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ABSTRACT

THE GROWTH, ENRICHMENT, AND DEHALOGENATING PROPERTIES OF *PYROCOCCUS* GBD AND *DESULFUROCOCCUS* SY

by
Siegfried Georg Mueller, Jr.

Recent investigations of volcanic smoker chimneys along the Atlantic Rift has lead to the discovery of hyperthermophilic bacteria. In particular, *Pyrococcus* GBD and *Desulfurococcus* SY were studied, optimizing growth conditions. This was achieved using elemental sulfur amendments, artificial sea water, and dehydrated marine broth. The microbes were then acclimated to enriched cultures containing 2,4,6 trichlorophenol in 0.0126 mM, 0.063 mM, and 0.126 mM amendments. The amended cultures showed dehalogenating properties and were subjected to a data qualifying program. This program indicated that the dehalogenation was a function of microbial growth. A mass balance experiment showed that both microbes were able to reductively dehalogenate 0.5 mM 2,4,6 TCP to 2,6 DCP, 2 MCP, and finally phenol in a quantitative manner.

THE GROWTH, ENRICHMENT, AND DEHALOGENATING PROPERTIES OF *PYROCOCCUS* GBD AND *DESULFUROCOCCUS* SY

by
Siegfried Georg Mueller, Jr.

A Thesis
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APPROVAL PAGE

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This thesis is dedicated to the loving memory
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CHAPTER 1

INTRODUCTION

For the past two and a half centuries, scientists have been peering into the microscopic world. In that time, they have discovered complex interactions between the microscopic and macroscopic organisms that define the environment today.

Historically, scientists have classified these organisms as bacteria or archea. To further define them, they were categorized as aerobic (oxygen utilizing) or anaerobic (oxygen intolerant) organisms. Even the temperature of the environment they were found in dictated a category, psycrophilic (up to 30 deg. C), mesophilic (20 to 45 deg. C), and thermophilic (up to 80 deg. C)(Lim 1989).

However, as technology advances, unexplored regions of the planet become objects of study. Recently, scientists have discovered unusual microbes in the smoker chimneys of submarine volcanoes. These organisms, now referred to as "hyperthermophiles", defied all previous conceptions of life. As stated by Hively (1993), "At 212 degrees, water turns to steam, and proteins- the very stuff of life- turn to glop (sic)....but a select group of creatures are just warming up."

This discovery, in turn, alters previous theories on evolution and adaptation. Prior beliefs dictated that organisms growing at high temperatures were modern adaptations to their mesophilic counterparts. The discovery of hyperthermophiles, however, changes this theory. These organisms, being ancient archea, could actually be the predecessors of modern mesophilic bacteria (Hively, 1993).

It is this adaptive cycle that is of interest to scientists investigating modern day, environmental problems. In particular, the biological treatment of hazardous waste is an area where an organism tolerant to very adverse conditions may prove useful.

Of the most recalcitrant chemicals in industry's waste streams, halogenated organics have proven the most difficult to remediate. Halogenated phenols, for instance, account for a great portion of organic wastes. Commonly used in preservatives, pesticides, and even industrial cleaners, chlorophenols can be found quite readily in industrial waste. For instance, 200 tons of the 22,000 tons of wastes buried at Love Canal by Hooker Chemical was 2,4,6 trichlorophenol (Wentz, 1989). Unfortunately, these chemicals have proven to be both persistent in nature and quite toxic to the human system.

CHAPTER 2

LITERATURE REVIEW

2.1 *Pyrococcus* Strain GB-D

According to Adams (1993) hyperthermophilic bacteria, or more properly archea, have been investigated since their discovery in the early 1980's. However, more recent field investigations into the black smoker chimneys of the Guaymas Basin have revealed new and less understood strains of hyperthermophiles. Jannasch et al. (1992) have enriched cultures recovered from the *Alvin* dives (specifically #1966) of January 1988; revealing new strains of *Pyrococcus*, previously not identified.

Specifically, *Pyrococcus* strain GB-D was recovered from a depth of 2020 m. This isolate can be described as an "obligately anaerobic, extremely thermophilic, sulfur dependent archeum (Jannasch et al. 1992)." Morphologically, this strain appears as an irregular coccoid gram negative cell, occurring singly or as diplococci. These organisms are motile within the temperature range of 75 to 95^{o} C, utilizing a polar flagella bundle for locomotion. According to Jannasch et al. (1992), GB-D will grow in a pH range of 5.5 to 8.3 with an optimum at 7.3. Doubling times for this organism have been recorded as 51, 36, and 60 minutes at 90, 95, and 100^{o}C, with an optimum temperature at 95^{o}C. According to Adams (1993) this classifies GB-D as a hyperthermophile.

Jannasch et al (1992) also specifically point out the dependence on sulfur that this organism expresses. No growth has been observed in cultures without sulfur amendments. *Pyrococcus* GB-D utilizes elemental sulfur as an electron

acceptor during it's growth phase. Throughout the growth phase, sulfide production has been measured and a correlation between growth cessation and sulfide levels of 12mM have been observed. Jannasch et al (1992) also notes that older cultures have had levels of sulfide as great as 20 mM, and can be identified by yellow tinting of the aqueous portion of the culture media by polysulfide formation.

Jannasch et al (1992) and Blumentals et al (1990) have also reported similar results of experiments using alternate electron acceptors. Both investigators have reported similar findings, whereby cystine has been the only acceptable alternative electron acceptor. All others apparently repress or do not facilitate GB-D's ability to grow.

Jannasch et al (1992) also reported on the antibiotic sensitivity of *Pyrococcus* GB-D. GB-D resists inhibition by vancomycin, streptomycin, and chloramphenicol while showing some sensitivity to rifampin. This is characteristic of archea, and is substantiated by Adams (1993) in work with other hyperthermophilic isolates. Other archea characteristics pertain to the presence of phytanyl glycerol diethers and diphytanyl diglycerol tetraethers (Jannasch et al, 1992; Adams, 1993; Brown et al,1990).

Pyrococcus GB-D was recovered from depths in excess of 2020m where pressures can exceed 300 ATM. These organisms, however, do not express hyper-barophillic (extreme pressure affinity) tendencies (Jannasch et al, 1992). Pressure, however, may play a role in achieving higher maximum and optimum growth temperatures by exceeding the boiling point of water. Studies by Jannasch et al (1992) showed a 5% decrease in doubling time by applying a 5 ATM pressure to the culture vessel and increasing maximum temperatures to

104°C. This tendency has also been documented by Adams (1993) comparing hyperthermolphillic isolates from shallow and deep volcanic vents.

Jannasch et al (1992) also investigated oxygen and metal tolerances of *Pyrococcus* cultures. Although these organisms are strict anaerobes, Jannasch et al (1992) found them tolerant to oxygen atmospheres at low temperatures (i.e. 2-4°C) and able to recover when enriched in hot anaerobic media in the presence of sulfur. However, free oxygen at elevated (growth) temperatures was extremely toxic. This can be attributed to the metabolic stasis and protein/enzyme rigidity at low temperatures (Jannasch et al, 1992; Adams, 1993; Hively, 1993).

Pyrococcus strain GB-D and GB-4 showed differential tolerances to excess metal enrichments. It was found that GB-4 was more tolerant to excess copper2+ and lead 2+ while growth inhibition was noted for GB-D. Hydrocarbon enrichments were also tested whereby finding that supplemental hydrocarbons (hexadecane and crude oil) had neither stimulatory or inhibitory effects. Jannasch et al (1992) also noted that after several weeks of incubation, these supplemental hydrocarbons were not metabolized as additional carbon sources.

2.2 *Desulfurococcus* Strain SY

Prior to the Guaymas dives of 1987 and 1988, investigations of black smoker chimneys at the 11°N East Pacific Rise volcanic vent sites produced scrapings that lead to the isolation of *Desulfurococcus* strain SY . These isolates belong to archea, and more specifically to the family *Desufurococcacae* (Jannasch et al, 1988)

Morphologically, strain SY can be described as "a gram negative irregularly coccoid organism ... occurring as single cells or diplococci (Jannasch et al, 1988)." This strain is also characterized by its lack of polar flagella bundles

and subsequent loss of motility. Strain SY also utilizes complex organic substrates such as yeast extract and tryptone for carbon sources. Jannasch et al(1988) also notes their ability to respire elemental sulfur while also being fermentative.

According to Jannasch et al (1988), elemental sulfur is not an absolute requirement for strain SY growth, but rather shows a "significant need" to achieve a maximum growth rate. It was found that doubling times for sulfur amended and unamended cultures were 2.9 and 3.2 hours, respectively. As pointed out by Zillig et al (1982), other strains of *Desulfurococcus* show a similar trend. In species *D. mobilis* and *D.mucosus*, cultures grew 10 times slower in the absence of sulfur and achieve cell densities five times lower than sulfur amended cultures. Jannasch et al (1988) found a similar pattern, whereby unamended SY cultures achieved only three to five percent of the maximum growth density in sulfur rich media.

Desulfurococcus SY shows a similar growth characteristic as *Pyrococcus* GB-D . Strain SY shows a sulfide production rate exponential to the growth rate, whereby maximum production rate coincides with the maximum growth rate. Inhibition and tolerance rates are significantly higher in strain SY, inhibition occurring at 170mM and tolerating anything below (Jannasch et al, 1988; Zillig et al, 1982).

Strain SY is also noted as requiring complex substrates containing nitrogenous compounds. Tests conducted by Jannasch et al (1988) utilizing individual and mixtures of amino acids and dipeptides did not support growth. Also, it was noted that their addition to low concentration yeast extract cultures of strain SY had no effect on growth rate at optimum conditions. This condition changed however at high yeast extract concentrations, suggesting that free amino

acids are not used as separate substrates but in a manner of "coutilizatiuon" or "co respiration", whereby the higher the substrate concentration, the higher the use of free amino acids (Jannasch et al, 1988).

Like *Pyrococcus*, *Desulfurococcus* cannot tolerate any free oxygen at growth temperatures (Jannasch et al, 1988) However, when cultures are cooled between 4 and 25 ^{o}C, they will recover when transferred to anoxic liquid suspensions at growth temperatures. Similarities were also seen between the two organisms pertaining to barophillic properties. The optimal temperature ranges for maximum growth of *Desulfurococcus* SY are unaffected by wide changes in pressure (ranging from 1atm to 300 atm) Strain SY also showed a high degree of sensitivity via growth inhibition to divalent copper and lead (Jannasch et al, 1992, Jannasch et al, 1988).

2.3 Sulfur, Polysulfides and Hyperthermophiles

It was previously indicated that sulfur is a vital key to growth in a hyperthermophilic environment. According to Blumentals et al (1990), bacteria-sulfur metabolism mechanisms can fall into two categories; 1) bacteria have to be in direct contact with solid sulfur to metabolize it or 2) bacteria uses a soluble sulfur compound formed by chemical or enzymatic reactions. Aerobic sulfur oxidation predominantly utilizes the direct contact method and actually secrete phospholipid surfactants to facilitate bacterial attachment. Conversely, anaerobic hyperthermophiles utilize soluble sulfur intermediates during sulfur metabolism (Blumentals et al, 1990; Jannasch et al , 1992).

The cell-sulfur interactions described by Blumentals et al (1990) were a result of experiments involving sulfur isolation via a dialysis bag within an incubated culture. Although direct cellular contact could not occur across the

barrier, hydrogen sulfide and methyl mercaptan production was still measured. Sulfide and polysulfide were measured in both inoculated and uninoculated cultures; however, concentrations increased faster in inoculated cultures. This observation, according to Blumentals et al (1990), suggests that organisms produces a sulfur solubilizing agent. Through the time course, however, the polysulfide concentration decreases as a result of bacterial reduction to hydrogen sulfide (Blumentals et al, 1990; Johnson and Wood, 1993). Through further investigation, it was found that "the sulfide produced by bacterial sulfur reduction serves as the sulfur nucleophile that further solubilizes sulfur" (Blumentals et al, 1990).

2.4 Thermo-Stable Enzymes and Proteins

Enzymes and their accompanying catalyzed reactions provide the biochemical basis for life processes. In hyperthermophilic organisms, enzymes have developed physiological adaptations that provide for thermostability. According to Brown et al (1990), the ability for *Pyrococcus* strains to grow in the presence of complex carbohydrates, polysaccharides, and starches indicates the presence and utilization of thermally stable amylolytic enzymes.

It has been found, however, that purified enzymes from hyperthermophiles are unstable at ambient temperatures (growth temperatures). Adams (1993) also suggests that cellular co-factors are produced to minimize heat denaturing of enzymes (in-vivo). Adams also points out that there is no "universal mechanism" between hyperthermophilic organisms pertaining to "cytoplasmic thermoprotectants".

One mechanism that has been isolated from P. furiosus deals with structural aberrations in the protein rubredoxin. Unlike its' mesophilic

counterparts, hyperthermophilic rubredoxin lacks an N-terminal methionine and glutamyl residue at the fifteenth position. The N-terminal becomes an integral part of a three stranded "beta-sheet" hydrogen network, that is tightly held together. The mesophilic N-terminal is much longer and is agitated violently by boiling water, thereby allowing the protein to rip apart (Adams, 1993; Hively, 1993).

2.5 The Reductive Dehalogenation Process

According to Mohn and Tiedje (1992), reductive dehalogenation can be defined as " the removal of a halogen substituent from a molecule with the concurrent addition of electrons to the molecule." This form of dehalogenation is the primary activity surrounding biological degradation in an anaerobic environment. Reductive dehalogenation is later broken down into two distinct processes. The first, hydrogenolysis, involves the removal of a halogen substituent and subsequent replacement with a hydrogen atom. The second process involves removal of adjacent halogen groups and subsequent bond formation between the affected carbon atoms. This process is often referred to as dihaloelimination or vicinal reduction.

As Mohn and Tiedje (1992) point out though, these processes are limited to the groups that they can act upon. Hydrogenolysis is capable of transforming alkyl and aryl halides while vicinal reduction is restricted to alkyl halides. These activities are also regulated by the availability of external electron acceptors. According to Sleat and Robinson (1984), the reduction of electron acceptors can be linked to the dehydrogenation reactions of aromatic catabolism.

