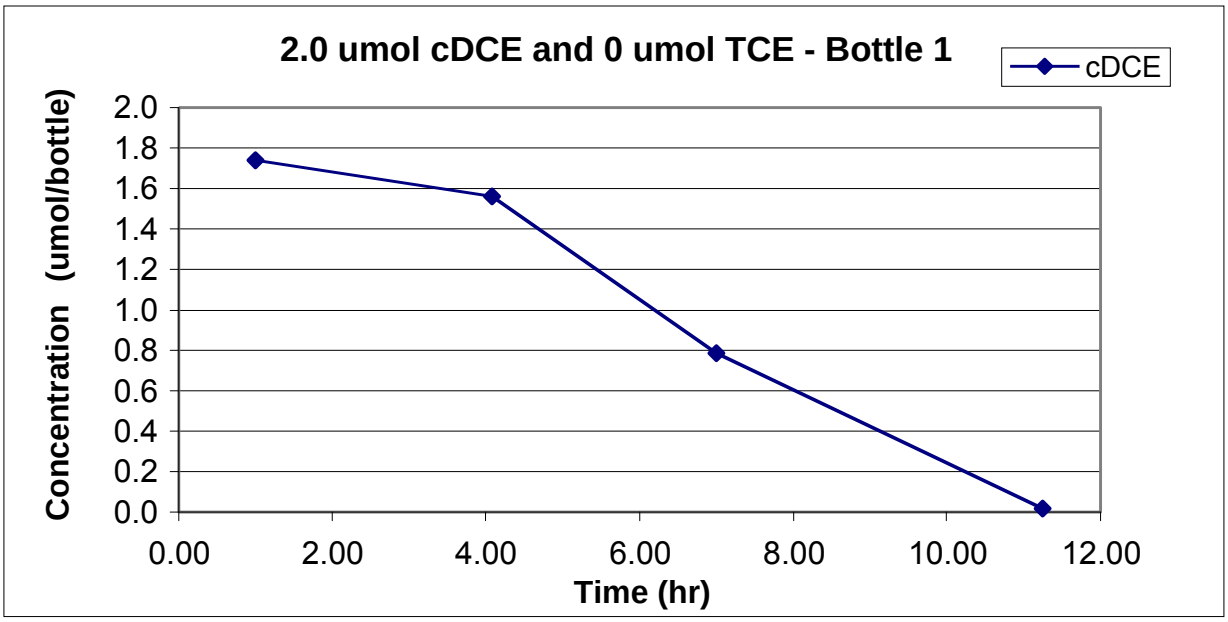


Figure 2.9 Degradation of cDCE Alone.

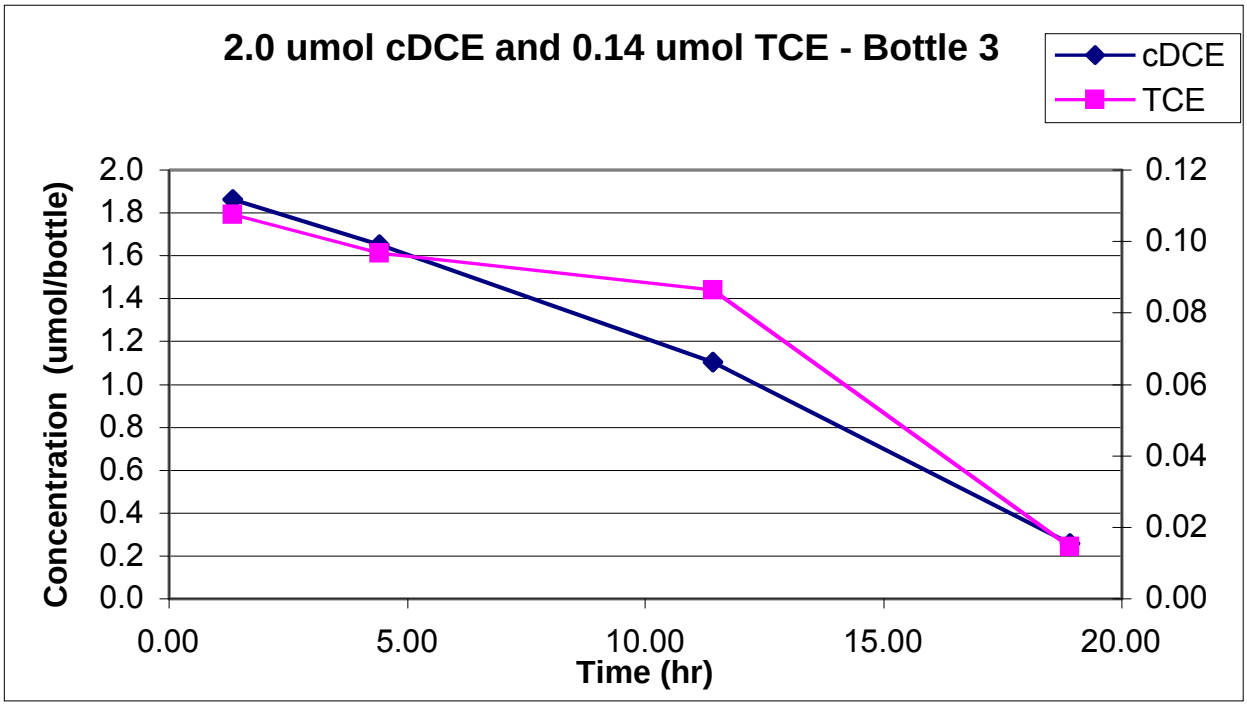


Figure 2.10 Degradation of cDCE and TCE at Lowest TCE Level.

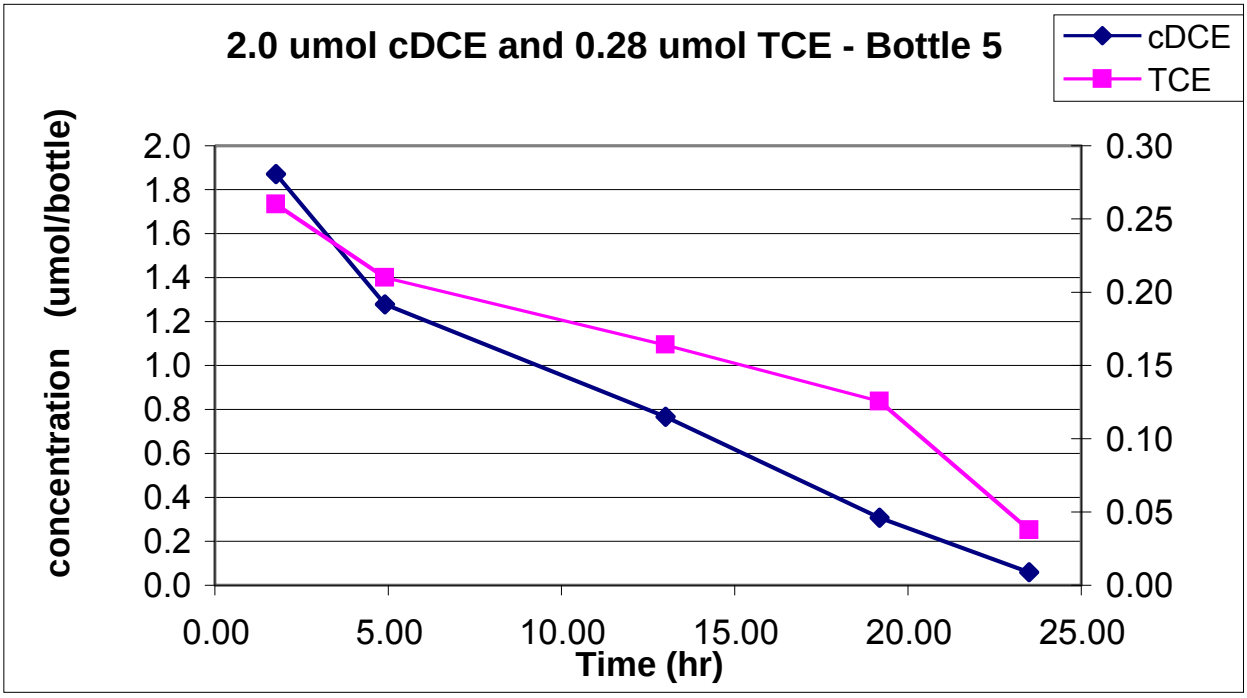


Figure 2.11 Degradation of cDCE and TCE at Medium TCE Level.

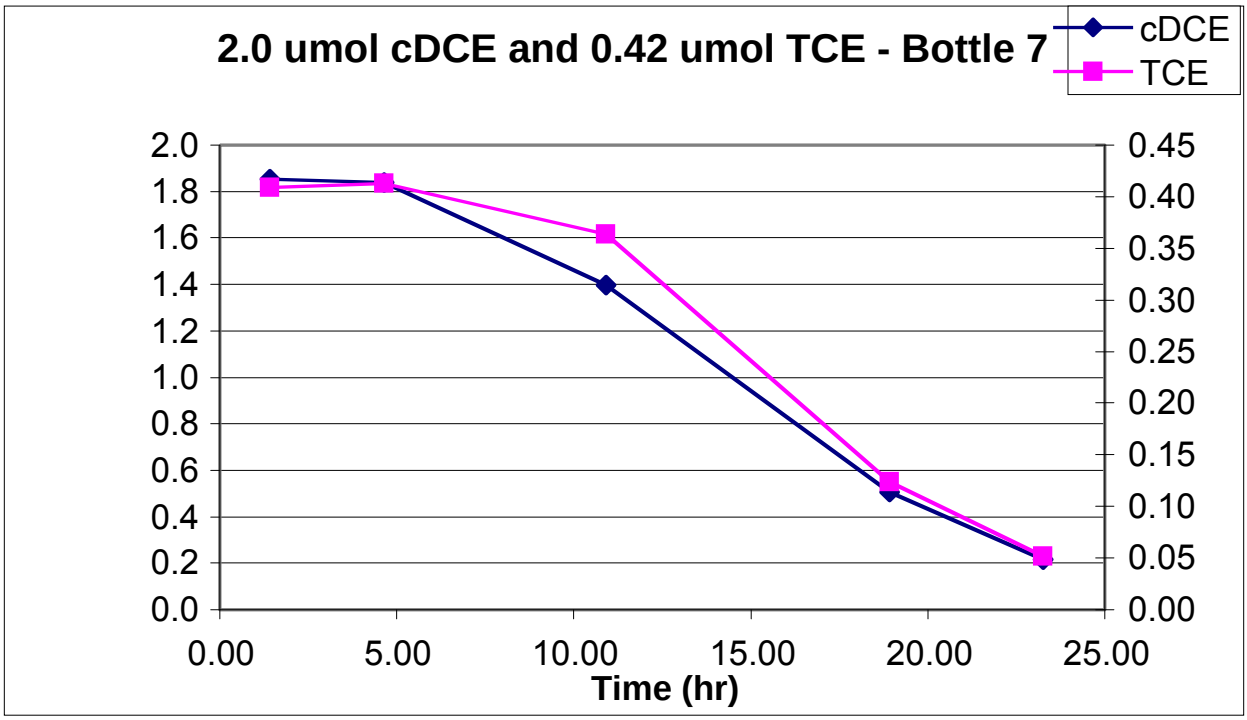


Figure 2.12 Degradation of cDCE and TCE at Highest TCE Level.

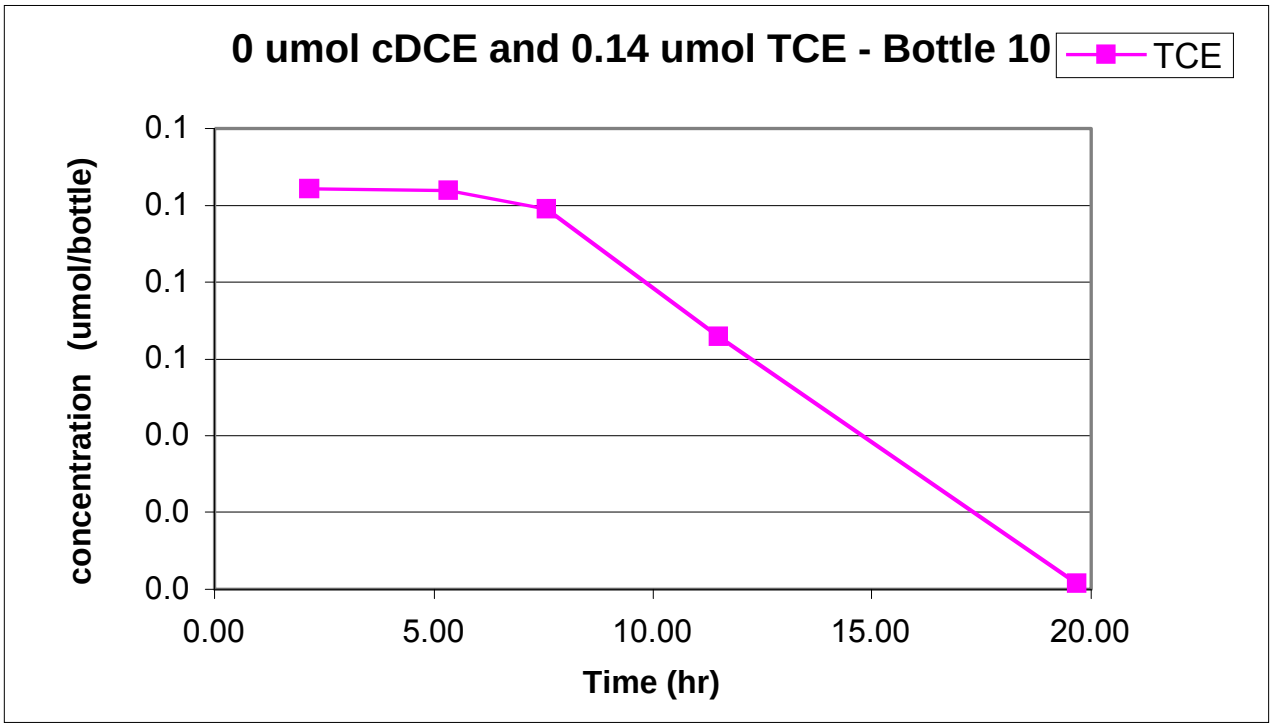


Figure 2.13 Degradation of TCE Alone at the Lowest TCE Level.

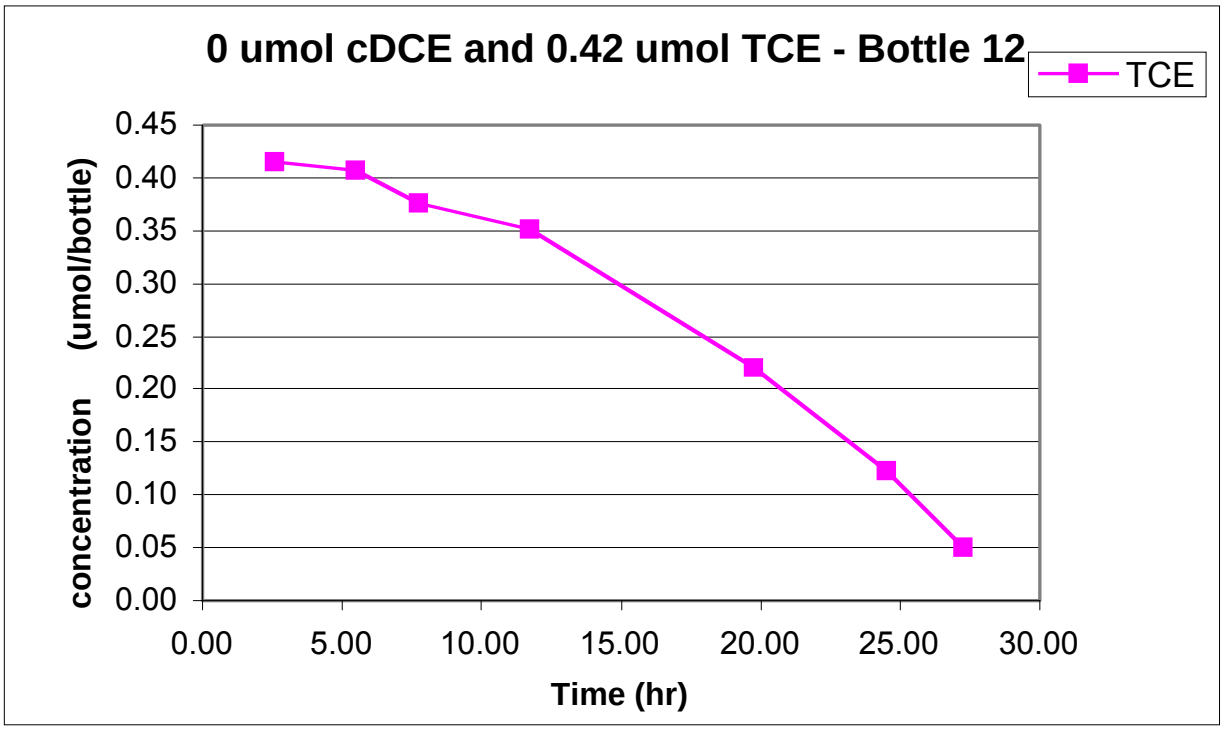


Figure 2.14 Degradation of TCE Alone at the Highest TCE Level.

As with VC/cDCE mixtures, degradations of cDCE and TCE appear to track each other in binary mixture – i.e., when the vertical scales are adjusted so that each starts out at the same position, their degradation curves nearly overlap so as to be identical (though perhaps not so near-perfectly as with VC and cDCE). Again, it is not a situation where one compound is degraded first or preferentially. Their "fraction remaining" values are virtually the same at any time.

In Figure 2.15 below, the results are presented in another way: The maximum rates of cDCE degradation are shown as function of initial, aqueous TCE concentration (taking into account partitioning to headspace). These actual, aqueous TCE concentrations are given in units of mg/l, since these units likely are more meaningful to practitioners than units of µM. It is apparent that TCE concentrations at or above about 150 ppb cause a 50% decline in cDCE degradation rate – yet it is also apparent from all of the foregoing data, that cDCE degradation nonetheless proceeds to completion and the effect of TCE upon cDCE degradation does not worsen at still higher TCE concentrations. Again, that's good news, from a bioremediation standpoint.

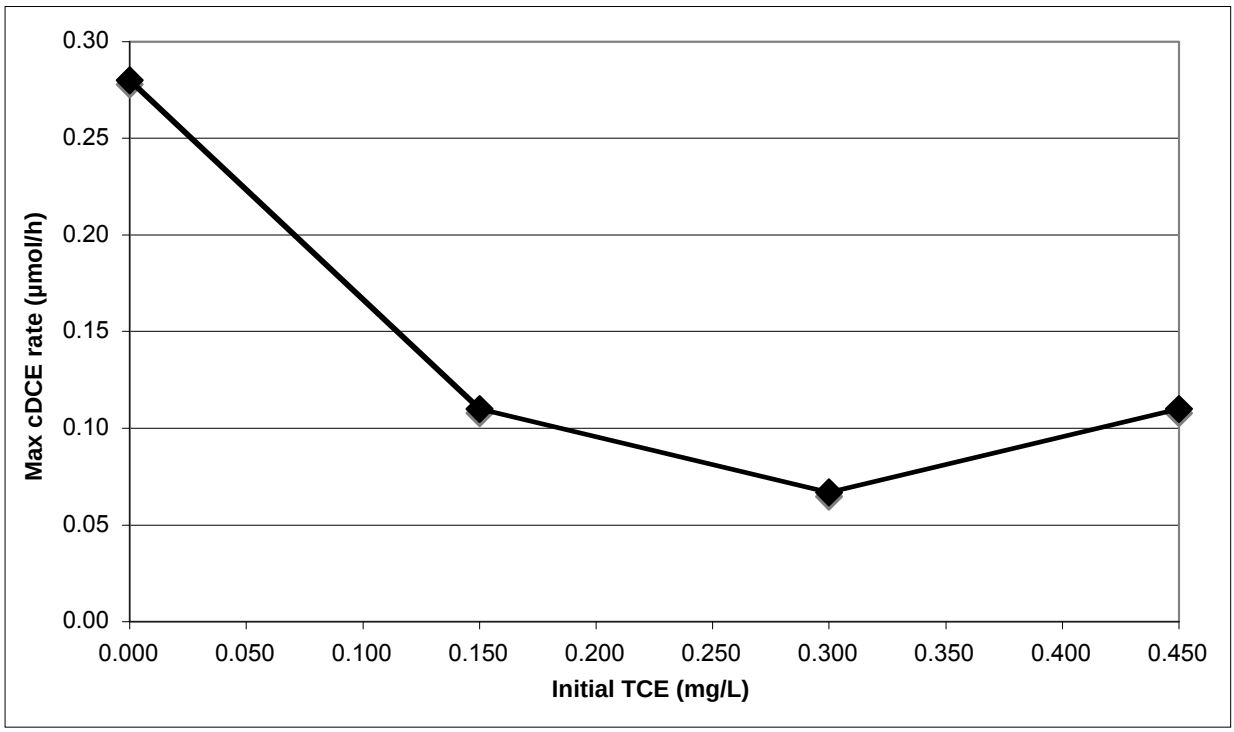


Figure 2.15 Maximum cDCE Degradation Rate as Function of Initial, Aqueous TCE Concentration.

In Figure 2.16 below, the maximum rate of TCE degradation is shown as function of initial, aqueous TCE concentration — with or without presence of cDCE. It is apparent that, over the range of TCE concentrations investigated, the TCE degradation rate was virtually first-order with respect to TCE concentration. It also appears that maximum rate of TCE degradation was little-influenced by the presence of cDCE. However, closer examination of the *patterns* of cDCE and TCE degradations shows evidence of complex mutual effects – i.e., in terms of lags and time-to-maximum degradation rates.

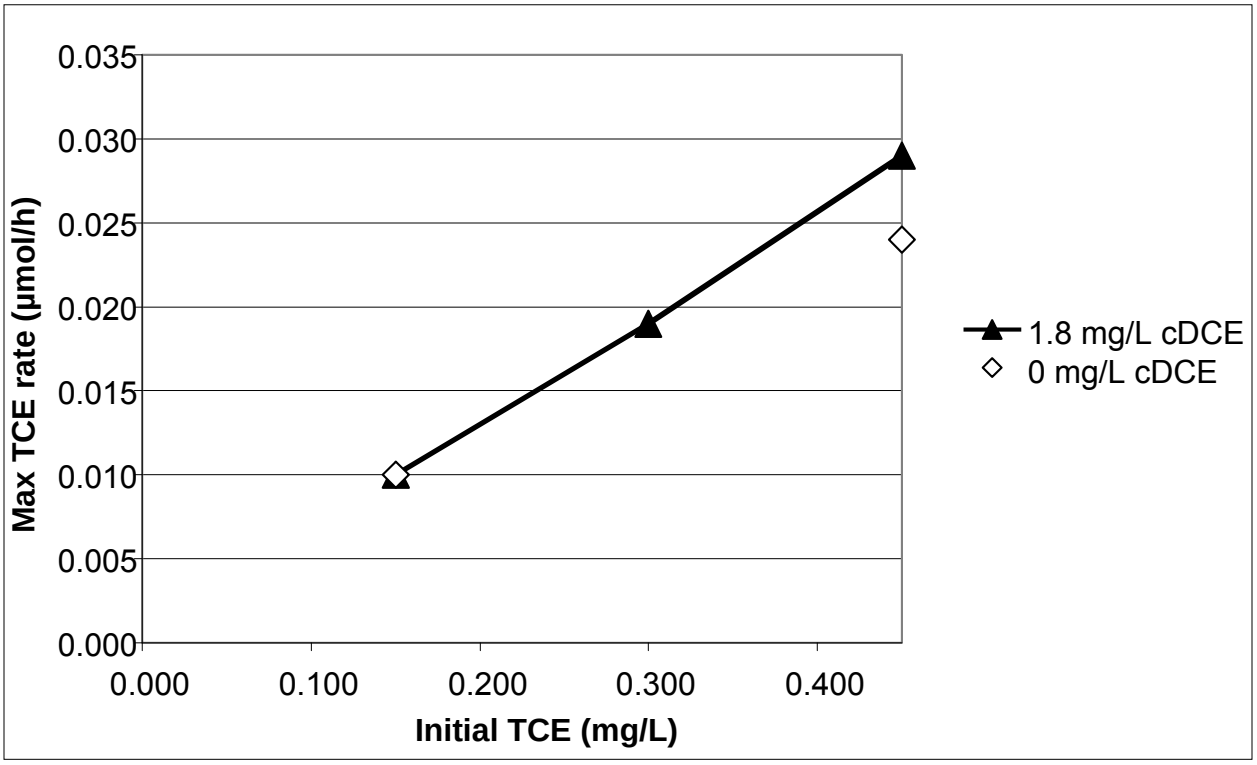


Figure 2.16 Maximum TCE Degradation Rate Versus Initial, Aqueous TCE Concentration — in Presence or Absence of cDCE.

1,2-Dichloroethane (1,2-DCA)

As with VC/cDCE mixtures and TCE/cDCE mixtures, degradations of cDCE and 1,2-DCA appear to track each other in binary mixture – i.e., when the vertical scales are adjusted so that each starts out at the same position, their degradation curves nearly overlap so as to be identical (though perhaps not so near-perfectly as with VC and cDCE). Again, it is not a situation where one compound is degraded first or preferentially. Their "fraction remaining" values are virtually the same at any time.

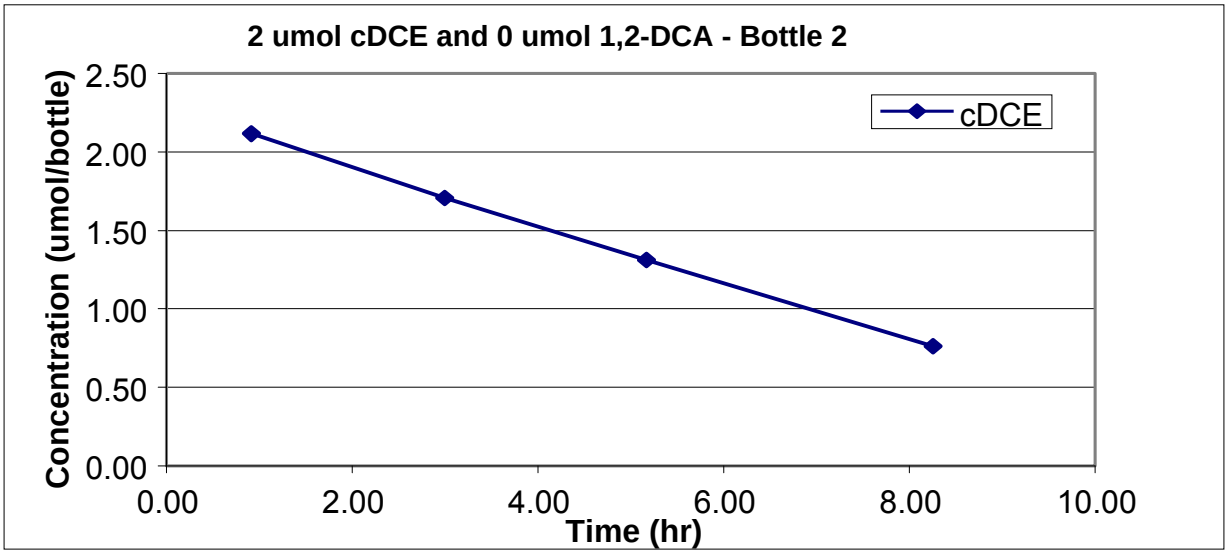


Figure 2.17 Degradation of cDCE Alone.

