Summary Report on the Aerobic Degradation of Diesel Fuel and the Degradation of Toluene Under Aerobic, Denitrifying and Sulfate Reducing Conditions

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DISCLAIMER

Introduction

The group of monoaromatic hydrocarbons known as BTEX (benzene, toluene, ethylbenzene, and the xylenes) and their presence in groundwater and soils is a widespread problem due primarily to the leakage of underground storage tanks at petroleum production wells, refineries, pipelines and distribution terminals (Fries et al., 1994). Guidelines have been established by the US Environmental Protection Agency for the cleanup of these chemicals in groundwater because of their carcinogenic potential (EPA, 1977). Although these aromatic hydrocarbons are relatively water soluble, they are in large part contained in the insoluble organic phase of the fuel which serves as a slow-release mechanism ensuring sustained, long-term contamination of groundwater sources (Hutchins et al,. 1991). Pump-and-treat technology is impractical for the restoration of aquifers contaminated with bulk fuel, because the dynamics of immiscible fluid flow result in prohibitively long time periods for removal of the organic phase (Wilson and Conrad, 1984).

The biodegradation of the components of BTEX under aerobic conditions is well established; oxygen is utilized for the activation of the ring and cleavage and serves also as the electron acceptor for complete oxidation (Gibson and Subramanian, 1984). In addition, aerobic biorestoration has been shown to be effective for many fuel spills (Thomas et al., 1987; Lee et al., 1988) However, at contamination sites involving soils and groundwater, oxygen is the limiting factor for the degradation of these components. This is due to the consumption of oxygen during aerobic hydrocarbon degradation, and the low water solubility of oxygen (Wilson et al., 1986).

The initial report of microbial mineralization of toluene and 1,3-dimethylbenzene in the absence of molecular oxygen was made by Kuhn and Schwarzenbach in 1986. They reported complete mineralization of these compounds under denitrifying conditions. Since, it has been widely reported that nitrate can serve as an electron acceptor during the anaerobic degradation of BTEX compounds by the processes of nitrate reduction and denitrification (Zeyer et al. 1986; Dolfing et al. 1990; Evans et al. 1991; Hutchins et al. 1991a; Altenschmidt and Fuchs, 1991). For example, organisms of the genus *Azoarcus,*

which are motile, gram-negative rods, were isolated and characterized by Fries et al., 1994 that were capable of toluene degradation under anaerobic, denitrifying conditions using nitrate as an electron acceptor. These denitrifying, toluene degrading isolates were common: these organisms were isolated from more than half the samples tested, from diverse sources including agricultural soils, compost, aquifer material, and contaminated soil samples from different regions of the world. An *in situ* field demonstration by Hutchins et al., 1991b provided a quantitative description of the effect of nitrate addition on the disappearance of BTEX in an aquifer contaminated with JP-4 jet fuel.

Summary of Accomplishments

Diesel Fuel Degradation by *Rhodococcus erythropolis*. The purpose of the initial subcontract awarded to NMSU was to determine kinetic rates of diesel fuel degradation by a novel strain of *Rhodococcus erythropolis* (designated A3, supplied by Jim Brainard, CST-3), a gram-positive, cocci-shaped bacterium. A3 was originally isolated from soil contaminated with used motor oil found outside of an automotive garage in Albuquerque, NM. Various other organisms that were capable of hydrocarbon degradation were also isolated from this soil. After initial testing, A3 was determined to have the greatest kinetic potential in terms of oil degradation. The reason this organism is so well suited to hydrocarbon degradation is probably due to its production of a biosurfactant, either membrane bound or extracellularly produced (unpublished data). Kinetic rates of degradation of A3 (substrate: diesel fuel) were to be made respirometrically by the measurement of oxygen consumption along with GC analysis, in shake culture and by biomass determination. Furthermore, studies were to be performed to determine the effects of media composition and diesel fuel loading rate on biodegradation. The results of this study are described here.

Over the course of ten experiments with the respirometer as the primary tool of analysis, A3 was seen to exhibit no ability to degrade diesel fuel at concentrations of 0.01, 0.1 and 1.0% (wt/wt) diesel fuel under conditions (sealed atmosphere) as are present in the respirometric reactors. It is believed that some of the shorter-chain, volatile organic constituents of the diesel fuel are inhibitory to A3 as in shake culture (where cultures are

open to the atmosphere) the organism seems capable of emulsifying the diesel fuel and increases in turbidity are always evident. However, in respirometric culture, A3 exhibited no ability to solubilize the diesel fuel and cultures appeared to remain static.

Variances in the media composition with regards to the type of media, vitamin addition and diesel fuel loading rates yielded no positive results except in batch culture. A new mineral salts medium, developed specifically for organisms of the genus *Rhodococcus* (Aiking et al., 1982), and vitamin B1 (thiamin) and/or myo-inositol were used and observed to greatly enhance the growth of A3 in batch culture. A3 seemed quite capable of tolerating loading rates of diesel fuel as high as 1.0% (wt/wt) in batch culture. Further experiments with the B1 and myo-inositol addition, suggested by Craig Vester, CST-3, were performed to ascertain the benefit of these compounds and clear results were obtained with regards to the beneficial effect of these amendments.

Tests were also performed in shake culture with GC headspace analysis and plate counting as the primary methods of diesel fuel degradation and biomass generation. The tests were performed in sealed (to the atmosphere) vials and samples were taken for plate counts and the headspace was analyzed for disappearance of volatile components of diesel fuel, indicating degradation. However, negative data was observed in these trials as well.

A3 was initially thought to be a good candidate for the degradation of toluene and initial aerobic degradation work was done with this organism while the subcontractor awaited the arrival of soil samples, when work would begin dealing with the enrichment for the degradation of toluene under aerobic, denitrifying, and sulfate-reducing conditions. The soil samples were obtained from two contaminated sites in Los Alamos county. One site was contaminated with gasoline (samples from two depths received) and the other with diesel fuel (sample from one depth received). Another soil sample (uncontaminated, from one depth) was also received. Several studies have been performed with these samples with regards to total numbers of bacteria present, most probable numbers (MPN) tests for denitrifying and sulfate-reducing organisms, and enrichments for the degradation of toluene under both denitrifying and sulfate-reducing conditions.

Positive results for the degradation of toluene by A3 were obtained with the respirometer. Two tests were performed to determine the ability of A3 to degrade

toluene. In addition to these tests, growth curves conducted with glucose were generated to determine the effect of the addition of B1 and myo-inositol on the growth of A3. The effect was clearly seen to be beneficial. During these tests, it was also ruled out that A3 might use the buffer HEPES (Sigma) as a source of carbon.

BTEX Degradation by Soil Isolates. Attention was then turned to the contaminated soil samples received from Craig Vester at Los Alamos. This most recent work (from May, 1995) is not funded by LANL but supported by Dr. Geoffrey Smith and the resources available to him in his environmental microbiology lab. These samples were tested for denitrification and sulfate-reducing activity by the most probable numbers assay (MPN) and positive results were seen in both conditions. This led to the initiation of enrichments of these soils to determine if toluene could be mineralized under aerobic, denitrifying (nitrate as electron acceptor) and sulfate-reducing (sulfate as electron acceptor) conditions. Positive results (degradation of toluene compared to sterile controls) were obtained and plans are set for enrichment transfers and a more accurate determination of the rates of toluene degradation on a per cell or per milligram protein basis. It is hoped that these final results may provide a means of remediating the contamination plumes in Los Alamos county by a technique such as the simple fertilization of the soils with the necessary electron acceptors (ie., nitrate and/or sulfate). Biodeg of BTEX compounds in soils under aerobic conditions is well studied but the problem that limits degradation at plume sites is most often the unavailability of electron acceptors. Oxygen becomes limiting at these sites because of its low solubility in water and its low rate of transport through saturated porous matrices like soil and sediments. It is for these reasons that stimulation of BTEX degradation under anaerobic conditions would be a very beneficial process.