The biological activity surrounding these processes in nature however are just now being understood.

2.6 Biological Activities

The degradative activity of anaerobic organisms on organic compounds has been studied quite thoroughly, and has actually been exploited in industry. Municipal sewage treatment plants and more recently, industrial bio-reactors have all exploited the ability of anaerobic micro-organisms to clean organic-laden waste streams.

However, this activity usually takes place under undefined conditions, using mixed cultures, and complex substrates. As pointed out by Mohn and Tiedje (1992), anaerobic degradation has been seen quite readily in mixed cultures, utilizing fairly simple alkyl halides to complex aryl halides as substrates to catalyze. Researchers have also found pure cultures (one species of organism grown in culture) able to degrade alkyl halides but have had very limited success in purifying anaerobic cultures that degrade aryl halides.

Dolfing (1990) has hypothesized that energy may be generated during reductive dechlorination that bacteria may be able to exploit. This hypothesis has been supported by thermodynamic data showing reductive dechlorination of 3-chlorobenzoate to benzoate as an exergonic reaction (Dolfing and Tiedje 1987). Dolfing (1990) found that ATP levels increased greatly in chlorobenzoate amended cultures and was able to derive energy from reductive dechlorination. Both Mohn and Tiedje (1992) and Dolfing (1990) were able to support these findings by sterilizing control samples. Both research groups found that by de-activating the biological component also inhibited dehalogenation.

2.7 Cell Free Activities

The biological components of interest were investigated to great detail by DeWeerd and Suflita (1990). Utilizing *Desulfomonile* tiedjei, a purified organism capable of dehalogenating aryl halides (Mohn and Tiedje 1992), cell extracts were prepared from a variety of conditions including cultures acclimated to chlorobenzoate. DeWeerd and Suflita (1990) found that both intact and cell free (extract) cultures dechlorinated 3-chlorobenzoate. However, the cell free (extract) cultures required prior inducement of the halogen substrate.

2.8 Induction and Acclimation

Researchers have found acclimation periods are required for organisms that are not in continuos contact with aryl halides of interest (Mohn and Tiedje 1992; Mueller et al 1991). Strujis and Rogers (1989) found that unacclimated sediment slurries spiked with di-chlorophenols had a lag time of four weeks before dehalogenation commenced. However, acclimated sediments began dehalogenating immediately and was complete within nine days. This was further substantiated by Pignatello et al (1983) where three weeks after dosing experimental stream channels, aquatic micro-flora populations adapted to penta-chlorophenol mineralization and became a major removal mechanism. Mikesell and Boyd (1986) further discovered a correlation between an organisms acclimation with specific substituent placement (i.e. ortho mono-chlorophenol vs. para mono-chlorophenol) and its ability to dechlorinate PCP completely. Holliger et al (1992) also found that by mixing sludges acclimated to specific isomers of mono-chlorophenol were able to completely degrade PCP. This effect was not observed in individual tests.

Researchers have identified four possible reasons for this "lag" period. Periods of acclimation can be a result of (a) genetic change, (b) induction, (c) diauxy responses (preferred substrate exhaustion), (d) cell growth from low densities (Linkfield et al. 1990). This was later observed by Goulding et al. (1988) when experimental results showed decreases in degradation rates in the presence of more easily metabolizable substrates.

2.8 Electron Acceptors

According to Dolfing (1990), reductive dechlorination occurs as a part of energy generating reactions. As stipulated by Mohn and Tiedje (1992), this can occur as a result of "...intracellular channeling of electrons or via inter specific competition for electron donors." This is supported by inhibition of dehalogenation by the addition of alternative/competitive electron acceptors, such as sulfate used by sulfate reducing bacteria (Mohn and Tiedje, 1992; Holliger et al, 1992).

Thermodynamically, this has been seen as an exogonic reaction due to bias for higher energy metabolites (Dolfing, 1990). As seen by Holliger et al (1992), reduction of highly chlorinated organics accumulated lesser chlorinated products. These were not utilized until the original parent substrates were entirely depleted. This process has been investigated utilizing Michaelis-Menten models on competitive inhibition (Suflita et al, 1983).

CHAPTER 3

EXPERIMENTAL APPARATUS AND PROCEDURES

3.1 Introduction

The hyperthermophilic organisms used in this study were *Pyrococcus* strain GB-D (*P.*GB-D) and *Desulfurococcus* strain SY (*D.*SY). Both organisms were acquired from Dr. Holger Jannasch of Woods Hole Oceanographic Institute, Woods Hole, MA 02543. These organisms were shipped in solution at ambient temperatures. Upon receipt, they were inoculated into fresh media and incubated; subsequently becoming the base culture for this investigation.

3.2 Objectives

This project had three primary purposes. First, culturing techniques were to be investigated. Due to their unusual origin, culturing hyperthermophiles under a variety of conditions was to be used in order to find an optimum growth media. These conditions included presence or absence of sulfur, artificial saltwater content, and growth under ambient or slightly elevated pressures.

The second primary purpose of this investigation was to investigate the effects of enriched media on hyperthermophilic bacteria and their acclimation abilities towards adverse environments. Of particular interest is the ability of these bacteria to reductively dehalogenate 2,4,6 trichlorophenol.

The third primary purpose for this investigation was to identify the possible dehalogenating processes, whether biological or abiological. This was to proceed utilizing several media variations to stimulate, retard, or eradicate growth of acclimated hyperthermophiles in enriched study vessels.

3.3 Materials List

Bacto-Marine Broth #2216- Difco (ingredients per liter: peptone 5g, yeast extract 1g, ferric citrate 0.1g,sodium chloride 19.45g, magnesium chloride .9g, sodium sulfate 3.24g, calcium chloride 1.8g, potassium chloride 0.55g, sodium bicarb 0.16g, potassium bromide 0.08g, strontium chloride 0.034g, boric acid 0.022g, sodium silicate 0.004g, sodium fluoride 0.0024, ammonium nitrate 0.0016, disodium phosphate 0.008g)

Instant Ocean- Aquarium Systems, Ohio

(2) Hot water baths- Precision All Steel Hot Baths, Model 183

glass serum bottles (crimp seal-125 ml), Wheaton Glass Co.

butyl rubber stoppers, Bellco Biotech

aluminum crimp seals (20mm), Bellco Biotech

elemental sulfur flowers, Fisher Chemical

syringes (1ml, 3ml, 5ml)- Becton Dickson

needles (21 gauge, 1 inch)- Becton Dickson

nitrogen/carbon dioxide gas (80:20), pre-purified-Matheson Gas

Coy anaerobic chamber (2-station soft side, auto air lock), Coy Laboratory Products

2,4,6 tri-chlorophenol (TCP), Fischer Scientific

2,6 di-chlorophenol (DCP), Fischer Scientific

2 mono-chlorophenol (MCP), Fischer Scientific

phenol, Fischer Scientific

methyl tert-butyl ether (HPLC grade)- Fischer Scientific

methanol (HPLC grade)- Fischer Scientific

- Envi-Chrom P Solid Phase Extraction Tubes (6 ml)- Supelco
- 2 ml crimp seal, auto sampler vials and Teflon coated crimp seals
- Waters Ultra-Wisp 715 Sample Processor
- Waters 600e System Controller
- Waters 484 Tunable Absorbance detector/IBM PC XT w/ Nelson Chromatographic Software
- Alltech SU Econosphere C8 Column and corresponding guard column
- Varian Star 3400 G.C. and Saturn Ion Trap M.S. w/ Compaq Pro-Linea 4/33
- J&W Scientific Capillary Column #122-5032 w/ DB 5 stationary

3.4 Preparation of Media

3.4.1 Preparation of Culturing Media

The basis of the culturing media utilizes #2216 Marine Broth (Difco) at half strength concentrations (19 g/L). This has been rehydrated in water processed through a Milli-RO reverse osmosis water station. This media is then boiled and de-gassed with a nitrogen-carbon dioxide mixture (80%/20%). The subsequent degassed media is decanted into 125 ml culture bottles under anoxic conditions and crimp sealed with butyl rubber stoppers. The media is then sterilized for 15 minutes in a high pressure sterilizer.

Artificial sea water (ASW) amended cultures utilize solutions of rehydrated ASW in full strength compositions (38 g/L). This is also added to Milli-RO water and sterilized prior to use. In a 3 liter flask, 19 g of 2216 broth is added to 500 ml of Milli-RO water. This is then brought up to 1 liter with full strength ASW and boiled and degassed. The resultant yield is half strength broth

in half strength ASW . This media is also decanted into 125 ml crimp seal vessels while continuously being degassed.

The prepared media is then sterilized at 121°C for fifteen minutes to insure culture purity and prolong media shelf life. Prior to inoculation, cultures requiring sulfur amendments are brought into a Coy anaerobic chamber. In the chamber, the vessels are disassembled and sulfur is aseptically transferred in 1% weight per volume additions. Due to the low melting point of sulfur, amendments can not be made prior to autoclaving. The crimp sealed vessels are then removed from the chamber for inoculation.

The culture bottles are inoculated on the bench top using aseptic and anoxic techniques. Syringes are degassed with pre-sterilized nitrogen/carbon dioxide pressurized bottles and the needles are flame sterilized over a gas burner. Transfers are made in 5 ml aliquots and then incubated at 95 oC in a hot water bath.

3.4.2 Growth Detection Via Pressure

As each of the hyperthermophiles are incubated at optimum growth temperatures, each strain evolves hydrogen sulfide gas at a specific rate. This gas, and other metabolic products contributing to head space content and pressure were measured as a function of increasing pressure versus time. The composition of the culture bottles appears in Table 4.1.

These bottles were prepared anaerobically, with a pressure gauge assembly piercing the stopper. The assemblies were composed of an 8x1/8(o.d.) inches copper tubing connecting a sensitive low pressure gauge (max. pressure < 30 P.S.I.). The bottles were incubated in the hot water bath, with the assemblies

Table 3.1 Pressure Bottle Composition

Composition	Bottle Number
49 ml media + 1ml Desulfurococcus + 1 % (wt/vol) sulfur	P.B.C.#1 @ 95 oC
49 ml media + 1 ml Pyrococcus + 1% (wt/vol) sulfur	P.B.C.#2 @ 95 oC
49 ml media + 1 ml Desulfurococcus + 1% (wt/vol) sulfur	P.B.C. #3 @ room temperature
49 ml media + 1ml Pyrococcus + 1 % (wt/vol) sulfur	P.B.C. #4 @room temperature
50 ml sterile media + 1%(wt/vol) sulfur	P.B.C. #5 @ 95 oC
49 ml media + 1ml Pyrococcus (no sulfur)	P.B.C. #6 @ 95 oC
49 ml media + 1 ml Desulfurococcus (no sulfur)	P.B.C. #7 @ 95 oC

contained under the gabled cover to maintain a constant temperature on the tubing.

3.4.3 Preparation of Acclimation/Enrichment Media

Media for acclimation vessels was prepared in a similar fashion as culturing media with salt amendments. However, volume was accounted for to insure correct enrichment concentrations. Half strength 2216 marine broth and half strength ASW was degassed and autoclaved in crimp sealed vessels.

Enrichment inoculations were made from a 12.6 mM (milli-molar) concentrated solution of 2,4,6 tri-chlorophenol (TCP). This solution was prepared with 2.5 g of TCP solubilized in 1.0 L of slightly basic water (w/ NaOH).

Acclimation vessels were made in three different TCP concentrations of 0.126mM, 0.063mM. and 0.0126 mM. These were prepared by measuring (volumetrically) 94ml media : 1ml TCP concentrate, 94.5ml : 0.5ml, and 94.9ml : 0.01ml, respectively; and autoclaving. Inoculates of 5 ml of *Pyrococcus* and *Desulfurococcus* were then added to the 0.0126 mM cultures and allowed to incubate for 24 hours. A 5 ml sample was then removed from the cultures, anaerobically, and inoculated to fresh, sterile, TCP free media with sulfur, and incubated for another 24 hours.

A 5 ml sample was then taken from the most recent culture and inoculated into the next higher concentration media bottle; and the process repeated to successively higher concentrations. Pressure was identified as a sign of growth, and the sample was analyzed with HPLC to identify TCP degradation.

3.4.4 HPLC Identification of TCP Dehalogenation

Although bacterial growth could be measured via pressure and sulfide production, the effects of growth on TCP concentration could only be measured utilizing High Performance Liquid Chromatography.

The qualitative measurements made from the HPLC results were used at this stage only to mark the disappearance of TCP and the emergence of possible products as a function of time. One milliliter samples were taken from enriched cultures just following inoculation and at given intervals during incubation. Each

set of samples was analyzed immediately after extraction to avoid chemical or abiotic degradation.

The samples were prepared by extracting 1 ml drawn from a cooled serum/culture bottle, using a sterile, degassed syringe. Samples were cooled to avoid TCP volatilization in the bottle head space. These samples were placed in micro centrifuge tubes, capped, and spun for 12 to 15 minutes. This time is sufficient to pellet out suspended sulfide particles, cells and cellular debris. This method is superior to micro-filtration due to TCP adherence to the filter media.

A volume of 0.5 ml was then taken from the supernatant and injected into HPLC vials. The sample was then acidified with 0.17 N hydrochloric acid, capped, and subsequently analyzed.

The HPLC conditions were as follows:

Table 3.2 HPLC Conditions

Make:	Waters Ultra-Wisp 715 Sample Processor 25 ul Injector Waters 600e System Controller Waters 484 Tunable Absorbance Detector	
Settings:	Pressure:	Isocratic
	Absorbance	280 nm
	Flow rate	1.00 ml/ min
	Eluent	50/50 methanol:water+1% acetic acid
	Column	Alltech Econosphere C8 SU Column & corrsponding guard column
Computer:	IBM P.C. XT	
Software:	Nelson Chromatographic Software	

3.4.5 Preparation of Media for a Data Qualifying Program

A data qualifying program (DQP) is an essential set of experiments used to eliminate other possible scenarios in a scientific manner. For hyperthermophilic work, a data qualifying program must be designed to eliminate outside biological and chemical activities as mechanisms in the dehalogenation of the chemical of interest. The program must also elucidate if 2,4,6 TCP dehalogenation is a result of microbial growth or a subsequent reaction to chemical substituents produced as a result of microbial growth. This process of elimination is achieved by tailoring the culture media for a specific result. The program appears in Table 4-3.