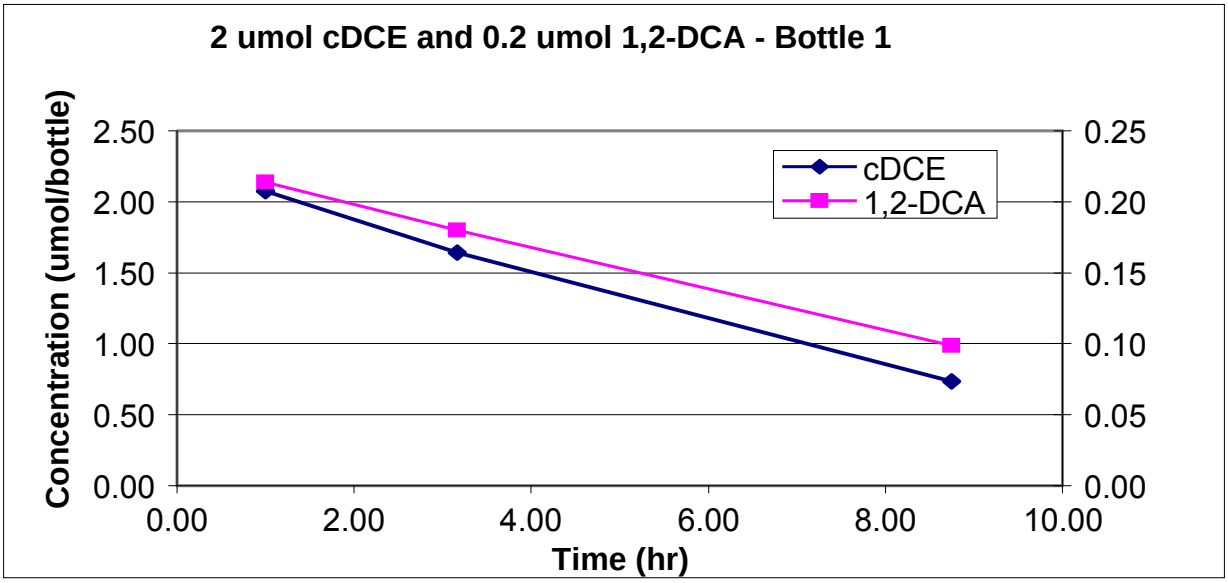


Figure 2.18 Degradation of cDCE and 1,2-DCA at Lowest 1,2-DCA Level.

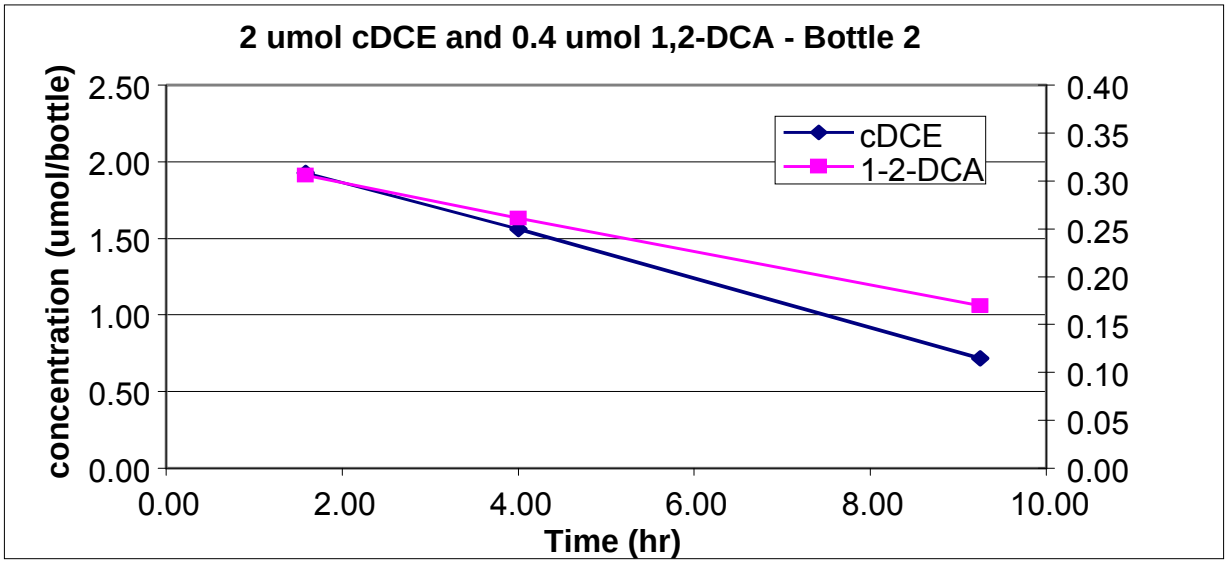


Figure 2.19 Degradation of cDCE and 1,2-DCA at medium 1,2-DCA level.

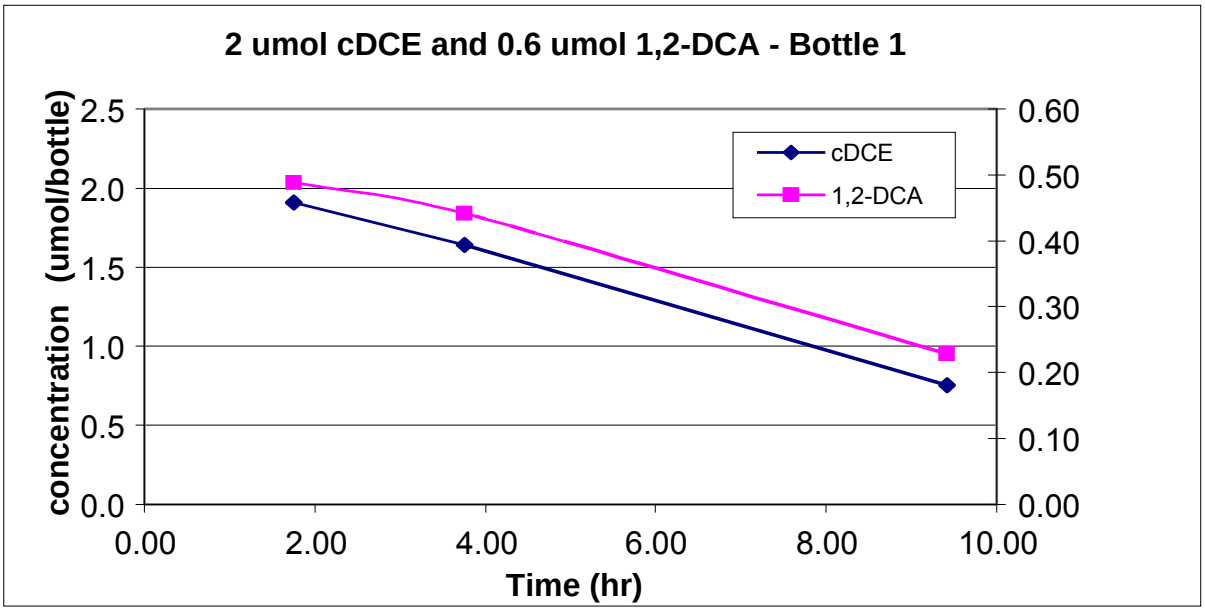


Figure 2.20 Degradation of cDCE and 1,2-DCA at highest 1,2-DCA level.

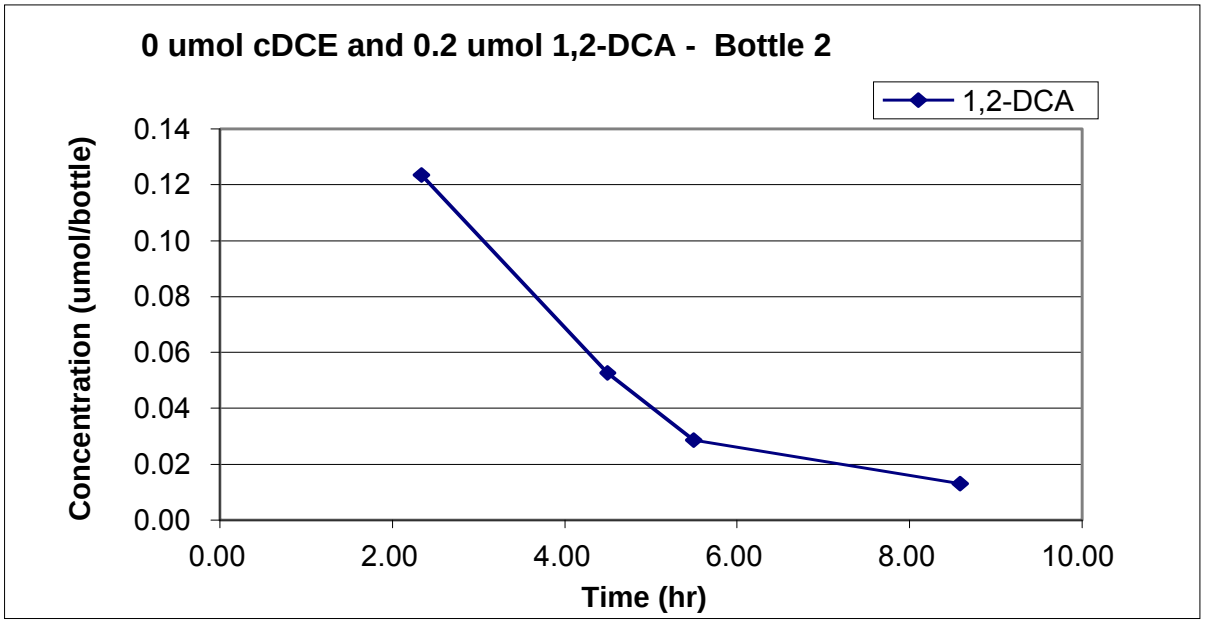


Figure 2.21 Degradation of 1,2-DCA Alone at the Lowest 1,2-DCA Level.

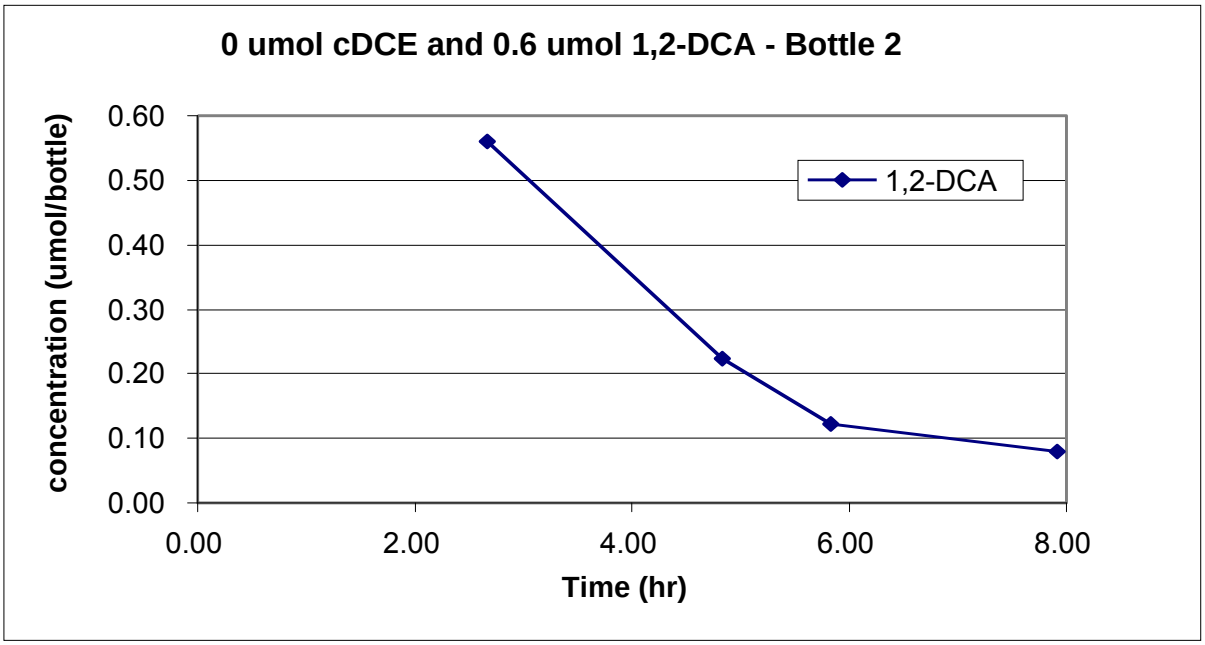


Figure 2.22 Degradation of 1,2-DCA Alone at the Highest 1,2-DCA Level.

In Figure 2.23 below, the results are presented in another way: The maximum rates of cDCE degradation are shown as function of initial, aqueous 1,2-DCA concentration (taking into account partitioning to headspace). These actual, aqueous 1,2-DCA concentrations are given in units of mg/l, since these units likely are more meaningful to practitioners than units of µM. Though it is apparent that the presence of 1,2-DCA at the concentrations employed (up to 0.6 mg/l) resulted in a decrease in cDCE-degradation rate, the effect was far less pronounced (at roughly the same molar concentrations of co-substrate) than was observed in binary mixtures of cDCE/VC or cDCE/TCE (i.e., compare Figures 2.7, 2.15, and 2.23). Again, that's good news, from a bioremediation standpoint.

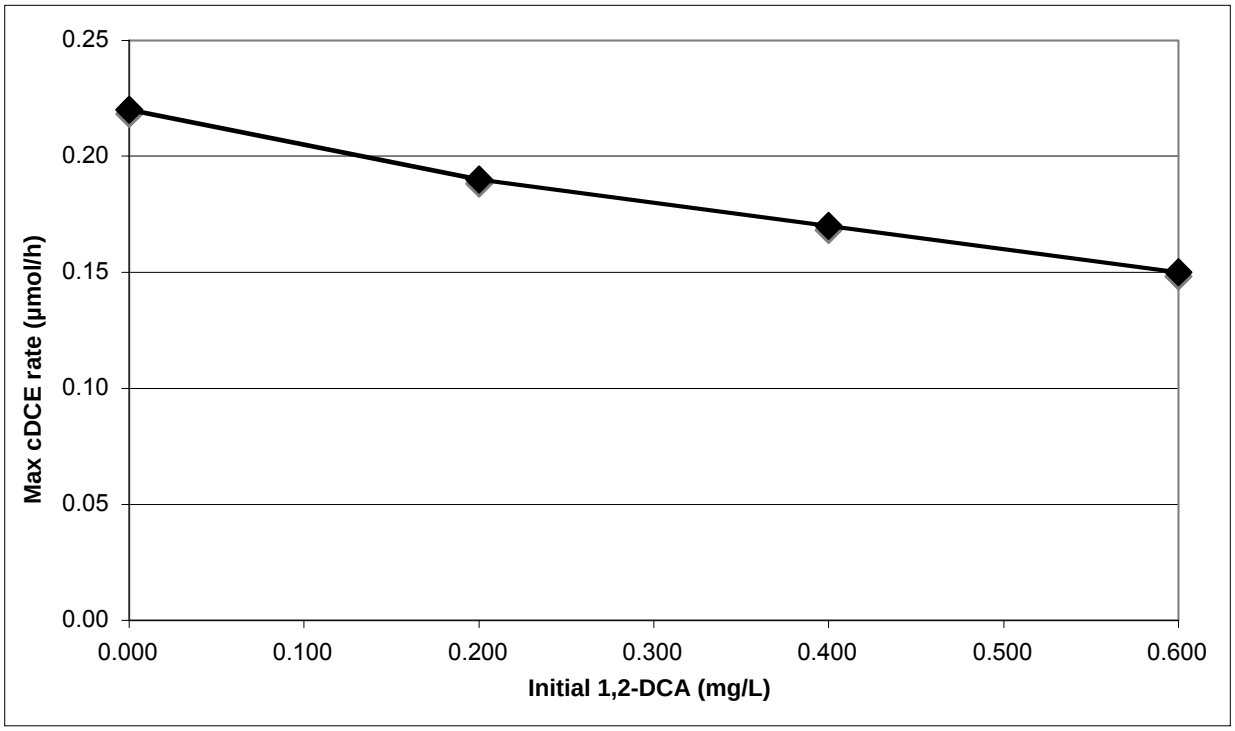


Figure 2.23 Maximum cDCE Degradation Rate as Function of Initial, Aqueous 1,2-DCA Concentration.

In Figure 2.24 below, the maximum rate of 1,2-DCA degradation is shown as function of initial, aqueous 1,2-DCA concentration—with and without presence of cDCE. It is apparent that, over the range of 1,2-DCA concentrations investigated, the 1,2-DCA degradation rate increased with increasing 1,2-DCA concentration—though not in perfect, linear fashion. It also appears that maximum rate of 1,2-DCA degradation was strongly influenced by the presence of cDCE—with rates of 1,2-DCA degradation markedly lower in presence of cDCE. Unlike with co-presence of VC or TCE, the 1,2-DCA degradation data suggest that competition exists between cDCE and 1,2-DCA degradations.

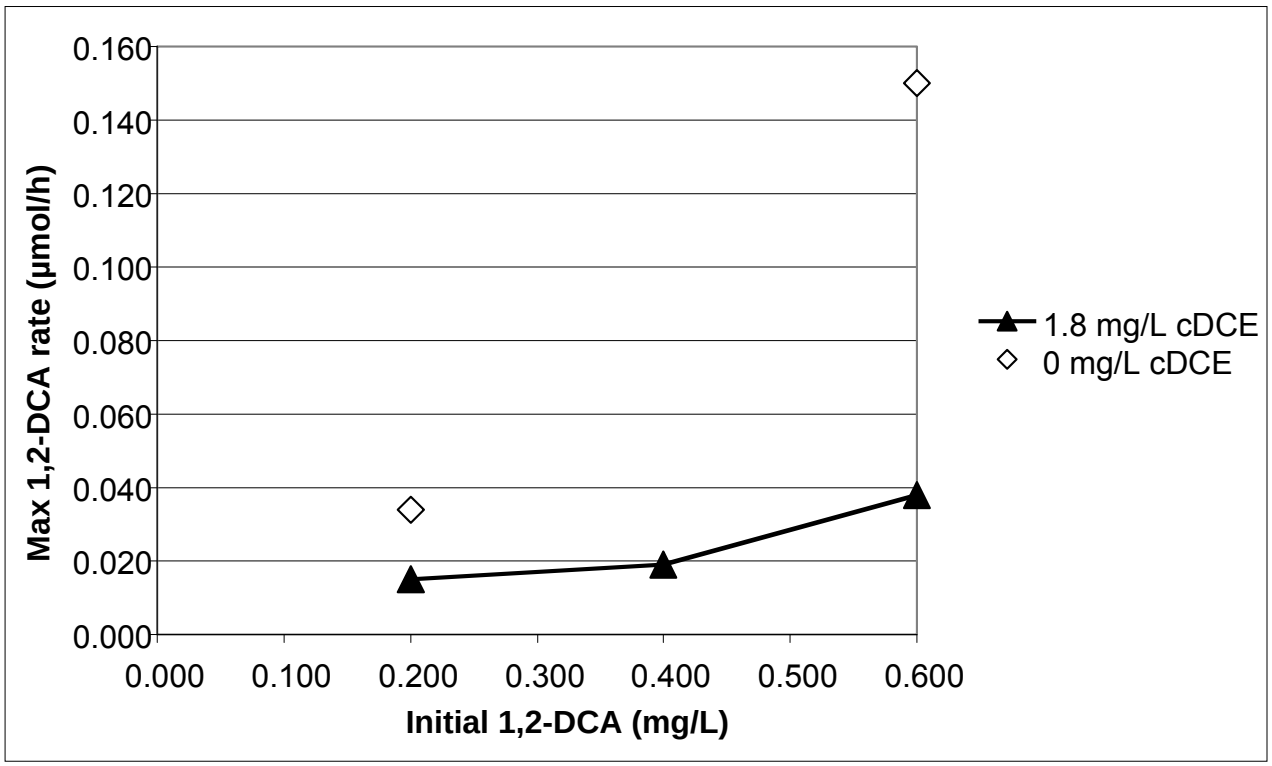


Figure 2.24 Maximum 1,2-DCA Degradation Rate Versus Initial, Aqueous 1,2-DCA Concentration — in Presence or Absence of cDCE.

2.4 Discussion

The good news, so far as bioremediation is concerned, is that although the co-presence of VC, TCE, or 1,2-DCA reduces the maximum degradation rate of cDCE, the rate remains substantial and cDCE can be completely degraded, as can the co-substrates.

The patterns of VC or TCE degradation in presence of cDCE suggest (but alone do not prove) some sort of parallel, co-metabolic degradation systems for cDCE and these co-substrates, with possibly some inhibitory/damaging byproducts of VC or TCE transformation. Co-presence of VC or TCE caused cDCE degradation rates to be halved, but the effect was not proportional to concentrations of VC or TCE. On the other hand, degradation of the co-substrate was either improved (VC) or unaffected (TCE) by the presence of cDCE.

This is in stark contrast to the patterns of 1,2-DCA degradation in presence of cDCE, which evidenced clearer signs of true competition: cDCE degradation was modestly inhibited by 1,2-DCA (but competitive inhibition would be expected to be modest, since molar concentrations of 1,2-DCA were far lower than those of cDCE) in a roughly linear decline with increasing 1,2-DCA concentration; and 1,2-DCA degradation was markedly inhibited by the very-much higher, cDCE concentration.

These results are consistent with our observation that JS666 can grow on 1,2-DCA, but not on VC or TCE. Different pathways are likely at work.

3. SUBTASK 1.3: ASSESS ABILITY TO SUSTAIN INDUCTION OF cDCE-OXIDIZING ENZYMES WHEN USING NONCHLORINATED CO-SUBSTRATES.

3.1 Objective:

The objective of this subtask was to assess the ability of JS666 to sustain induction of cDCE-oxidizing enzymes when using nonchlorinated co-substrates. Ethanol has previously been shown to be a good growth substrate for JS666 (Tim Mattes personal communication), but whether ethanol repressed, stimulated or had no effect on cDCE degradation was unknown.

3.2 Methods:

Ethanol was added to serum bottles in varying ratios to cDCE (1:1, 2:1, and 1:2) on a molar basis. All bottles containing ethanol degraded cDCE more rapidly than control bottles without ethanol and the final optical density of bottles containing ethanol was almost twice that of control bottles.

Succinate, acetate, ethanol and 2-chloroethanol were added to serum bottles at the same carbon concentration as in 40 µmol cDCE. After growth on the alternate substrate, cDCE was added to the bottles, and cDCE disappearance was followed. cDCE-only control bottles took 3 days to degrade the initial cDCE addition and 2 days per later additions. Growth on succinate took 1 day, but the initial cDCE addition required 6 days for degradation to be complete, and subsequent additions required 2 days. Growth on acetate required 2 days, and 5 days for degradation of the first addition of cDCE, and 1-2 days for subsequent additions. Growth on ethanol required 3 days, and 1-2 days for degradation of subsequent additions of cDCE. 2-Chloroethanol cultures did not grow. After 8 days, the average OD_{600} was 0.094, 0.094, 0.106, and 0.159, in the DCE, succinate, acetate, and ethanol fed cultures, respectively.

The ability of JS666 to degrade cDCE in the presence of ethanol was examined in more detail. Cultures were grown on cDCE, then aseptically harvested by centrifugation. The supernatant was discarded and the pellet was suspended in ½-MSB. Equal volumes of the suspension were used to inoculate 160 ml serum bottles containing 50 ml of ½-MSB. The bottles were fed mixtures of cDCE and ethanol as shown in the table below. The four intermediate mixtures (Bottles B-E) were very similar and degraded cDCE at increasing rates during a 45 h (30 h for Bottle E) period at the end of which all the cDCE was exhausted. Bottle A (cDCE only) was similar to the faster bottles, but the lag period before more rapid cDCE consumption was about 12 h longer. cDCE degradation was slowest in Bottle F. At 45 h, all bottles were spiked with 3 µl cDCE and

analyzed 1 and 7 h after the spike. All bottles showed immediate consumption of cDCE, despite some bottles having been without cDCE for up to 2 days (last column Table). The results indicate that moderate amounts of EtOH do not inhibit cDCE degradation, but whether there is a stimulatory effect on a per cell basis cannot be determined from these data. In the aggregate, cDCE degradation was faster with EtOH in the culture. Differences in OD_{600} among the bottles at 45 h were negligible. The most significant result is that short periods of starvation for cDCE in the presence of ethanol do not result in an immediate loss of the enzymes responsible for cDCE catabolism. The result suggests that the organism could tolerate a variable cDCE supply under field conditions.

Table 3.1 Initial Amounts of cDCE and EtOH in 160ml Serum Bottles.

Bottle	µl cDCE	µl EtOH	mmol cDCE	mmol EtOH	h cDCE starvation
A	3	0	40	0	0
B	3	1	40	17	0
C	3	2	40	35	0
D	3	3	40	52	0
E	2	3	27	52	15
F	1	3	14	52	24
G	0	3	0	52	46

Dense suspensions of cells were grown with varying ratios of cDCE:EtOH and monitored frequently. Additional cDCE:EtOH was added whenever the cDCE was consumed. After 4 days, the cells were harvested by centrifugation, and the cell pellet was transferred to fresh serum bottles. After addition of cDCE, the bottles were shaken at room temperature for 1 hour after which the cDCE concentration was measured. The bottles were shaken at room temperature for 2 more hours; then cDCE was measured again.

3.3 Results:

Table 3.2 presents the growth and activity of the various cultures. All the bottles started with the same size inoculum; therefore the final protein content reflects how much biomass accumulated after 4 days growth on the DCE:EtOH mixtures. The 3:2 (v:v) ratio yielded the most biomass and the fastest cDCE consumption per bottle; however, the no ethanol control had the highest specific activity for cDCE degradation.

Table 3.2 Biomass Growth + cDCE Consumption.

Bottle:	A 3:0	B 3:1	C 3:2	D 3:3
µmol DCE T_0	33.13	39.15	38.34	47.42
µmol DCE T_1	24.96	23.82	19.62	36.99
T_1-T_0 (min)	135	131	130	118
µmol/min/bottle	0.06	0.12	0.14	0.09
mg protein/ml	0.58	1.8	3.1	2.6
µmol/min/mg protein	0.10	0.07	0.05	0.03

3.4 Acetonitrile as an Alternate Growth Substrate

Cyclohexanecarboxylic acid (CHCA) was reported to be a good growth substrate for JS666 (Tim Mattes, personal communication). In a preliminary experiment, cells fed CHCA grew more rapidly than cells without CHCA and were able to degrade cDCE following growth on CHCA. The growth experiment was repeated using higher concentrations of CHCA with a more concentrated stock solution of CHCA in MeCN. A smaller cell yield on more CHCA led to an investigation of MeCN, the solvent used to make the stock solution of CHCA, as a growth substrate.