Methods

Aerobic Degradation of Diesel Fuel-Respirometry. Utilizing an eight reactor, pneumatically controlled respirometer (N-Con, Larchmont, NY) obtained from Los Alamos, a total of 10 experiments designed to measure the oxygen uptake of cultures of A3, mineralizing diesel fuel as a sole source of carbon, were performed. Upon delivery of the respirometer to New Mexico State University, it was in need of repair. With the assistance of Dr. Fernando Cadena (Civil Engineering, NMSU), the instrument was rebuilt a total of three times and all leaks in the system were sealed. The instrument was then calibrated and tested with cultures of A3 grown on Nutrient Broth (Difco). Three of these tests were performed to implement measures to control the contamination of the cultures as the instrument is not well designed for pure culture work.

The first four respirometer experiments were performed with Bushnell-Haas (Difco) broth, a medium designed for hydrocarbon degradation. Experimental manipulations during these trials were the variation of loading rates of diesel fuel (0.01, 0.1 and 1.0%) and the addition of vitamin B1 to cultures. Positive controls (inocula plus nutrient broth) were included in all but the first and second runs with the respirometer. Negative controls (no inocula plus nutrient broth) were included in all but the first respirometer run.

The next series of respirometer runs included drastic increases in inoculum size (including the addition of whole batch culture that had evidenced solubilization of diesel fuel and turbidity), the addition of myo-inositol to cultures, and the pre-volatilization of diesel fuel before addition to cultures to remove possibly toxic components of the diesel fuel.

Aerobic Degradation of Diesel Fuel-Shake Culture. Duplicate cultures were prepared in flasks under two conditions at a concentration of 0.1% diesel fuel. One set of cultures was incubated capped and therefor sealed to the atmosphere (with enough headspace for adequate oxygen). The other set of cultures was incubated with loose-fitting aluminum foil. The cultures were incubated for four days (96 hours).

In a second experiment, six 240-mL Voavials were prepared and amended with 60 mL of MSM + B1 and myo-inositol. Four vials were capped with teflon-lined septa (two for plate counts and two for negative controls (without substrate) for background plate

counts) and two were capped with Mininert valves for headspace sampling and GC analysis. Cultures were plated after inoculation and before the addition of diesel fuel (0.1%) for a zero timepoint. Zero timepoints were also taken after the addition of diesel fuel for headspace analysis. A standard curve for diesel fuel analysis was also generated to give accurate determinations of diesel fuel concentration in the cultures.

Aerobic Toluene Degradation by A3. Two respirometer experiments were performed with A3 to determine is ability to utilize toluene as a sole source of carbon. The prepared reactor cultures contained the *Rhodococcus* specific mineral salts medium, B1 and myo-inositol. Toluene concentrations were varied at 20, 100, and 1000 ppm. Positive controls containing 0.1% glucose and toxicity tests containing 100 and 1000 ppm toluene plus 0.1% glucose were also prepared. Inocula for both experiments came from a culture of A3 adapted to toluene at a concentration of 10 ppm.

Effect of HEPES, B1, and myo-inositol addition on the Growth of A3. A shake culture test was set up to determine whether A3 had a biochemical need for B1 and/or myo-inositol and also to test whether A3 could utulize HEPES buffer as a carbon source (as HEPES was the buffer used in the A3/toluene degradation respirometer experiments). Samples were measured for optical density by spectrophotometry at 540nm. Phosphate buffered MSM was used in 100 mL volumes with the following amendments:

negative control (inoculated with no carbon source)
negative control (inoculated with the addition of myo-inositol and B1)
0.1% glucose
0.1% glucose + myo-inositol and B1
24mM HEPES
24mM HEPES + myo-inositol and B1
0.1% glucose + 24mM HEPES
0.1% glucose + 24mM HEPES + myo-inositol and B1

Subsequent plating of all cultures revealed pur cultures of A3.

Aerobic and Anaerobic Toluene Degradation by Contaminated soils.

Sample Designation:

10c	Uncontaminated soil from a depth of 0-10 feet
27g	Gasoline contaminated soil from a depth of 0-27 feet
68g	Gasoline contaminated soil from a depth of 60-80 feet
10d	Diesel fuel contaminated soil from a depth of 0-10 feet

Denitrifying Bacteria (DNB) MPNs. A total of three, three-tube MPN trials were performed on all soil samples to obtain an estimation of the number of denitrifying organisms present in the soils. 1/2 concentration nutrient broth (Difco) was amended with 5mM potassium nitrate (electron sink) and 9 mL was distributed into Hungate tubes (containing Durham tubes to trap nitrogen gas produced from the reduction of nitrate) and autoclaved with black butyl septa and caps. Septa were puncutred with 27 ga. Needles during autoclaving to allow venting of oxygen. After removing the tubes from the autoclave, they were immediately flushed with sterile nitrogen and evacuated under vacuum. This process (flush/evacuate) was repeated four times to ensure the removal of oxygen from the headspace of the tubes.

In the first MPN series, dilutions of 1g of each soil sample were made from 10E-01 through 10E-05. The second set contained dilutions of 10E-04 through 10E-08 and the third set contained dilutions of 10E-05 through 10E-11. Extraction media for the first two MPNs was Bushnell-Haas broth that contained 0.1% Tween 80 in the first extraction tube (10E-01). In the third MPN set, initial extraction of bacteria was made by blending 20 mL samples for 20 seconds, placing them on ice for 2 minutes, and blending them again for 20 seconds. These samples were then serially diluted to a final dilution of 10E-11. Subsamples of 1 mL of these dilutions were then inoculated into the corresponding Hungate tube (Tiedje et al., 1982).

Sulfate Reducing Bacteria (SRB) MPNs. Starkey's media was prepared at 5mM sulfate, 31mM lactate and 31mM butyrate (carbon sources). The ingredients for the rest of the media preparation are as follows (in g/L):

KH2PO4	3.5
NH4Cl	1.0
K2HPO4	0.5
MgSO4 7H20	2.0
CaCl2 2H2O	0.1
Mohr's Salt	0.010

Sulfate reducing activity was identified by the production of sulfide and the formation of a black precipitate (FeS). The same process to extract the soils was followed as is listed for the protocol for DNB MPNs except that the extraction media was Starkey's without the lactate or butyrate. The initial extraction tube contained 0.1% Tween-80. Dilutions were

carried out from 10^{-1} to 10^{-5}. Tubes were allowed to incubate for four weeks before reading.

Aerobic, DNB and SRB Enrichments. Triplicate samples were prepared for each of the four soils under both denitrifying and sulfate-reducing conditions. For all samples, BSM 337 (Poindexter, 1992) was prepared and amended with sulfate or nitrate as was appropriate. The media preparation is as follows (expressed in millimolar concentrations):

Chemical	Aerobic	DNB	SRB
KH2PO4	1	1	1
Na2HPO4	1	1	1
MgSO4 7H2O	0.8	0.8	0.8
FeSO4	0.007	0.007	0.007
MnCl2	0.0018	0.0018	0.0018
Na2MoO4	0.0021	0.0021	0.0021
(NH4)4SO4	3.8	0	3.8
Na2SO4	0.4	0	0.4
KNO3	5	5	0

14 mL of appropriate BSM 337 was added to serum vials and loosely capped with crimp-top, teflon-lined septa. Samples were then autoclaved. Upon removal from the autoclave, septa were crimped on tight immediately and samples were flushed with nitrogen and evacuated four times to remove oxygen from the headspace. After this process, reducing agent (248uM Na2S and 496uM cysteine) was added to the sulfate-reducing conditions and resazurin (indictor) was added to both denitrifying and sulfate-reducing conditions. 300 mg of each soil sample was then added to each vial under a sterile stream of nitrogen to keep oxygen from entering the vials.

Vials were then incubated overnight and then analyzed by GC headspace analysis for any volatile organic components. Toluene was then added to these samples at an initial concentration of 5ppm. Samples were analyzed by GC for toluene two hours later for a zero time and again at 24 hours. Eight days later, samples were spiked with 10 ppm toluene, analyzed two hours later and again 24 hours later. This procedure was repeated and the samples analyzed at 2, 12 and 24 hours post-spike.