The basis of the DQP media is half strength marine broth in artificial salt water, as prepared in the aforementioned sections. Each microcosm requiring inoculation received 5ml aliquots of fully-grown (high-density) culture. Those microcosms requiring TCP amendments were standardized at 1 mM concentrations.

3.4.5.1 Description of DQP Microcosms

The description of each microcosm, intended purpose, and physical make up are as follows:

Bottles 101,201-These microcosms were composed of 45 ml of sterile media and a 5 ml inoculation of fully grown culture. The purpose of these cultures is to establish an analytical baseline of normal interferences for a growing culture with no TCP amendments.

Bottles 102, 103, 202, 203-These microcosms were prepared with 41.07 ml of sterile media, 5 ml inoculation, and 3.93 ml of TCP stock solution. Bottles 102 and 202 were amended with 1% (wt/vol) elemental sulfur. These bottles

Table 3.3 Data Qualifying Program for Hyperthermophiles

Inoculated Microcosm

Pyrococcus GBD

Bottle Amendments	Bottle Number
Inocula + sulfur, no TCP	101
Inocula + sulfur + TCP	102
Inocula + TCP, no sulfur	103
Inocula + TCP + hydrogen head space	104
Inocula + TCP + sulfur, cold	105
Maximum Growth + TCP + sulfur	106
Inocula + SDS + sulfur + TCP**	107
Inocula + sulfur + TCP, no SDS**	108
Maximum Growth + TCP + SDS**	109

Desulfurococcus SY

Bottle Amendments	Bottle Number
Inocula + sulfur, no TCP	201
Inocula + sulfur + TCP	202
Inocula + TCP, no sulfur	203
Inocula + TCP + hydrogen head space	204
Inocula + TCP + sulfur, cold	205
Maximum Growth + TCP + sulfur	206
Inocula + SDS + sulfur + TCP**	207
Inocula + sulfur + TCP, no SDS**	208
Maximum Growth + TCP + SDS**	209

Uninoculated Microcosms

Bottle Amendments	Bottle Number
TCP + Sterile Supernatant	301
TCP + Sterile Supernatant + sulfur	302
TCP + sodium sulfide (hot)	303
TCP + sodium sulfide (cold)	304
TCP + hydrogen head space + Supernatant	305
TCP + Methyl Mercaptan (MM)**	306
TCP + Di-Thio-Threotal (DTT)**	307

**These tests were run side by side to each other but separate from the program.

were grown side by side in order to establish a link between bacterial growth or inhibition in the presence and absence of sulfur; and the subsequent effects on TCP.

Bottles 104,204- These microcosms were prepared with 41.07 ml of sterile media, 5 ml inoculation, and 3.93 ml TCP stock solution. The head space has been pressurized to 10 PSI over normal with sterile hydrogen gas. The purpose of these microcosms was to utilize an alternate electron acceptor (other than elemental sulfur) and record the effects on TCP concentrations.

Bottles 105,205- These bottles were prepared with 41.07 ml sterile media, 5 ml inoculation, and 3.93 ml TCP stock solution. The bottles were also amended with 1% (wt/vol) elemental sulfur, however, they were incubated cold (at ambient room temperature). These microcosms were used to show the effects on growth and TCP concentrations at temperatures far below normal incubation temperature, and possibly account for abiotic/chemical reactions taking place between the microcosm components themselves.

Bottles 106,206- These microcosms were composed of 46.07 ml of mature culture from a previous growth experiment. These bottles were then supplemented with fresh sulfur and 3.93 ml of TCP stock. The purpose of these bottles was to elucidate the effects of a fully grown culture on TCP concentrations.

Bottles 107,207- These bottles were composed of 41.07 ml sterile media, 3.93 ml TCP stock, 5 ml active culture, and supplemented with 5 % (wt/vol) sodium do-decyl sulfate (SDS) and 1% sulfur. Bottles 108 and 208 were run under identical conditions without SDS supplements. Sodium do-decyl sulfate was used as a lysing agent capable of completely destroying the cell membranes of the bacteria. The purpose of these side-by-side tests was to compare the effects

of an active culture versus a de-activated culture on the concentration of TCP.

Bottles 109,209- These microcosms were made using 46.07 ml of fully grown culture, 3.97 ml TCP stock, and 1% sulfur /5% SDS (wt/vol) amendments. The purpose of these bottles was to show if non-living metabolic by-products, manufactured during the growth stage and left in solution, had any effects on TCP concentration.

Bottles 301,302- These microcosms were composed of 41.07 ml sterile media, 3.93 ml TCP stock, and 5 ml of cell free supernatant. The supernatant was prepared by anoxically extracting 6 ml of a cooled, active culture in the anaerobic chamber. This sample was then centrifuged for 10-12 minutes to pellet out any cellular material. Carefully, 5ml was decanted from the top and inoculated into the serum bottle. Microcosm 302 was also supplemented with 1 % (wt/vol) sulfur. The purpose of this set up was to account for any cell free activity from the supernatant saturated with growth products, at growth temperatures.

Bottles 303,304- These bottles were composed of 41.07 ml sterile marine broth, supplemented with 3.93ml TCP stock and 5 ml sodium sulfide. The purpose of these microcosms was to show possible chemical activity using strong reducing agents. Bottle 304 was incubated at ambient room temperature to show any links between optimum growth temperatures (>90^{o}C)and chemical reducing ability.

Bottle 305- This microcosm was composed of 46.07 ml cell free supernatant, 3.93 ml TCP stock, and pressurized to 10 PSI with sterile hydrogen gas. This microcosm was set up to show any cell free activity using hydrogen gas and the effects on TCP concentrations.

Bottles 306, 307- These microcosms were prepared using 42.9 ml sterile media, 3.9 ml TCP stock, and 3.2 ml methyl mercaptane (MM) or; 46.07ml sterile media, 3.93 ml TCP stock and 0.003g di-thio-threotal (DTT); per bottle, respectively. These microcosms are prepared using strong organic reducing agents, void of hyperthermophilic bacteria, in order to observe their effects on TCP concentration. These chemicals were also selected on the basis of there radical reactivity at high temperatures. These samples were also processed at pH 3.0 and pH 1.0, in order to observe other chemical states of TCP-degraded products.

3.5 Degradation Mass Balance

Utilizing a data qualifying program necessitates a mass balance experiment. The mass balance accounts for, in a quantitative manner, products formed and lost from the system. In dealing with the reductive dehalogenation of 2,4,6 TCP, the amount of DCP, MCP, and phenol formed must be in proportion to the initial concentrations of TCP. If the product is degraded past phenol, then a net loss must be accounted for as a function of time.

The mass balance cultures were composed of 43.1 ml sterile marine broth, 5 ml inoculation of mature culture (*Pyrococcus* or *Desulfurococcus*), 1.9 ml of 2,4,6 TCP stock, and amended with 1% (wt/vol) sulfur. This standardized the TCP concentration at 0.51 mM. A sample was taken immediately after inoculation; and the cultures, subsequently, incubated at 95 deg. C. The cultures were then sampled at 7 days, 21 days, and 42 days. Each sample was processed in a similar manner as the DQP and extracted into MTBE. These samples were then refrigerated and analyzed with the standards on the GC-MS in one sitting.

The samples were processed at the same time to eliminate variation due to daily fluctuations in the GC-MS.

3.6 The Phenol Extraction Process

Although HPLC could identify TCP and possible degraded products, the process falls short due to its lack of specificity and susceptibility to interferences from the media. For positive identification of TCP loss and the identification of degraded products, an organic extraction process was utilized in tandem with gas chromatography-mass spectrophotometry.

The extraction process used was chosen for simplicity, accuracy and reproducibility. This procedure utilized a Supelco "ENVI-Chrom P" solid phase extraction tube (SPE). The packing consists of styrene divinyl-benzene copolymer beads. These beads have active, aromatic sites on the resin bead that is attractive to phenolic compounds. After sample elution, the phenols can then be recovered separately and analyzed.

The process used in these studies is as follows:

1) Place the extraction tube in a vacuum recovery system.
2) Wash and activate the packing with 6 ml of methyl t-butyl ether.
3) Prior to the tube draining completely of MTBE, add 6 ml of pure methanol.
4) Prior to the tube draining completely of methanol, add 6 ml of deionized water (*for samples processed at pH 3.0 and pH 1.0, the D.I. water must be normalized at those pH's prior to elution*).
5) Add 5 ml of culture supernatant and vacuum suction no

greater than 5 ml per minute.

6) Vacuum dry the tube for 1 minute and prepare the sample receptacle.

7) Add 2 ml of MTBE to soak the packing in the tube with no suction.

8) After 1 minute, add 3 ml of MTBE and vacuum suction the solution no greater than a drop wise rate.

9) Seal the sample receptacle and process immediately, or refrigerate for a later time.

3.7 Sample Analysis

After processing through a SPE tube, the samples were analyzed with a gas chromatograph - mass spectrophotometer. This method was chosen an the basis of accuracy and precision, and also the ability to positively identify the degradation products.

Table 3.4 Gas Chromatograph-Mass Spec. Conditions

Make	Varian Star 3400
Settings	
Column	J&W Scientific Capillary Column #122-5032 30mx.35mm
Phase	D B 5 -- 0.25 micron film
Temp.	Ramp from 40°C to 250°C in 38 minutes
Mass Detector	Saturn Ion Trap
Computer	Compaq Pro-linea 4/33
Software	Varian Saturn gc.ms Revision C
Program	EPA Protocol 625 (analysis of phenols)
Autosampler	Leap Technologies 105 slot

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Growth and Productivity

As stated earlier, the organisms of interest were *Pyrococcus* GBD and *Desulfurococcus* SY. These are both classified as hyperthermophilic, sulfur reducing, obligate marine anaerobes. They were obtained as pure cultures from Woods Hole Oceanographic Institute, Woods Hole, MA. The growth of these organisms was identified via pressure build up in the culture vessel. These gasses, according to Jannasch et al (1992), are composed primarily of hydrogen sulfide. This evolution is a result of the reduction of elemental sulfur amended to the culture and other trace sources in the marine broth. Mercaptan and fatty alcohols could also compose a portion of the gas pressure.

4.1.1 *Desulfurococcus* SY Variations

Pressure bottles were set up in a side by side experiment, where temperature and sulfur amendments were varied. In pressure bottle composition #1, a marine broth + sulfur + Inocula culture was incubated at 95 ^{o}C for four hours. As can be seen in Table 1 and Figure 1, internal pressure in the head space of the culture vessel slowly, but steadily increases. This increase in pressure ceases at 14 PSI, which is achieved after 240 minutes of incubation. The color of the culture solution also has taken on a yellowish tint due to sulfide accumulation.

Comparatively, however, pressure bottle composition # 3 and # 7 showed very little increase in head space pressure. Culture composition #7 had marine broth and Inocula, but was not sulfur amended. Incubated at 95 ^{o}C for several

hours, this culture evolved very little gas and growth appeared limited., achieving a maximum pressure of 2 PSI. These results can be seen on Table 2 and Figure 2. The solution did not take on a characteristic yellow tint.

Culture composition # 3 was sulfur amended but was incubated at ambient room temperature. This culture did not evolve any growth evident gasses. This can be seen on Table 3 and Figure 3, where after 240 minutes of study, there was never a head space pressure increase.

4.1.2 *Pyrococcus* SY Variations

Pressure bottle composition # 2 included sulfur amendments and was incubated at 95 ^{o}C. The evolution of head space gas was very rapid, and after only 105 minutes achieved a maximum pressure of 14 PSI (please refer to Table 4 and Figure 4). The culture media also took on the yellowish tint of dissolved sulfide.

In pressure bottle composition #6 and #4, similar trends were seen as with PBC #2 and #3. In PBC # 6 (Table 5 and Figure 5), sulfur unamended and incubated at 95 ^{o}C, a slight increase in head space pressure occurs, but slowly and maximizes at 2 PSI. In PBC #4 (Table 6 and Figure 6) the Pyrococcus culture will also not grow at sub-optimal temperatures, and never evolves head space gasses.

4.1.3 Sterile Controls

Sterile control cultures were set up using marine broth and elemental sulfur. This sterile culture, PBC # 5, was incubated at 95 ^{o}C to account for moderate temperature abiotic chemical reactions of the media, that may add to head space gas. As can be seen in Table and Figure 7, after several hours of incubation, there

is no appreciable increase in head space pressures. A minor elevation of pressure is do in part to increasing the temperature of a confined system.

4.2 2,4,6 TCP Enrichment Program

Culture enrichment is utilized in many cases to allow bacteria to develop in an environment alien to its natural surroundings. The organisms may simply grow and exist with the enrichment or possibly interact with the amendment. This process in turn fosters properties in the bacteria not normally expressed in non-enriched environments. This experiment dealt primarily in 2,4,6 trichlorophenol, due in part to its' recalcitrance in nature and widespread contamination. The possible effects on TCP that were to be investigated were degradation (simply breaking down of the compound, nonspecifically) or dehalogenation (successive elimination of chlorine from the phenol ring).

4.2.1 TCP Degradation via *Pyrococcus* GBD

In the following microcosms, 2,4,6 TCP was added in increasing concentration. Low levels of compound (0.0126 mM) were used to initiate the enrichment series, due to the unknown lethal concentration limits of the compound on the *Pyrococcus* strain.

The enrichment series was initiated with 0.0126 mM TCP. This compound, as can be seen in Table 8 and Figure 8, was observed over 28 days. In the course of time, HPLC identification of this compound showed a slow rate of degradation. At the end of the study period, *Pyrococcus* GBD had not successively eliminated the TCP enrichment.

Conversely, the enrichment series made from the original culture became increasingly effective at eliminating TCP from the culture. As can be seen in

comparing Table 9/Figure 9 and Table 10/Figure 10, up to ten times the initial concentration of TCP could be degraded from the system by rapid selection of TCP tolerant populations of *Pyrococcus*. Although by products could be found in increasing concentration down field of TCP on the HPLC output, these compounds could not be properly identified, nor were they found in exact stoichiometric amounts.