Cultures were grown with cDCE only, MeCN only, MeCN + cDCE, and with no C source. cDCE disappearance was monitored along with OD_{600}. cDCE disappearance was slower when MeCN was present in the culture than when cDCE was the only carbon source. When OD_{600} reached a plateau for the MeCN only culture, cDCE was added to all cultures once daily to bring all cultures to 1.3 mM cDCE. Cultures were grown for 1 week, harvested and transferred to fresh medium. After a second week, cultures were harvested, transferred to fresh medium and the specific activity with cDCE was determined. The specific activity of MeCN + cDCE grown cells was equal to the specific activity of cDCE only cells. MeCN grown cells were half as active (Table 3.3).

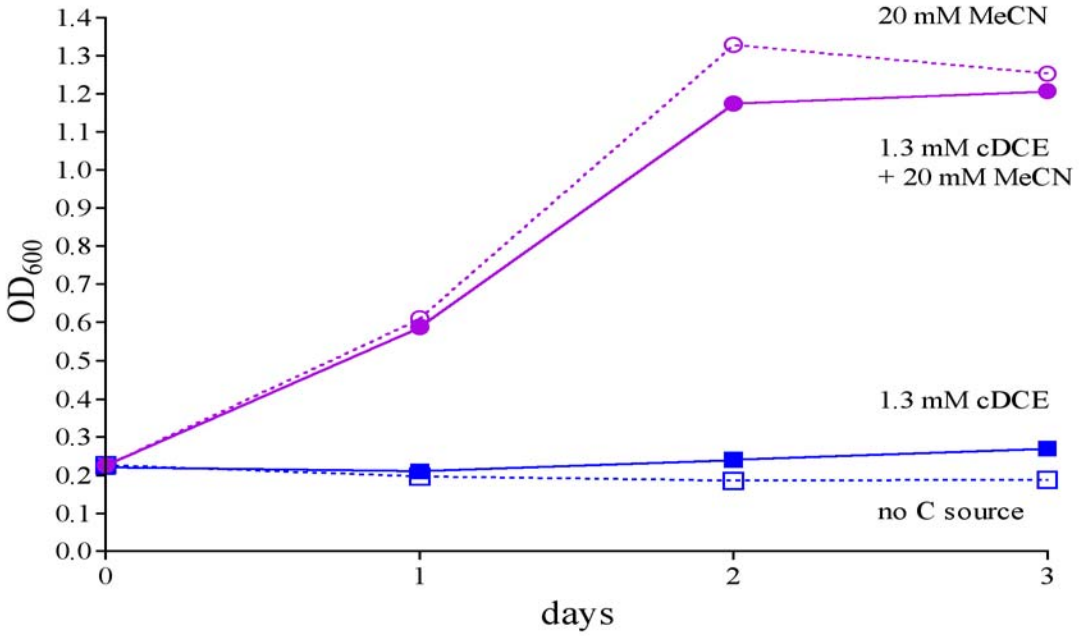


Figure 3.1 Growth of JS666 on cDCE and MeCN.

Table 3.3 Activity of JS666 Grown on Various Substrates.

Culture	cDCE	cDCE+MeCN	MeCN	No C
µmol/min/mg protein	0.0839	0.0817	0.0363	Not detected
µmol/min/bottle	0.1259	0.1716	0.1017	

The result indicates that MeCN can be added as a co-substrate to get a large initial cell mass without long-term loss of the ability to grow on cDCE. Because the ability to grow on MeCN is rarer than the ability to grow on ethanol, it should be easier to maintain purity in cultures grown with MeCN than in cultures grown with ethanol. The next steps are to determine the optimum amount of MeCN to provide as co-substrate for cDCE, and to test the system in a bioreactor.

3.5 cis-1,2-Dichloroethane as a Growth Substrate

The JS666 genome suggests that JS666 has the pathway to degrade DCA. Coleman had previously reported that JS666 did not grow on DCA (Coleman, Mattes et al. 2002), but Coleman tested the ability to grow on DCA at only one concentration, 800 µM (40 µmol/bottle), the standard concentration that he used for growth on DCE. DCA was tested at a series of concentrations from 800 µM to 100 µM. Disappearance was followed by GC. Very slow disappearance was seen at 800 and 400 µM. Disappearance reached a plateau in the 800 µM bottles after approximately a quarter of the DCA was gone. DCA in the 400 µM bottles took 4 days to degrade, but only a single subsequent addition was degraded. After a 1-day lag, DCA was no longer detected in the headspace of the 200 and 100 µM bottles. Seven additions of DCA at 200 µM were made before degradation slowed. Optical density doubled from 0.03 to 0.06 in the 200 µM DCA serum bottles but did not increase in the 800 µM serum bottles. The data indicate that JS666 grows on DCA, but at much lower concentrations of DCA than of DCE. The concentration is also much lower than that reported for a *Pseudomonas* that was isolated on 1 mM DCA (Hage and Hartmans 1999); however, the same authors reported that an attempt to enrich DCA-degrading bacteria from a variety of sources using 1 mM DCA resulted in no isolates and concluded that DCA-degrading bacteria are not widespread. Based on our results, an alternate interpretation might be that DCA concentrations in the mM range are too toxic for most DCA-degrading bacteria. Estimates of the dimensionless Henry's constant for DCA at 22°C is much lower than for DCE (0.034 and 0.147 for DCA and DCE, respectively) (U.S. Environmental Protection Agency 2006). This suggests that DCA is more toxic than DCE at comparable total concentrations because much more DCA is dissolved in the aqueous phase.

4. SUBTASKS 1.4 TO 1.7: SUSTENANCE OF cDCE OXIDATION ACTIVITY IN MIXED AND PURE CULTURE

4.1 Introduction

Subtasks 1.4 through 1.7 (i.e., Subtask 1.4 – Sustenance of cDCE oxidation activity in mixed culture, subsurface environments; Subtask 1.5 – The effect of co-presence of other degradable organics on sustenance of cDCE oxidation activity in mixed-culture, subsurface material; Subtask 1.6 – The effect of other degradable organics on JS666 alone; and Subtask 1.7 – The sustenance of cDCE oxidation activity in a rich, mixed-culture environment containing a wide variety of potential competitors) were investigated in a series of microcosm studies that are best considered together, because common methods were used for all. Additional studies – not part of the statement of work but considered by the investigators to be critically important–are presented concerning the effect of JS666 inoculum level on performance in bioaugmented microcosms constructed from subsurface material from the SRS.

4.2 Materials

4.2.1 Program of Study

The intention of the experiments performed as described here was to assess the survivability of JS666 in subsurface materials and to determine under what conditions this culture could be applied for successful bioaugmentation. Microcosm experiments were performed under "ideal" conditions, and then systematically challenged with inhospitable conditions and other potential barriers. cDCE degradation was monitored, and because this organism would later be used in field tests of bioaugmentation, a molecular probe was applied to track growth or die-off of JS666 within some microcosms, a test of the probe's efficacy. Microcosms were constructed with subsurface materials from five sites: SRS; Hill AFB; Robins AFB; Ft. Lewis; and Aerojet. Additionally, microcosms were constructed using two dilutions of primary sewage effluent: unautoclaved (contributing both complex organic substrates as well as competing and/or predatory microbes) and autoclaved (contributing only complex organics).

4.2.2 Media -- MSM

Carbon-free MSM modified from Hartmans *et al.* (Table) was used to sustain JS666. The trace metals solution (TMS) was added after the MSM was autoclaved and cooled to prevent precipitation. To maintain sterility, TMS was passed through a 0.2-µm filter prior to addition to the MSM. The pH of resulting MSM was approximately 7.1 to 7.2, and the buffering capacity of the media was due to the 20-mM concentration of phosphate.

Table 4.1 Minimal Salts Medium (MSM).

KH_2PO_4	0.95 g/L
K_2HPO_4	2.27 g/L
$(NH_4)2SO_4$	0.67 g/L
TMS[1]	2 ml/L

[1]*Trace Metal Solution (TMS) contained per liter of distilled water: 60 g $MgSO_4 \cdot 7H_2O$, 6.37 g EDTA, 1.0 g $ZnSO_4 \cdot 7H_2O$, 1.0 g $CaCl_2 \cdot 2H_2O$, 1.0 g $FeSO_4 \cdot 7H_2O$, 1.0 g $NaMoO_4 \cdot 2H_2O$, 1.0 g $CoCl_2 \cdot 6H_2O$, and 1.0 g $MnSO_4 \cdot H_2O$. TMS was prepared by dissolving EDTA in water, adjusting the pH to 6.5 with H_2SO_4 then adding the remaining ingredients. TMS was filter-sterilized and stored at 4ºC in the dark. Final pH was ~2-3.*

4.2.3 Media -- ½MSB

In an attempt to increase the buffering capability of the medium, a second type of medium was used in some experiments. A one-to-one dilution of Stanier's Mineral Salts Medium (½ MSB) was used (Table 4.2, Stanier *et al*. 1966). This media was used at half-strength and had 40 mM phosphate, which is double that of MSM.

Table 4.2 Stanier's Mineral Salts Medium (Full Strength).

Na_2HPO_4	5.64 g/L
KH_2PO_4	5.44 g/L
Hutner's Base[1]	20 ml/L
$(NH_4)2SO_4$	1 g/L

[1]*Hutner's Base contained per liter of distilled water: 10 g nitrilotriacetic acid (NTA), 14.45 g $MgSO_4$, 3.33 g $CaCl_2 \cdot 2H_2O$, 0.00925 g $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 0.099 g $FeSO_4 \cdot 7H_2O$, and 50 ml of Metals 44. Hunter's Base was prepared by dissolving NTA in 150 ml of water with 7.5 g of KOH. Each of the other ingredients were dissolved separately and added. The pH of the solution was adjusted to 6.6-6.8 and the volume was adjusted to 1L. Metals 44 contained per liter of distilled water: 2.5 g EDTA, 10.95 g $ZnSO_4 \cdot 7H_2O$, 1.54 g $MnSO_4 \cdot H_2O$, 5 g $FeSO_4 \cdot 7H_2O$, 0.392 g $CuSO_4 \cdot 5H_2O$, 0.248 g $Co(NO_3)_2 \cdot 6H_2O$, 0.177 g $Na_2B_4O_7 \cdot 10H_2O$*

4.2.4 *cis*-1,2-Dichloroethene

Neat cDCE (99%) was purchased from TCI America, Portland, Oregon. The cDCE was filter-sterilized into a 25-ml serum bottle, sealed with a Teflon-coated butyl-rubber septum and crimp-sealed with a tear-off aluminum cap.

4.2.5 Soil and Groundwater Types

The moisture content of each type of soil was found by heating the soil samples and analyzing them gravimetrically. Empty crucibles were baked for one hour at 104ºC to assure complete drying. The crucibles were tare-weighed after they had cooled in a desiccator. Approximately

10- to 15-gram of wet soil samples were added to triplicate crucibles, and they were weighed again before being baked at 104ºC overnight. The samples were allowed to cool overnight in a desiccator before being weighed again. The difference in weight was used to calculate the amount of moisture contained in the soil. In order for microcosms to be uniform from treatment to treatment, dry soil weight as calculated from native material weight was used in their preparation.

The native pH was determined for each of the soils used in preparing the microcosms by mixing 50% (by dry weight) soil with distilled water (dH_20). pH measurements were taken after the slurry had equilibrated, and were made with an Accumet micro-electrode with a calomel reference.

All soil and groundwater samples were shipped on ice to Cornell and stored at 4ºC in the dark for later use. Available characteristics and constituents are listed here.

Savannah River Site Soil and Groundwater

Approximately 10 liters of Savannah River Site (SRS) soil and twenty liters of groundwater were collected on January 18, 2005 in Aiken, South Carolina. The site was selected to be in an aerobic zone where reductive dechlorination had apparently ceased and was stalled at cDCE. Groundwater was taken from well CRP44. The soil samples were taken at a depth of 4-5 feet below ground surface, 8 feet north of well CRP44A. The soil was homogeneous silty sand. The following data were available from the site prior to sampling (Table , Jennings *et al.* 2005).

Table 4.3 Savannah River Site Soil Characteristics and Constituents.

Parameter	Average	Parameter	Average
cis-1,2-DCE, mg/L	0.530	Nitrite, mg/L	<0.1
PCE, mg/L	<0.005	Chloride, mg/L	5.5
TCE, mg/L	<0.005	TOC, mg/L	<5
VC, mg/L	<0.005	Specific Conductance, µS/cm	26
Ethene, mg/L	<0.005	Methane, mg/L	0.140
DO, mg/L	3.7	Temp, ºC	25.1
Sulfate, mg/L	4.3	pH	5.1
Nitrate, mg/L	<0.1	ORP, mV	122

DO – dissolved oxygen, ORP – oxidation-reduction potential, uS/cm - microSeimens per centimeter, mV – millivolts

Table 4.4 SRS Groundwater Parameters – Field-Measured January 18, 2005.

Parameter	Average
Temp, ºC	14.8
pH	4.4
DO, mg/L	0.9
ORP, mV	122

DO – dissolved oxygen; ORP – oxidation-reduction potential

Robins AFB Soil

Subsurface material obtained from Robins AFB was taken from approximately 100 feet west of the BIA8 well. Groundwater characteristics from the time of sampling are available in Table 4.5 (Watling, 2007). There were seven successive cores taken from this location, and contained approximately 700 g or 1400 g each. All samples were taken at a depth of 20-30 feet. The microcosms were prepared using the shallowest sample. The native pH of the soil was found to be 6.40, as measured in our lab, and the material was fine-to-coarse-grained sands with interlayered silts and clays. The soil did not appear to be heterogeneous from core to core.

Table 4.5 Robins Site Characteristics.

Parameter	Average	Parameter	Average
max PCE, mg/L	<0.02	max Fe, mg/L	0.04
max TCE, mg/L	0.46	pH	4.2-5.8
max cis-1,2-DCE, mg/L	0.30	Conductivity, µS/cm	24-63
max VC, mg/L	0.05	ORP, mV	165-335
max Sulfate, mg/L	<5	DO, mg/L	6-11
max Nitrite, mg/L	0.40	Depth to water, ft bgs	6-9
max Nitrate, mg/L	0.40		

ft bgs = feet below ground surface; uS/cm = microSeimens per centimeter

Hill AFB Soil

There was approximately 700 g of material in each of the three cores received from Hill AFB. All samples were obtained near the abandoned monitoring well U1-175, taken at 80-82 ft bgs, and two from 85-87 ft bgs. Microcosms were prepared from one of the cores taken at 85-87 ft bgs. This soil consisted of sand and gravelly sand with silts and had a pH of 8.47, as measured in our lab. The other cores consisted of large rocks and hard clay, and thus were unusable. The characteristics and constituents of the groundwater as measured at extraction are available in Table 4.6 (Watling, 2007).

Table 4.6 Hill AFB Groundwater Characteristics.

Parameter	Average	Parameter	Average
max PCE, mg/L	--	max Fe, mg/L	1.94
max TCE, mg/L	--	pH	6.6-7.6
max cis-1,2-DCE, mg/L	0.086	Conductivity, µS/cm	1132
max VC, mg/L	--	ORP, mV	64.5-335
max Sulfate, mg/L	42.6	DO, mg/L	3.6-10.5
max Nitrite, mg/L	0.2	Depth to water, ft bgs	81
max Nitrate, mg/L	103.9		

Aerojet Soil

The samples of Aerojet subsurface material were taken from well #3651 at a depth of 113 ft bgs. They were fine- to coarse-grained sands, with interlayered gravel, silts, and clays. The data in Table 4.7 were available from groundwater sampling (Watling, 2007).

Table 4.7 Aerojet Site Groundwater Characteristics.

Parameter	Average	Parameter	Average
max PCE, mg/L	<0.002	max Fe, mg/L	0.20
max TCE, mg/L	0.41	pH	6.6
max cis-1,2-DCE, mg/L	0.0035	Conductivity, µS/cm	325
max VC, mg/L	ND	ORP, mV	63.0
max Sulfate, mg/L	3.4	DO, mg/L	2.01
max Nitrite, mg/L	ND	Depth to water, ft bgs	92
max Nitrate, mg/L	11.0		

Ft. Lewis Groundwater

Approximately 16 liters of groundwater was received from North Wind, Inc., courtesy of Tamzen Macbeth. The samples were taken at Fort Lewis, Washington. The groundwater had a native pH of 6.90 and exhibited little to no buffering capacity.

IAWTP Primary Effluent

Primary effluent was collected from the Ithaca Area Wastewater Treatment Plant on October 12th, 2006. It was stored at 4ºC in the dark for later use.

Culturing Techniques

The initial culturing of JS666 revealed that when this bacterium was grown with cDCE as a sole carbon source, there was a long lag time before degradation begun. Additionally, when JS666 was grown on cDCE alone, culture densities remained low. The level of cDCE required to maintain dense cultures was toxic to the organism. Because of these two conditions, the culturing technique described below was developed to facilitate high growth and to shorten lag time. This technique involved growing the culture initially with a co-substrate. However, despite success growing the culture to high density and having a small lag time when switched to cDCE alone, there were unforeseen consequences. This technique yielded a culture that was unable to maintain growth on cDCE alone, and therefore a second technique was developed. Early experiments were conducted with cultures grown via the first technique, but later experiments employed the second technique.

Cultures Exhibiting Cometabolic-Like Behavior

Because JS666 is a slow-growing organism, to obtain enough biomass for experiments it was grown using 10 mM of succinic acid in addition to 40 µl of cDCE. These cultures were inoculated with frozen stocks of JS666. These stocks were prepared by suspending concentrated culture in MSM and 15% glycerol as a cryo-protectant and flash-freezing in liquid nitrogen. Each 1-ml frozen pellet was thawed on ice and resuspended in 1 liter of MSM with 40 µl of neat cDCE, and 10 mM of succinic acid (disodium salt, ACROS). This culture was grown in a custom-made 2-liter glass bottle (1 liter of headspace) with a standard, 20-mm serum-bottle top, sealed with Teflon-coated butyl rubber septum, and crimp-sealed with a tear-off aluminum cap. The cDCE was delivered through the flame-sterilized septum with a syringe (VICI). The culture was amended with above-ambient oxygen, which was passed through a sterile syringe packed with glass wool and delivered through a sterile, 25-gauge needle. For large cultures, oxygen was delivered as a 30-second burst at 10-psig, as large amounts of oxygen were consumed while grown with succinic acid. When almost all of the cDCE delivered was degraded, the cultures were given another 40-µl spike. After approximately half of the second spike of cDCE was consumed, the culture was washed twice. This involved spinning down the culture for 10 minutes at 7000 RPM in a cooled centrifuge. Then, the supernatant was decanted under a hood and the pelleted culture resuspended in approximately 30 ml of MSM. Next, the culture was then spun down again at 10,000 RPM for 4 minutes and the supernatant decanted. Finally, the culture was resuspended and used to inoculate experimental culture bottles (Figure 4.1).

To determine the amount of MSM required to resuspend the pelleted culture to the correct concentration (V_c, concentrate volume), Equation 4.1 was used. The experiments were carried out in 160-ml glass serum bottles, which contained 100 ml of MSM and 4 µl of neat cDCE as a sole carbon source, and at a biomass concentration of an optical density at 600 nm (OD_{600}, see enumeration techniques, of approximately 0.3. These smaller cultures were also amended with excess oxygen delivered as a 5-second burst at 10 psig for each culture.

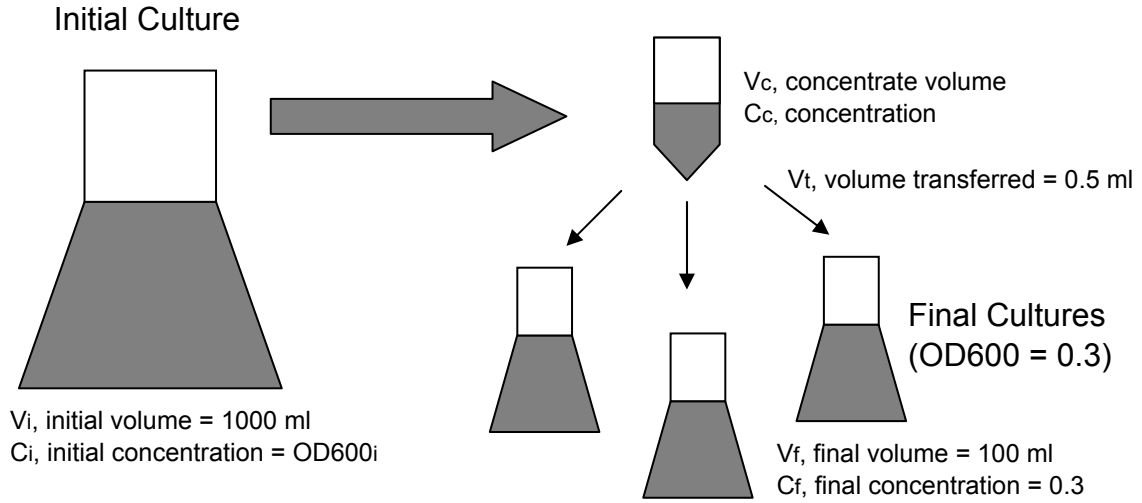


Figure 4.1 Obtaining the Correct OD600.

$$V_C = \frac{C_i V_i}{C_f V_f}(V_t)$$ **Equation 4.1 to determine the volume of concentrate, *Vc***

All cultures were stored at 22ºC in the dark on an orbital shaker at 160 RPM. The 160-ml serum bottles were inverted to help prevent volatilization through the stopper.

5% Transfer Cultures

Transfer cultures were established as a way to maintain the culture with cDCE as the sole carbon source. This was accomplished by inoculating one liter of MSM with one ml of frozen stock, 40 µl of cDCE, and without succinic acid or any other co-substrate. After approximately one and a half spikes of cDCE were degraded, 5 ml of culture was transferred into approximately 95 ml of fresh MSM with 4 µl of cDCE in a 160-ml glass serum bottle sealed with a Teflon-coated butyl-rubber stopper and aluminum crimp-cap. These cultures were monitored and re-fed before cDCE had been completely depleted. After 2-3 spikes of cDCE were degraded, 5 ml of this culture was transferred using a disposable, sterile, gas-tight syringe and disposable sterile needle into 95 ml of fresh, sterile MSM. This technique assured that the cDCE was never exposed to an alternative carbon source. Cultures were stored inverted at 22ºC in the dark on an orbital shaker at 160 RPM. All cultures were grown with ambient levels of oxygen.

Verification of Purity

To maintain a pure JS666 culture, aseptic techniques were utilized. As an assurance that these techniques were properly executed, purity was verified by visual identification. After any handling that could have potentially contaminated the culture, before any inoculation, or after

any 5% transfer, a small amount of culture was streak-plated on one-quarter strength trypticase soy nutrient agar as a non-selective medium. Colony morphology was confirmed once by microscopy and restriction fragment length polymorphism. JS666 forms tight, yellowish-white colonies that are slow growing. Any deviation from this morphology or growth pattern suggested contamination. Contamination was seen on two occasions and was easily recognized by its variant morphology. It was filamentous with black spores on the first occasion and dark orange with darker spots on the second. In each case, the culture line was discarded.

4.3 Methods

4.3.1 Microcosm Setup

The microcosm studies were designed to address possible hurdles in the survival of JS666 in a variety of subsurface materials. These included abiotic factors in the soil, presence of indigenous microorganisms, micronutrient and metals requirements, alternate co-substrates, buffering capacity of soil or groundwater, inoculation level, and level of cDCE administered. Abiotic factors of the soil were addressed by creating microcosms with different levels of soil (i.e., 5, 15, or 50 grams (equivalent dry weight) soil). For each treatment, media or dH_20 was added to yield a total of 50 ml of liquid, accounting for the moisture content in each type of soil. Groundwater or soil that was not amended with media demonstrated the material's native ability to buffer against HCl production as cDCE was degraded and also its ability to provide necessary trace metals and micronutrients. Biotic factors, such as predation and competition from indigenous microbiota, were addressed by using autoclaved soil to prepare some microcosms. To autoclave the soil, each serum bottle of soil was autoclaved for approximately three hours at 120ºC and above-ambient pressure. After two days, the bottles were autoclaved for a second time to kill or stall sporulating microorganisms. Additionally, primary effluent was used to amend some microcosms, representing a rather severe condition of possible competition and/or predation.
Alternative co-substrates that were administered in some microcosms included succinic acid, ethanol, or autoclaved primary effluent. All microcosms were prepared aseptically under a laminar-flow hood, with an autoclaved spatula and funnel. Autoclaved media or dH_20 water was then added aseptically and 2.3 µl of cDCE was delivered via syringe through flamed septa. Each experiment was carried out at 22ºC in the dark.

Ten microcosm experiments were carried out to address the above questions. These were as follows:

- 50 grams dry weight SRS soil amended with either MSM or SRS groundwater, at elevated oxygen levels, inoculated with transfer culture at two different levels, agitated at 160 RPM on an orbital shaker.

- 5, 15, or 50 grams dry weight SRS soil, autoclaved or raw, amended with ½ MSB, inoculated with frozen stock culture with ethanol, agitated at 60 RPM on an orbital shaker.
- 5, 15, or 50 grams dry weight SRS soil, autoclaved or raw, amended with ½ MSB, inoculated with transfer culture, agitated at 60 RPM on an orbital shaker.

- 5, 15, or 50 grams dry weight Robins soil, autoclaved or raw, amended with ½ MSB, inoculated with transfer culture, agitated at 60 RPM on an orbital shaker.

- 5, 15, or 50 grams dry weight Hill soil, autoclaved or raw, amended with ½ MSB, inoculated with transfer culture, agitated at 60 RPM on an orbital shaker.

- 50 ml of Ft. Lewis groundwater or 45 ml amended with 5 ml of 10X MSM, inoculated with transfer culture, agitated at 60 RPM on an orbital shaker.

- 50 grams dry weight Aerojet soil amended with MSM or dH_20 water, inoculated with transfer culture, agitated at 60 RPM on an orbital shaker.

- 50 grams dry weight SRS soil amended with MSM, inoculated at 1X, 0.1X and 0.01X previous levels with transfer cultures, spiked with 1C, 0.1C and 0.01C previous cDCE levels, agitated at 60 RPM on an orbital shaker.

- 50 ml of MSM amended with either 1% or 10 % autoclaved or raw primary effluent, inoculated at 1X, 0.1X previous levels with transfer cultures, agitated at 60 RPM on an orbital shaker.

50 grams dry weight SRS soil amended with MSM and either 1% or 10% raw primary effluent, inoculated at different levels, inoculated at 1X, 0.1X previous levels with transfer cultures, agitated at 60 RPM on an orbital shaker.

4.3.2 Analytical Methods

<u>Analyte Measurements – Gas Chromatography</u>

A Perkin-Elmer Autosystem model GC was used to measure cDCE, oxygen, and carbon dioxide. The GC was equipped with both a flame-ionization detector (FID) and a thermal-conductivity detector (TCD). Samples were separated by an 8-ft by 1/8-in stainless-steel column packed with 1% SP-1000 on 60/80 Carbopack B (Supelco) and carried through the column by high-purity nitrogen gas (Airgas) at 30 ml/min. The fuel to the FID was a mixture of medical grade air (Airgas) and hydrogen (Airgas) at 450 and 45 ml/min, respectively. The TCD received nitrogen as both the carrier gas and the reference gas at 30 ml/min. Samples of 100 µl were taken aseptically from culture headspace with a gas-tight syringe and sterile needle (Vici). Initially, the sample was sent to the TCD where carbon dioxide and oxygen were measured. After 0.76

minutes, a switching valve then directed the flow to the FID to measure the cDCE, which eluted after 5.8 minutes. The column was kept at 90ºC for 2.5 minutes before the temperature was raised to 145ºC at a rate of 30ºC/min. The temperature was held at 145ºC for an additional 2.17 minutes. The measurements were analyzed by Turbochrome software (Perkin Elmer), which was also used to program and run the method described here. To determine the quantity of cDCE that corresponded to peak areas, standards were made with known amounts of cDCE put into bottles with the same headspace and liquid volumes as the unknown samples. Standards on pure oxygen and pure carbon dioxide were also created. This was done by purging 160-ml glass serum bottles sealed with a butyl-rubber stopper and aluminum crimp-cap with nitrogen gas before adding a known volume of either oxygen or carbon dioxide with a gas-tight syringe.