Sterile controls were also prepared at the time of the second addition of toluene to insure that any loss of toluene in the GC analysis would not be due to escape from the vials or by sorption to soils. This was done by adding 300mg of each of the four soils to

14.7 mL of BSM 337, autoclaving, waiting 24 hours and then autoclaving again. These samples were spiked with 10 ppm toluene and analyzed concurrently with the active samples although sampling of these preparations did not begin until the second addition of toluene to the Aerobic, DNB and SRB enrichments.

A standard curve for toluene analysis was also prepared to judge variance in GC analysis. Samples prepared for the standard curve were prepared in 30-mL serum vials with teflon lined septa with 15 mL distilled water and a 15 mL headspace. Concentrations of toluene in the standards were 10, 5, & 1 ppm. All GC analysis of enrichments, sterile controls and standards was performed on an HP 6890 Gas Chromatograph using a **** column and the detection method was FID. All sample volumes (headspace) were 10 uL.

Acridine Orange Direct Count Microscopy (AODC). The same dilution series of all soil samples used for MPN set I were also used for AODCs to determine total numbers of bacteria present in the samples.

The procedure for the first set of AODCs were performed as follows: 50 uL of acridine orange was added to 500uL of the 10^{-1} to 10^{-3} dilutions and incubated at room temperature for 5 minutes. 500 uL of the stained preparation was filtered with Nuclepore (0.2uM) polycarbonate black filters after the filters were rinsed with 0.1% Tween 20. The filters were then viewed under epifluourescent microscopy.

For the second set of AODCs, the dilution series used for the DNB MPN III set was refrigerated and saved for staining. 100 uL of acridine orange and 400 uL of phosphate buffer (pH 7.2) was added to 500 uL of the 10^{-2} dilution of the 10d sample and incubated for five minutes. Duplicate filters were treated identically except for the last step where under one condition, the filter was dried for 10 minutes under vacuum and air dried for 5 minutes, then rewetted with phosphate buffer before mounting to a slide under immersion oil and examined by epifluorscent microscopy. This treatement is recommended by Bitton et al., (1993). The other treatment was the normal procedure where filters were removed from the vacuum apparatus after the liquid had been pulled through. It is thought that the difference in the rewetting vs. not may have some effect on the color of the cells as viewed through the microscope (green vs. orange respectively),

thus, making it easier to identify cells against a background of soil matter that is invariably stained orange.

Results

Aerobic Degradation of Diesel Fuel - Respirometry and Shake Culture. Figure 1 depicts the type of data that the respirometer produces. This run was made with two concentrations of nutrient broth (4 and 2g/L). Averaging the total oxygen uptake of the triplicate reactors yields data for both experimental conditions that compare well to each other. The 2g/L nutrient broth reactors average 480 mg/L total oxygen uptake where the reactors that contain a two-fold increase in nutrient broth average 800 mg/L oxygen uptake. As can be seen, the total increase in oxygen uptake is also roughly two-fold. This data provided a sound basis for beginning work with the degradation of diesel fuel.

Figure 2 shows data obtained from the third run with the respirometer where attempts were made to degrade diesel fuel as a sole carbon source using A3 as the organism. Diesel fuel concentration in this run is 0.1%. As is evident, the only reactor that exhibited oxygen uptake in large amounts is the positive control that contained nutrient broth with A3 as the innoculum. All other reactors, negative control included, showed only transient initial oxygen uptake. At 70 hours, sterile yeast extract was added to three (6,7, & 8) of the diesel fuel reactors at a concentration of 100 ppm. These reactors proceeded to show oxygen uptake after about 40 hours more incubation time, indicating the presence of live organisms capable of respiration. The other two diesel fuel only cultures (4 & 5) exhibited no more oxygen uptake during this time. Subsequent plating of all reactors showed that A3 was still the sole organism present in all reactors, including reactors 4 and 5.

Further experiments were conducted to ascertain whether lower concentrations of diesel fuel in reactor culture would be more appropriate (less toxic). Vitamin B1 was also added to these cultures and concentrations of diesel fuel tested were 0.05 and 0.01%, the lowest concentration being two orders of magnitude lower than what was initially tested (1.0%) in the pilot run with diesel fuel. These tests yielded no positive data towards the degradation of diesel fuel. The addition of myo-inositol to cultures also had no effect, nor

did the switching of the medium used (Bushnell-Haas to *Rhodococcus* MSM). Also tested was whether we could evaporate some of the more volatile and therefor more toxic components of the diesel fuel, making the mix of hydrocarbons less toxic to the organisms. This was done by autoclaving neat diesel fuel and then placing it in a 55° water bath for one hour to further drive off volatile components. The respirometer test run with this diesel fuel also yielded no positive results.

Along with this study a parallel experiment was run in shake culture (the first shake experiment). The aerated cultures showed emulsification of the organic phase of diesel fuel early on (44 hrs) whereas the capped cultures did not. By 96 hours, no visible organic phase could be further seen in the aerated cultures while the capped cultures showed no signs of emulsification or turbidity. [This lead to the notion that using even evaporated diesel fuel in the respirometer reactors somehow interferred with the degradation of it.]

In addition to the above experiments, a respirometer experiment was performed using much larger inoculum (10% as opposed to 1 and 3%) sizes. Also included in this run was a 100% inoculum of a culture of A3 grown on diesel fuel in shake culture that was thought to be in log or near-log phase growth. None of the reactors showed any more oxygen uptake than other experiments previously conducted, including the culture that was thought to be in log phase growth.

Aerobic Toluene Degradation byA3. The two respirometry experiments conducted with A3 and toluene as a sole source of carbon yielded encouraging results. Figure 3 depicts the results of on of the experiments performed with A3 and toluene. As can be seen, oxygen uptake was observed in reactors that contained toluene as a sole carbon source. Reactor 2 is a negative control that was inoculated without any carbon source (glucose or toluene).

Effect of HEPES, B1, and myo-inositol addition on the Growth of A3. Figure 4 depicts the results of the test run to determine whether HEPES could be used by A3. Also included in this test were conditions that would allow the determination of whether A3 either needed or fared better with either myo-inositol or B1 as micronutrients.

Aerobic and Anaerobic Toluene Degradation by Contaminated soils.

Denitrifying MPNs and Sulfate Reducing MPNs. Both types of incubations of soil samples yielded positive results. Gas production (in the case of the denitrifying MPNs) and FeS formation (in the case of the sulfate reducing MPNs) were seen in the MPN trials. These positive results justified the incubations for the enrichments of organisms that could degrade toluene under nitrate-reducing and sulfate reducing conditions.

Aerobic, DNB and SRB Enrichments. Figure 5 shows the standard curve generated to compare GC analysis of Aerobic, DNB and SRB enrichments. Upon the first addition of toluene (at 5 ppm) to all enrichment sets analysis (at t= 24 hours) by GC showed that toluene was present in similar concentrations except for the 27g sample, where levels of toluene were three times lower than all other samples (both DNB and SRB samples), indicating higher activity in this sample (Figures 6 & 7). In the aerobic enrichments, toluene had already disappeared in all four samples (Figure 8).

Upon the second addition of toluene (at 10 ppm) to the samples, analysis one and a half days later showed the near complete disappearance of toluene in all of the DNB enrichments (figure 7) as well as in the SRB enrichments (figure 7). The aerobic samples showed the most activity with the complete disappearance of toluene in the 10c, 27g, and 10d samples (figure 8). The only sample that contained any toluene at 1.5 days was the 68g sample. It was prior to this second addition of toluene that the sterile controls discussed above were started. Therefor, the data represented in figures 6, 7, & 8 is somewhat misleading as the curve for the sterile controls should be transposed ahead by 14 days.