4.2.2 TCP Degradation via *Desulfurococcus* SY

Similar to the *Pyrococcus* experiments, *Desulfurococcus* was also subjected to an enrichment series. Amendments of 0.0126 mM TCP were made to initial cultures and observed. The effects, as can be seen on Table 11 and Figure 11, reflect the *Pyrococcus* experiments previously mentioned. With *Desulfurococcus*, TCP was slowly degraded over the course of 28 days. Increasing amounts of by product were observed accumulating down field.

Increasing the enrichment concentration and successively inoculating from low concentration cultures to high concentration fostered the ability for Desulfurococcus to degrade TCP. As can be seen in Table 11/Figure 11 and Table 12/Figure 12, over the course of the experiment, the rate of TCP disappearance increased, and successively larger amounts of by-product can be observed accumulating down field.

4.2.3 Degradation vs. Dehalogenation

At this stage, HPLC identification of by products was difficult due to the interferences from the complex media and the super imposing of multiple compounds as a result of incomplete column separation. Dehalogenation could

not be proven, nor could it be discounted at this stage, without precise identification of the by-products formed.

The effects on TCP appeared to be biological or biochemical in origin, dependent upon the growth of the organism. The distinction to be made between biological and biochemical being: biological defines the metabolic or enzymatic use of TCP by an organism; while biochemical defines the effect of chemical agents, manufactured during the growth phase of the organism, on TCP independent of the organisms growth. As can be seen on Table and Figure 14, sterile controls composed of sterile media, sulfur and TCP showed no appreciable degradation even at incubation temperatures. Consequently, there were no by-products formed

4.3 The Data Qualifying Program

There were several main objectives to the data qualifying program (please refer to Table 4-3). First, the DQP was used to identify if the TCP degradation was actually a dehalogenation process by positively identifying all growth products. Second, the DQP was to identify if the loss of TCP could be attributed to biological, biochemical, or abiotic chemical reactions.

The DQP could achieve such accuracy and precision due to the extraction and analysis technology employed. The extraction process involved a solid phase extraction media, which selectively separated out phenol or phenol-like groups. The analysis involved gas chromatography in tandem with a mass spectrophotometer, utilizing the software that could positively identify the analyzed compounds.

4.3.1 Results of Bottles 101 and 201

These bottles were inoculated with *Pyrococcus* and *Desulfurococcus*, respectively. These organisms were cultured in the presence of sulfur but not TCP for the duration of the program. The intent of these cultures was to establish a baseline of possible biological interferences that may impose upon the phenol analysis regions.

The results of this study showed that neither *Pyrococcus* nor *Desulfurococcus* produce substances that, when extracted, appear to interfere within TCP, DCP, MCP or Phenol ranges (please refer to Figure 15). The cultures did grow to maximum density, and at the end of 36 days, appeared to have a yellowish tint to the culture liquid, indicating sulfide production at elevated levels.

4.3.2 Results of Bottles 102 and 202

Bottle 102: This culture was composed of sterile media, elemental sulfur, organism innocula, and standardized at approximately 1mM TCP. Immediately after inoculation, the sample was analyzed and showed to have a TCP concentration of 1.03 mM. As can be seen in Table 15 and Figure 16, at 0 days and 4 days, there is no sign TCP degradation. However, between days and 22 days, dehalogenation occurs. This is evident by the appearance of the dehalogenated products di-chlorophenol, mono-chlorophenol, and phenol. At day 22, DCP concentrations are at their highest, and subsequently are subjected to further dehalogenation for the duration of the program. At approximately day 35 of the program, the concentrations of TCP and DCP are less than their dehalogenated substituents.

Bottle 202: This culture, much like bottle 102, was composed of sterile media, *Desulfurococcus* innocula, elemental sulfur and standardized to approximately 1 mM TCP. Immediately after inoculation, the culture was sampled and TCP concentrations were found to be 0.99 mM, initially. Referring to Table 16 and Figure 17, it can also be seen that there was no appearance of DCP, MCP or Phenol at the start of the program. However, between 4 days and 22 days of the project dehalogenation commenced. Dehalogenated products such as DCP, MCP and Phenol were now identified in appreciable quantities. At the 36 day mark, Phenol and MCP appeared in greater quantities than TCP and DCP.

4.3.3 Results of Bottles 103 and 203

These cultures were set up identical to cultures 102 and 202, but are sulfur unamended. Neither culture 103 nor 203 grew, even at growth temperatures, in the absence of sulfur. There appeared to be no high temperature reactions between the media, innocula, and TCP. Referring to Figures 17, 18 and Tables 18,19; TCP concentrations started at 0.986 for both cultures and ended at 0.985 and 0.998, respectively. This increase is due in part to a slightly higher extraction recovery.

4.3.4 Results of Bottles 104 and 204

Bottles 104 and 204 were set up using sterile media, *Pyrococcus* and *Desulfurococcus* innocula, respectively; and TCP. A pressurized hydrogen head space was established in place of a sulfur amendment in attempts to culture the hyperthermophiles an alternative electron acceptor. As can be seen in Table 19, 20 and Figures 20,21; the organisms were unable to grow to any appreciable densities. This lack of growth is reflected in the lack of dehalogenation.

4.3.5 Results of Bottles 105 and 205

Bottles 105 and 205 set up in an identical fashion as bottles 102 and 202. Bottles 105 and 205 however, were incubated at ambient room temperature. This provided a control for growth dependence. It has already been established that both *Pyrococcus* (bottle105) and *Desulfurococcus* (bottle 205), are metabolically inactive below optimum growth temperatures (95 deg C). As can be seen in Table 21/ Figure 22, the culture was sampled immediately after inoculation and found to contain 0.997 mM TCP. At the end of 45 days, the TCP concentration was 0.996 mM. The *Pyrococcus* could not grow at such a low temperature, and was unable to dehalogenate the TCP.

Bottle 205 showed similar results. Referring to Table 22 and Figure 23, the initial concentration of TCP was 0.985 mM. Also unable to grow and metabolically inactive, the *Desulfurococcus* culture had a concentration of 0.983 mM TCP at the end of 36 days.

4.3.6 Results of Bottles 106 and 206

These bottles were composed of previously grown cultures, resupplemented with fresh elemental sulfur, and standardized to approximately 1mM TCP. The primary purpose of these cultures was to elucidate the effects of a mature culture on TCP.

As can be seen from Table 23 and Figure 24, immediately after the addition of TCP, bottle 106 was sampled and found to contain only 0.972 mM TCP. This quickly under went dehalogenation, and by the fourth day showed DCP, MCP, and Phenol in appreciable quantities. By the 36 day, all of the TCP

had been dehalogenated, DCP had reached a maximum concentration and began to decline, and MCP and Phenol were continuing to become more concentrated.

In Table 24 and Figure 25, bottle 206 shows a similar trend, however, the initial rate of dehalogenation is slower. By the end of the 36 day, 0.1 mM of TCP still remained in the microcosm, and higher concentrations of DCP, MCP and Phenol are also evident.

In both microcosms, it appears that having an initial high density of cells, as found in a mature culture, increases the rate at which initial dehalogenation will take place. This coincides with the slower rate seen in freshly inoculated cultures such as bottles 102 and 202, where cell populations are low.

4.3.7 Results of Sodium Do-Decyl Sulfate Bottles (107, 108, 109, 207, 208,209)

These microcosms were run separate from the DQP, but side by side to each other for comparison. Amended cultures 107,109,207 and 209 contained 5% (wt/vol) sodium do-decyl sulfate (SDS), a strong detergent that is capable of lysing cell membranes. This agent was selected to halt cellular activity due to the hyperthermophyles resistance to other popular antibiotics.

Bottles 107 and 207: (refer to Table 25/26&Figure 26/27) This microcosm was established with sterile media, elemental sulfur, *Pyrococcus* and *Desulfurococcus* innocula (respectively), and standardized to approximately 0.5 mM TCP. Immediately after inoculation, the SDS agent was added, the sample shaken vigorously over the span of several minutes, and sampled. The beginning concentration of bottle 107 was 0.55mM; bottle 207 was 0.54 mM. After 14 days of incubation at optimum growth temperatures, no dehalogenation or degradation had taken place in either microcosm. No growth was evident in either culture, and no sulfide was seen accumulating in the solution. Final

concentrations of TCP were : bottle 107--0.55mM TCP, bottle 207--0.54 mM TCP

Bottle 108: (refer to Table 27 and Figure 28) This culture was established as a comparison microcosm. The culture was composed of sterile media, *Pyrococcus* innocula, elemental sulfur, and standardized to approximately 0.5 mM TCP. This culture was not subjected to SDS lysing and was allowed to incubate at optimum growth temperatures. Much like previously studied cultures, initial TCP concentrations were 0.51 mM; and rapidly dehalogenated over the 14 day incubation to a final concentration of 0.11 mM. Dehalogenated products such as DCP, MCP, and Phenol show a characteristic increase, as can be seen in Figure 28.

Bottle 208: This culture was also grown as a comparison microcosm. This culture was composed in a similar fashion to bottle 108, however, it was inoculated with **Desulfurococcus**. As seen in Table 28 and Figure 29, the initial concentration of the TCP amendment was 0.52mM. Over the 14 day incubation, TCP was dehalogenated to approximately 20% of the initial concentration. Dehalogenated products were also recovered in increasing concentrations.

Bottles 109 and 209: These bottles utilized mature cultures of *Pyrococcus* and *Desulfurococcus*, respectively. The cultures were standardized at approximately 0.5 mM TCP and immediately lysed with 5% SDS. The microcosms were then sampled immediately. Referring to Table 29 and Figure 30, bottle 109 had an initial concentration of 0.51 mM TCP. After 14 days of incubation at optimum growth temperatures, no dehalogenation or degradation had taken place, and a final concentration of 0.51 mM was measured.

Bottle 209 also had an initial concentration of 0.52 mM TCP. After 14 days of incubation, the lysed culture also failed to dehalogenate the TCP and had

a final concentration of 0.52 mM (refer to Table 30 and Figure 31). In both cultures the absence of living organisms eliminated the dehalogenation process, and also showed that it is the activity of the organism itself, and not a chemical by-product of growth that dehalogenates the TCP.

4.3.8 Results of Bottles 301 and 302

These bottles were composed primarily of sterile media, cell free innocula, and standardized to approximately 1mM TCP. The primary purpose of these cultures was to account for cell free activity with regards to the concentration of TCP. Referring to Table 31 and Figure 32, bottle 301 was incubated at optimum growth temperatures for 36 days. During that time period, the concentration of TCP was virtually unaffected. Similarly, bottle 302 was supplemented with sulfur and also incubated for 36 days. As can be seen on Table 32 and Figure 33, the TCP concentration was also unaffected, eliminating the possibility of cell free activity in these cultures.

4.3.9 Results of Bottles 303 and 304

Bottles 303 and 304 were composed of sterile media, sodium sulfide, and standardized to approximately 1 mM TCP. The effects of strong reducing agents on TCP concentrations is unknown, and these cultures attempt to simulate high sulfide concentrations in a cell free environment. As can be seen in Tables 33/34 and Figures 34/35, sodium sulfide does not affect TCP concentrations, nor does it appear to reduce it at all (even at optimum growth temperatures).

4.3.10 Results of Bottle 305

Bottle 305 was composed primarily of cell free supernatant, standardized to 1 mM of TCP, and pressurized with hydrogen gas. This culture was an attempt to supply the microcosm with growth by products (from the organisms and left in solution) and an alternative electron acceptor. However, as can be seen in Table 35 and Figure 36, the concentration of TCP remains unaffected by the complex media, even after 36 days at optimum growth temperatures.

4.3.11 Results of Bottles 306 and 307

These cultures were also cell free, but amended with methyl mercaptan and di-thio-threotal. These organic reducing agents were selected on the basis of strength and probable presence in a sulfur reducing bacterial culture. However, even at incubation temperatures and extended periods of time, they had no effect on TCP concentration. This can be seen on Tables 36/37 and Figures 37/38.

4.4 Mass Balance Results

The two culture bottles set up for the mass balance procedure were composed of sterile Marine broth, mature culture innocula, elemental sulfur, and standardized to approximately 0.51 mM TCP. Samples were taken immediately after inoculation and preserved for analysis at a later time.

Standards were also made, paying exacting attention to concentration and standardization. The standards were composed of phenol, 2-monochlorophenol, 2,6 dichlorophenol, and 2,4,6 trichlorophenol. Each compound was standardized to precisely 0.5 mM, 0.25 mM, and 0.10 mM concentration. Each solution was subjected to an MTBE extraction and analyzed with the GC-MS. The results

were then plotted, subjected to a linear regression and used as references for the analyzed data. (refer to Table 40 and Figures 41,42,43,44)

4.4.1 Results of Mass Balance Culture 1 (*Desulfurococcus* SY)

Referring to Table 38 and Figure 39, it is easily seen that through the course of time, 0.499 mM TCP is reductively dehalogenated from to DCP, MCP and Phenol. The rate of dehalogenation of TCP, as can be seen on Table 37, is 0.0105 mM/day over the course of 42 days. The sum total of the dehalogenated products at the end of the experiment was 0.495 mM ([TCP]+[DCP]+[MCP]+[Phenol]) . This result indicates that the amount of TCP and subsequent dehalogenated products are quantitatively conserved throughout the system. This also lends evidence that mineralization or further degradation of phenol is not taking place in this system at the 42 day mark.

4.4.2 Results of Mass Balance Culture 2 (*Pyrococcus* GBD)

Referring to Table 39 and Figure 40, the dehalogenation of TCP can be seen quite readily. With an initial concentration of 0.499 mM, the resultant concentration of 0.070 mM reflects a rate of dehalogenation of approximately 0.102 mM/day for 42 days. The sum total of the dehalogenated products was 0.51 mM at the termination of the experiment. Although slightly higher, this result indicates that the TCP and dehalogenated products were conserved over the 42 day period. This event also lends evidence that further mineralization or degradation has taken place at this point. The increased total is a result of slightly higher recovery rates from the MTBE extraction.