Analyte Measurements – Ion Chromatography

Ion chromatography was employed to measure succinic acid and chloride ions. These were measured on a Dionex system that consisted of a CD25 Conductivity Detector, an Autoselect AS50 Autosampler and Chromatography Compartment, and a GS50 Gradient pump. The entire system was controlled remotely by PeakNet 6 software. Samples of 0.3-ml were extracted from cultures using a sterile disposable 1-ml gas-tight syringe, and passed through a 0.2-µm Teflon-coated filter. These were loaded into 2-ml glass sample vials that were then placed in the autosampler tray. The tray remained stationary while the sampling needle arm moved to each vial and extracted 0.25 µl of sample. The sample was carried through an Ionpac AS14A 4-mm x 250-mm analytical column by a step-gradient eluent of NaOH in distilled-deionized water (DDI). The initial concentration of NaOH eluent was 7.5 mM. This was held constant for 3.5 minutes, after which it was stepped up over 1.5 minutes to 40 mM and held there for the remaining 3 minutes. Eluent was purged with nitrogen gas for 30 minutes prior to use, and was stored under pressure of helium gas to maintain oxygen-free solutions. This was important, as any oxygen trapped in the column would lead to erroneous results. Chloride ions eluted at 2.02 minutes, while succinic acid came out at 2.90 minutes. The sampling needle was flushed with DDI water between samples. The GS50 gradient pump controlled the step-gradient eluent, and the CD25 conductivity detector measured and quantified the ionic analytes. PeakNet 6 software analyzed the measurements. The system was also equipped with an Anion Self-Regenerating Suppressor (ASRS-ULTRA), which suppressed eluent conductivity and increased sensitivity of the measurements.

Enumeration Techniques

OD_{600}

Optical density of cultures at 600 nm (OD_{600}) was measured with an Eppendorf Biophotometer. The biomass concentration of more dense cultures could be estimated from this technique. These included mature transfer cultures and cultures grown to high density with a co-substrate.

A 1-ml aliquot of culture was aseptically sampled and put into a standard plastic cuvette and placed in the Biophotometer to be analyzed.

Fluorometry with Picogreen Reagent

DNA was extracted from pure cultures or from microcosms using a MoBio UltraClean Microbial DNA or UltraClean Soil DNA Kit, respectively. DNA extractions were stored at -20ºC until later use. The total concentration of DNA in each sample was quantified with fluorometry using the PicoGreen reagent that binds double-stranded DNA. A Fluoroskan Ascent spectrophotometer measured fluorescence of Picogreen bonded to double-stranded DNA at an excitation wavelength of 485 nm and emission wavelength of 538 nm. A standard curve of known lambda DNA concentrations allowed unknown sample concentrations to be interpolated. The standards were prepared from stock lambda DNA and diluted to 1, 0.1, 0.01, and 0.001 ng DNA/µl. An appropriate amount of Picogreen reagent was diluted 1:200 in TE buffer (10 mM Tris-HCl and 1 mM EDTA at pH 8), and a100-µl aliquot of the diluted reagent was added to each well of a 96-well, black polystyrene plate (Corning). Another 100 µl of sample or standard was added to each well. Samples were prepared by diluting the unknown samples 1:50 in TE buffer. The plate was shaken at 1200 RPM, and then incubated for five minutes before fluorescence was measured.

QPCR

The fluorometrically measured concentration of DNA extracted from pure JS666 cultures was assumed to be entirely comprised of JS666 DNA. From this, JS666 standards were created for real-time quantitative polymerase chain reaction (QPCR). Measured concentration of DNA was converted to copies of target gene per microliter of sample by using the following equations (Equation 4.1, Equation 4.2),

$$MW\left(\frac{g}{mol}\right) = (bp) \times 660\left(\frac{daltons}{bp}\right)$$ **Equation 4.1. Estimation of Average Molecular Weight of Double-Stranded DNA**

Where the size of the JS666 genome is 5.9 Mb as reported by the Joint Genome Institute Microbial Sequencing Program,

$$\left(\frac{copies}{\mu l}\right) = \frac{6.02 \times 10^{23}\left(\frac{copies}{mol}\right) \times C_{DNA}\left(\frac{g}{\mu l}\right)}{MW\left(\frac{g}{mol}\right)} \text{f}$$

Equation 4.2. Conversion of Concentration of Double-Stranded DNA to Copy Number

and there is one copy of the target (isocitrate lyase) gene per JS666 organism. The JS666 standards were aliquoted for single use to avoid degradation caused by repetitive freeze/thaw and stored at -20ºC for later use.

The JS666-specific primers for QPCR were developed by Laura Jennings (2005) and have been demonstrated by conventional PCR to be specific. They target the isocitrate lyase gene, which is a functional gene in the glyoxylate cycle and produce an amplicon of 139 base pairs (Table 4.8). Primers were created by IDT in Portland, Oregon and freeze-dried for shipment. They were reconstituted upon arrival and diluted to a 60-µmolar concentration.

Table 4.8 JS666 Specific Primers for Quantitative Real-Time PCR.

Target Gene	**Sense Primer**
isocitrate lyase	AceA 276F 5'-TGCCGCTGACAACAACAC-3'
	Anti-Sense Primer
isocitrate lyase	AceA 414R 5'-ATCAATGCCTTTGGAGTGC-3'

The copy number of the isocitrate lyase gene and therefore the amount of JS666, was quantified using a real-time PCR thermocycler (iCycler Detection System, BIO RAD) and the intercalation agent SYBR Green (BIO RAD). The reaction was set up by creating a master mix of reagents according to the recipe in Table 4.9. The master mix was put into aliquots that were large enough for triplicates of each reaction. DNA template was added to the aliquots to increase reproducibility by reducing pipetting errors. The aliquots with DNA template were loaded onto a 96-well reaction plate (BIO RAD), sealed with optical tape (BIO RAD), and centrifuged before being placed in the iCycler.

Table 4.9 Real-Time PCR Recipe.

Component	**Volume per Reaction (µl)**	**Final Concentration**
SYBR Green[1] (2X)	13.3	1X
Primer 1 – AceA 276F (15 µM)	1.25	0.75 µM
Primer 2 – AceA 414R (15 µM)	1.25	0.75 µM
Molecular Grade Water	6.38	-
DNA Template	6.2	-
Total Volume	**25 µl**	

[1]Contains 100 mM KCl, 40 mM Tris-HCl (pH 8.4), 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), iTaq DNA Polymerase (50 units/ml), 6 mM $MgCl_2$, SYBR Green 1, 20 nM fluorescein, and stabilizers.

To initiate the reaction, the iCycler was heated to 50ºC for two minutes before the temperature was ramped to 95ºC for three minutes. The iCycler was then programmed to run 40 cycles of 15 seconds at 95ºC to denature the DNA followed by 1 minute at 63ºC to allow for annealing and extension. Fluorescence was measured after every cycle. A melt curve was completed following the amplification reactions to confirm the specificity of the primers and the reactions. The melt curve followed as the PCR products were denatured by tracking their fluorescent emission while the temperature in the iCycler was ramped by 0.5ºC every 10 seconds. As the PCR products were denatured, there was a sharp decrease in fluorescent emission that marked their melting temperature. Primer dimers would have formed much shorter amplicons, and therefore would melt at a lower temperature then product. As such, they are easily distinguished from actual product. Additionally, had a second product formed, it would have been distinguished by a different melting temperature.

QPCR Applied to Soil Systems

Soil extractions of DNA also contain large amounts of humic acids, which are inhibitory to the PCR reaction and vary with soil type. To overcome this inhibition, DNA extracts from soil are diluted. To determine what dilution-fold was most effective for the PCR reaction, the following procedure was performed for each soil. A known amount of JS666 was used to inoculate a soil sample. Next, a DNA extraction was performed. This DNA was diluted 10-, 20-, 50-, 100-, and 200-fold and the copy number was measured using QPCR. These were compared against the expected amount of DNA as determined by a liquid extraction performed on the same inoculum.

Conventional PCR

Conventional PCR was used to test the specificity of the JS666 primers. Reactions were carried out to amplify DNA from pure JS666 cultures, *Escherichia coli*, and primary effluent. Both universal primers (Table 4.10, Hays 2002) and JS666-specific primers (Table 4.8) were applied. Additionally, negative controls were run. The reaction was carried out in an Eppendorf Mastercyler Gradient thermocycler. The reaction mix was prepared as in Table 4.11. Fifty microliters of reaction mix with the appropriate primers and DNA template was put into 0.2 ml PCR tubes (Fisher). The thermocycler was warmed to 94ºC prior to starting the reaction cycles. The cycles started at 94ºC for 3.5 minutes. Next, the reaction was cooled to 55ºC and held for 30 seconds. Finally, the thermocycler was warmed to 72ºC for one minute. These temperatures and times allowed for denaturation, annealing, and extension, respectively. This protocol was repeated for 30 cycles and then held at 4ºC.

Next, get electrophoresis was performed to separate the PCR product, using a 1% agarose gel, made by mixing molecular-grade agarose with Tris-Borate-EDTA buffer (TBE). Because GoTaq buffer contains loading dye, 5 µl of PCR product was loaded directly onto the gel. Four microliters of 1 kilo-base-pair, and 25-sbase-pair ladders were also loaded to size the universal primer and JS666 specific primer products, respectively. These were loaded with 6X loading

dye (15% ficoll, 0.125% bromophenol blue, and 0.125% xylene cyanol). The gel was run at 50 V for approximately 1 hour. After this time, the gel was stained with ethidium bromide and visualized with ultra-violet radiation. Gel electrophoresis was also used to resolve QPCR product when melt curves were not sufficient to demonstrate primer specificity.

Table 4.10 Universal Primer Sequence.

Target Gene	Sense Primer	
Universal 16S rRNA	8F	5'-AGAGTTTGATCCTGGCTCAG-3'
	Anti-Sense Primer	
Universal 16S rRNA	1492R	5'-GGTTACCTTGTTACGACTT-3'

Table 4.11 Conventional PCR Recipe.

Component	Volume per Reaction (µl)	Final Concentration
5X Green GoTaq Buffer	10	1X
$MgCl_2$	5	5mM
dNTPs	1	0.2mM/dNTP
Primer 1 (15 µM)	0.83	0.25 µM
Primer 2 (15 µM)	0.83	0.25 µM
Molecular Grade Water	31.1	-
Taq Polymerase	0.25	1.25 Units
DNA Template	1	<0.5 ug
Total Volume	**50 µl**	

4.4 Results and Discussion

Microcosm studies were conducted to ascertain whether or not JS666 is a viable candidate for bioaugmentation. Unless this organism can survive and degrade cDCE in subsurface materials, it cannot be considered for use in bioaugmentation. The initial microcosm study was conducted in SRS soil with either SRS groundwater or MSM. The microcosms that contained only subsurface material from SRS were as similar to actual conditions as could be replicated in bench-top studies with the exception that a higher water-to-soil ratio was employed than the in situ condition. The results were less than favorable and are described below. To determine what conditions were inhibiting degradation or survivability, subsequent microcosm studies were conducted in what could be considered ideal treatments, and then successively challenged. Ten studies were conducted in total, and six different subsurface materials were used.

4.4.1 Initial Microcosm Study

The initial microcosm study was conducted to determine the survivability of JS666 in a controlled soil system as a surrogate for field experiments. It was conducted using 50 grams (dry weight) SRS soil amended with either MSM or SRS groundwater. These bottles had above ambient oxygen levels and were agitated at 160 RPM on an orbital shaker. They were inoculated with transfer culture at either 4% or 8% v/v, which were approximately 5×10^{7} to 1×10^{8} cells/ml as calculated by Live/Dead microscopy cell counts. Degradation was observed in all no-soil control bottles (i.e., JS666 inoculated into MSM-only), and uninoculated soil-soils showed no degradation (Figure 4.2). JS666 was unable to degrade cDCE in an unbuffered soil system with groundwater, possibly due to the prohibitively low pH of approximately 5.0. Partial degradation was observed in the soil microcosms amended with MSM and inoculated with 4% transfer (Figure 4.3). Additionally, one bottle of SRS soil and MSM inoculated with 8% transfer showed complete degradation, but this was not observed in its duplicate (Figure 4.4).

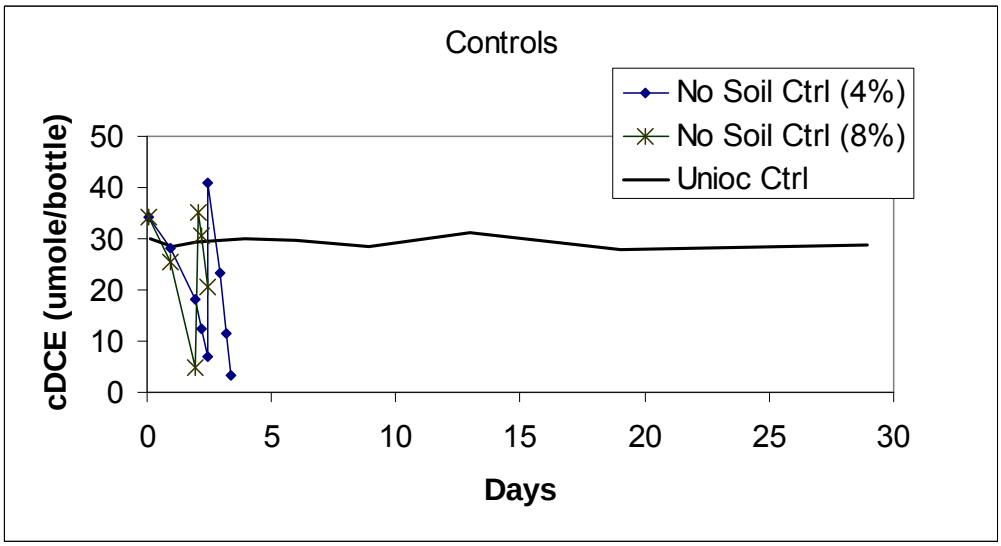


Figure 4.2 No-Soil (MSM) Controls at Two Inoculum Levels (4% and 8% v/v) and uninoculated-soil SRS soil controls in MSM. Single Replicates are Shown for Clarity, but Duplicate Bottles Behaved Similarly.

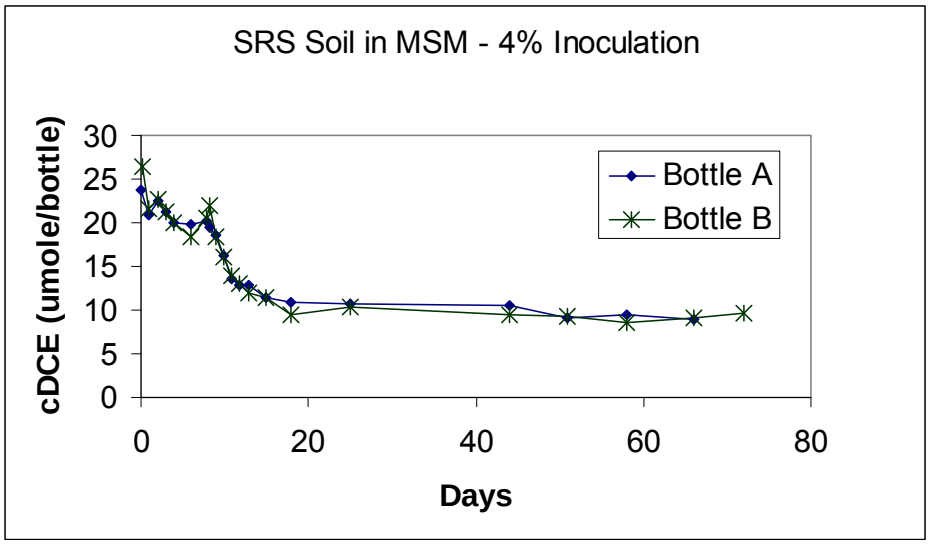


Figure 4.3 SRS Soil in MSM Inoculated With 4% Transfer Culture.

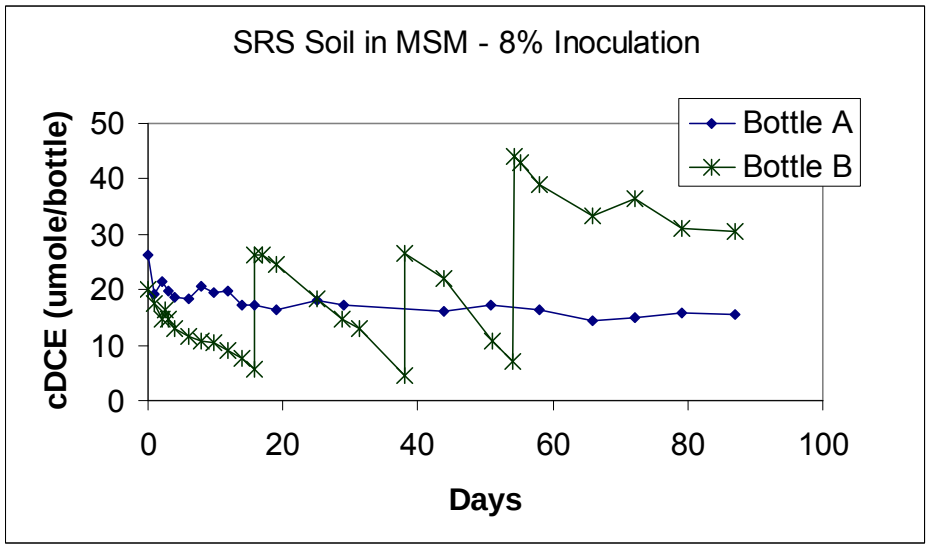


Figure 4.4 SRS Soil in MSM Inoculated With 8% Transfer Culture.

Why complete degradation was not observed in the replicate was not then understood. Any degradation in the bottles amended with MSM was presumably aided by buffering and/or micronutrient supplementation from the medium. Additionally, the inoculum level employed in this study was approximately four orders of magnitude higher then what has been demonstrated to be effective in soil systems (Ramadan *et al.* 1990). However, it was not above what has been observed as effectual in our planktonic cultures. Nevertheless, dense biomass concentration has been shown to correlate with rapid loss of degradation ability (Jennings 2005), so this could have also been a factor here.

The results from the supposed replicates of the 8% transfer bottles are indeed curious (Figure 4.4). For added insight, the molecular probe based on the isocitrate lyase gene of JS666 was applied to DNA extracted from these bottles. The copy numbers of JS666 within these microcosms were found through quantitative real-time PCR (qPCR) and are presented in Figure 4.5 It is apparent that the number of surviving JS666 cells in bottle A was two orders of magnitude lower than the inoculum level, while bottle B showed less than a one-order-of-magnitude drop in JS666 level. Both DNA extractions were diluted 1:50 and also 1:100 before being applied to the qPCR assay. This was to assure that no matrix effects were interfering with the PCR reaction. The results were virtually identical if we corrected for dilution, demonstrating that potential PCR inhibitors were sufficiently diluted out (data not shown). Assumptions have to be made about how the final JS666 levels came about, as we have no data on their temporal variations. It is possible that each bottle suffered a massive die-off initially, and only one was able to recover. Alternatively, perhaps both bottles experienced a slow, prolonged die-off, but this was more pronounced in one bottle than in its replicate. Regardless if these or other explanations describe what transpired, there was still a strong correlation between presence of JS666 and degradation of cDCE, which appears to validate the utility of the molecular probe. In order to investigate speculations as to why this microcosm study failed, further studies were conducted.

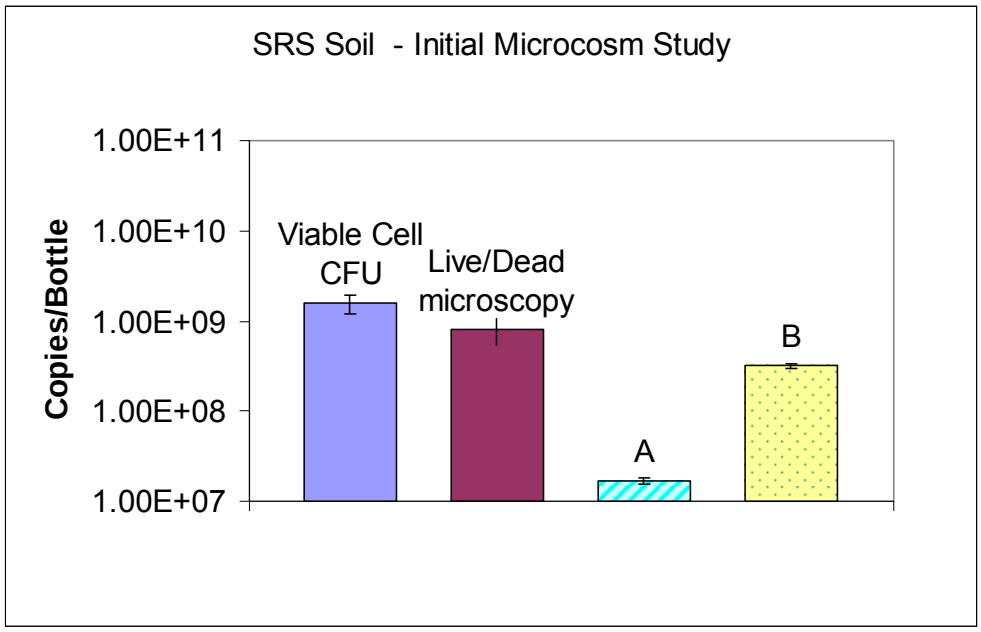


Figure 4.5 Quantification of JS666 Levels in Bottles A and B at the Conclusion of the Study (ca. 90 days)**, compared with initial, inoculated levels. Both A and B were run at a 1:50 dilution. The first two bars depict inoculated levels in these two bottles, based on assays of the inoculum source culture via plate counts** (CFU/bottle) **and live-dead, direct microscopic counts** (cells/bottle)**.**

4.4.2 SRS Soil with Ethanol and Cometabolic-like JS666 Microcosms

Given the lack of reliable success in the previous study, the next microcosm study was intended to ascertain whether JS666 could serve as an effective bioaugmentation agent in presence of ethanol as cosubstrate. It was inoculated with a target concentration of $4x10^5$ cells JS666 per ml of liquid medium in the microcosm, which was about three orders of magnitude less then in the previous, unsuccessful study. Unless stated otherwise, all microcosms from this point forward used this same level of inoculation. The particular inoculum for this microcosm study came from a culture that was grown on ethanol and was exhibiting cometabolic-like behavior with respect to cDCE degradation. Therefore, these microcosms were amended with 10 mM ethanol, in addition to the usual 30.5 µmoles of cDCE. Each microcosm was amended with ½ MSB as the culture medium and was agitated at 100 RPM on an orbital shaker. Since the previous microcosm study was not reliably successful, this one was prepared in a manner to allow investigation of whether abiotic or biotic factors in SRS soil were problematic. If factors in the SRS soil were problematic, then one might expect that the amount of SRS soil used to construct a microcosm would matter, and so they were constructed at differing soil levels of 5, 15 and 50 grams dry weight. Abiotic factors would include presence of inhibitory conditions; biotic factors would include presence of predators or competing microbes. To separate biotic from abiotic factors, microcosms were prepared with autoclaved soil in different amounts. Biotic factors would also include competition for the supplied ethanol, a readily available carbon source.

There was no degradation observed in the inoculated control bottles containing ½ MSB in absence of soil (Figure 4.6). However, there was degradation seen in microcosms prepared with autoclaved soil (Figure 4.8). There was slow, but partial degradation in raw soil inoculated with JS666 and amended with MSM and ethanol, and no degradation was observed in uninoculated-soil controls (Figure 4.7). Since microcosms constructed from autoclaved soil performed better than did those constructed from raw soil, it is therefore possible that biotic factors in SRS soil could be controlling the success of JS666 as a bioaugmentation agent.

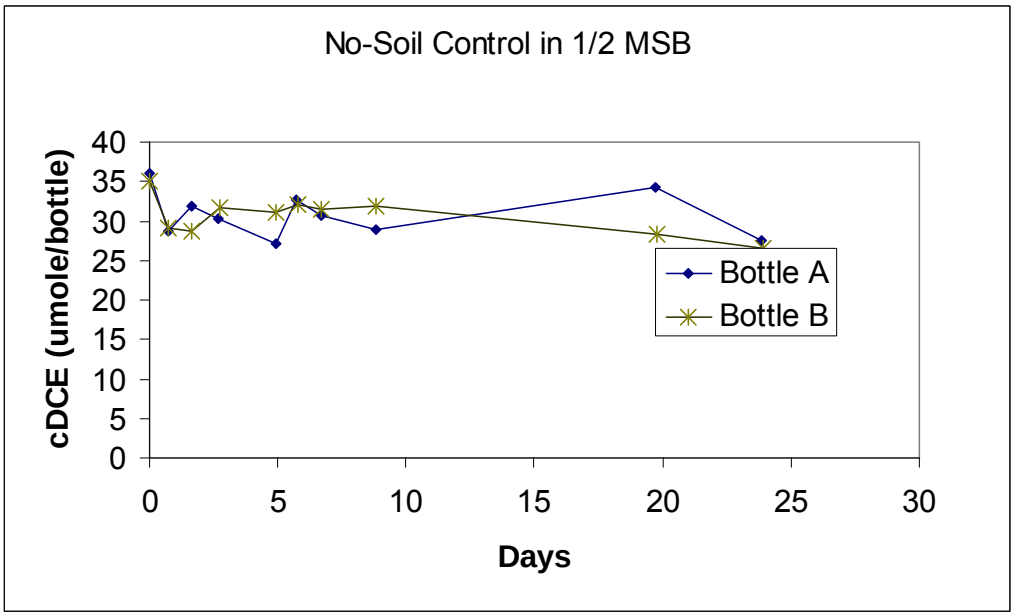


Figure 4.6 No-Soil (½ MSB) Inoculated Control with 10 mM Ethanol.