With the third spike of 10ppm toluene to the samples, it was determined that more timepoints for GC analysis should be taken within the first 24 hours of the post-spike period. As can be seen in Figure 6, it seems that toluene just about completely disappears in all but a couple of samples within 24 hours. The only samples that retained toluene at 24 hours were one (of triplicate samples) of the DNB 68g samples, one of the SRB 27g samples and the 68g aerobic toluene sample. From this 10 ppm spike on day 20 and the following series of analyses we were, however, able to obtain an estimated rates of

degradation relying on the zero timepoint (t=2hrs) and the 12 hour timepoint. The results are as follows in Table 3 below:

Sample		Rate of Degradation (mg/L/hr) *
ATOL	10c	0.21
	27g	indeterminate **
	68g	0.58
	10d	0.64
DNB	10c	0.68
	27g	indeterminate **
	68g	0.64
	10d	0.73
SRB	10c	0.66
	27g	0.7
	68g	0.88
	10d	0.6

* Extrapolated below lowest standard on standard curve (1ppm) for all samples.
** All sample values zero at t= 12 hours post-spike.

These rates were determined by comparing average peak areas to the standard curve seen in Figure 5.

AODCs. Currently, the method development described above in the methods section of this work is still underway. Previous results for AODCs were limited and difficult to interpret due to the background (soil particles) being stained orange along with the microorganisms.

Discussion

After the succesful refitting of the respirometer and the positive data obtained in the nutrient broth experiments, it was disappointing to find that no respirometric data of value could be obtained testing A3 and diesel fuel. There are three possible explanations to the negative results seen with diesel fuel in the respirometer. One is that some of the shorter chained, volatile components of the diesel fuel are toxic to the organisms. This is the most likely possibility as all carrying cultures (used for inocula) evidenced the solubilization/emulsification of the diesel fuel as well as turbidity within a short frame of time (roughly 48 hours from inoculation). Also, the tests conducted in shake culture of the two conditions where one set of cultures were sealed and the other left open to the

atmosphere lead to fairly conclusive results. In theory, if the toxicity hypothesis is accepted, the cultures that are capped should not show any growth or turbidity as the toxic, volatile components are unable to be blown off into the atmosphere, thus eliminating them from contact with the organisms. Likewise, the cultures that are left open to the atmoshpere would be expected to bloom with growth after these components have volatilized and been removed from the matrix.

Another possibility that would explain the negative results is that diesel fuel, in some way, interferes with the operation of the respirometer. This could be true if the volatile components in the diesel fuel were to, upon capping the reactors, volatilize and fill the headspace of the reactors with a positive pressure. Since the respirometer relies on the detection of a negative pressure inside of the reactors (generated from the transport of available oxygen in the headspace to the liquid phase of the media), any negative pressure produced by the equilibration of oxygen between the gaseous and liquid phases in the reactor could be equalized by the further volatilization of organic compounds. This scenerio is unlikely however, because toluene (a very volatile compound) does not produce this effect when tested in the respirometer. In either case, no kinetic rates of degradation of diesel could be produced despite best efforts.

Very good data was obtained on the degradation of toluene by A3 utilizing the respirometer. Figure 3 shows the data obtained from the first experiment. As can be seen, A3 showed an ability to withstand and degrade concentrations of toluene as high as 100 ppm. Reactors that contained both 0.1% glucose and 100 ppm toluene (7 & 8) showed very similar rates and total amounts of oxygen uptake when compared to cultures that contained glucose alone, indicating that 100 ppm had no detrimental (toxic) effects on the organisms. A subsequent respirometer experiment that raised toluene concentrations to 1000 ppm showed that that amount of toluene could not be tolerated by A3 (at least not without prior adaptation). Reactor 2 in figure 3, which was a replicate of reactor one that contained 0.1% glucose as a positive control, was shut down due to a leak in the reactor flask. The data obtained from reactors 3 through 6 show that A3 is capable of mineralizing toluene as a sole source of carbon.

The shake culture experiment was performed to ascertain whether A3 could utilize the buffer HEPES as a source of carbon. It was thought that if this were the case, respirometer results from the toluene experiments would be inaccurate as A3 may use HEPES preferentially over toluene as a source of carbon. This did not turn out to be true however. As can be seen in figure 4, it was shown that A3 did not use HEPES as a source of carbon (H and H+ cultures where HEPES was the only source of carbon). A previous experiment designed to test this also gave the same results. Samples in the previous experiment were prepared in duplicate. Also determined by the experiment shown in figure 4 was that A3 indeed has some requirement for myo-inositol and/or B1. By comparison of the glucose (G) and glucose + HEPES (GH) containing cultures with and without myo-inositol and B1 it can be seen that in cultures that contained a usable carbon source (glucose) plus the amendment of B1 and myo-inositol growth of the organisms reached log phase much earlier.

It is believed that the best information provided by the results of the SRB MPNs is to say that anaerobic, sulfate reducing bacteria are present in all of the soil samples. It was feared that numbers would be low, however, and that MPN results may be inconclusive without modification of the method to concentrate the sample. On the other hand, it was hoped that sulfate reducers present would be capable of degrading toluene, which appears to be the case.

All four samples of each set of enrichment conditions showed remarkable activity in the area of toluene degradation upon the first addition of 5 ppm toluene. This is the reason that adequate GC analysis was not performed to obtain rates of degradation after the first two additions of toluene to the samples. The disappearance of toluene simply occurred more rapidly than what was anticipated. It is thought that this may be due to the fact that three of the samples tested had been previously exposed to gasoline and diesel fuel, both of which contain toluene. Table 3 shows that one of the lowest rates of degradation occurred in the 10c sample which was taken from uncontaminated soil. To prove this, adequate sampling should have been performed upon the first addition of toluene to the enrichments however. Also, it is believed that a worn plunger in the syringe used to sample the vials during the first analysis for toluene may have contributed to low

and variable peak areas. The third addition of toluene did, however, yield data that could be used to determine rough estimates of degradation rates. The problem with the rates listed in Table 3 above is that the levels of toluene found at t= 12 hours (post-spike) fall below what could be reliably determined by the standard curve generated. Because of the linearity of the standard, however, a simple extrapolation could be made to render concentrations lower than what was detected at the lowest standard concentration. A more adequate curve for following the degradation of toluene must be generated, avoiding the circumstance where sampling for GC analysis yields peak areas to low to reliably generate rates of degradation. This initial data is encouraging for further testing.

Rates of degradation (taken from analysis of the third toluene addition) among the three conditions tested compare favorably with one another in that they ranged from 0.21 to 0.88 mg/L/hr or greater (for indeterminate samples). A seemingly narrow range of degradation. Confidence is high that the anaerobically prepared enrichments (denitrifying and sulfate-reducing) are indeed quite anaerobic as the indicator to colorimetrically determine those conditions (resazurin) indicates roughly -50mV in some of the denitrifying vials and close to -100mV in almost all of the sulfate reducing vials.

Figures 6, 7, and 8 show each of the enrichment conditions plotted against the sterile controls that were prepared as described above (it must be kept in mind that the sterile control curves should be transposed ahead). As can be seen, the peak areas found for the sterile controls, though variable, do not seem to be decreasing as a general trend. These controls were set up to mimic the live samples with respects to septa puncturing and frequency of sampling. As more data is accumulated in the live controls, the sterile controls will be statistically analyzed to produce a standard error and range of variation of GC data.

Overall, work has proceeded well toward the goals outlined in the introduction previously with regards to the degradation of toluene under aerobic, denitrifying and sulfate-reducing conditions. Future work will include more accurate rates of degradation for all of the enrichment conditions which will be on a per cell basis and accurate determinations of original numbers of total and denitrifying bacteria present.

Preliminary evidence suggests that there may be high numbers of target organisms present in the soil plumes that could be enhanced via *in situ* fertilization techniques, thus leading to the economical and effective cleanup of the contaminated sites.