CHAPTER 5

CONCLUSIONS

5.1 Growth and Productivity

- Desulfurococcus strain SY is a hyperthermophilic, obligate anaerobe.
- Pyrococcus strain GBD is also a hyperthermophilic, obligate anaerobe.
- An optimum temperature of 95 deg. C is required for both strains to grow. Below this temperature, both strains are metabolically dormant.
- Cultures that were sulfur unamended showed an insignificant degree of growth, and did not evolve characteristic gasses present in an actively growing culture.
- As can be seen in sterile controls, the components of the media used to grow the bacteria will not produce evidence of growth.

5.2 TCP Enrichment

- Both Pyrococcus and Desulfurococcus were able to acclimate to TCP in their cultures.
- Both organisms, as a function of time, began expressing a tendency to degrade the TCP amendments.
- As subcultures were made of each organisms into higher concentrations of TCP, their ability to degrade also increased.

- This effect was substantiated by sterile controls, held under identical conditions as live cultures, inability to effect a change on TCP amendments.

5.3 The Data Qualifying Program.

- The DQP was able to solidify the organisms ability dehalogenate TCP.
- Actively growing cultures without TCP amendments did not produce any interferences that may clash with the output range of TCP or it's constituents.
- Cultures that were able to grow with TCP and sulfur amendments showed active dehalogenation
- Cultures that were not sulfur amended or were incubated at sub-optimal temperatures did not actively dehalogenate and TCP concentrations remained unaffected throughout the experiment.
- Alternate electron acceptors, such as hydrogen gas, did not stimulate growth, and did not allow TCP to be dehalogenated.
- The SDS amended cultures solidified the dependence of active growth on TCP dehalogenation; where, a comparison of active cultures dehalogenating and lysed cultures maintaining a constant concentration of TCP.
- Fully matured cultures that were subjected to lysing did not retain the ability to dehalogenate. This indicates that chemicals produced as a result of growth are not responsible for dehalogenation, but rather the organism itself in a metabolically active state.

- Experiments using cell free cultures were unable to affect a change in TCP concentrations.
- Strong chemical and organic reducing agents were used to simulate the reducing environment of an actively growing culture, but proved to be unable to reduce the TCP amendments.

5.4 The Mass Balance

- Culture established the mass balance experiments actively and rapidly dehalogenated 2,4,6 TCP.
- According to the identification via mass spectrophotometer, the dehalogenated products were in the form of 2,6 DCP, 2 MCP, and phenol. Trace amounts of the isomers 2,4 DCP and 2,5 DCP which are products of the contaminant 2,4,5 TCP.
- Desulfurococcus SY had a rate of TCP disappearance of 0.0105 mM/day, and produced DCP, MCP, and Phenol in stoichiometric amounts.
- Pyrococcus SY had a rate of TCP disappearance of 0.0102 mM/day. and also produced DCP, MCP, and Phenol in stoichiometric amounts.
- At the termination point of the mass balance experiments, all of the dehalogenated components could account for the amount of TCP lost. Mineralization or further degradation of phenol was not seen at the 42 day mark.

APPENDIX A

APPENDIX OF TABLES

Table 1 Pressure Bottle Composition #1 (Desulfurococcus SY)

Time(minutes)	Pressure (PSI)
0	0
15	2.5
30	2.5
45	6.5
60	7
75	7.5
90	8.5
105	8.5
120	9
135	9.5
150	10
165	11
180	11.5
195	13
210	13.2
225	13.5
240	14

Table 2 Pressure Bottle Composition #7 (Desulfurococcus, no sulfur)

Time (Minutes)	Pressure(PSI)
0	0
30	1
60	1
120	1
180	2
240	2

Table 3 Pressure Bottle Composition #3 (Desulfurococcus, room temp)

Time (minutes)	Pressure (PSI)
0	0
60	0
120	0
180	0
240	0

Table 4 Pressure Bottle Composition #2 (Pyrococcus SY)

Time (Minutes)	Pressure (PSI)
0	0
15	7
30	11
45	12.5
60	13
75	13.1
90	13.5
105	14
120	14
135	14
150	14
165	14
180	14
195	14
210	14
225	14
240	14

Table 5 Pressure Bottle Composition #6 (Pyrococcus, no sulfur)

Time (minutes)	Pressure(PSI)
0	0
30	1
60	1
120	1.5
180	2
240	2

Table 6 Pressure Bottle Composition #4 (Pyrococcus at room temp.)

Time (Minutes)	Pressure(PSI)
0	0
60	0
120	0
180	0
240	0

Table 7 Pressure Bottle Composition #7 (sterile media at 95 deg C)

Time (Minutes)	Pressure(PSI)
0	0
30	1
60	1
120	1
180	1
240	1

Table 8 HPLC Identification of TCP Degradation (Pyrococcus at 0.0126 mM)

Days	Area	Unk. 1	Unk.2	Unk.3
0	25000	0	0	0
7	23000	1000	0	0
17	22000	1200	1150	700
21	12000	3400	3200	2200
28	6000	5400	5000	4500

Table 9 HPLC Identification of TCP Degradation (Pyrococcus at 0.063 mM)

DAYS	AREA	Unk.1	Unk.2	Unk.3
0	128000	0	0	0
7	99800	22000	9800	2400
17	65000	45000	17000	8500
21	2300	51000	27000	16500
28	0	48000	28000	25000

Table 10 HPLC Identification of TCP Degradation
(Pyrococcus at 0.126 mM)

DAYS	Area TCP	Unk.1	Unk.2	Unk.3
0	248000	0	0	0
7	150000	100000	50000	20000
17	100000	145000	100000	75000
21	75000	160000	125000	122000
28	4120	125000	130000	131000

Table 11 HPLC Identification of TCP Degradation (Desulfurococcus at 0.0126 mM)

DAYS	Area	Unk 1	Unk 2	Unk 3
0	26000	0	0	0
7	19500	1000	0	0
17	14000	5500	2500	1000
21	11500	8000	5600	5000
28	8000	8200	6500	6000

Table 12 HPLC Identification of TCP Degradation (Desulfurococcus at 0.063 mM)

DAYS	Area TCP	Unk.1	Unk.2	Unk.3
0	130000	0	0	0
7	95000	17000	10500	1000
17	72000	55000	19000	5500
21	5500	54000	30000	17000
28	500	50000	32000	23000

Table 13 HPLC Identification of TCP Degradation (Desulfurococcus at 0.126 mM)

Days	TCP Area	Unk 1	Unk.2	Unk.3
0	250500	0	0	0
7	175000	18000	10000	5000
17	120000	40000	35000	17000
21	80000	82000	85000	45000
28	45000	79000	90000	65000

Table 14 HPLC Identification of TCP Degradation (Sterile Media at 0.126 mM)

Days	Area TCP
0	255000
7	254000
17	255000
21	255000
28	250000

Table 15 DQP Bottle 102

Time(days)	[TCP](mM)	Area
0	1.026624	8792000
4	1.024748	8776000
22	0.23061	2002000
36	0.120528	1063000

Time(days)	[DCP](mM)	Area
0	0	0
4	0	0
22	0.25458	2199000
36	0.13803	1199000

Time(days)	[MCP](mM)	Area
0	0	0
4	0	0
22	0.113465	988200
36	0.153349	1334000

Time(days)	[Phenol](mM)	Area
0	0	0
4	0	0
22	0.057669	497700
36	0.170555	1464000

Table 16 DQP Bottle 202

Time(days)	[TCP](mM)	Area
0	0.997902	8547000
4	0.957104	8199000
22	0.262497	2274000
36	0.114431	1011000

Time(days)	[DCP](mM)	Area
0	0	0
4	0	0
22	0.266002	2297000
36	0.219615	1899000

Time(days)	[MCP](mM)	Area
0	0	0
4	0	0
22	0.101562	885000
36	0.149313	1299000

Time(days)	[Phenol](mM)	Area
0	0	0
4	0	0
22	0.055602	480000
36	0.160391	1377000

Table 17 DQP Bottle 103

Time (days)	[TCP](mM)	Area
0	0.986413	8449000
4	0.978206	8379000
22	0.988288	8465000
45	0.985944	8445000

Table 18 DQP Bottle 203

Time (days)	[TCP](mM)	Area
0	0.986764	8452000
4	0.998605	8553000
22	0.997315	8542000
36	0.998839	8555000

Table 19 DQP Bottle 104

Time (days)	[TCP](mM)	Area
0	0.921114	7892000
4	0.88313	7568000
22	0.916776	7855000
36	0.904936	7754000
36	0.90646	7767000

Table 20 DQP Bottle 204

Time (days)	[TCP](mM)	Area
0	0.972696	8332000
4	0.973634	8340000
22	0.968828	8299000
36	0.970117	8310000

Table 21 DQP Bottle 105

Time(days)	[TCP](mM)	Area
0	0.997667	8545000
4	0.98993	8479000
22	0.994736	8520000
36	0.99626	8533000

Table 22 DQP Bottle 205

Time (days)	[TCP](mM)	Area
0	0.985709	8443000
4	0.981137	8404000
22	0.979496	8390000
36	0.983834	8427000

Table 23 DQP Bottle 106

Time(days)	[TCP](mM)	Area
0	0.972696	8332000
4	0.294619	2548000
22	0.082778	741000
36	0.070352	635000

Time(days)	[DCP](mM)	Area
0	0	0
4	0.123928	1078000
22	0.10866	947000
36	0.079988	701000

Time(days)	[MCP](mM)	Area
0	0	0
4	0.045045	395000
22	0.096141	838000
36	0.127398	1109000

Time(days)	[Phenol](mM)	Area
0	0	0
4	0.027798	242000
22	0.052681	455000
36	0.115999	997000

Table 24 DQP Bottle 206

Time(days)	[TCP](mM)	Area
0	0.961794	8239000
4	0.933658	7999000
22	0.214783	1867000
36	0.100129	889000

Time(days)	[DCP](mM)	Area
0	0	0
4	0	0
22	0.289779	2501000
36	0.257028	2220000

Time(days)	[MCP](mM)	Area
0	0	0
4	0	0
22	0.116441	1014000
36	0.206867	1798000

Time(days)	[Phenol](mM)	Area
0	0	0
4	0	0
22	0.066817	576000
36	0.172074	1477000

Table 25 DQP Bottle 107

Time	[TCP](mM)	Area
0	0.552532	4748000
7	0.547726	4707000
14	0.551477	4739000

Table 26 DQP Bottle 207

Time (days)	[TCP](mM)	Area
0	0.54456	4680000
7	0.54796	4709000
14	0.54585	4691000

Table 27 DQP Bottle 108

Time(days)	[TCP](mM)	Area
0	0.517714	4451000
7	0.241864	2098000
14	0.112907	998000

Time(days)	[DCP](mM)	Area
0	0	0
7	0.11507	1002000
14	0.246888	2133000

Time(days)	[MCP](mM)	Area
0	0	0
7	0.024053	213000
14	0.116095	1011000

Time(days)	[Phenol](mM)	Area
0	0	0
7	0.011676	104000
14	0.089831	773000

Table 28 DQP Bottle 208

Time(days)	[TCP](mM)	Area
0	0.522286	4490000
7	0.236589	2053000
14	0.101301	899000

Time(days)	[DCP](mM)	Area
0	0	0
7	0.131387	1142000
14	0.261923	2262000

Time(days)	[MCP](mM)	Area
0	0	0
7	0.029935	264000
14	0.130858	1139000

Time(days)	[Phenol](mM)	Area
0	0	0
7	0.012261	109000
14	0.126746	1089000

Table 29 DQP Bottle 109

Time(days)	[TCP](mM)	Area
0	0.517714	4451000
7	0.493447	4244000
14	0.510445	4389000

Table 30 DQP Bottle 209

Time(days)	[TCP](mM)	Area
0	0.52592	4521000
7	0.524513	4509000
14	0.524748	4511000

Table 31 DQP Bottle 301

Time(days)	[TCP](mM)	Area
0	1.004115	8600000
4	1.003294	8593000
22	1.003529	8595000
36	1.003763	8597000

Table 32 DQP Bottle 302

Time (days)	[TCP](mM)	Area
0	0.985709	8443000
4	0.983365	8423000
22	0.983599	8425000
36	0.984185	8430000

Table 33 DQP Bottle 303

Time(days)	[TCP](mM)	Area
0	0.986178	8447000
4	0.991454	8492000
22	0.987116	8455000
36	0.980785	8401000

Table 34 DQP Bottle 34

Time (days)	[TCP](mM)	Area
0	0.98864	8468000
4	0.988757	8469000
22	0.987585	8459000
36	0.987116	8455000

Table 35 DQP Bottle 305

Time (days)	[TCP](mM)	Area
0	0.986413	8449000
4	0.973869	8342000
22	0.98653	8450000
36	0.982661	8417000

Table 36 DQP Bottle 306

Time(days)	[TCP](mM)	Area
0	0.494736	4255000
7	0.501184	4310000
14	0.497433	4278000

Table 37 DQP Bottle 307

Time(days)	[TCP](mM)	Area
0	0.51619	4438000
7	0.517011	4445000
14	0.516424	4440000

Table 38 Mass Balance Culture 1

Time(days)	[TCP](mM)	Area
0	0.499874	4398946
7	0.326651	2874579
21	0.166787	1467781
42	0.054647	480948

Time(days)	[DCP](mM)	Area
0	0	0
7	0.148088	918372
21	0.169394	1050468
42	0.157695	977937

Time(days)	[MCP](mM)	Area
0	0	0
7	0.03842	176581
21	0.085906	375545
42	0.168305	720796

Time(days)	[Phenol](mM)	Area
0	0	0
7	0.022508	50008
21	0.048745	108253
42	0.116096	257773

Table 39 Mass Balance Culture 2

Time(days)	[TCP](mM)	Area
0	0.499066	4391831
7	0.323583	2847585
21	0.197365	1736862
42	0.070065	616623

Time(days)	[DCP](mM)	Area
0	0	0
7	0.131114	813131
21	0.212814	1319673
42	0.16936	1050255

Time(days)	[MCP](mM)	Area
0	0	0
7	0.021757	106762
21	0.101828	442260
42	0.181353	775467