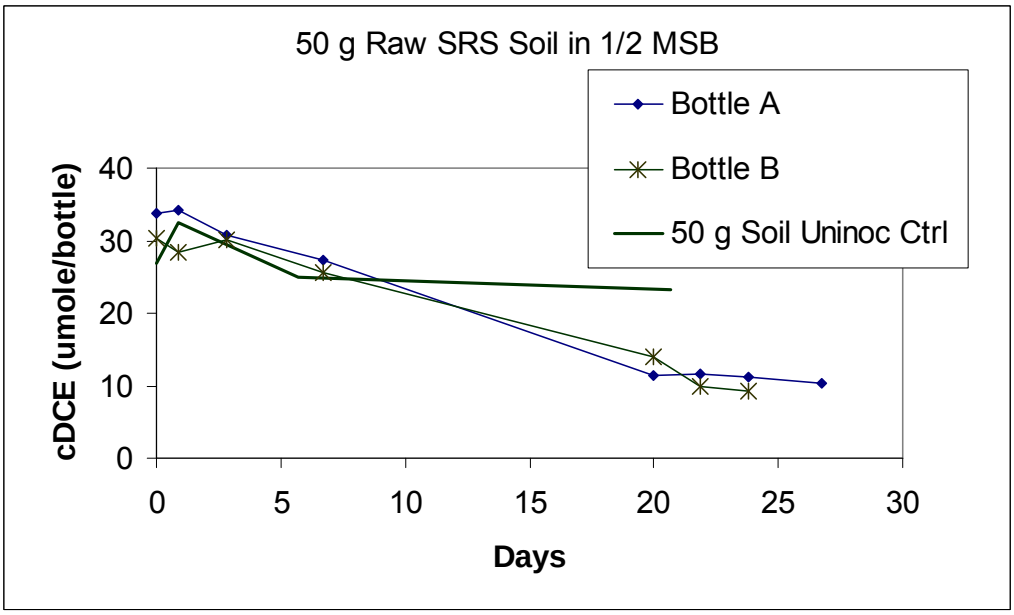


Figure 4.7 50 g Raw SRS Soil Inoculated with JS666 and Amended with ½ MSB and 10 mM Ethanol. Similar rates were seen in 5 and 15 g of soil, but only 50 g is shown here for clarity.

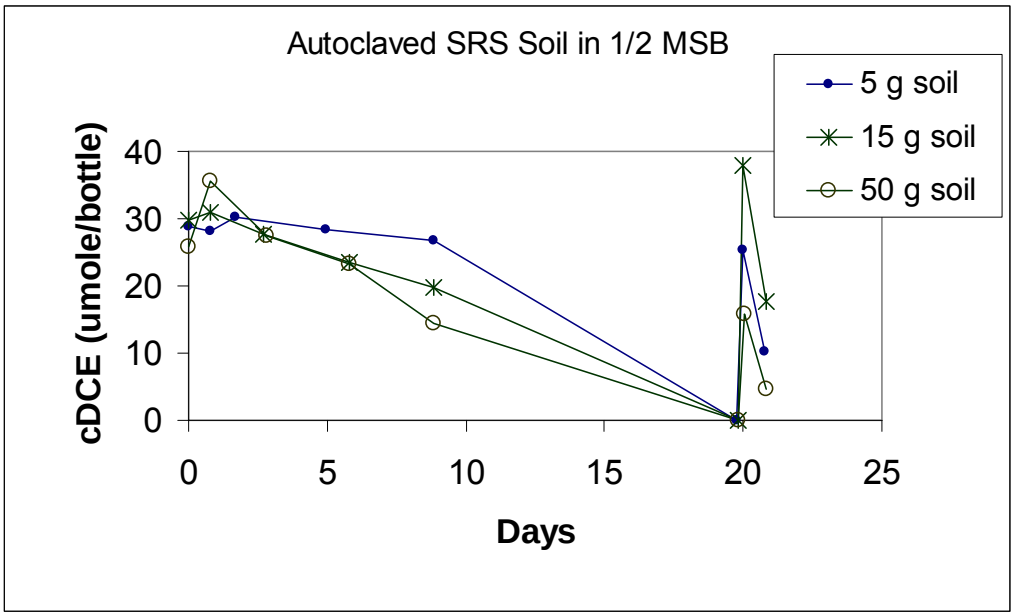


Figure 4.8. Autoclaved SRS Soil Inoculated with JS666 and Amended with ½ MSB and 10 mM Ethanol. Results for 5, 15, and 50 grams of soil. Duplicates behaved similarly (not shown).

4.4.3 SRS Soil Microcosms With Transfer Culture

The next microcosm study was designed to investigate the same factors as the previous study. However, this study was carried out using transfer culture that was grown on cDCE-only and was clearly exhibiting growth-coupled, cDCE degradation. The microcosms therefore were not amended with an alternative co-substrate. Again, factors of the SRS soil were examined through the use of different soil levels of 5, 15, and 50 g dry weight with both raw and autoclaved soil. The cultures were amended with ½ MSB and agitated at 100 RPM on an orbital shaker. Once more, there was no degradation observed in the inoculated, no-soil control bottles (data not shown). cDCE degradation was seen in inoculated microcosms prepared with raw, but not autoclaved soil (Figure 4.9, Figure 4.104.10), opposite of what was observed in the previous study. This suggests that JS666 is able to compete in a system where cDCE is the main available carbon and energy source. However, at this time we did not understand why microcosms prepared with autoclaved soil failed to degrade cDCE. Additionally, no degradation was observed in uninoculated-soil controls (Figure 4.94.9). We hypothesized some constituent of raw soil, either physical or chemical, supported growth and degradation. It is possible that the success of this experiment, relative to the first microcosm, was attributed to lower oxygen levels and slower agitation. These factors both caused both chemical and physical stress upon the organism that would have impeded its success in the microcosm. Moreover, the lower inoculation level might have also contributed to degradation. Though these speculations have not been investigated, lower inoculation level could have allowed viable organisms to find protection with in the soil matrix from predation, phages or inhibitory chemicals.

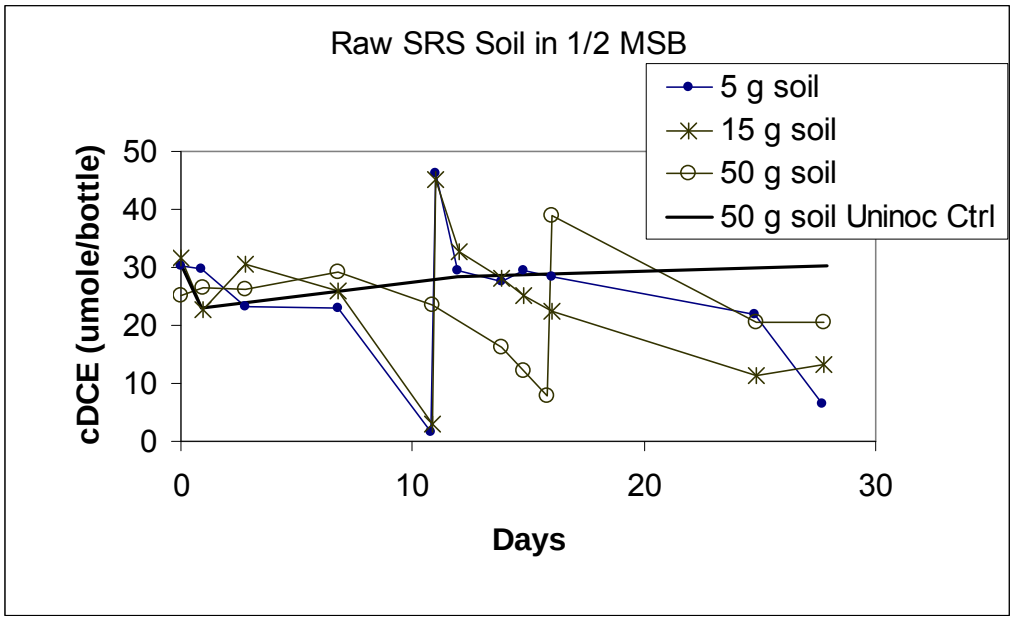


Figure 4.9 JS666-Inoculated SRS Soil in ½ MSB. Results for 5, 15, and 50 g soil. Duplicates behaved similarly (not shown)**.**

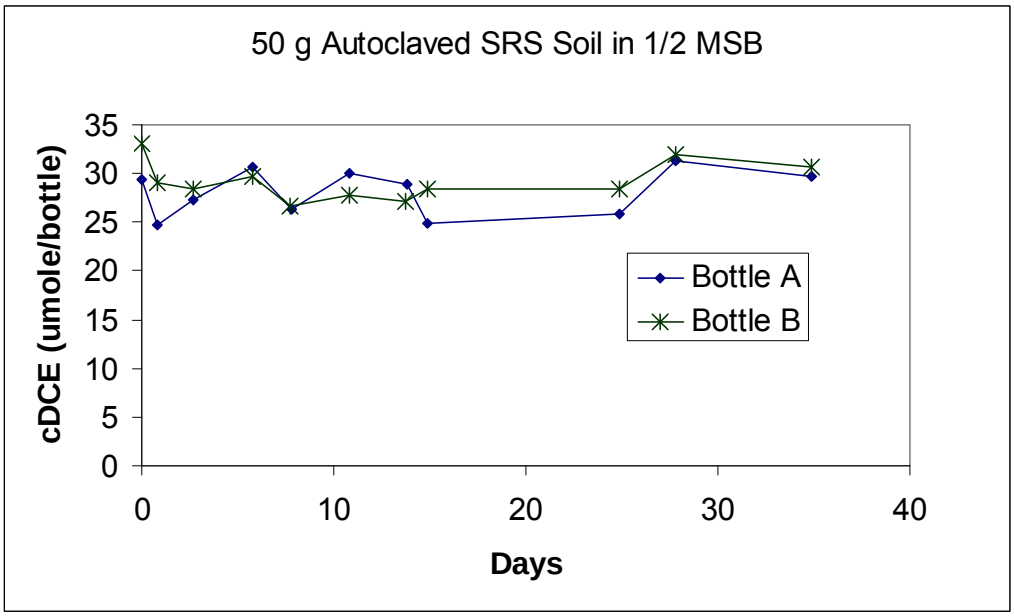


Figure 4.10 Autoclaved SRS Soil in ½ MSB Inoculated with JS666 Transfer Culture. Results Shown for 50 g Soil for Clarity, However the 5 and 15 g Soil bottles Behaved Similarly.

4.4.4 Robins Soil Microcosms

The success of the previous study led us to test the ability of JS666 to survive and degrade cDCE in various other types of available subsurface materials. Because no evidence from the previous studies indicated that level of soil had any bearing on the success of JS666, all subsequent microcosms were prepared with 50 g dry weight soil and inoculated with cDCE-degrading transfer culture at the target level of 4×10^5 cells JS666 per ml of liquid medium. The biotic factors of the soil were examined by preparing some of the microcosms with autoclaved soil. Within 9 days, all inoculated microcosms prepared with raw soil and ½ MSB had degraded the added cDCE (Figure 4.11). Inoculated microcosms prepared with raw soil, dH_2O and pH-neutralized prior to inoculation slowly and incompletely degraded cDCE over 35 days (Figure 4.12). None of the autoclaved microcosms amended with either ½ MSB or dH_2O evidenced any significant cDCE degradation (Figure 4.134.13). No degradation was observed in uninoculated-soil controls (Figure 4.11, Figure 4.12). Additionally, there was no degradation seen in any of the inoculated no-soil controls prepared with ½ MSB (Figure 4.14). The continued poor performance of JS666 in ½ MSB (in absence of soil) suggests that this medium is not a particularly good one (Figure 4.64.6, Figure 4.14). That JS666 performed better in ½ MSB when also provided with soil suggests that the soil provides something of benefit to JS666.

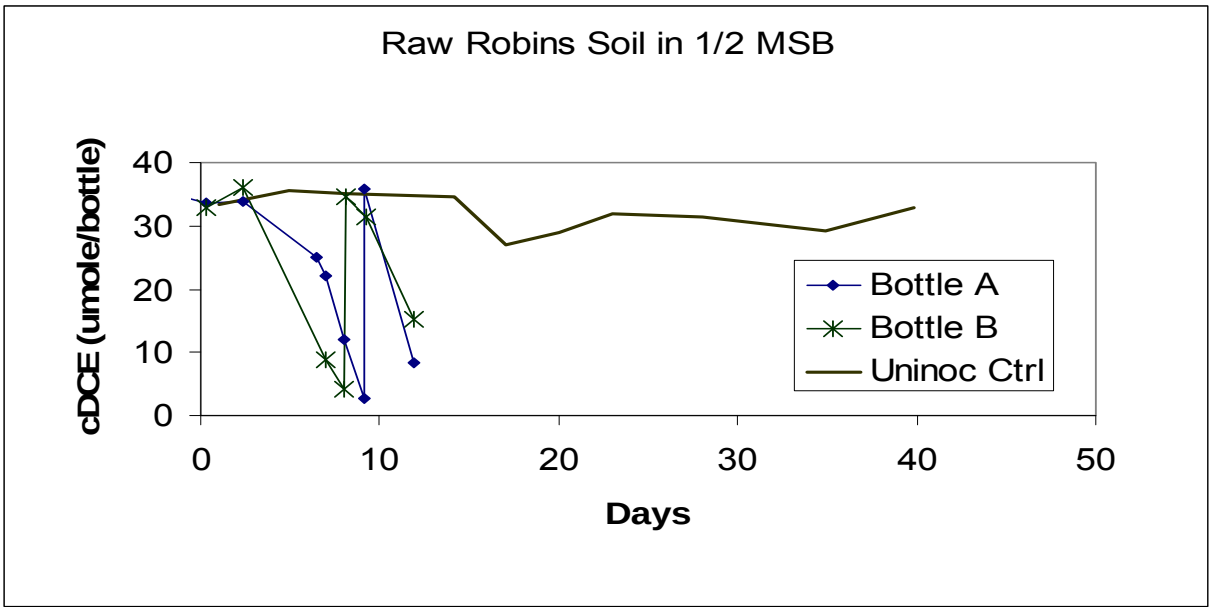


Figure 4.11 Robins Soil Inoculated with JS666 Amended with ½ MSB.

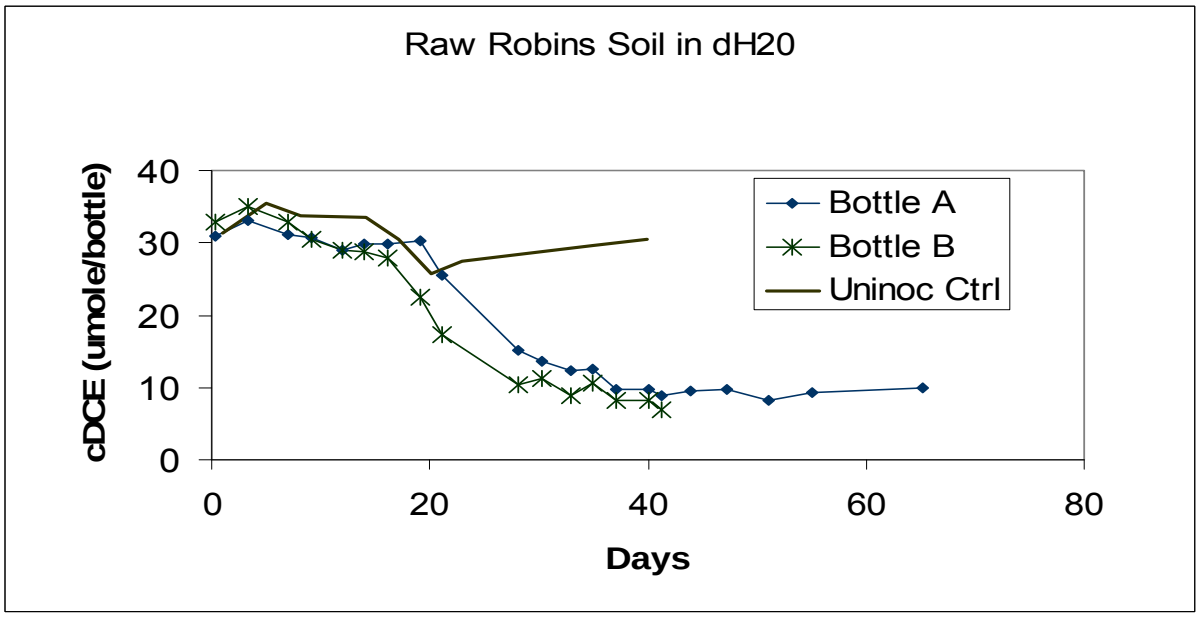


Figure 4.12 Robins Soil Inoculated with JS666 Prepared with dH_2O.

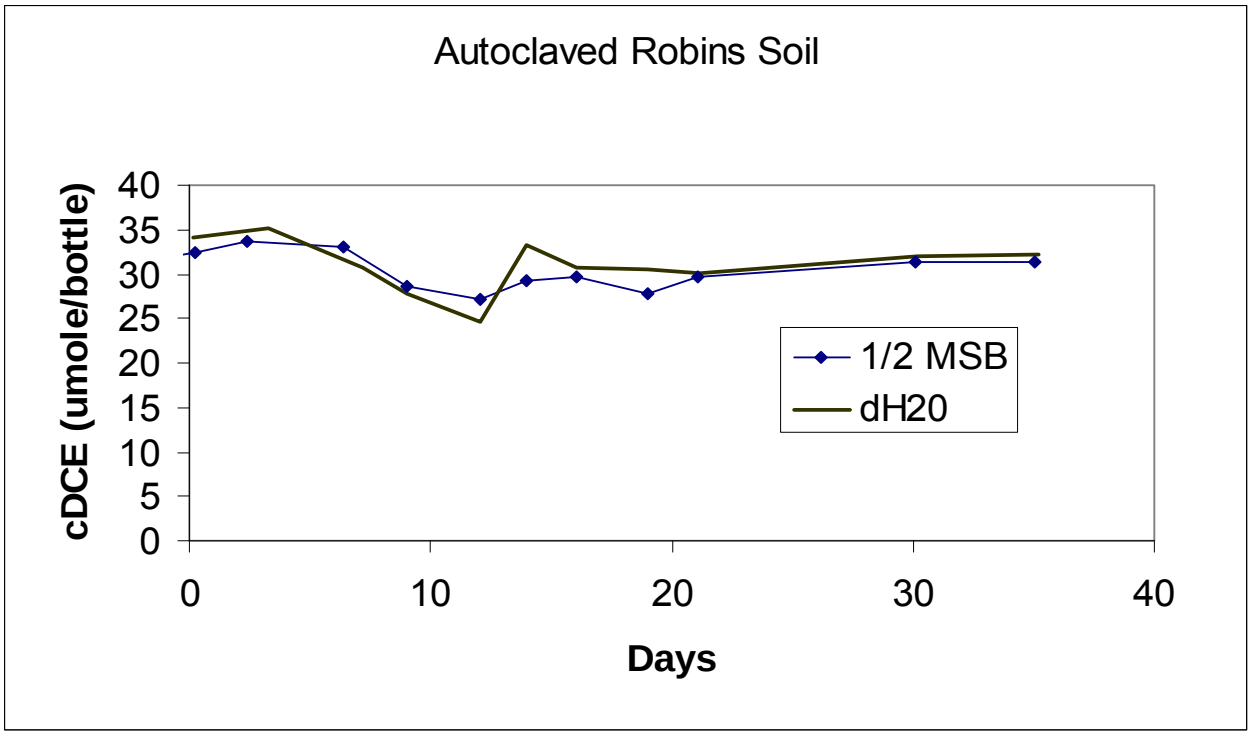


Figure 4.13 Inoculated 50 g Autoclaved Robins Soil in Either ½ MSB or dH_2O Inoculated. Duplicates Behaved Similarly (not shown)**.**

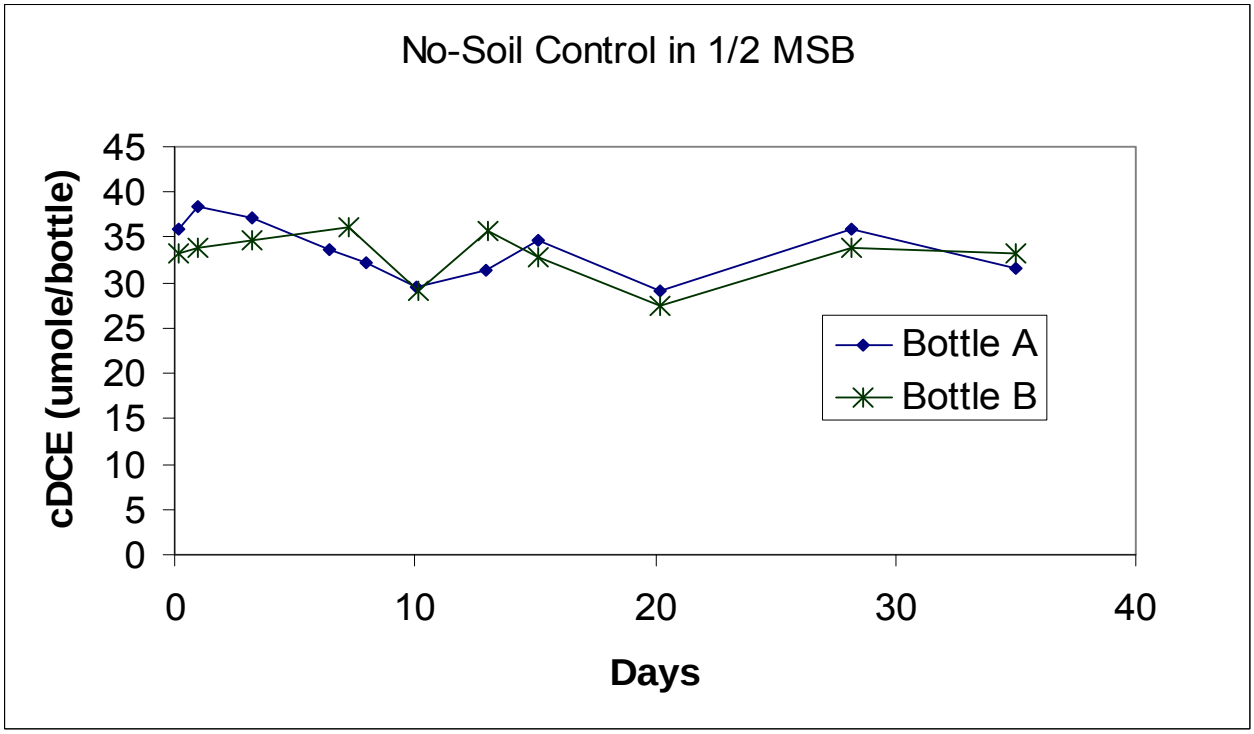


Figure 4.14 Inoculated No-soil Control in ½ MSB.

4.4.5 Hill Soil Microcosms

Microcosms were prepared with soil from Hill AFB. These microcosm bottles were prepared in the same manner as the previous ones conducted with Robins soil.

As with the Robins microcosms, within 9 days all inoculated microcosms prepared with raw Hill AFB soil and ½ MSB had degraded the added cDCE (Figure 4.154.15). Within 40 days, inoculated pH-neutralized microcosms prepared with raw soil and dH2O completely degraded the cDCE (Figure 4.154.15). No degradation was observed in uninoculated-soil controls, nor was it seen in microcosms prepared with inoculated autoclaved soils (Figure 4.154.15, Figure 4.164.16). There was insufficient soil to create uninoculated-soil controls in dH_2O as well as in ½ MSB. We chose to create uninoculated-soil control bottles in ½ MSB assuming that enhancing the conditions of organisms present would more likely lead to degradation were it to occur in uninoculated-soil microcosms.

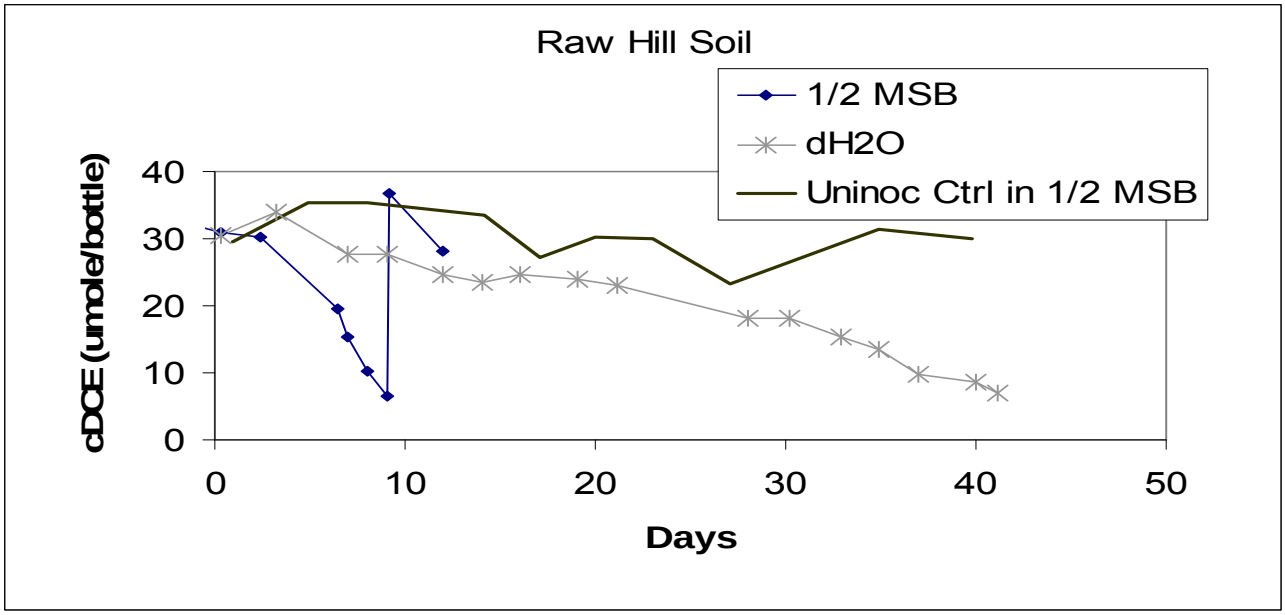


Figure 4.15 Inoculated 50g Hill Soil Amended with ½ MSB or dH_2O. Duplicates Behaved Similarly (not shown)**.**

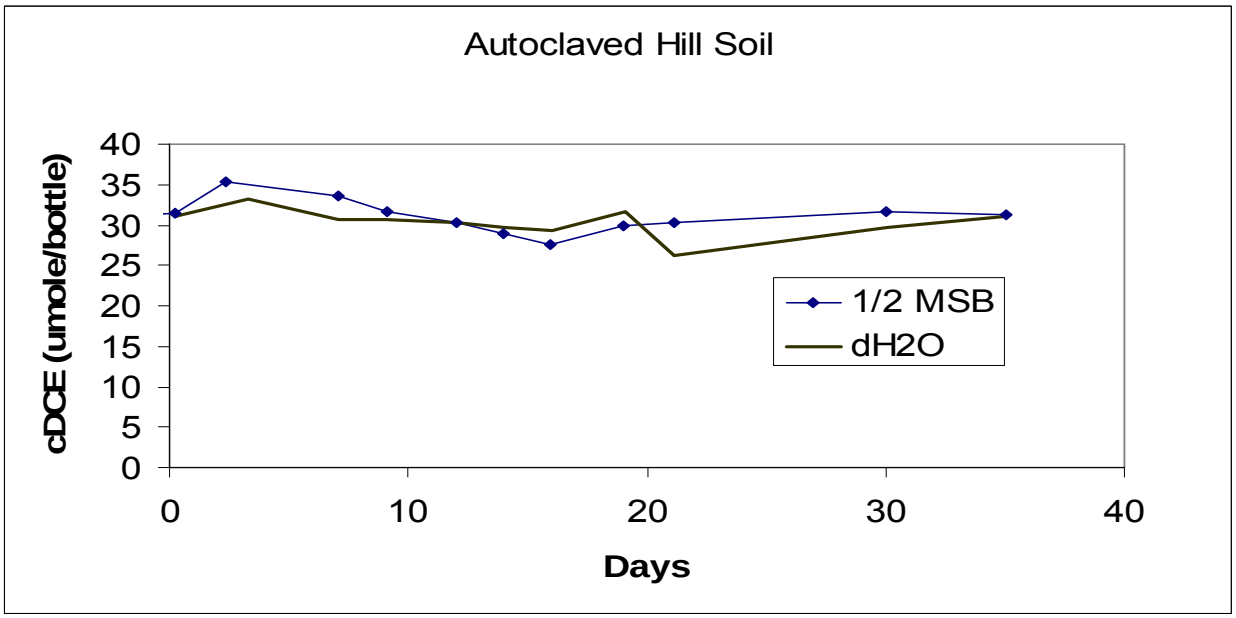


Figure 4.16 Inoculated 50 g Autoclaved Hill Soil in ½ MSB or dH_2O. Duplicates Behaved Similarly (not shown)**.**

Other studies investigating how to best sterilize soil revealed that autoclaving soil significantly alters its properties, both chemically and physically. It has been shown to liberate manganese, as much as 120 times the original value, and possibly other toxic materials (Skipper and Westerman 1973). Additionally, it can alter nutrient availability of nitrogen and potassium, lower the pH of the soil, and change the surface area of the material (Abou-Shanab *et al.* 2003, Peterson 1962, Skipper and Westerman 1973, Wolf and Westerman 1989). As such, autoclaved soil becomes an inhospitable environment for microorganisms. Additionally, the media used (½ MSB) is not ideal for growing JS666, which caused the inoculated no-soil controls of this and the previous three treatments to fail and perhaps stressed the organism causing it to perform more poorly (Figure 4.174.17). Despite this, microcosms prepared with raw soil in ½ MSB were able to degrade successfully, further validating that soil systems are actually beneficial to the organism. Moreover, because microcosms that were prepared using raw soil were consistently successful at degrading cDCE, no further studies were conducted with microcosms prepared with soil sterilized by any means. In the light of this information, microcosms that were prepared using autoclaved soil were not considered in final analysis and conclusions.