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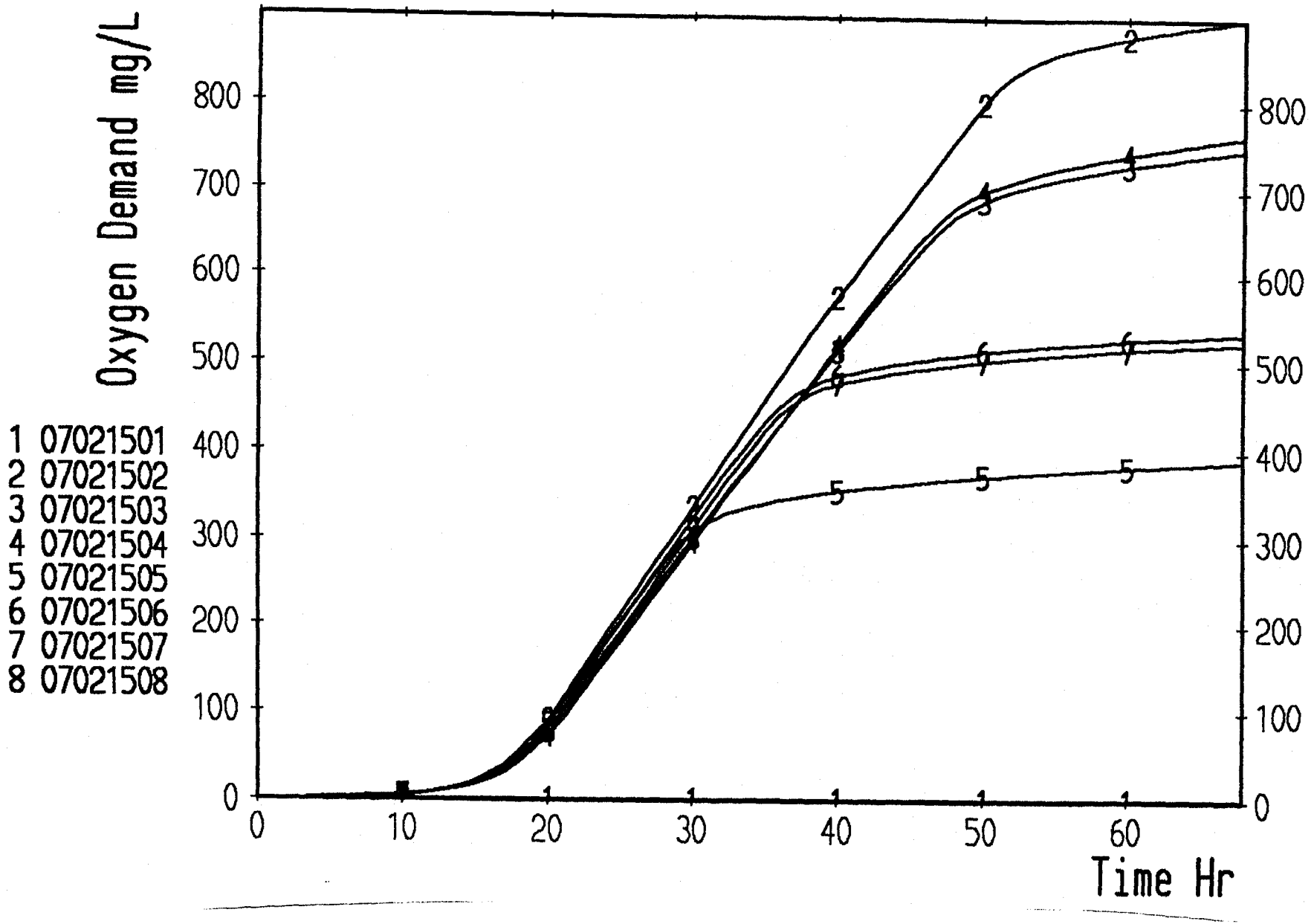

Figure 1. Respirometric data from initial respirometric experiment. Reactor 1 is a negative control, reactors 2-4 are 4 g/L nutrient broth and reactors 5-7 are 2g/L nutrient broth.

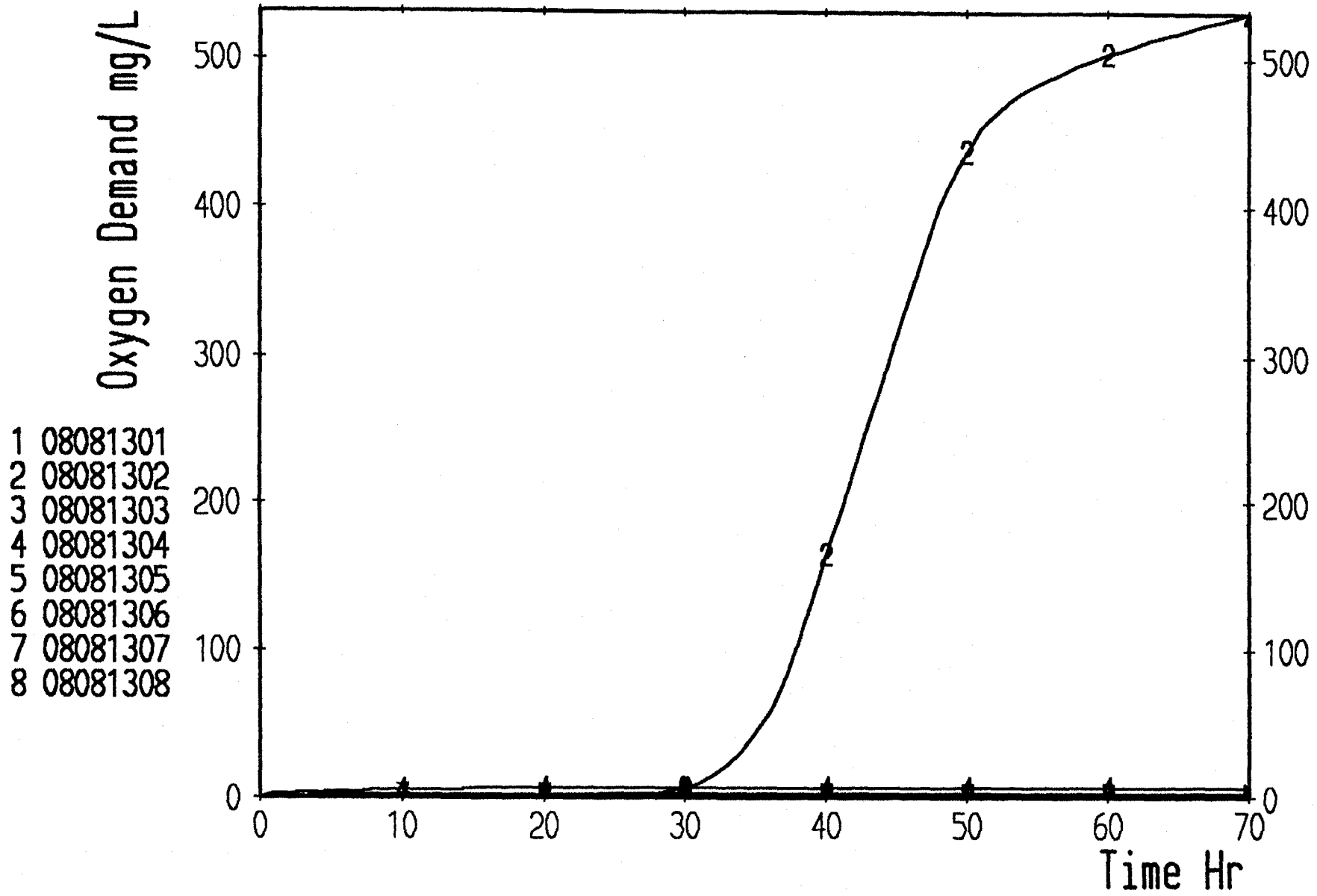

Figure 2. Representative respirometric data from diesel fuel degradation experiments. Reactor 1 contains nutrient broth and 0.1% diesel fuel. Reactor 2 is a positive control containing nutrient broth. Reactor 3 is a negative control containing 0.1% diesel fuel and reactors 4-8 are live samples containing 0.1% diesel fuel.