Time(days)	[Phenol](mM)	Area
0	0	0
7	0.024978	55492
21	0.060656	134697
42	0.10461	232274

Table 40 Analysis of Standards

2,4,6 TCP Analysis

Conc. (mM)	Area
0.5	4400176
0.25	2200159
0.1	880055
slope=	8800000
y-intercept=	51.4

2,6 DCP Analysis

Conc.(mM)	Area
0.5	3101555
0.25	1550992
0.1	620445
slope=	6200000
y-intercept=	226

2 MCP Analysis

Conc.(mM)	Area
0.5	2082082
0.25	1023614
0.1	408416
slope=	4190000
y-intercept=	15600

Phenol Analysis

Conc.(mM)	Area
0.5	1109593
0.25	554887
0.1	221918
slope=	2220000
y-intercept=	40

APPENDIX B

APPENDIX OF FIGURES

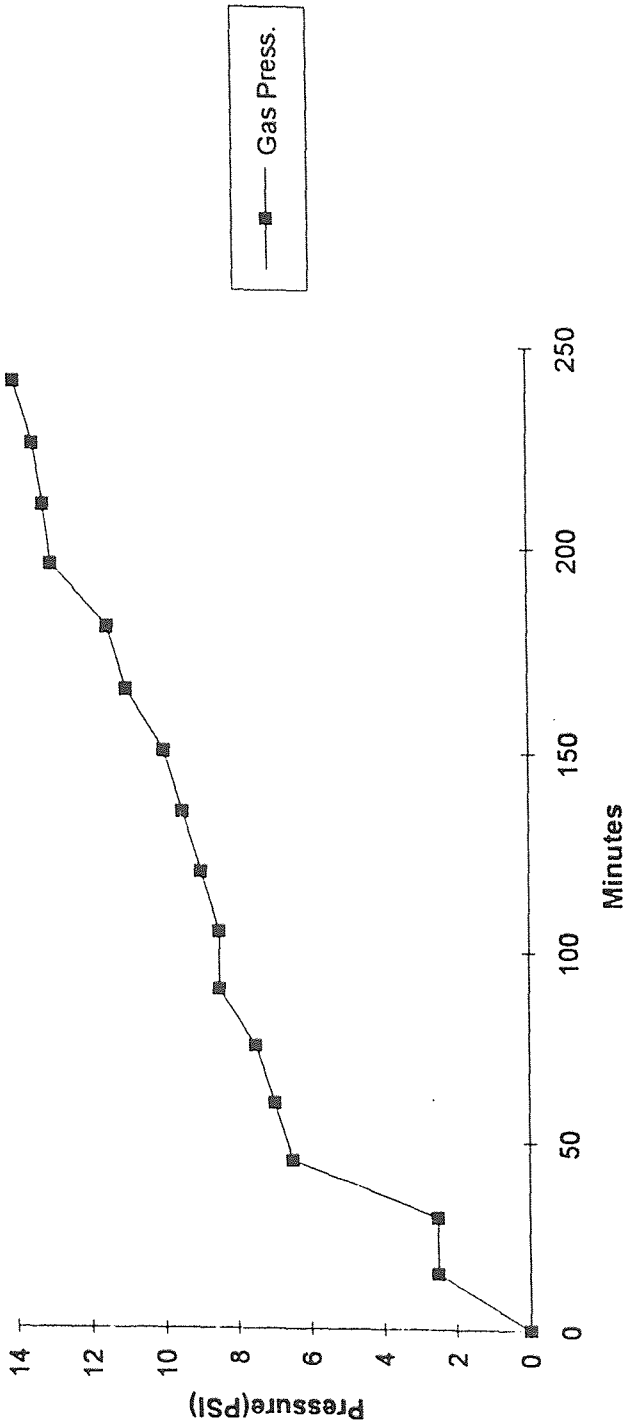