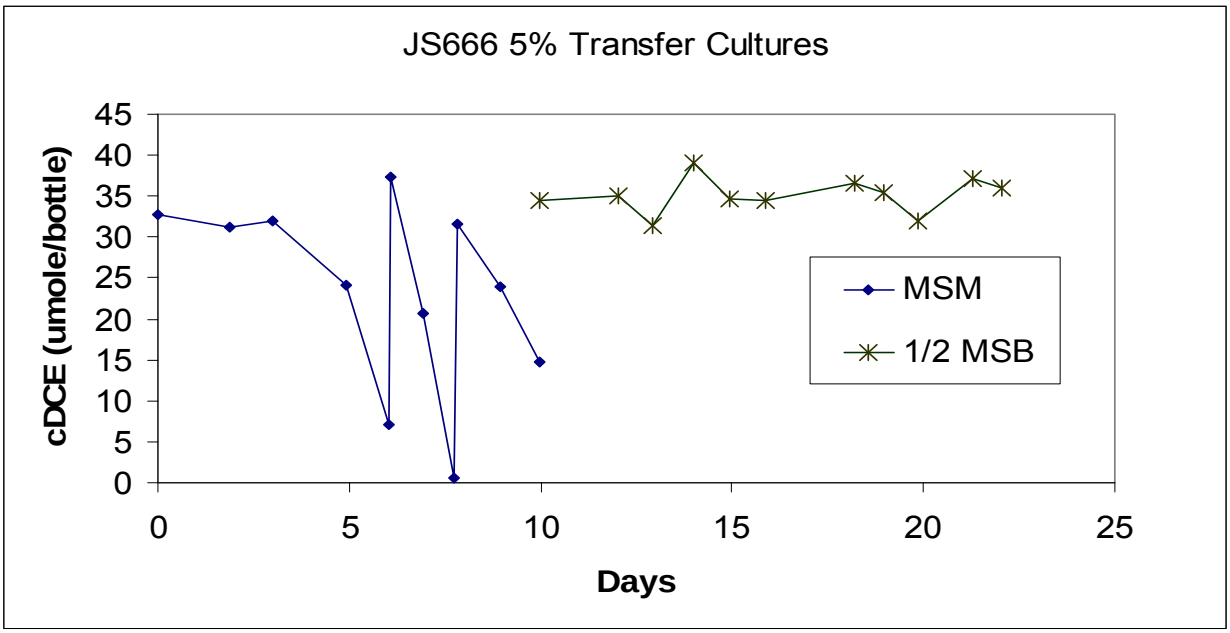


Figure 4.17 5% Transfer Culture Started in MSM and Switched to ½ MSB. Duplicate Culture Performed Similarly (not shown)**.**

4.4.6 Ft. Lewis Groundwater Microcosms

The next microcosm study was prepared with groundwater from Ft. Lewis, WA that was supplemented with micronutrient and buffer by adding 5 ml of 10X MSM to 45 ml of groundwater. Additionally, bottles were prepared with groundwater that was not amended. All inoculated microcosms used cDCE-degrading transfer culture and were agitated at 100 RPM on an orbital shaker. Since switching back to the better, MSM, degradation was observed in all inoculated MSM-only controls, and no degradation was seen in the uninoculated-groundwater control microcosms that were either amended or not (Figure 4.184.18, Figure 4.194.19). This indicated that degradation was due to JS666 and not native organisms in the groundwater. There was full degradation within 12 days in the inoculated microcosms that were amended with MSM (Figure 4.204.20). One of the groundwater microcosms that had no amendment was able to fully degrade the added cDCE; however, this result was not replicated in the duplicate bottle (Figure 4.214.21).

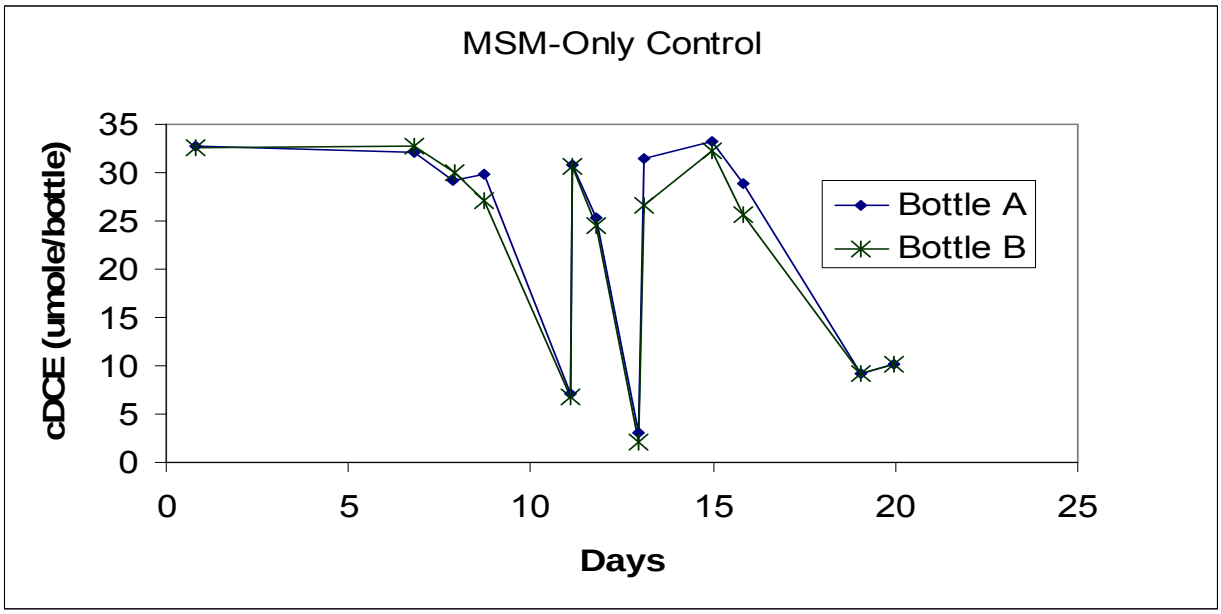


Figure 4.18 Inoculated MSM-only Control.

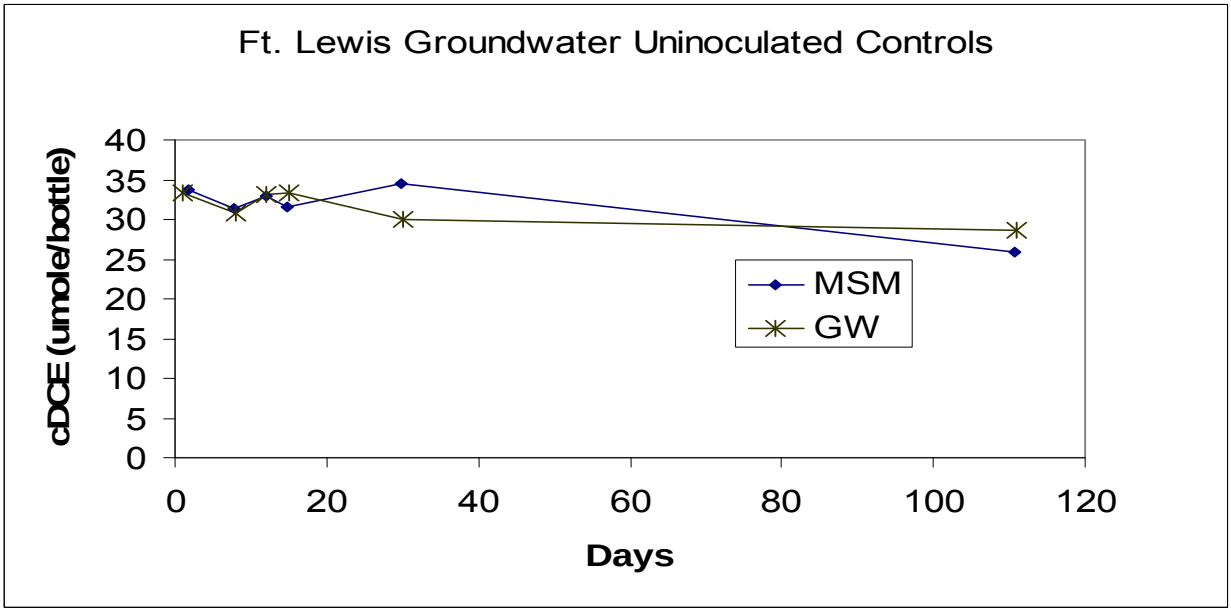


Figure 4.19 Ft. Lewis Groundwater Uninoculated Controls With 10X MSM or With Groundwater Only (GW). Duplicates Behaved Similarly (not shown)**.**

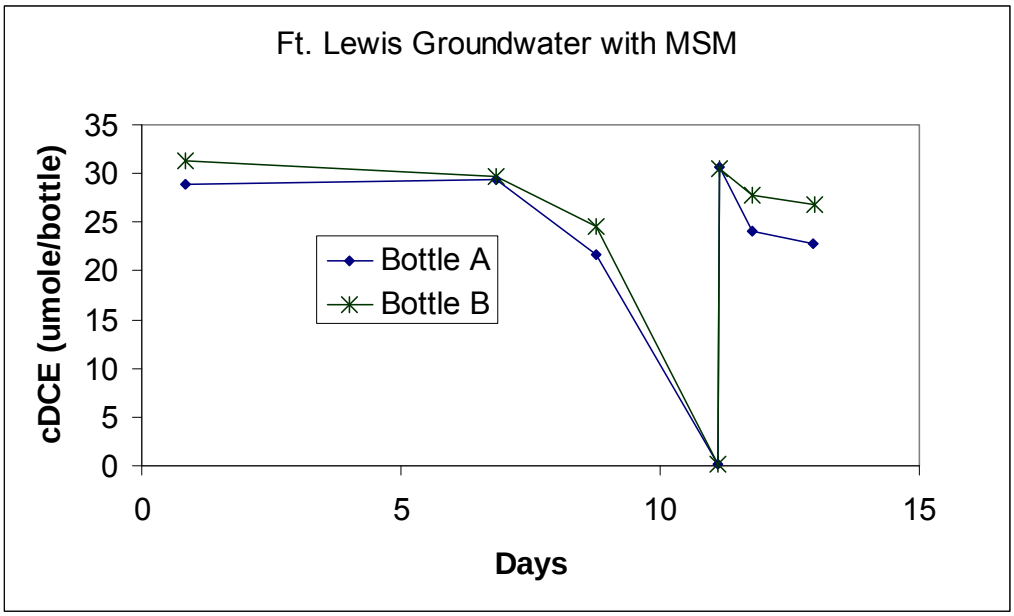


Figure 4.20 Inoculated Ft. Lewis Groundwater Amended with MSM.

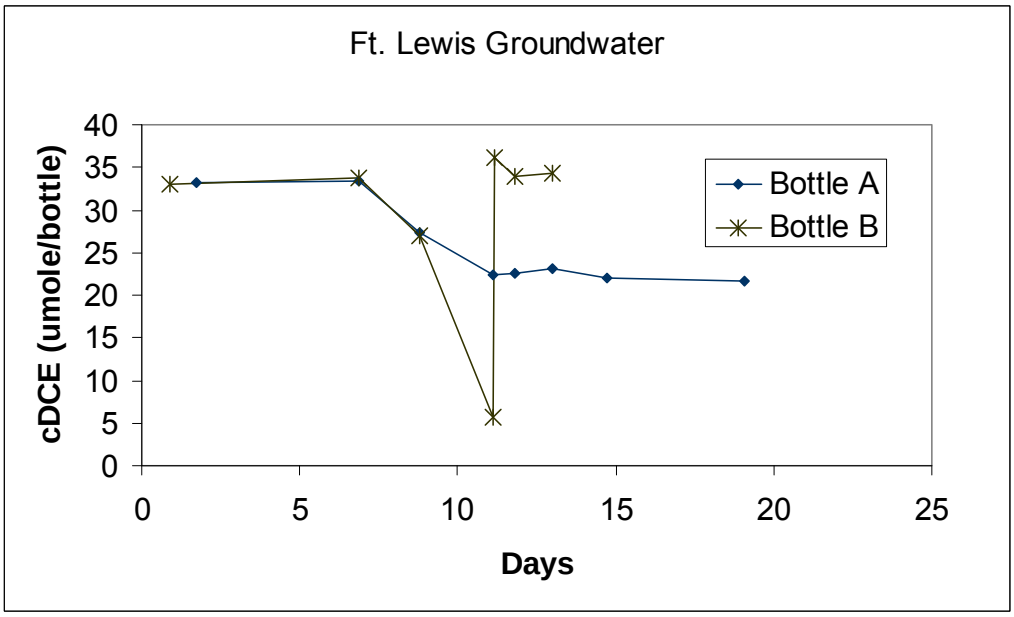


Figure 4.21 Inoculated Ft. Lewis Groundwater With No Added Buffer or Micronutrients. Results were not Replicated and so Duplicates are Shown.

4.4.7 Aerojet Soil Microcosms

Microcosms were prepared with 50 g dry weight soil from Aerojet. Soil with and without MSM amendment was inoculated with cDCE-degrading transfer culture. There was degradation in all inoculated no-soil MSM controls (Figure 4.22), and no degradation in uninoculated-soil controls (Figure 4.234.23), indicating a healthy inoculum source and that the degradation observed in inoculated Aerojet microcosms was caused by the added JS666 and not by indigenous organisms in the subsurface (Figure 4.244.24, Figure 4.254.25). These data taken in conjunction with the data from the Ft. Lewis microcosms, indicates that subsurface material exhibiting conditions favorable to JS666, most notably aerobic with circumneutral pH, supports degradation without any further amendment.

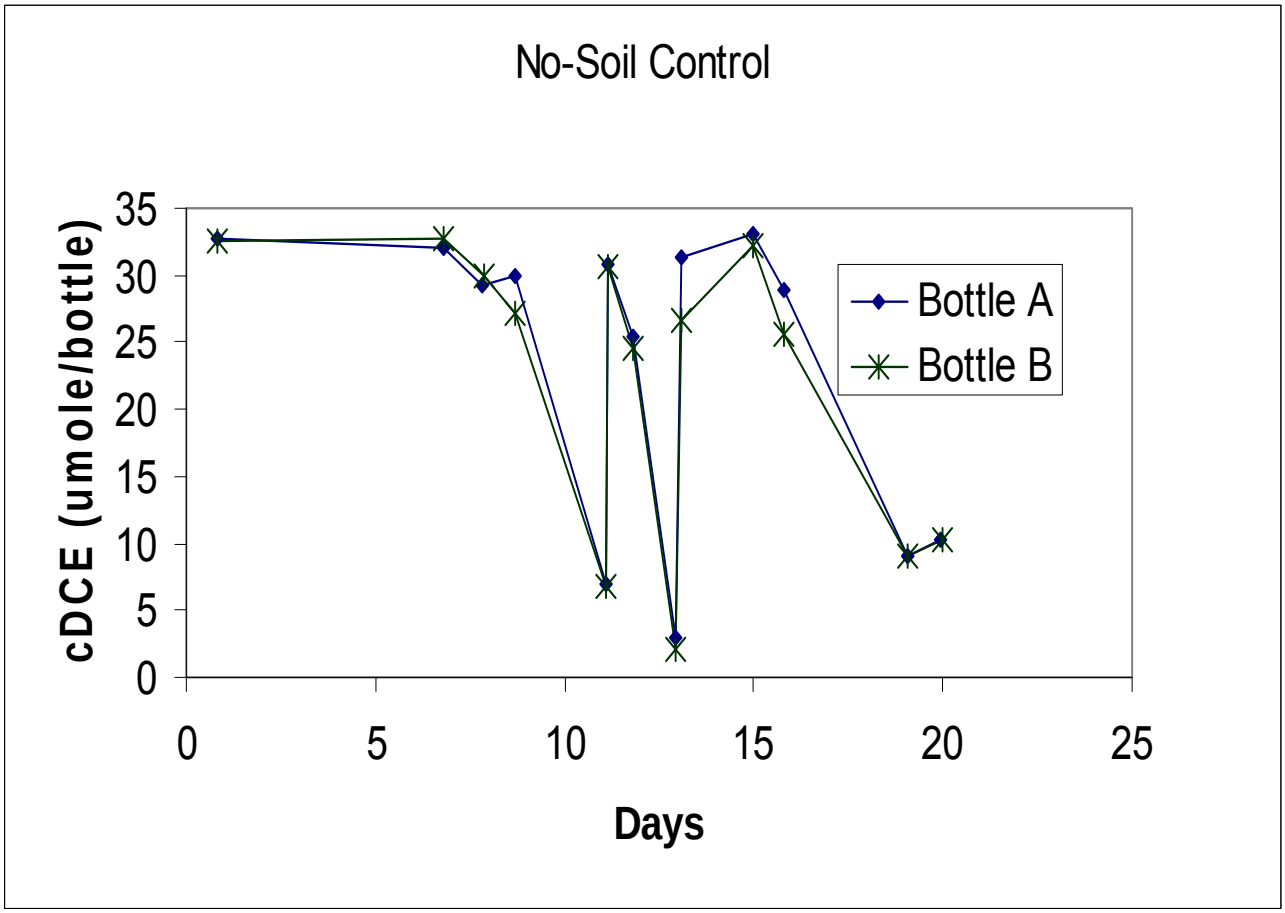


Figure 4.22 Inoculated No-Soil Control in MSM.

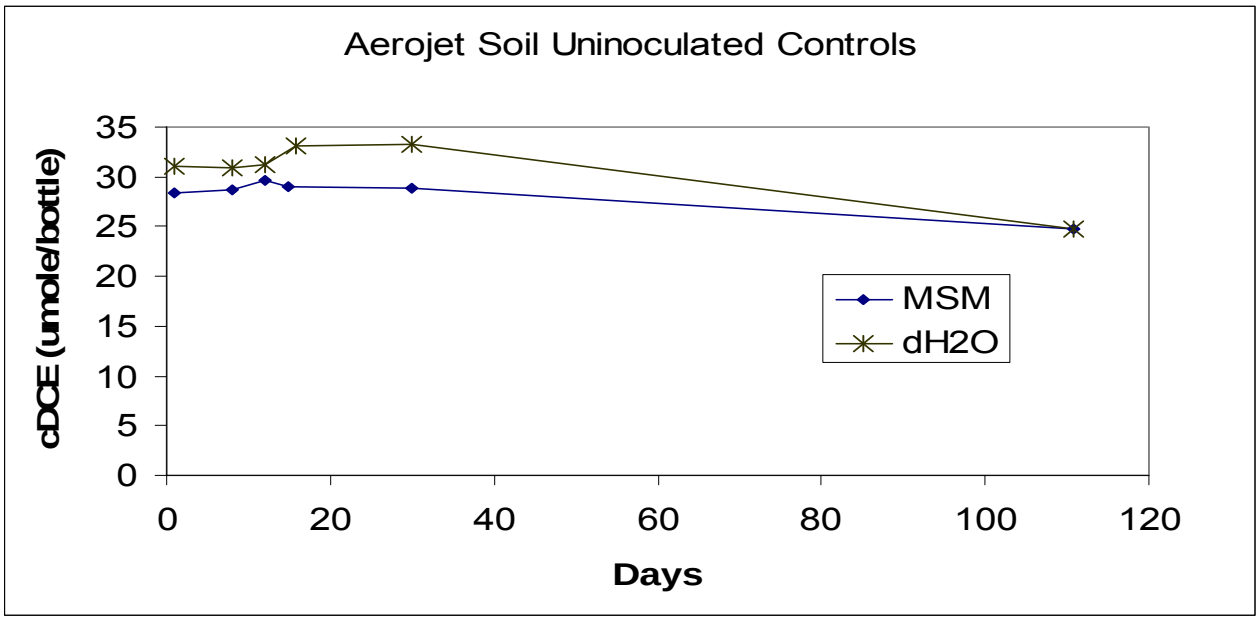


Figure 4.23 50 g Uninoculated Aerojet Soil Prepared with Either MSM or dH_2O. Duplicates Behaved Similarly (not shown)**.**

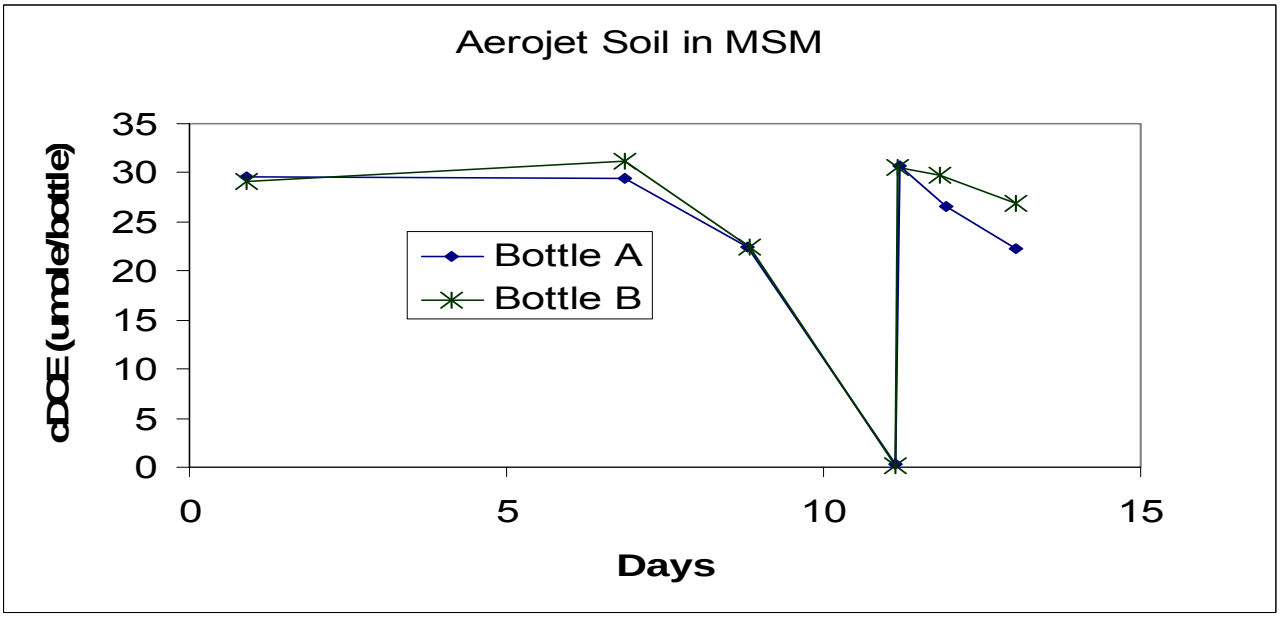


Figure 4.24 Aerojet Soil Prepared with MSM and Inoculated with JS666 Transfer Culture.

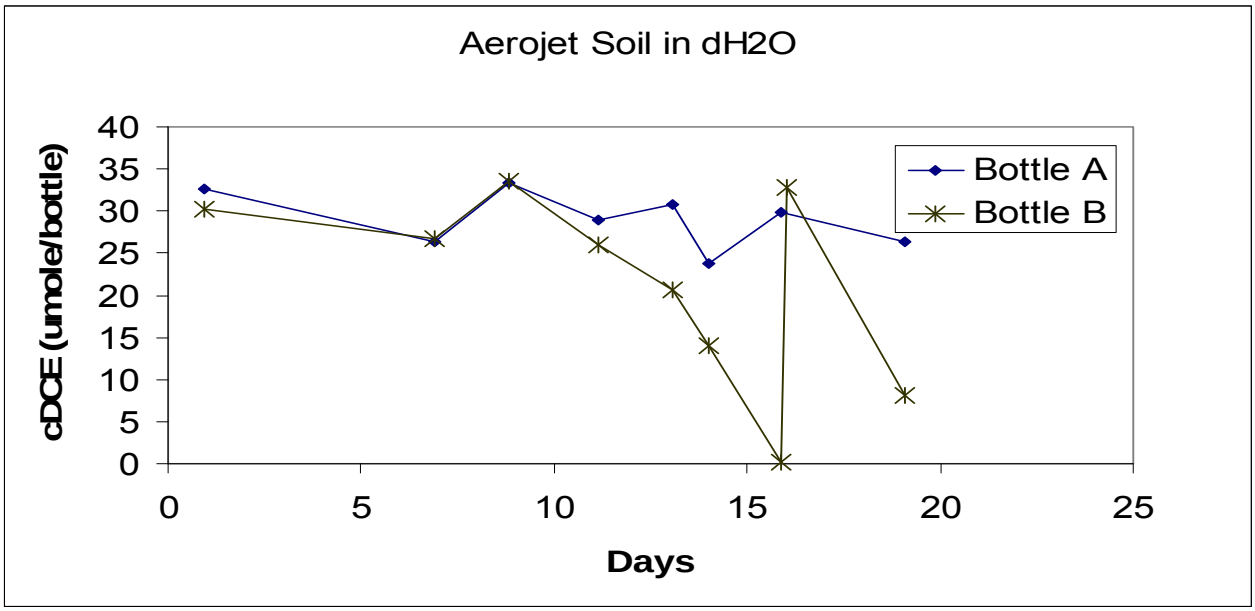


Figure 4.25 Aerojet Soil Inoculated with JS666 Prepared with dH_2O. Degradation Observed in One Replicate.