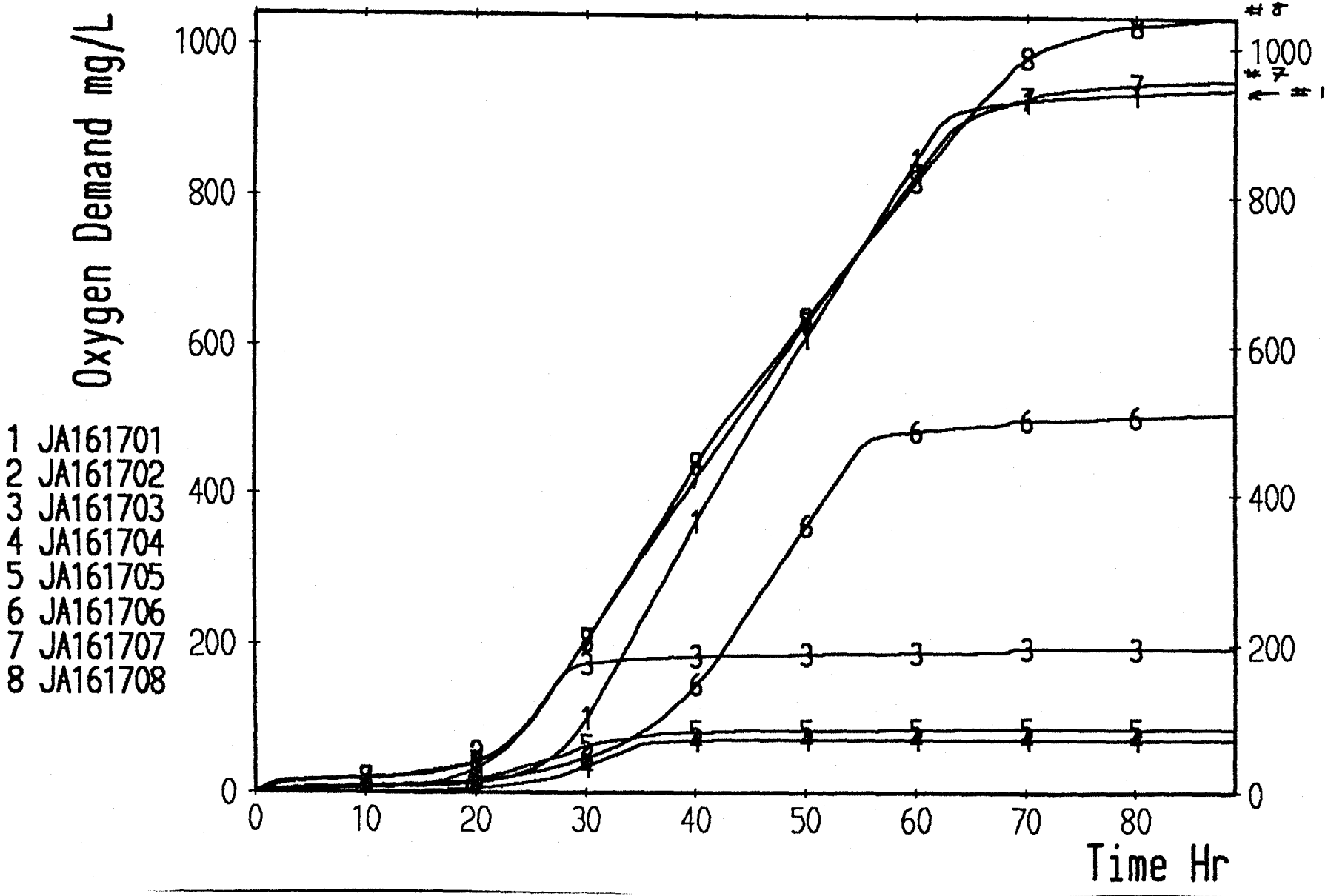

Figure 3. Respirometric data from the initial toluene degradation (A3) experiment. Reactor 1 contains 0.1% glucose (positive control), 3 and 4 contain 20 ppm toluene, 5 and 6 contain 100 ppm toluene and 7 and 8 contain 100 ppm toluene plus 0.1% glucose.

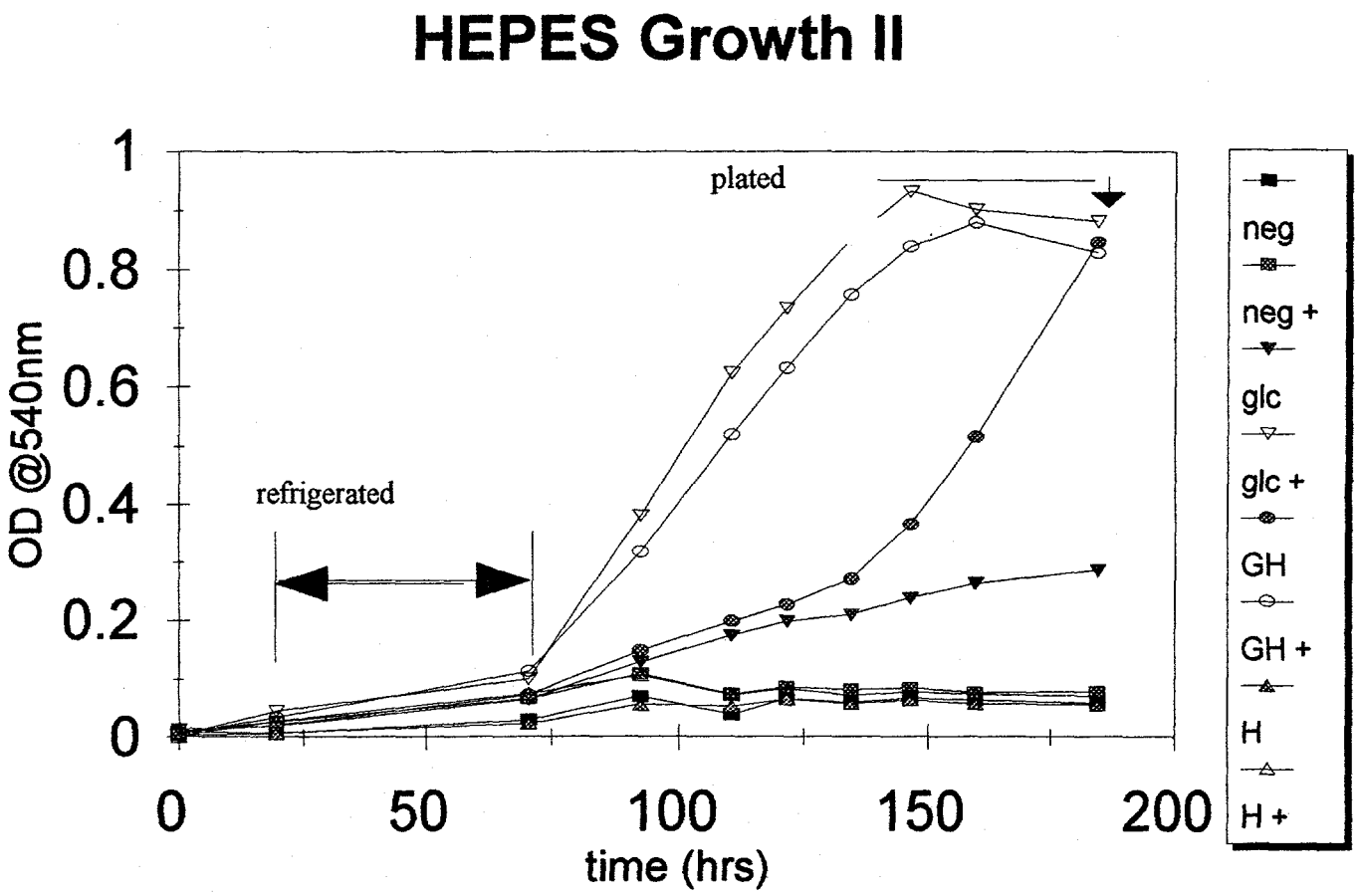

Figure 4. Results from B1/myo-inositol experiment. neg= negative control, neg+ = negative control plus amendments, glc= glucose, glc+ = glucose plus, GH = glucose plus HEPES, GH+ = same plus amendments, H= HEPES, H+ = HEPES plus