Figure 1 PBC 1- *Desulfurococcus* SY at 95 deg. C

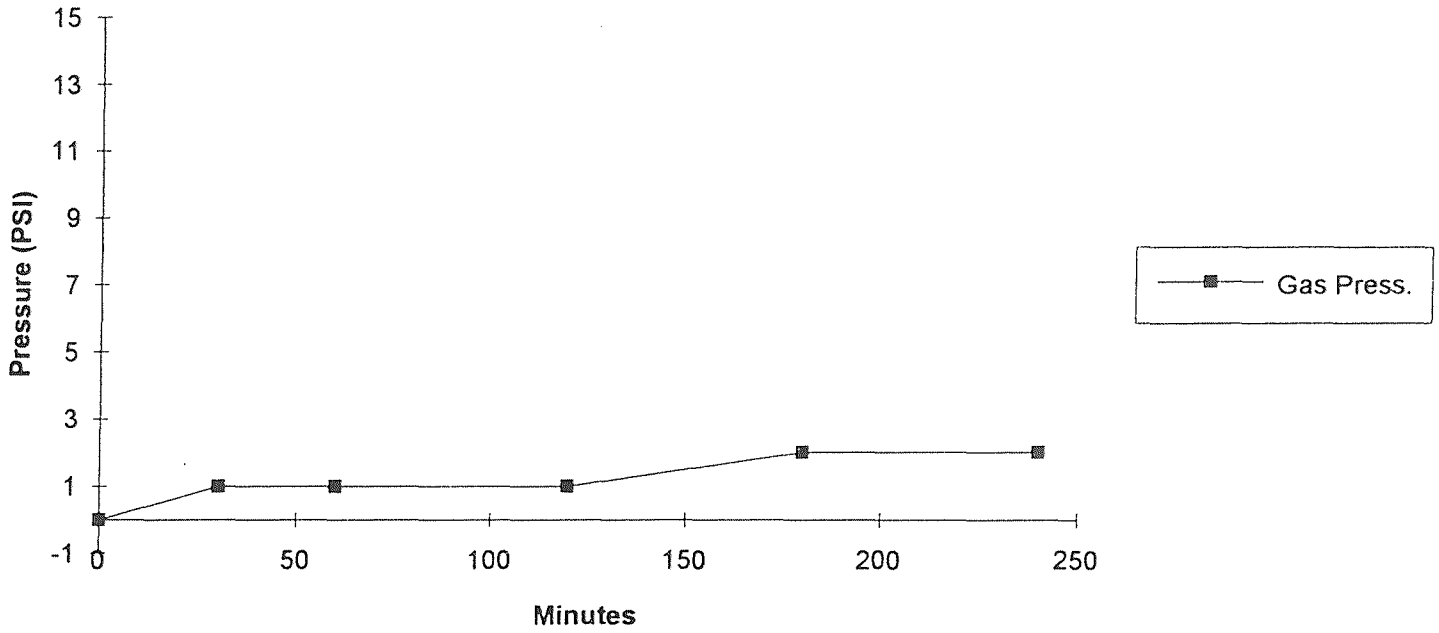


Figure 2 PBC 7- *Desulfurococcus* SY at 95 deg.C- no sulfur

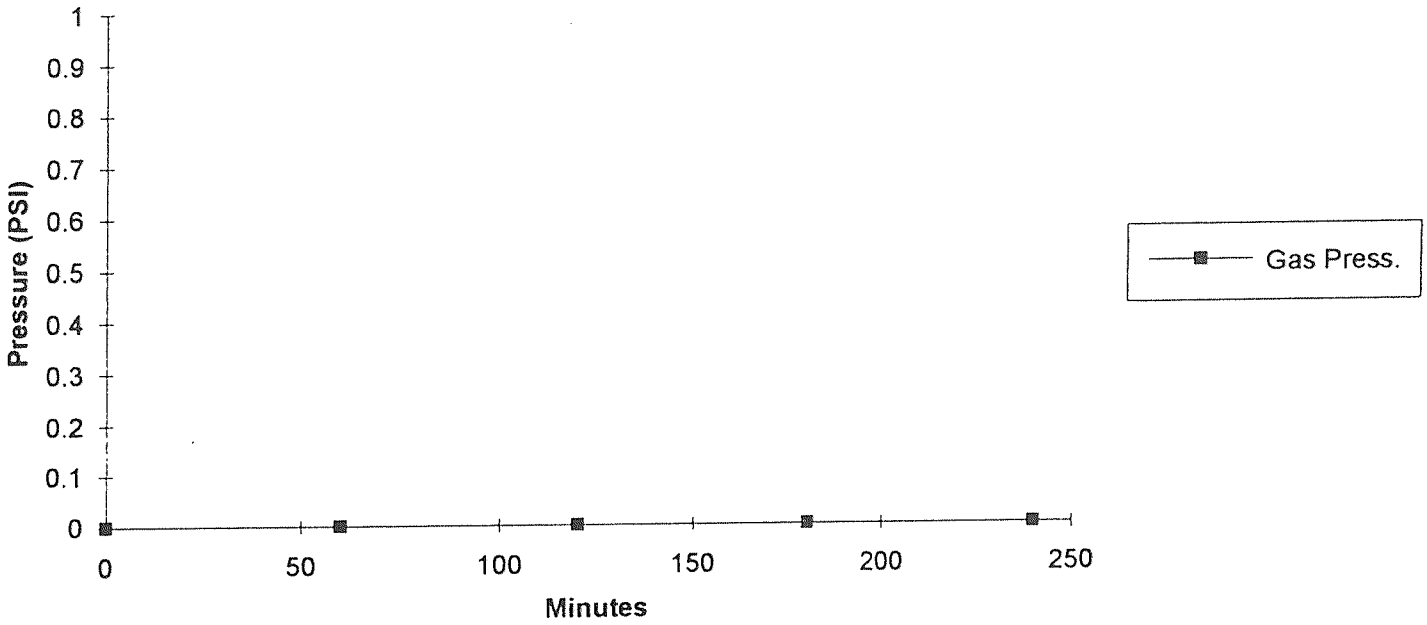


Figure 3 PBC 3- *Desulfurococcus* SY at Room Temperature

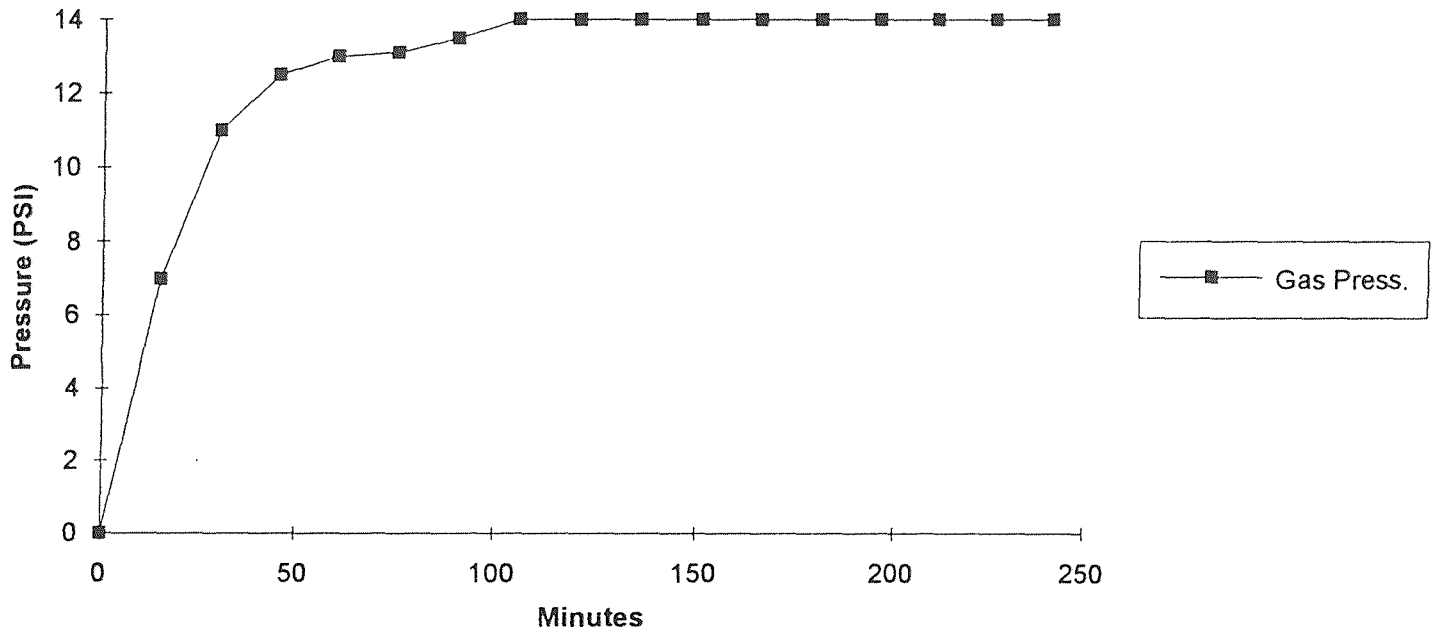


Figure 4 PBC 2- *Pyrococcus* GBD at 95 deg.C

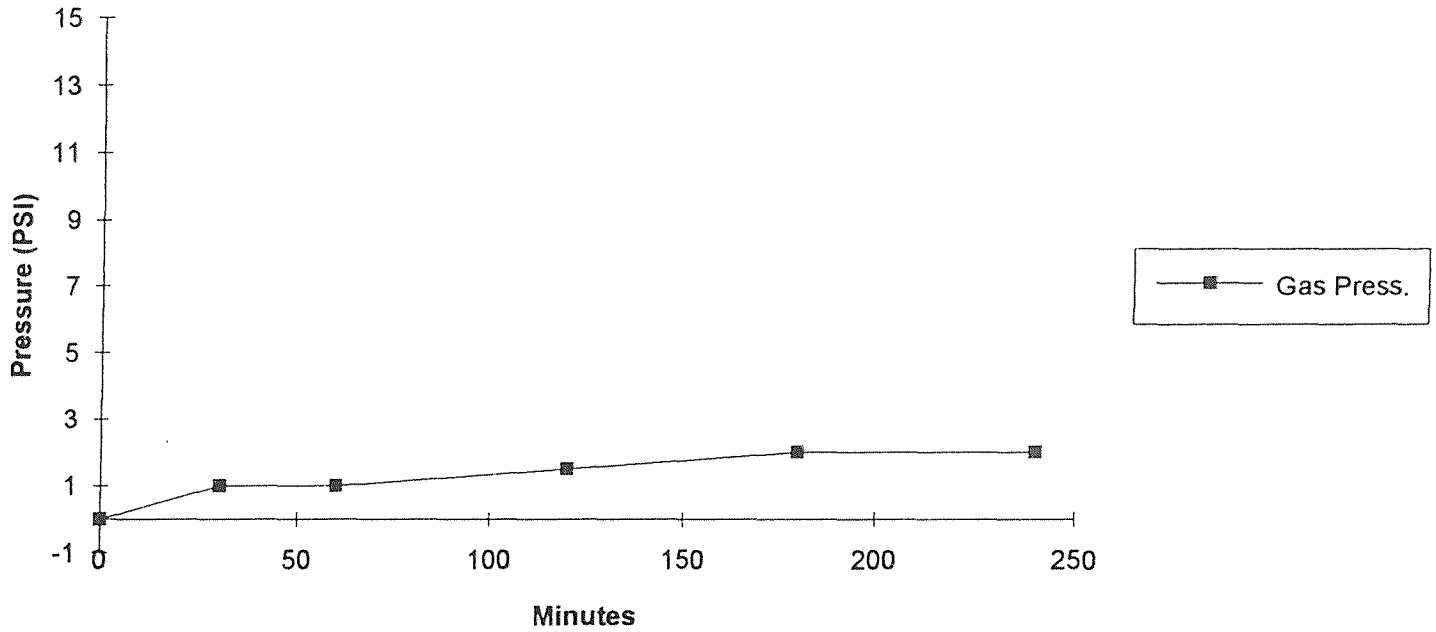


Figure 5 PBC 6- *Pyrococcus* GBD at 95 deg. C- no sulfur

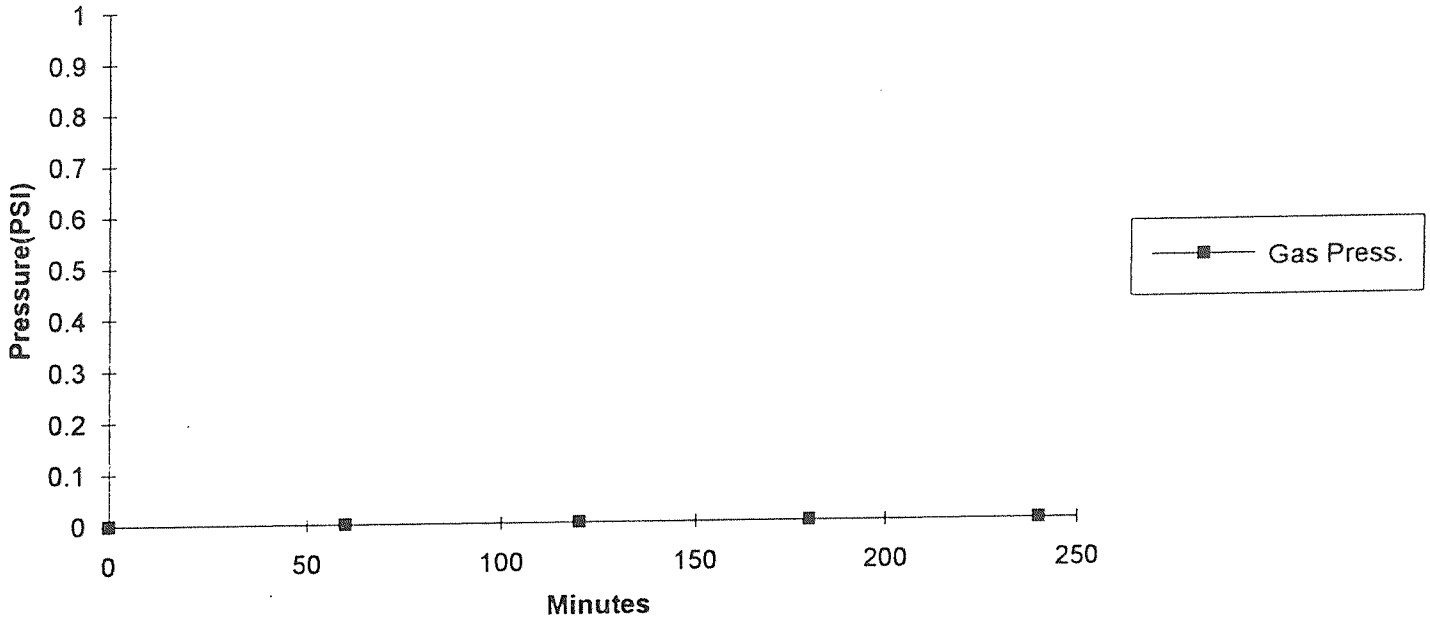


Figure 6 PBC 4- *Pyrococcus* GBD at Room Temperature

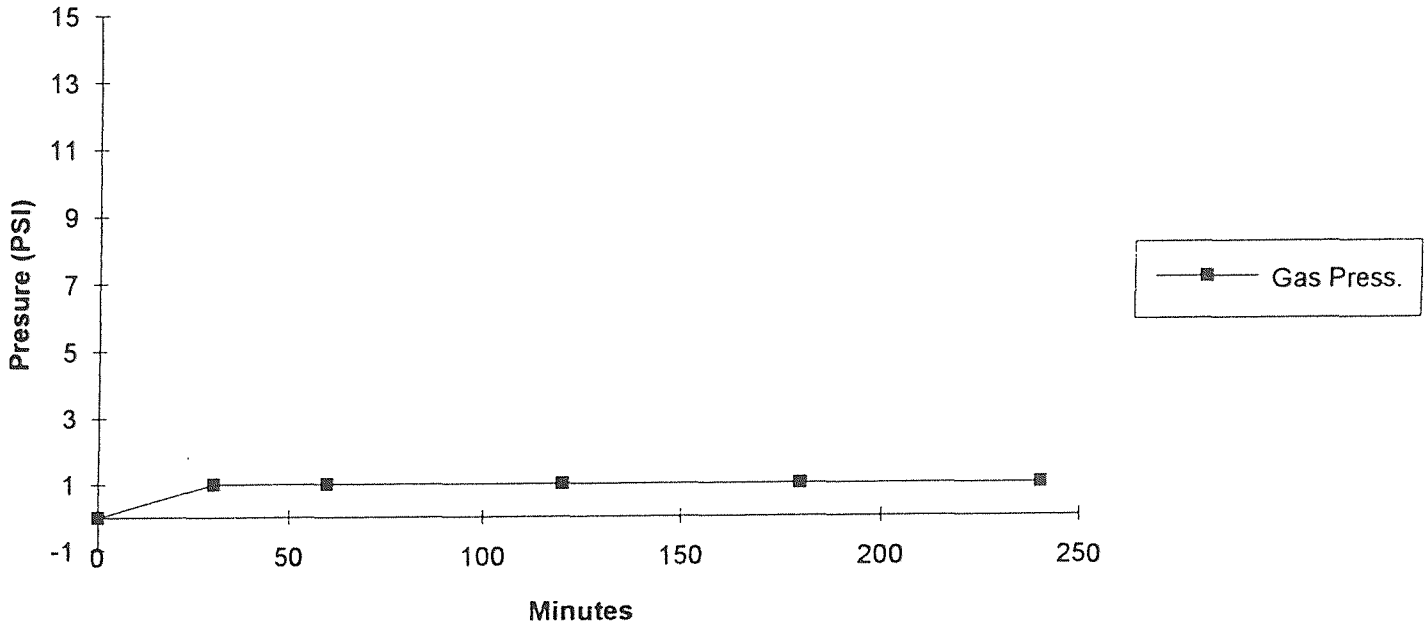


Figure 7 PBC 5-Sterile Media + Sulfur at 95 deg.C