4.4.8 SRS Soil Microcosms at Three Inoculation Levels and Two cDCE Levels

The success of bioaugmentation could be dependent on inoculum size. Therefore, the next study examined the possible effects of inoculum level. Ramadan *et al*. suggested an inoculation level for bioremediation of a density on the order of 4×10^4 to 4×10^5 cells per ml (Ramadan *et al.* 1990). Our successful microcosms had all been inoculated with a culture density close to 4×10^5 cells per ml. To address the question of what mass of culture would be necessary to inoculate a field site, we inoculated 50 g SRS soil amended with MSM at 1/10th (0.1X) and 1/100th (0.01X) of this level, as well as at the normal level (1X). All inoculations were made with cDCE-degrading transfer culture. Additionally, it was already known that JS666 was able to degrade massive amounts of cDCE. However, what was not known is what level of cDCE needs to be present for JS666 to begin degrading. To address this, microcosms were prepared at 1/100th the customary level of cDCE (0.01C) as well as at the standard level (1C, corresponding to a nominal concentration of 59 mg/L). Large quantities of SRS soil were still available for use, so this was the subsurface material that was employed and was amended with MSM. Degradation was observed in all no-soil (i.e., MSM-only) controls, at 1C cDCE, and all levels of inoculation (Figure 4.264.26). There were successively slower degradation rates of cDCE the smaller the inoculum level. Similar degradation trends were observed in the soil microcosms with 1C cDCE and smaller inoculum levels, and no degradation was exhibited by the uninoculated-soil controls (Figure 4.274.27).

In the no-soil (i.e., MSM-only) controls with 0.01C cDCE, there was degradation at inoculum levels of 1X, 0.1X, and in one of the 0.01X bottles (Figure 4.284.28). For the soil microcosms at 0.01C cDCE, degradation was observed at all inoculation levels, and none was seen in the uninoculated-soil controls (Figure 4.294.29). To be conservative, an inoculation size of 0.1X (approximately 4×10^4) is recommended for field applications. Low levels of cDCE are difficult to measure precisely. Because of this, degradation trends appear noisy. However, it is clear that JS666 is able to degrade these lower levels to below detection, which is below 15 nmoles/bottle nominal concentration.

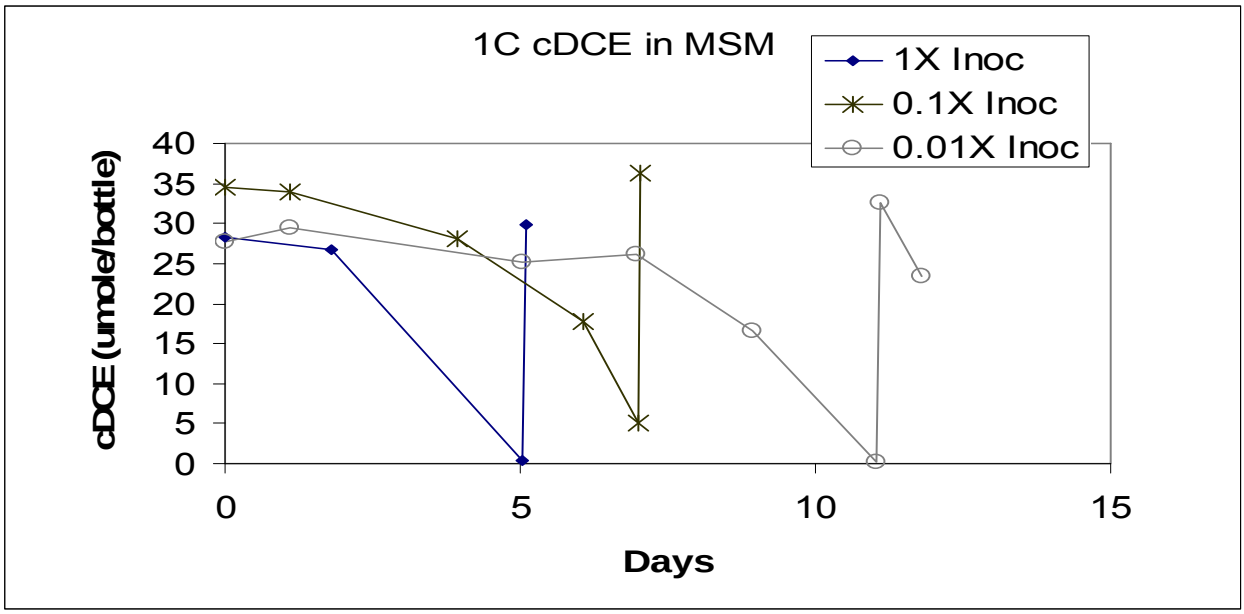


Figure 4.26 MSM Inoculated at Three Levels. For reference, 1C Corresponds to Approximately 60 mg/L Nominal Concentration, and 1X Corresponds to Approximately 4×10^5 **cells/ml. Duplicates Behaved Similarly** (not shown)**.**

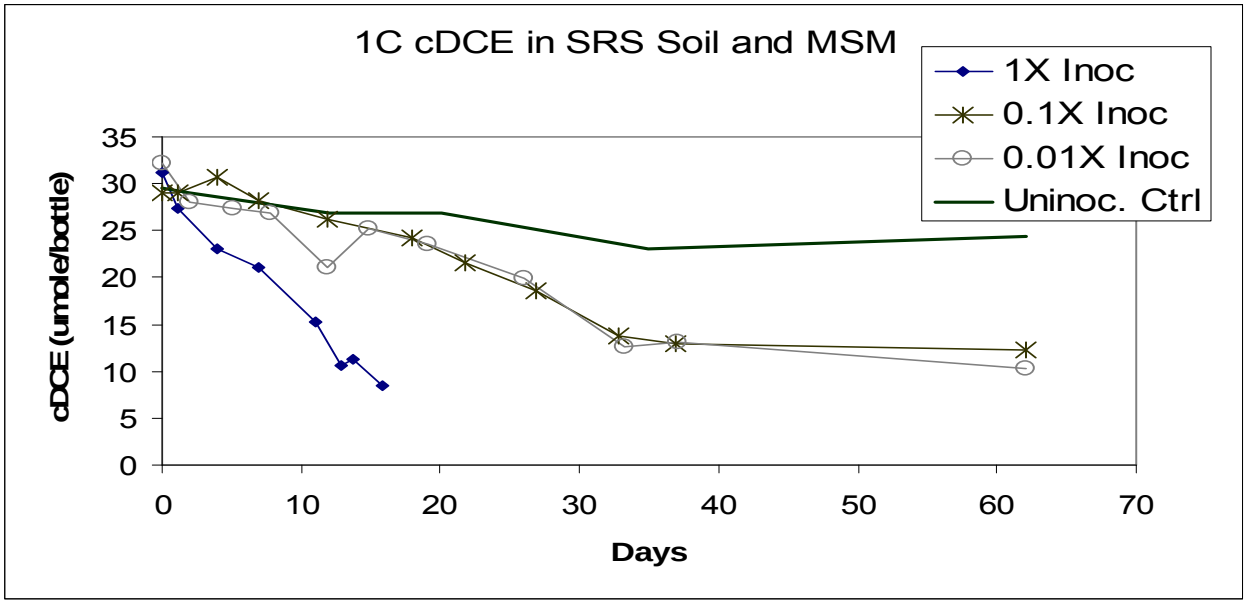


Figure 4.27 SRS Soil Amended With MSM and Inoculated at Three Different Levels. For Reference, 1C Corresponds to Approximately 60 mg/L Nominal Concentration, and 1X Corresponds to Approximately 4×10^5 **cells/ml. Duplicates Behaved Similarly** (not shown)**.**

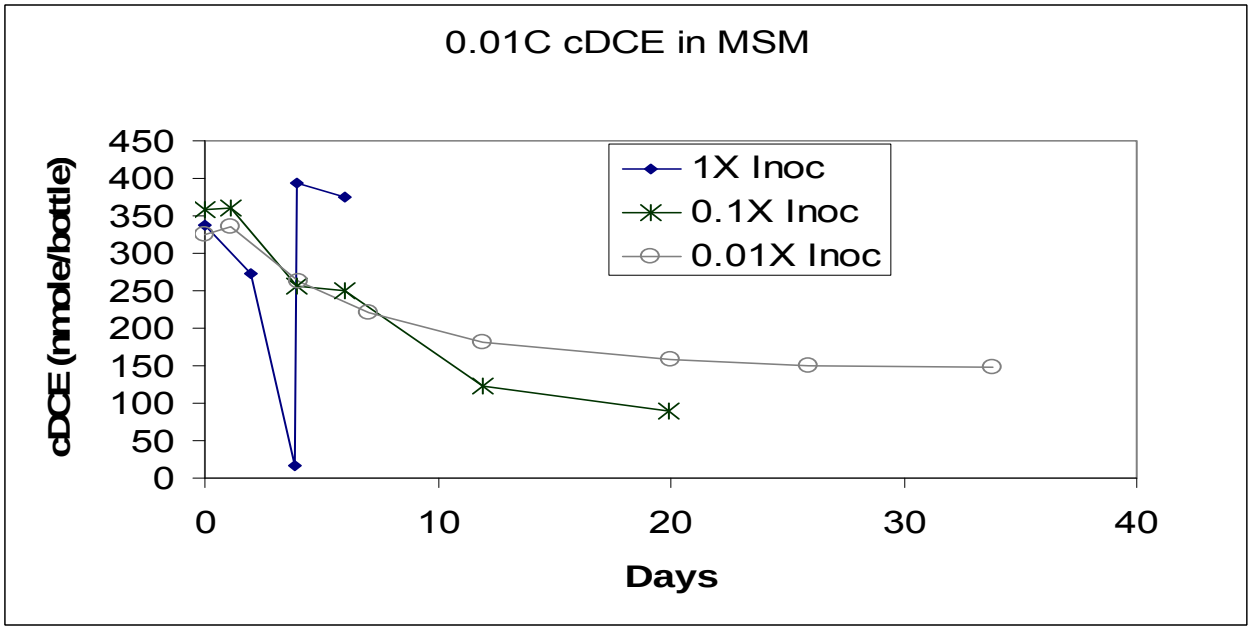


Figure 4.28 MSM Inoculated at Three Levels and Fed 1/100th of Normal cDCE Level. For Reference, 0.01C Corresponds to Approximately 0.60 mg/L Nominal Concentration, and 1X Corresponds to Approximately 4×10^5 **cells/ml. Duplicates Behaved Similarly** (not shown)**.**

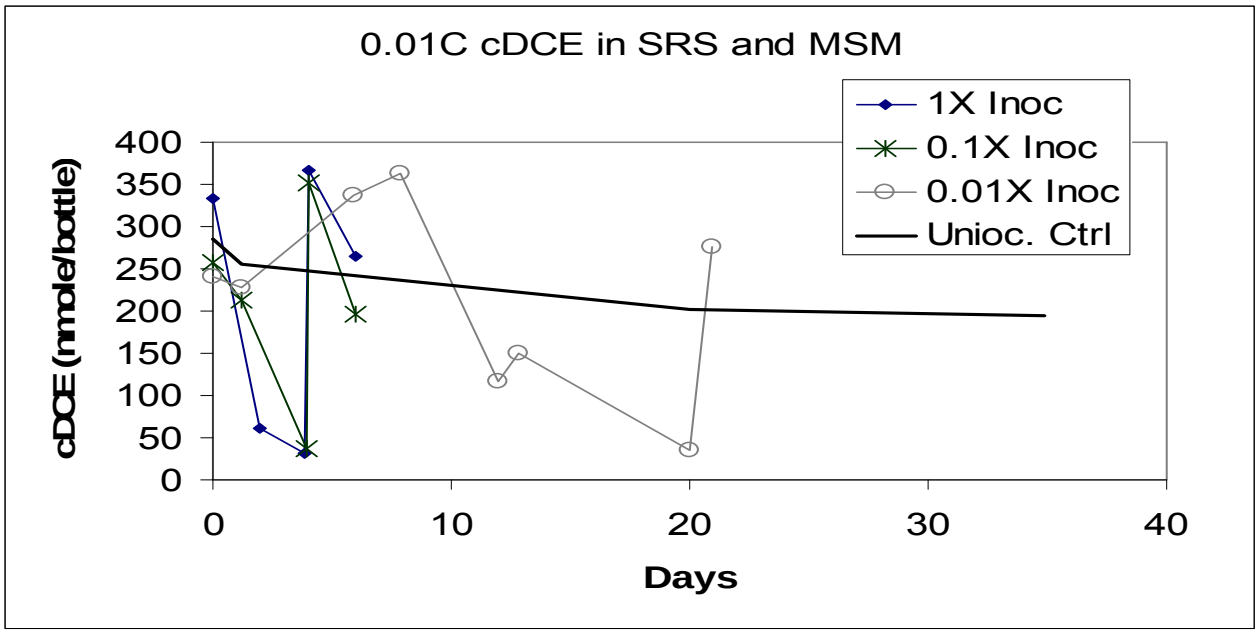


Figure 4.29 SRS Soil Amended with MSM Inoculated at Three Levels and Fed 1/100th of Normal cDCE Level. For Reference, 0.01C Corresponds to Approximately 0.60 mg/L Nominal Concentration, and 1X Corresponds to Approximately 4×10^5 **cells/ml. Duplicates Behaved Similarly** (not shown)**.**

4.4.9 Primary Effluent Microcosms

The previous microcosms demonstrated that JS666 is able to degrade cDCE in all subsurface materials investigated. The subsequent microcosm study aimed to further challenge JS666 by inoculating cultures in the presence of other microorganisms and a rich mixture of carbon sources. MSM was amended with either 1% or 10% (v/v) raw or autoclaved primary effluent from a local municipal wastewater treatment plant and inoculated at two different levels, 1X and 0.1X, and fed 60 mg/L nominal concentration of cDCE. It should be noted that even at 1%, the presence of raw effluent was a rigorous test of the ability of JS666 to survive in a mixed culture expected to contain predators, as well as competing bacteria and phages.

All microcosms prepared with either 1% or 10% autoclaved primary effluent were able to degrade cDCE, regardless of the initial inoculation level (Figure 4.30, Figure 4.31). This demonstrates that even in the presence of a mixture of alternative — and most likely preferable — carbon sources, JS666 is able to degrade large amounts of cDCE.

In microcosms prepared with 1% raw primary effluent, rapid and complete degradation of cDCE was observed in microcosms with low inoculum levels and eventual degradation was seen at the high inoculum levels (Figure 4.32). Partial degradation was observed in the microcosms that had lower inoculum levels and high concentrations of primary effluent, and slow partial degradation was observed with high-concentration inoculum (Figure 4.33). Though this is counter intuitive,

it was reproduced and further work done with the molecular probe has confirmed that these are the true inoculation levels. It is possible that the predatory response of organisms such as protozoa require a minimum amount of substrate (i.e JS666) to be stimulated, and so lower inoculation levels actually can be more successful at remediation. MSM controls showed that the inoculum was viable and healthy and that the lower inoculation level required more time to degrade a spike of cDCE (Figure 4.34).

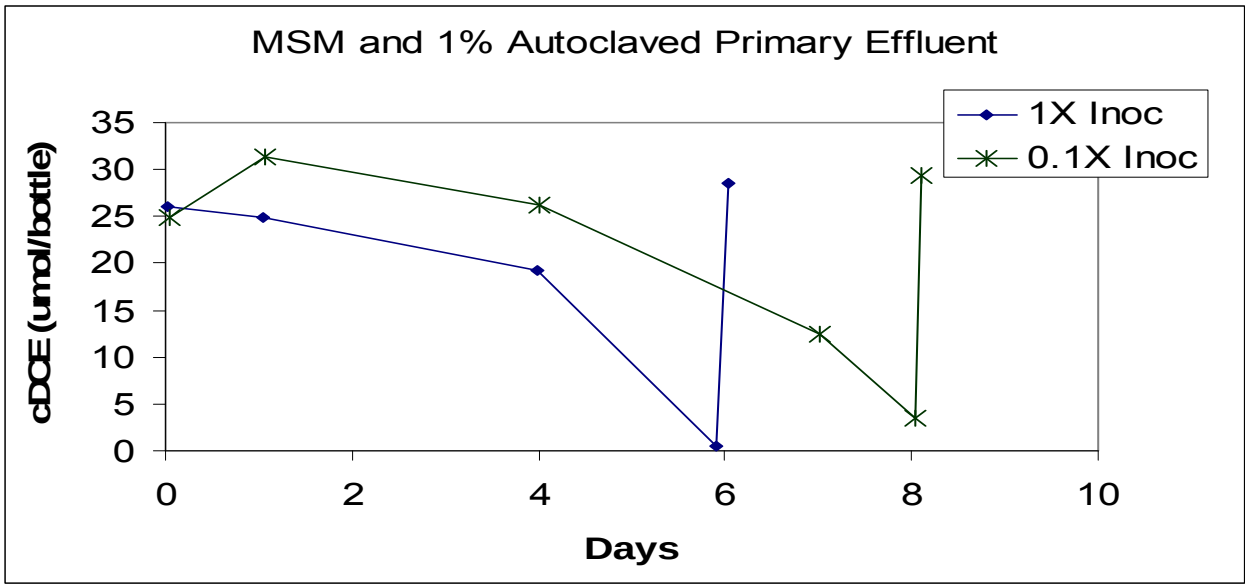


Figure 4.30 MSM Amended with 1% (v/v) Autoclaved Primary Effluent and Inoculated with JS666 at Two Levels. Duplicates behaved similarly (not shown). For reference, 1X corresponds to approximately 4×10^5 cells/ml.

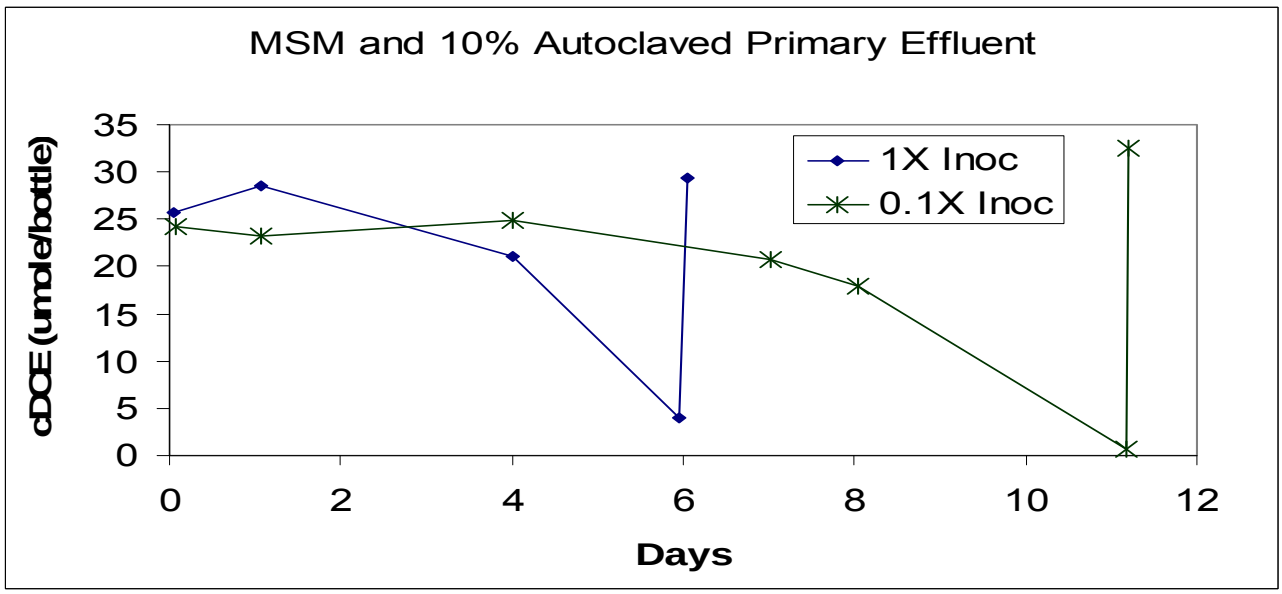


Figure 4.31 MSM Amended With 10% (v/v) Autoclaved Primary Effluent and Inoculated at Two Levels. Duplicates Behaved Similarly (not shown)**. For reference, 1X Corresponds to Approximately** 4×10^5 **cells/ml.**

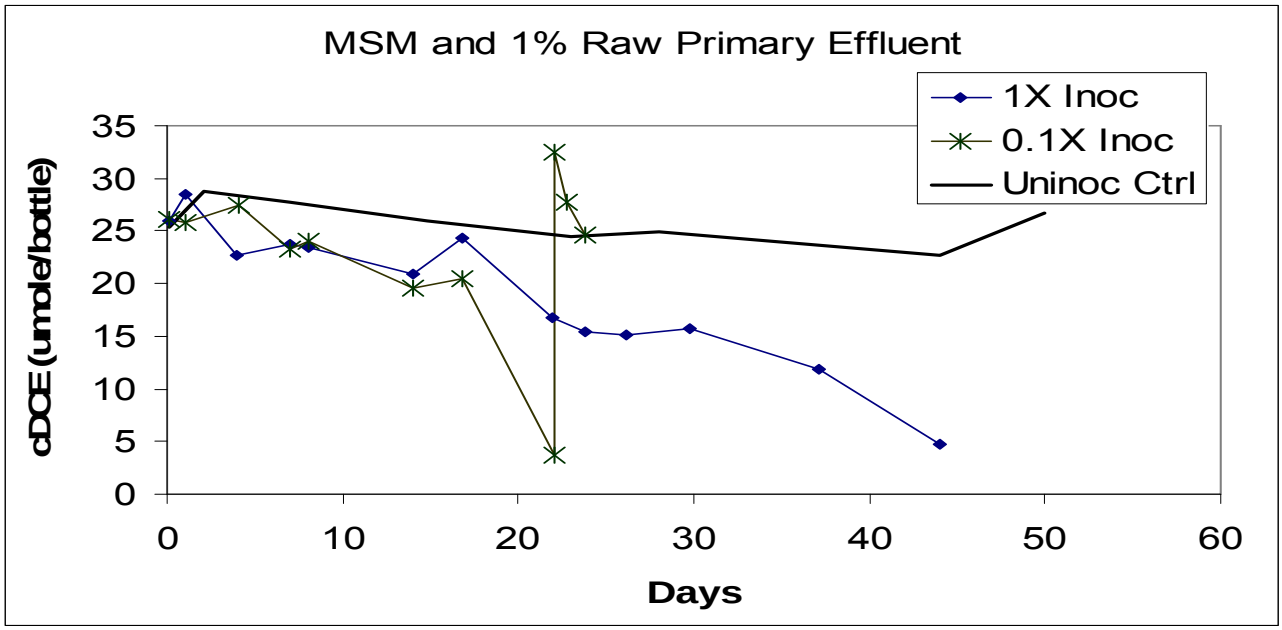


Figure 4.32 MSM Amended with 1% Raw Primary Effluent. For Reference, 1X Corresponds to Approximately 4×10^5 **cells/ml. Duplicates Behaved Similarly** (not shown)**.**

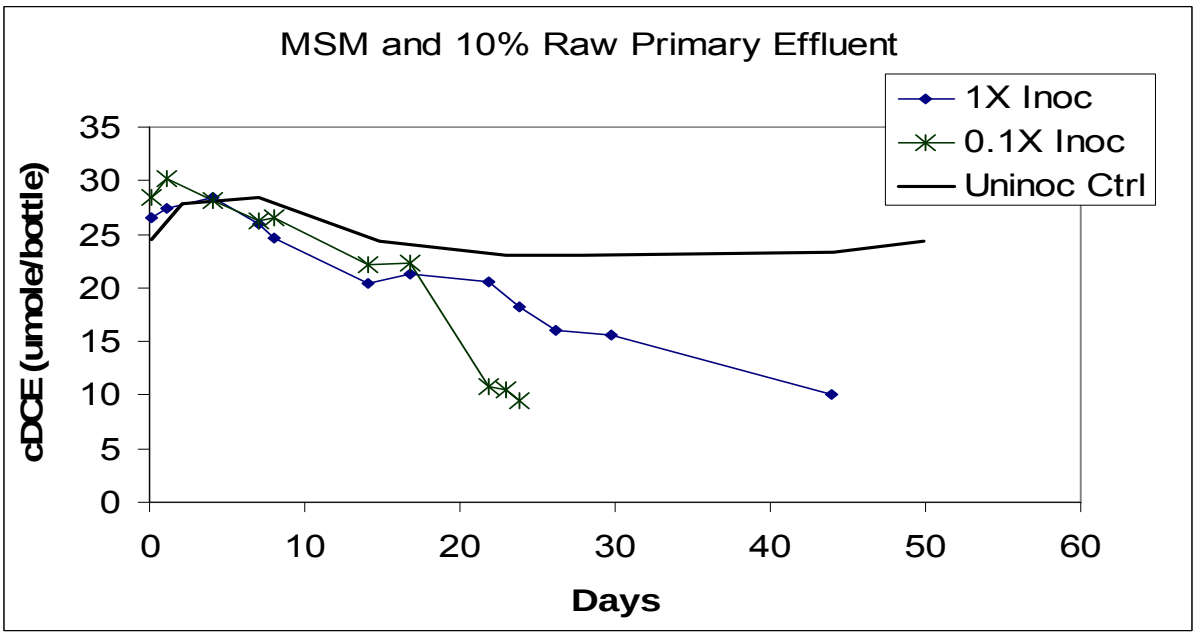


Figure 4.33 MSM amended with 10% Raw Primary Effluent. For Reference, 1X Corresponds to Approximately 4×10^5 **cells/ml. Duplicates Behaved Similarly** (not shown)**.**

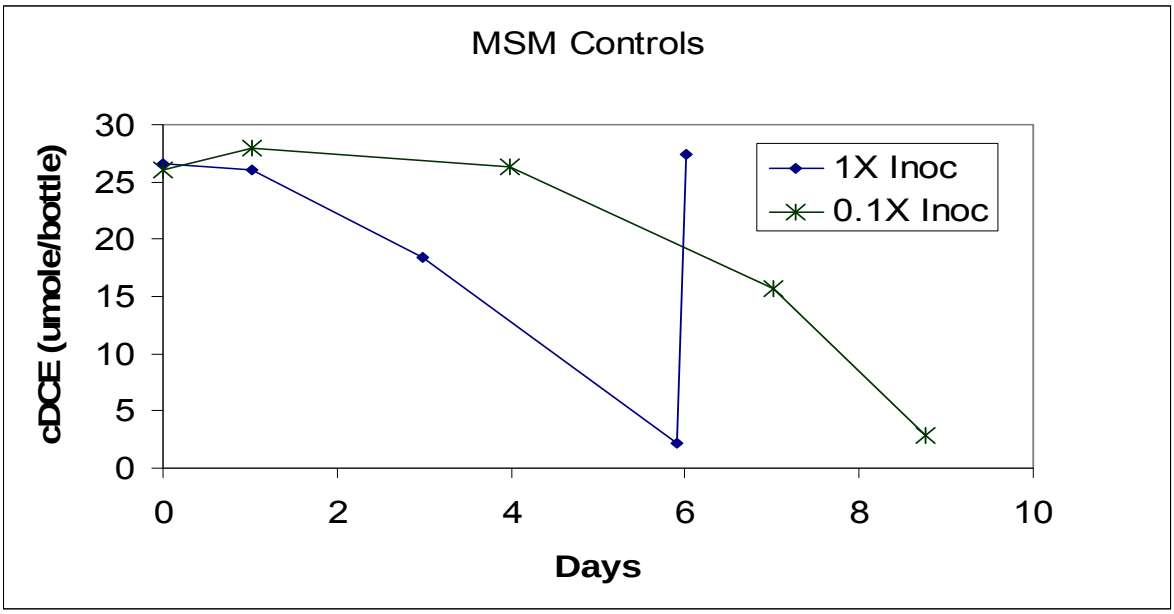


Figure 4.34 MSM Controls Inoculated at Two Levels. For reference, 1X Corresponds to Approximately 4×10^5 **cells/ml. Duplicates Behaved Similarly** (not shown)**.**

4.4.10 Primary Effluent and SRS Soil Microcosms

Finally, the objectives and methods of the previous study were applied to a soil system. Fifty grams SRS soil was employed and amended with MSM and either 1% or 10% (v/v) raw primary effluent. However, the organisms in the primary effluent were probably not as active as they had been in the previous study. The primary effluent has been stored in the dark, at 4ºC in a sealed bottle for approximately three weeks. These storage conditions most likely led to anaerobic conditions, which could have negatively affected some organisms. Similar results were observed in this microcosm study as in the previous study conducted without soil; however, lower inoculation levels required more time to degrade the same amount of cDCE than did the higher inoculation levels (Figure 4.354.35, Figure 4.364.36, and Figure 4.374.37). One can speculate that the soil offers niches for JS666 to effectively hide and escape predation, even at the high concentration of raw primary effluent, or that the soil offers some other type of protection. van Veen *et al.*(1997) report that soil can provide "microhabitats" that can be protective habitats from adverse conditions, including chemicals such as chloroform. In fact, the higher percentage of primary effluent seemed to help complete degradation at the low inoculation level. This could be due to the presence of a rich carbon source or other nutrients that aided the growth of JS666.