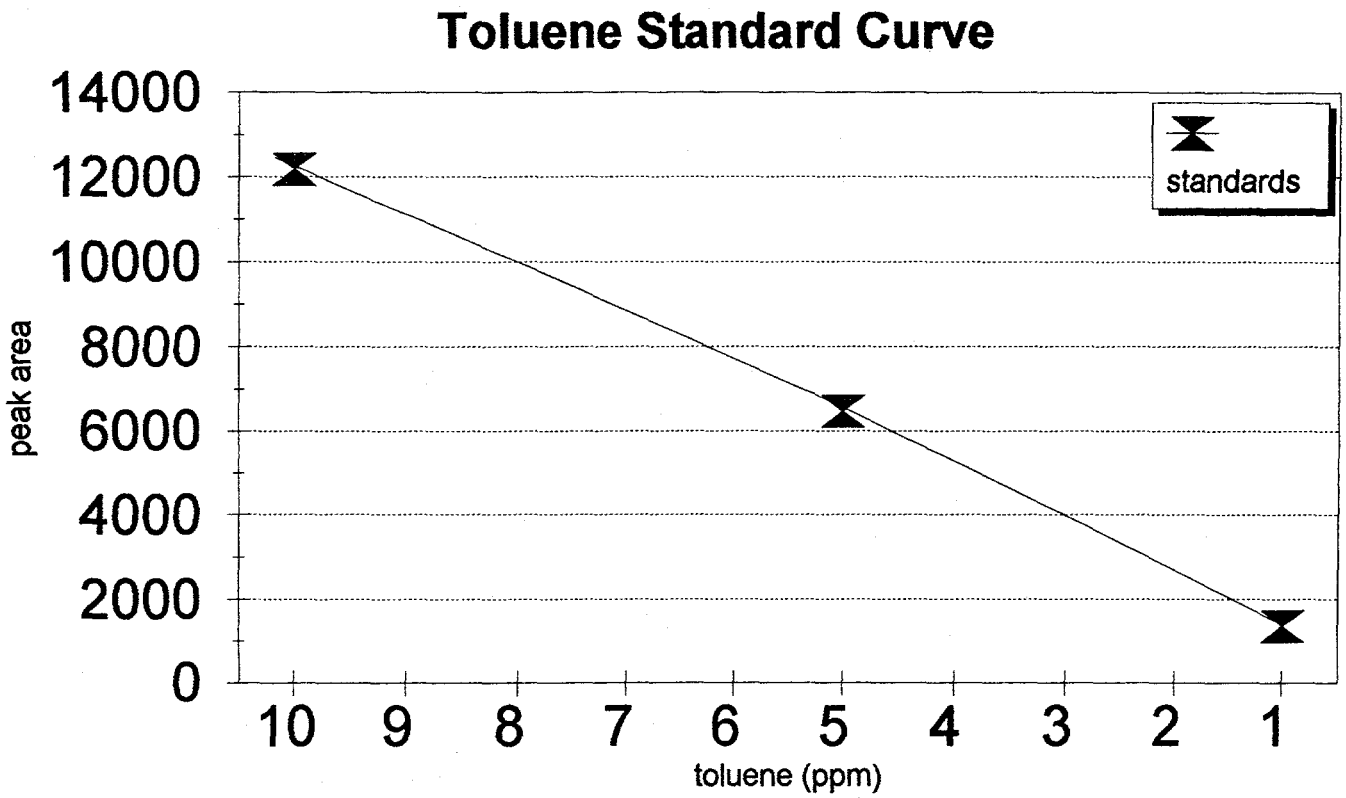

Figure 5. Standard curve for toluene analysis. Concentrations are 10, 5, and 1 ppm and are not logarithmic. Peak areas from averages of peak areas taken over eight days. All samples taken post-plunger (syringe) replacement.
R squared = 0.9989

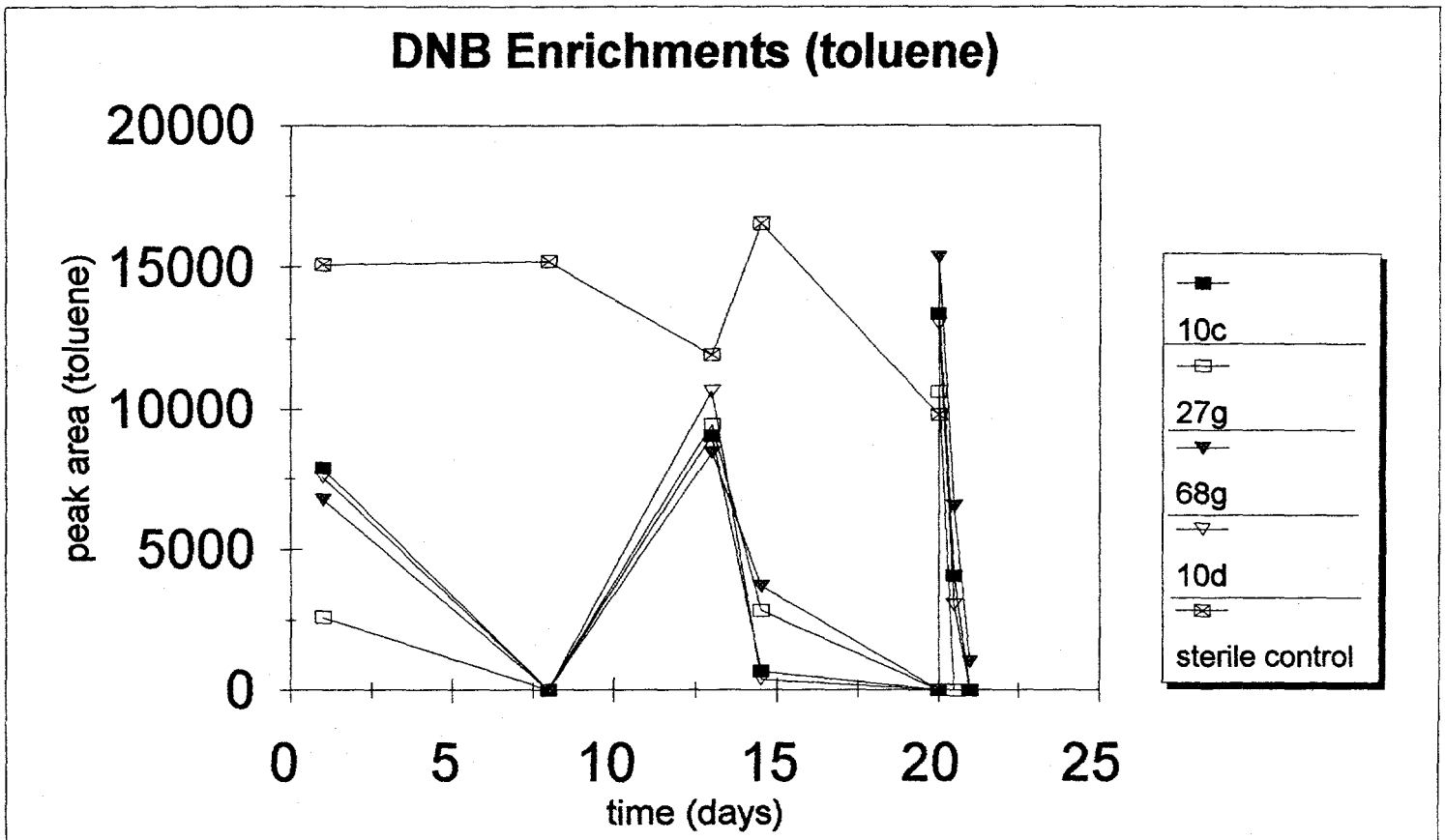

Figure 6. GC analysis of toluene enrichments under denitrifying conditions. 10c through 10d samples are averages of triplicate samples.

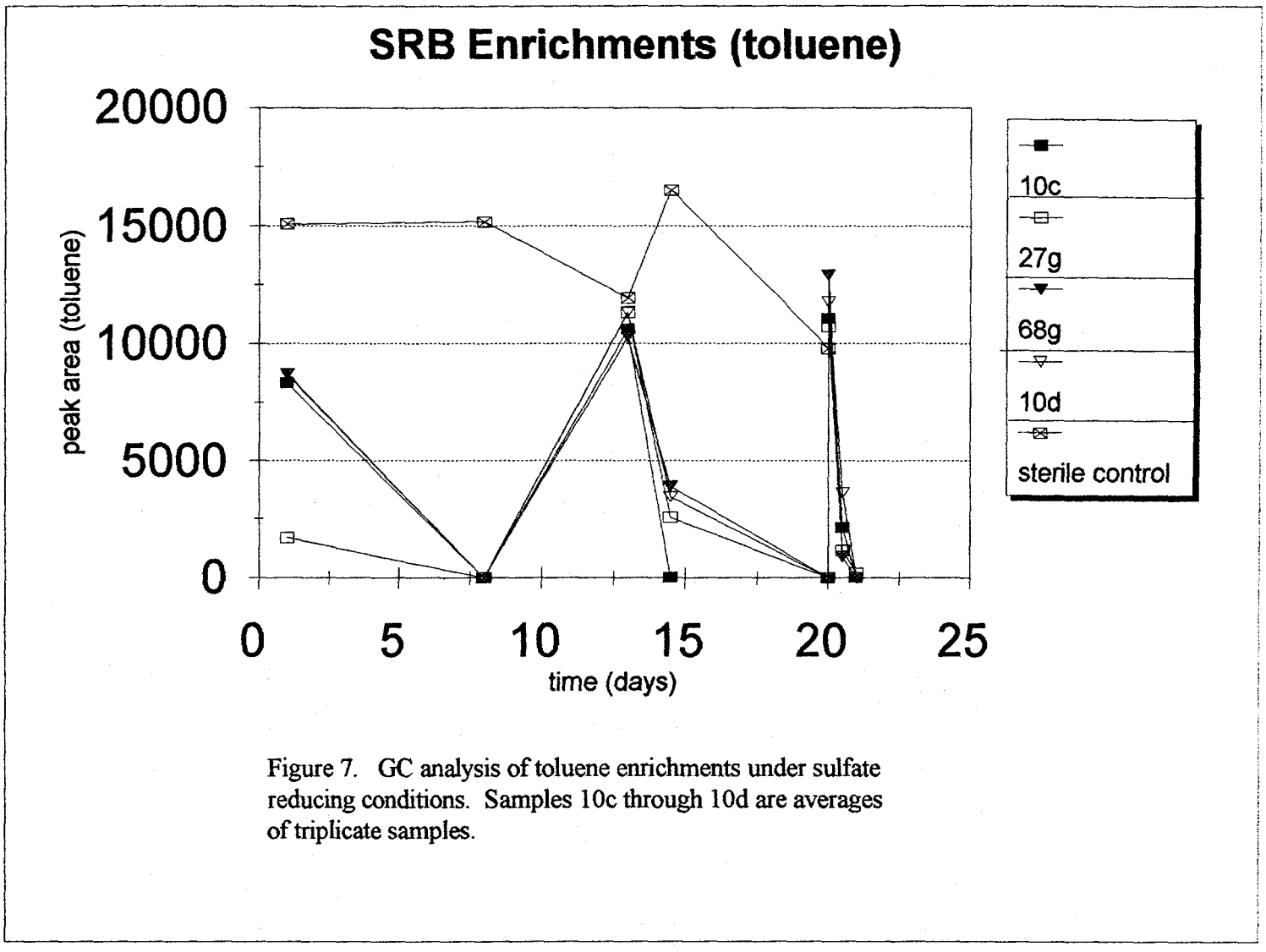

Figure 7. GC analysis of toluene enrichments under sulfate reducing conditions. Samples 10c through 10d are averages of triplicate samples.

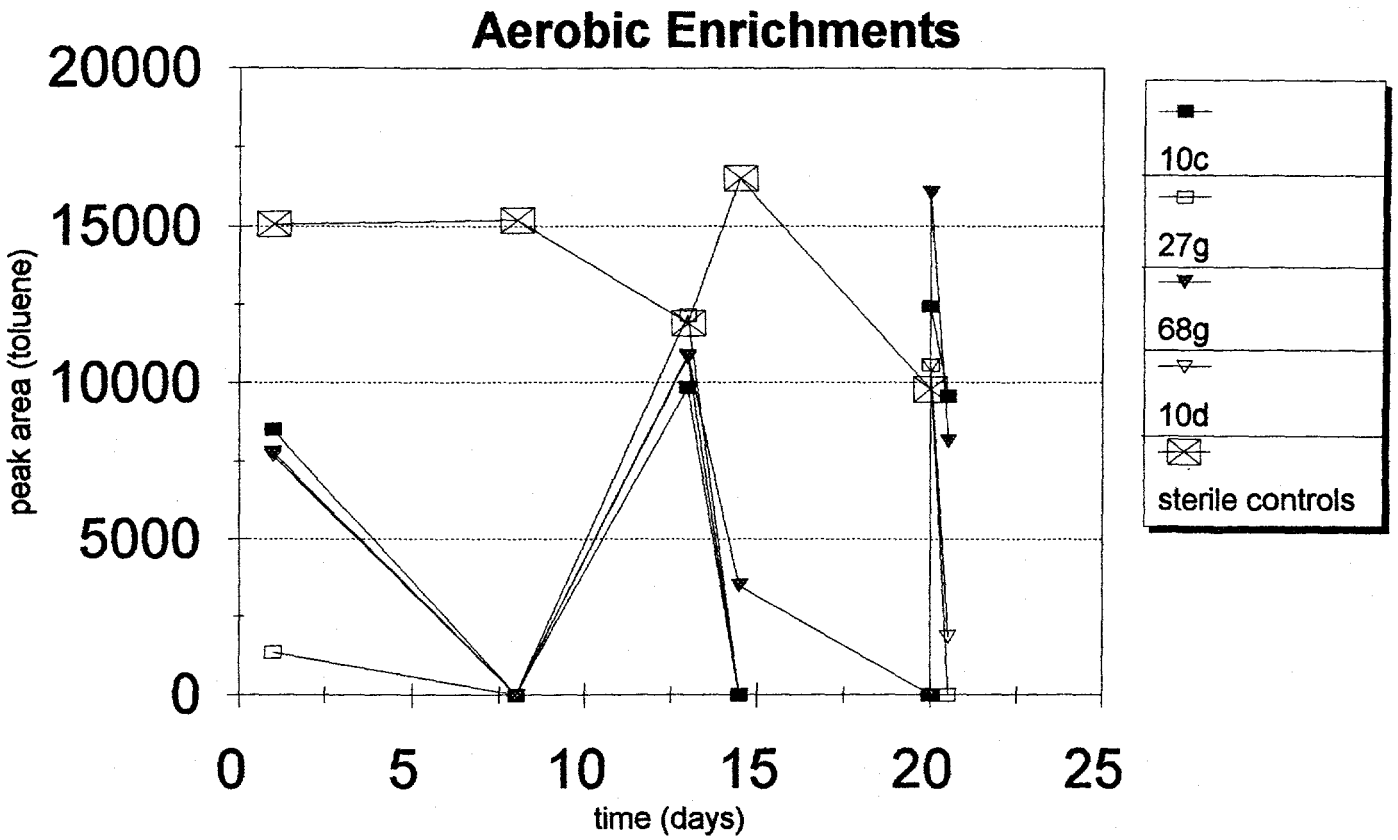

Figure 8. GC analysis of toluene enrichments under aerobic conditions. 10c through 10d samples are single samples and the sterile controls are averages of four sterile samples (10c through 10d).