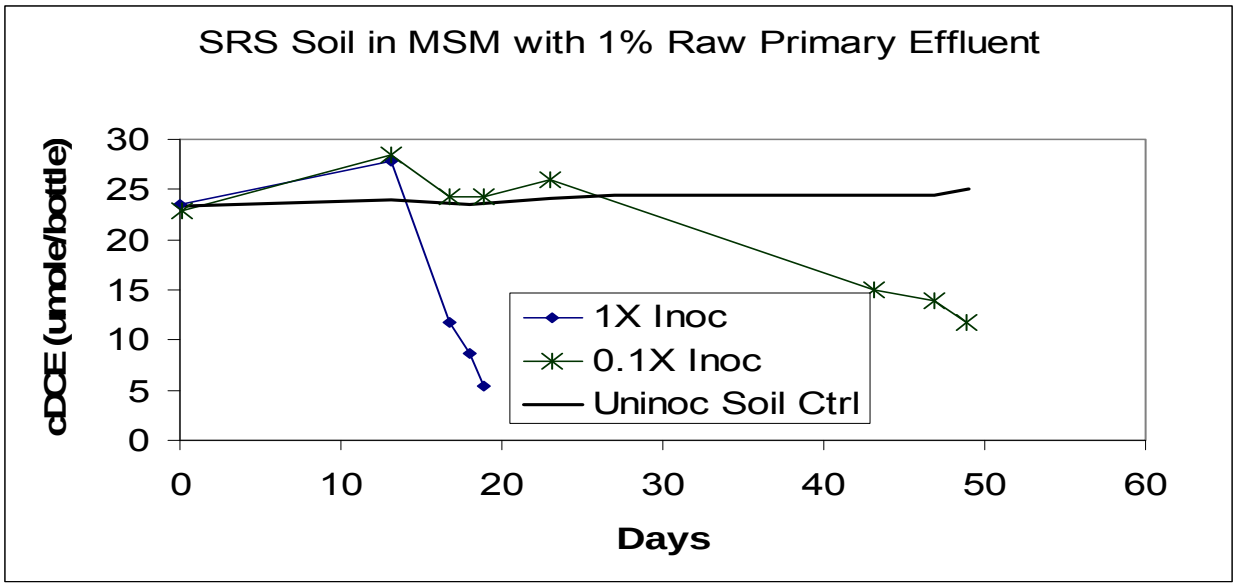


Figure 4.35 SRS Soil Amended With MSM and 1% Raw Primary Effluent and Inoculated at Two Levels. For reference 1X Corresponds to Approximately 4×10^5 **cells/ml. Duplicates Behaved Similarly** (not shown)**.**

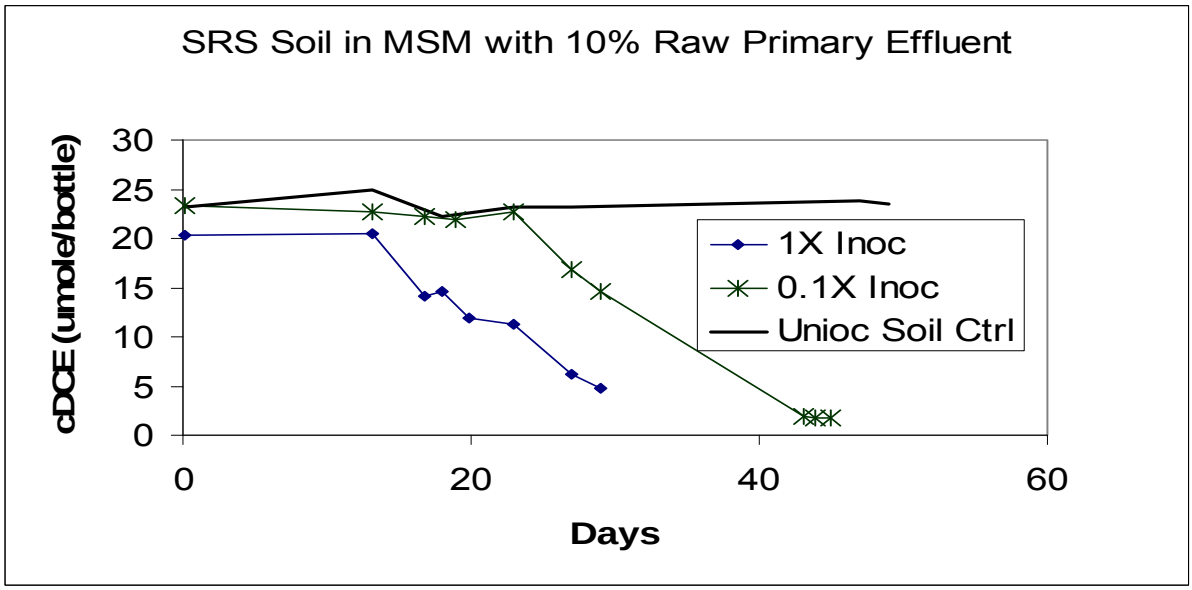


Figure 4.36 SRS Soil Amended with MSM and 10% Raw Primary Effluent and Inoculated at Two Levels. For Reference 1X Corresponds to Approximately 4×10^5 **cells/ml. Duplicates Behaved Similarly** (not shown)**.**

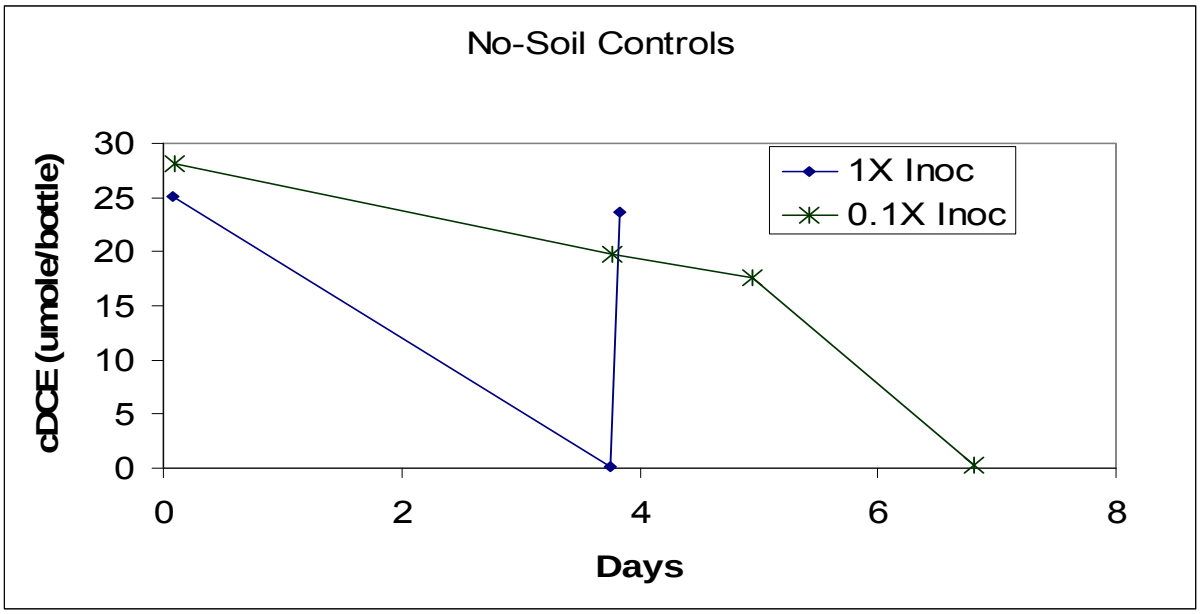


Figure 4.37 No-soil MSM Controls Inoculated at Two Levels. For reference 1X Corresponds to Approximately 4×10^5 **cells/ml. Duplicates Behaved Similarly** (not shown)**.**

5. SUBTASK 1.8. ADDITIONAL MICROBIAL ECOLOGY WORK.

5.1 Objective:

The objective of Subtask 8 was to determine whether the ability to degrade cDCE is transmissible. In spite of the presence of at least two possible means of gene horizontal transfer, that is the two megaplasmids and the Mu-like bacteriophage, the ability to degrade cDCE is not readily transmissible between bacteria under laboratory conditions.

5.2 Material and Methods

5.2.1 Growth Conditions

JS666 was grown on ½ MSB media containing cDCE as described above. For growth on rich media, cDCE was omitted and media was supplemented with 10mM succinate and 0.1% yeast extract (MSY medium).

5.2.2 Plasmid Curing by Growth in Non-selective Media

Cultures were transferred ten times (5% inoculum) under non-selective conditions (MSY medium), and then diluted and plated onto rich media (¼ Tryptic Soy Agar) plates. Single colonies arising on plates were used to inoculate 5 mL of MSY media, and grown to early stationary phase. Approximately 10^7 cells were embedded in Low Melting Temperature agarose (Sigma), and the cells were lysed by proteinase K and SDS. The stability of the megaplasmids when JS666 was grown in the absence of cDCE was confirmed by Pulsed Field Gel Electrophoresis (PFGE). PFGE was performed with a Bio-Rad CHEF-DR II system using the conditions described by (Krum and Ensign, 2001 #3).

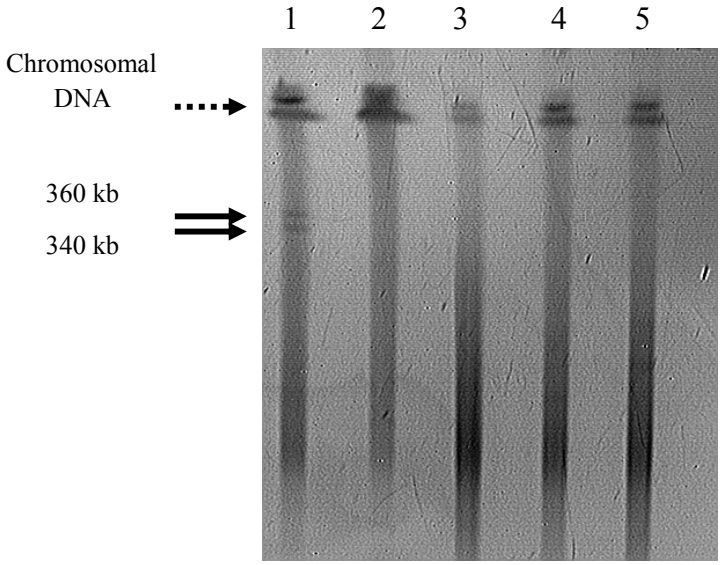


Figure 5.1 PFGE Analysis of Megaplasmid Content of JS666. *Lane 1*, JS666 DNA Subcultured at Least Ten Times in the Absence of cDCE. *Lanes 2-5*, Extraneous Samples from other Bacteria. The *Broken Arrow* Shows the Position of Chromosomal DNA. The *Solid Arrows* Show the Position of the 360 kb and 340 kb Megaplasmids.

5.2.3 Plasmid Curing by DNA Damaging Agents

Next, attempts were made to cure JS666 of its megaplasmids by subculturing on MSY medium in the presence of the DNA damaging agents, acridine orange (AO), ethidium bromide (EtBr), or mytomicin C (MMC). JS666 was subcultured 1:40 into MSY medium with increasing concentrations of AO, EtBr, or MMC. Experiments were performed in duplicate. After one week, growth was measured.

Table 5.1 DNA Damaging Agents Tested.

Compound	Minimum Inhibitory Concentration (μg/ml)
Acridine Orange	1.6
Ethidium Bromide	1.6
Mytomicin C	0.8

JS666 cultures grown in the presence of subinhibitory concentrations of each agent (0.8 μg/ml AO, 0.8 μg/ml EtBr; 0.4 μg/ml MMC) were subcultured again onto MSY medium with no added inhibitor. After 1 week, serial dilutions were made and the dilutions were plated on ¼ TSA. After about one week, several hundred single colonies arose on the ¼ TSA plates. Approximately 300 colonies total (about 96 from each treatment) were screened by PCR followed by gel electrophoresis for the presence of the megaplasmids.

PCR Screen for the Megaplasmids

A PCR screen for unique genes on each of the megaplasmids was developed. The genes chosen were: a putative acriflavin resistance gene, Bpro_5560, on the 340 kb megaplasmid; and a putative phenylalanine/histidine ammonia-lyase, Bpro_5198, on the 360 kb megaplasmid (Table 5.2).

Table 5.2 Target Genes and Primers for Megaplasmids.

Target Gene	Location	Amplicon Size	Primers
acriflavin resistance protein, Bpro_5560	340 kb megaplasmid	904 bp	Forward: 5'-TGGCGAATCTCTTTGTCACCTCCA-'3 Reverse: 5'-TTACTACCGGCTTTCTTGGCCACT-'3
phenylalanine/histidine ammonia-lyase, Bpro_5198	360 kb megaplasmid	529 bp	Forward: 5'-GCGCAATTGGTGGTGCAAATCAAC-'3 Reverse: 5'-GCTCAGCTTGCAGGCGTAAATCAA-'3

All tested JS666 colonies arising from the AO, EtBr, or MMC treatments (about 300) were positive for the presence of both the 340kb and 360 kb megaplasmids. A negative control of the related bacterium, *Polaromonas napthalenivorans* strain CJ2, was negative for both megaplasmid markers (data not shown). A representative gel of 5 AO treated colonies (Figure 5.2) shows the presence of both megaplasmid markers.

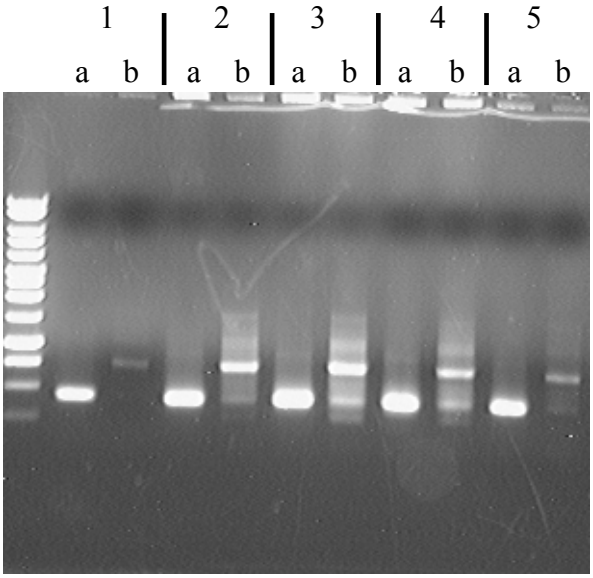


Figure 5.2 Representative Gel of 5 AO – Treated Colonies.

JS666 megaplasmids are stable in the presence of acridine orange. *Lanes 1, 2, 3, 4, and 5*: Five individual JS666 colonies treated with acridine orange as described above. *Lanes 1-5a:* PCR for 360 kb megaplasmid gene; *Lanes 1-5b:* PCR for 340 kb megaplasmid gene.

Conclusions, Analysis of the Megaplasmid of JS666

JS666 contains 2 megaplasmids of about 340kb and 360kb in size. Our long term goal was to determine if the genes involved in the degradation of cDCE were located on the megaplasmid(s), and if so, were they transmissible to other bacteria. The megaplasmids of JS666 were found to be stable during growth on media lacking cDCE and were also stable during growth in the presence of DNA damaging agents. The presence of predicted plasmid stabilization genes (such as partition and segregation genes, and toxin/antitoxin genes) on each megaplasmid (www.jgi.doe.gov) likely accounts for the inability to cure the megaplasmids from JS666.

5.2.4 Conjugal Transfer of the Genes for cDCE Degradation

Polaromonas napthalenivorans strain CJ2, a close relative of strain JS666, was tested for growth with cDCE as the sole carbon and energy source. Computer annotation suggests that strain CJ2 has the necessary genes for 1,2-dichloroethane degradation (www.jgi.doe.gov). Therefore, it is possible that strain CJ2 could grow on cDCE. Strain CJ2 showed no detectable growth on MSB

medium supplemented with 100 μM cDCE after 9 days of incubation, confirming that strain CJ2 lacks the genes for cDCE growth. A control culture of strain JS666 inoculated in the same medium showed noticeable growth (O.D.600nm of 0.1) after 7 days.

Mating experiments were performed in an attempt to transfer the cDCE degradation ability from JS666 ($cDCE^+$, $rifampicin^{sens}$) to *P. naphthalenivorans* CJ2 ($cDCE^-$, $rifampicin^{resist}$). Five mL cultures of each strain were grown to early stationary phase on MSY medium, spun down, mixed in a 1:1 ratio, and then spotted onto the surface of a 0.2 micron filter on the surface of a ¼ TSA plate. Mating mixtures were left overnight at room temperature. Mating mixtures were then resuspended in ½ MSB medium supplemented with cDCE and 50 μg/mL rifampicin. Neither growth nor cDCE disappearance occurred, as compared to uninoculated controls, indicating that the ability to degrade cDCE was not transmissible from JS666 to CJ2 in this experiment.

Next, the transfer of the cDCE degradation genes from JS666 to strain CJ2 in the presence of cDCE was tested. Mating mixtures of the two cultures were spotted on the surface of solid minimal media (1/2 MSB) with cDCE added to the headspace. After 1 week, mating mixtures were collected, resuspended in ½ MSB then spread onto the surface of 1/2 MSB + 50 μg/mL rifampicin plates, and finally the plates were incubated under a headspace of cDCE, or on the bench-top (no cDCE). After approximately three weeks, small colonies appeared on plates incubated in the presence of cDCE (about 10^9 per mL); however, colonies of the same size appeared on plates incubated in the absence of cDCE (also about 10^9 per mL). Two hundred were screened for growth on cDCE using a 96-well microplate assay as follows. Colonies were taken from plates incubated with cDCE and transferred to wells (in a 96-well microplate array) containing fresh ½ MSB supplemented with 15 mg/mL bromothymol blue. Plates were incubated with a headspace of cDCE. None of the colonies tested grew, as measured by a color change, after two months of incubation.

<u>Conclusions- No Conjugal Transfer of the Genes for cDCE Degradation</u>

Horizontal gene transfer of the genes for cDCE degradation from JS666 to CJ2 was not demonstrated in the lab. This may be due to the difficulty of induction/expression of the genes for cDCE degradation in a new host (CJ2) after growth on rich media, or it may be due to the presence of multiple genes, on several replicons, all of which must be successfully transferred to obtain a $cDCE^+$ host. Elucidation of the pathway and genes for cDCE degradation by JS666 will enable a definitive answer to this question.

5.2.5 Phage Transduction

JS666 has several genes that are annotated as phage Mu proteins. Wild type phage Mu is a temperate bacteriophage of *E. coli* and has been shown to integrate randomly in its host, and to transduce host DNA (about 2-5 kb) to other strains of Enterobacteria. The JS666 Mu-like phage genes are located on the chromosome, in two divergently transcribed *ca.* 15 kb operons (for a

total of about 30 kb; http://genome.ornl.gov/microbial/bpro_js666/). The presence of multiple genes, which together appear to encode all the necessary phage assembly and structure functions, suggests that JS666 harbors at least one bona fide Mu-like phage.

Table 5.3 Mu-like Phage Genes in JS666.

Mu-like Phage Genes in JS666	Locus Tag Number
Phage Mu tail fibre protein	Bpro_3731
Hypothetical (no COG)	Bpro_3732
Phage Mu baseplate protein	Bpro_3733
Phage Mu protein gp36	Bpro_3734
Phage Mu baseplate assembly protein gp45	Bpro_3735
Phage Mu tail protein gpP	Bpro_3736
Phage Mu DNA circulation protein	Bpro_3737
Hypothetical (no COG)	Bpro_3738-Bpro_3740
Phage Mu tail sheath protein gpL	Bpro_3741
Hypothetical (no COG)	Bpro_3742
Phage Mu protein gp37	Bpro_3743
Phage Mu protein gp36	Bpro_3744
Hypothetical (no COG)	Bpro_3745-Bpro_3748
Phage Lambda repressor	Bpro_3749
Phage Lambda repressor	Bpro_3750
Hypothetical (no COG)	Bpro_3751
Phage Mu Integrase	Bpro_3752
Phage Mu RNA helicase	Bpro_3753
Hypothetical (no COG)	Bpro_3754-Bpro_3771
Phage Mu transcriptional regulator	Bpro_3772
Hypothetical (no COG)	Bpro_3773-Bpro_3774
Amidase	Bpro_3775
Hypothetical (no COG)	Bpro_3776-Bpro_3777
Transcriptional regulator	Bpro_3778
Phage exported protein	Bpro_3779
Hypothetical phage proteins	Bpro_3780-Bpro_3781
Phage Mu protein gp28	Bpro_3782
Phage Mu protein gp29	Bpro_3783
Phage Mu protein gp30	Bpro_3784
Phage Mu protein gpG	Bpro_3785

Mu-like phage genes annotated in JS666 are shown in Table 5.3. These genes are arranged in two divergently transcribed operons at approximately position 4.0 Mb on the chromosome.

Detection of Phage in Culture Supernatants

Fifty ml liquid culture of JS666 on MSY medium was grown to early stationary phase and then filtered through a 0.2 micron filter to remove cells. Next, the filtrate was treated by incubating with 100 ug/mL DNase and RNase for 4 hours at 37ºC to digest free (not phage encapsulated) nucleic acid. Phage nucleic acid, encapsulated in protein, should remain undigested. Remaining nucleic acid, presumably phage, was precipitated by ZnCl and concentrated by ethanol precipitation as previously described {Santos, 1991 #608}. The presence of phage nucleic acid was tested by gel electrophoresis. Unfortunately, no DNA was detected by gel electrophoresis (data not shown).

Detection of Phage-mediated Transduction of Genetic Markers

We tested if the putative phage of JS666 is able to transfer genetic markers between strains of JS666 via transduction as described previously {Rapp, 1987 #609}. We isolated a spontaneous ethidium bromide resistant and a spontaneous nalidixic acid resistant derivative of JS666 (designated JS666 strain $EtBr^{resist}$ and JS666 strain Nx^{resist}) by plating 10^9 cells of JS666 onto the surface of MSY plates supplemented with either 3.2 µg/mL EtBr or 50 µg/mL nalidixic acid. For phage-mediated transduction assays, the wild type strain JS666 was the recipient. Phage was prepared by collecting supernatants from 10 mL cultures of JS666 strain $EtBr^{resist}$ or JS666 strain Nx^{resist} and passing the supernatants through a 0.2 micron filter. Phage was incubated with 10 mL of wild type JS666 overnight at room temperature and then plated onto MSY media containing 3.2 µg/mL EtBR or 50 µg/mL nalidixic acid.

Table 5.4 Antibiotic-resistance of Donor Phenotype.

Donor phenotype	Anitbiotic-resistant cfus per mL	
	- filtrate	+ filtrate
$EtBr^{resist}$	0.2	0.4
Nx^{resist}	2.4	26.4

Culture supernatants of JS666 strain Nx^{resist} are apparently able to transfer the nalidixic acid resistant phenotype to wild type JS666 at a frequency at least 10-fold higher than the frequency of spontaneous mutation rate of wild type JS666. This suggests that the JS666 phage is indeed active. However, no evidence for the transfer of the ethidium bromide resistance phenotype between strains was obtained. It is unlikely the JS666 phage can transfer the genes for cDCE degradation, as the results described above for the conjugal transfer of the genes for cDCE degradation were not designed to rule out the possibility of phage, and were negative at any rate.

5.2.6 Conclusions

- The two megaplasmids were not demonstrated to be transmissible. We were unable to cure the megaplasmids from JS666 by growth on nonselective media, or by growth in presence of the DNA damaging agents acridine orange, ethidium bromide, or mitomycin C. As we were unable to cure wild-type JS666 of its plasmids, we were therefore unable to demonstrate conjugation between wild type JS666 and a plasmid-cured derivative of JS666.
- Experiments were performed to transfer the cDCE degradation ability from JS666 to *P. naphthalenivorans* CJ2 by conjugation on solid surface of rich media. No evidence for transfer of cDCE degradation genes to *P. naphthalenivorans* CJ2 was found.
- The presence of a Mu-like phage was tested. The apparent transfer of nalidixic acid resistance to nonresistant JS666 by culture supernatant indicates an active phage.

6. IMPLICATIONS OF RESEARCH

Results of these experiments lend promise to the eventual commercial-scale use of JS666 as a bioaugmentation agent. Cells that have been stored or stockpiled over a short period of time can rapidly recover the ability to degrade cDCE, lending promise to the culture's in-situ activity following transport to and subsequent injection at a suitable field site. Kinetic assays have also allowed for estimation of biodegradation rates (e.g., cDCE degrades at 120 ug/L/day at 23°C using a 4 x 10^4 cells/mL inoculum). Because JS666 can co-metabolize trans-1,2-dichloroethene (cDCE), TCE, VC, 1,2-DCA, or ethene while growing on cDCE, its application can be expanded to sites containing not only cDCE but mixtures of cDCE and these other co-contaminants. Furthermore, microcosms studies conducted with a range of aquifer materials suggest that JS666 will survive and remain active in subsurface environments. Experiments with various inoculum levels suggest that 10^4 cells/mL will be an effective dose of JS666. Unfortunately, no evidence was found to suggest that the ability to degrade cDCE can be transferred from JS666 to indigenous bacteria. As such, when assessing bioaugmentation with JS666 as a remedial alternative it is necessary to ensure that the conditions at the site are suitable for JS666 to thrive. Ideal conditions for JS666 include pH conditions above 6.5, dissolved oxygen levels from >0.01 to <8 mg/L, and low conductivity (<15 mS/cm).

7. REFERENCES

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