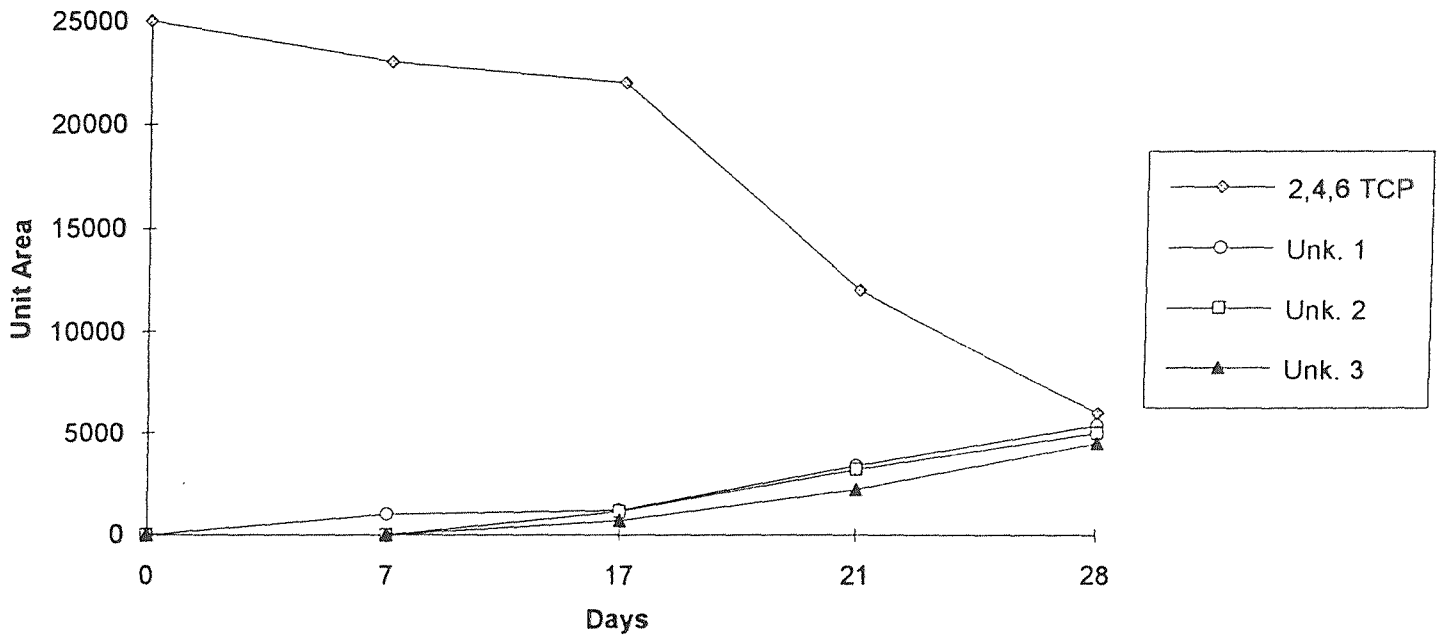


Figure 8 2,4,6 TCP Degradation Vs. Time

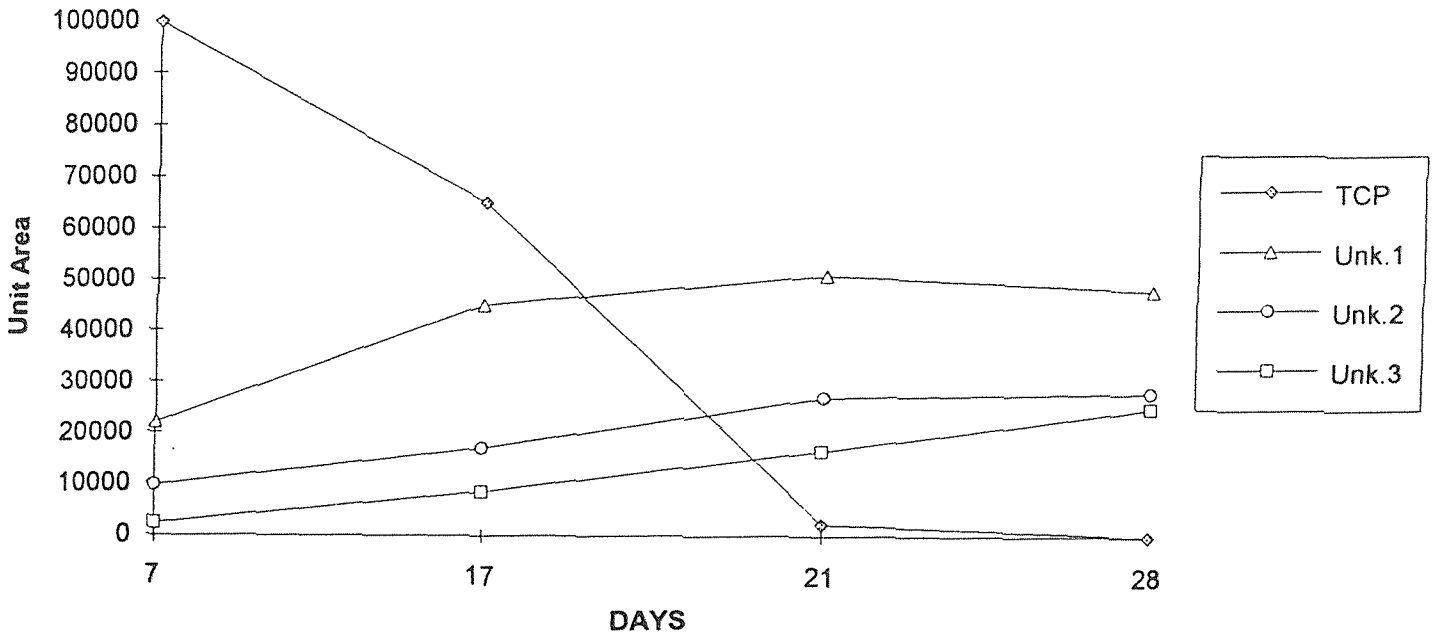


Figure 9 2,4,6 TCP Degradation Vs. Time

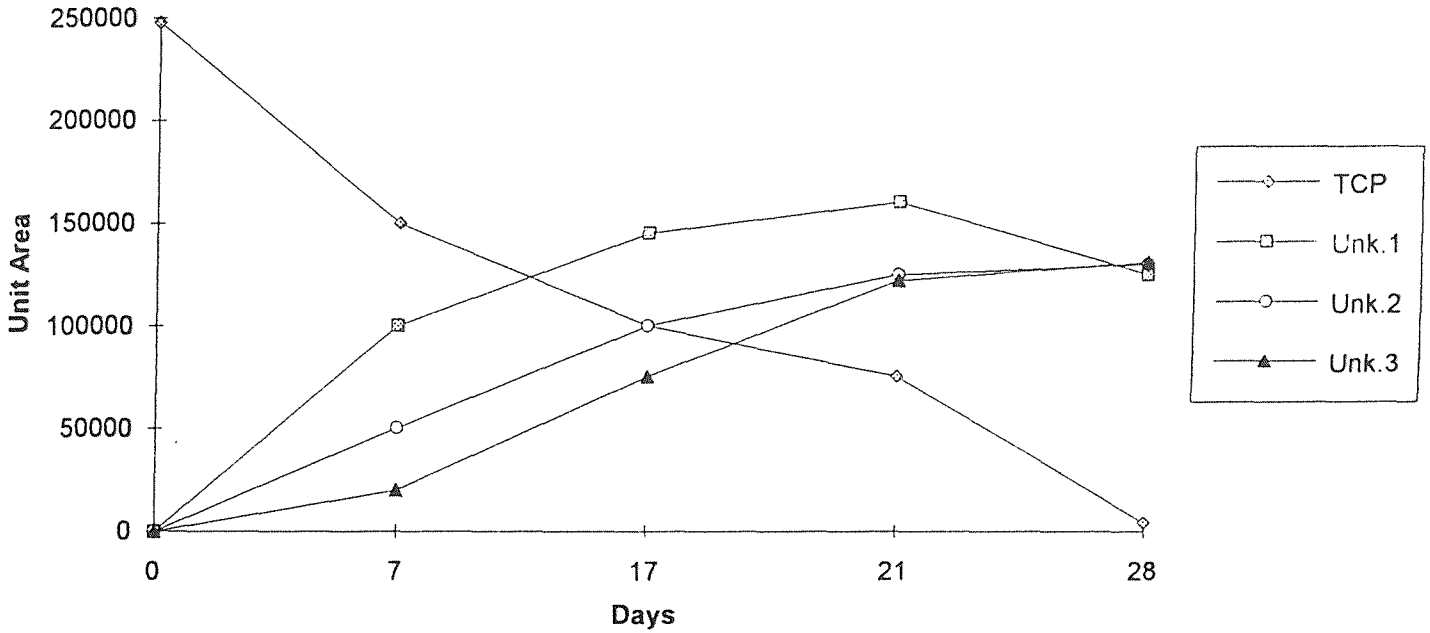


Figure 10 TCP Degradation Vs. Time

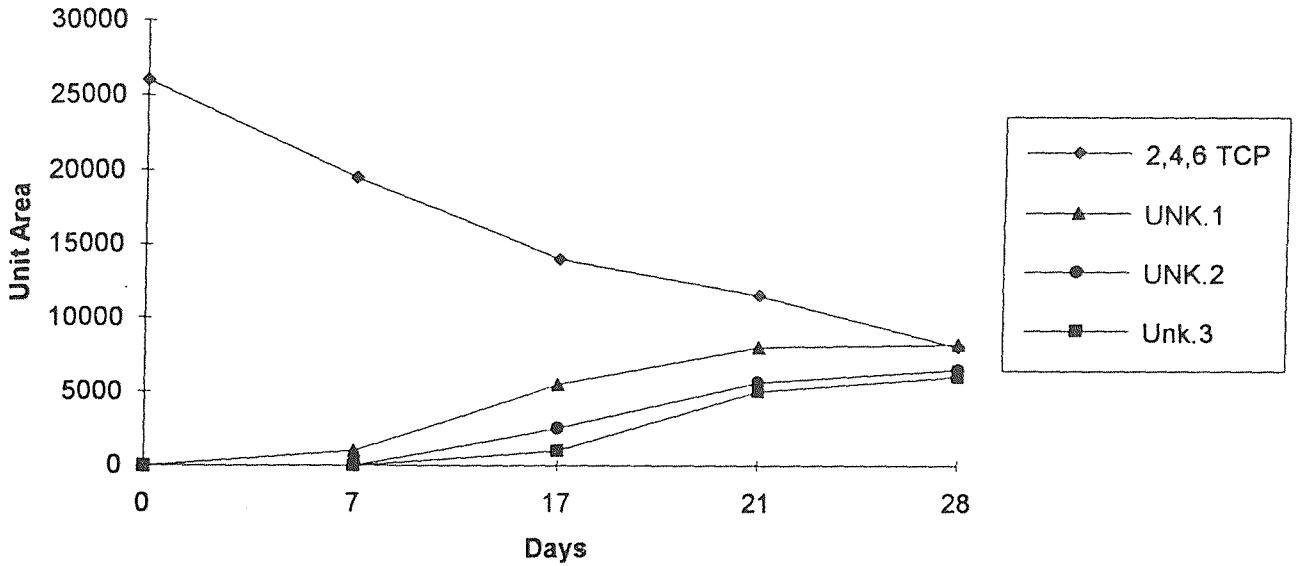


Figure 11 TCP Degradation vs. Time

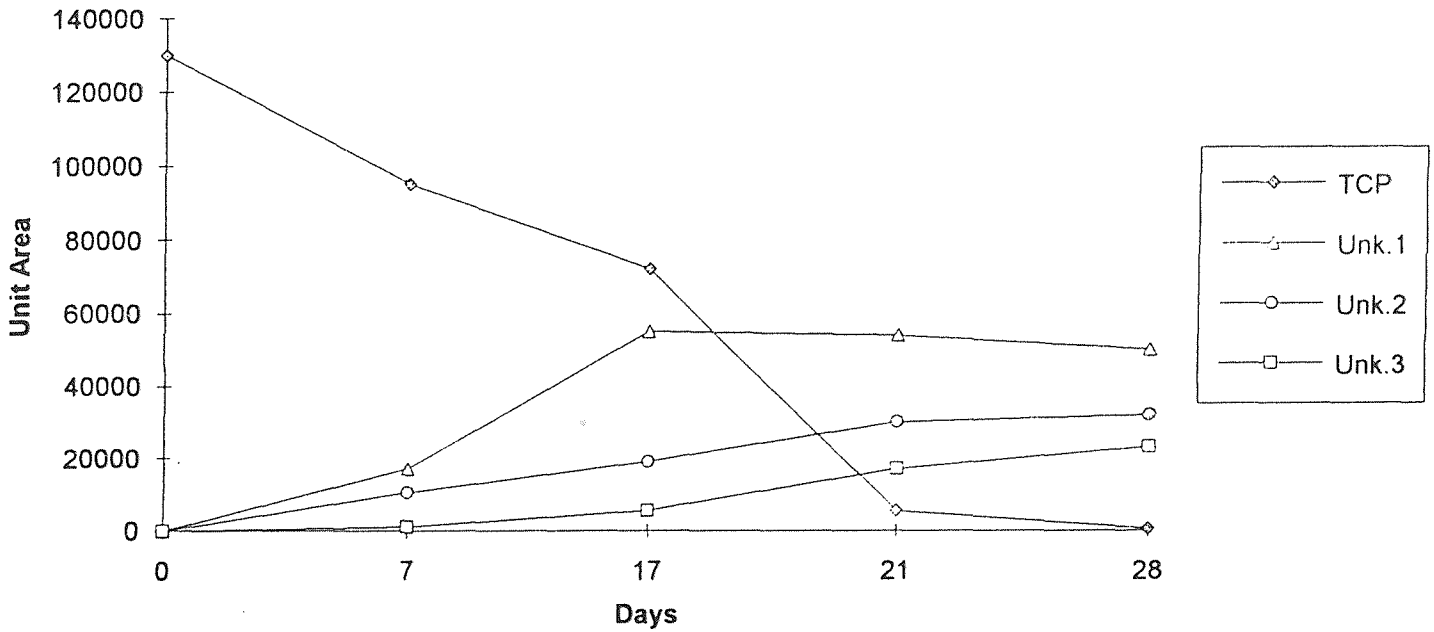


Figure 12 2,4,6 TCP Degradation vs. Time

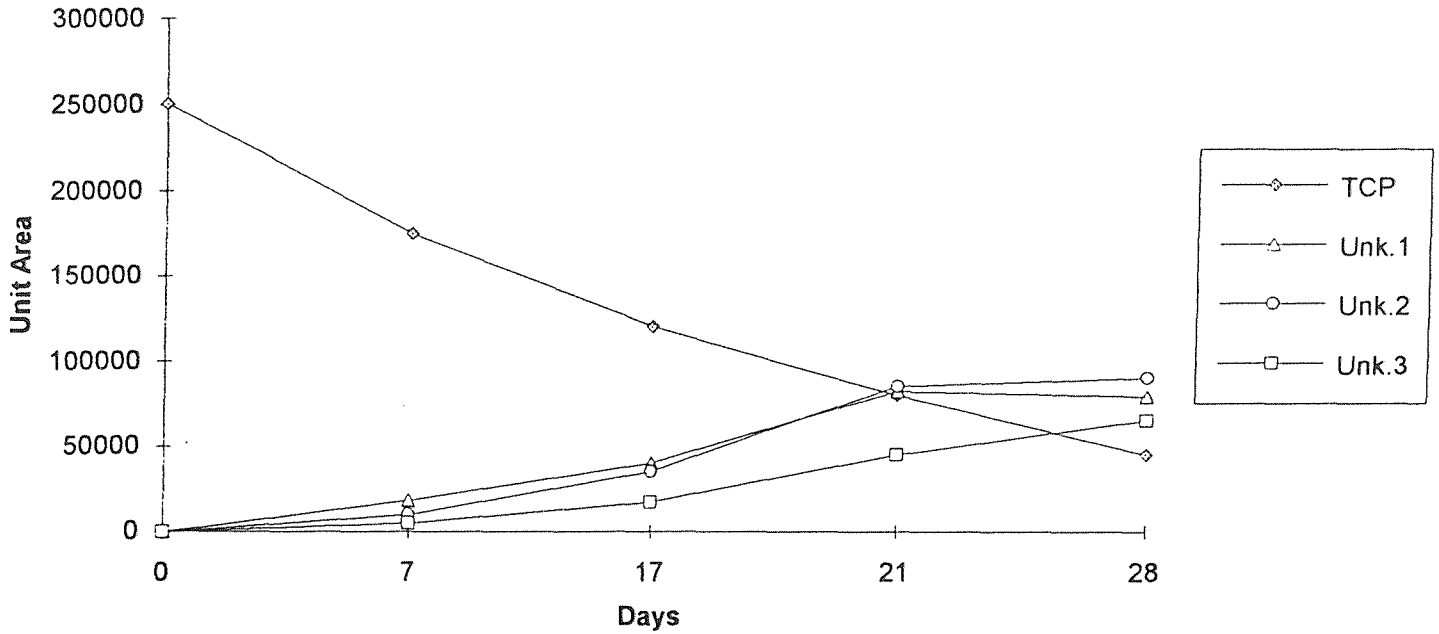


Figure 13 2,4,6 TCP Degradation Vs. Time

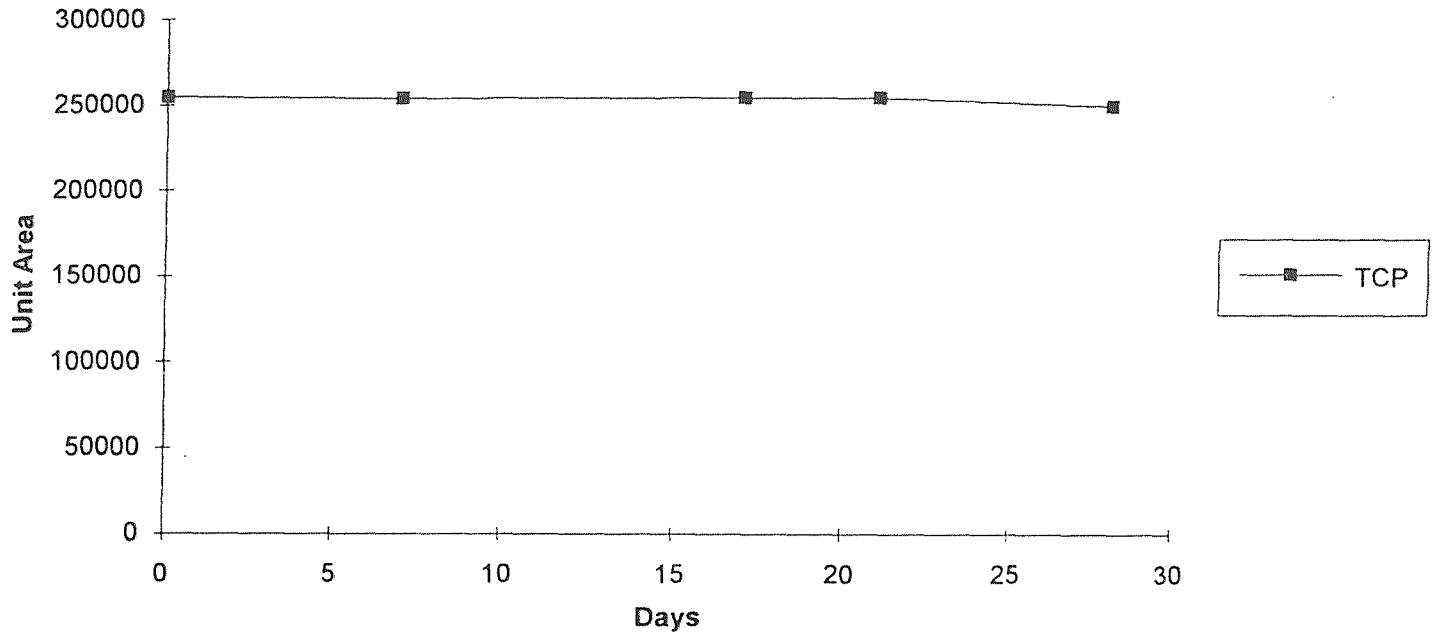


Figure 14 2,4,6 TCP Degradation Vs. Time (Sterile Control)

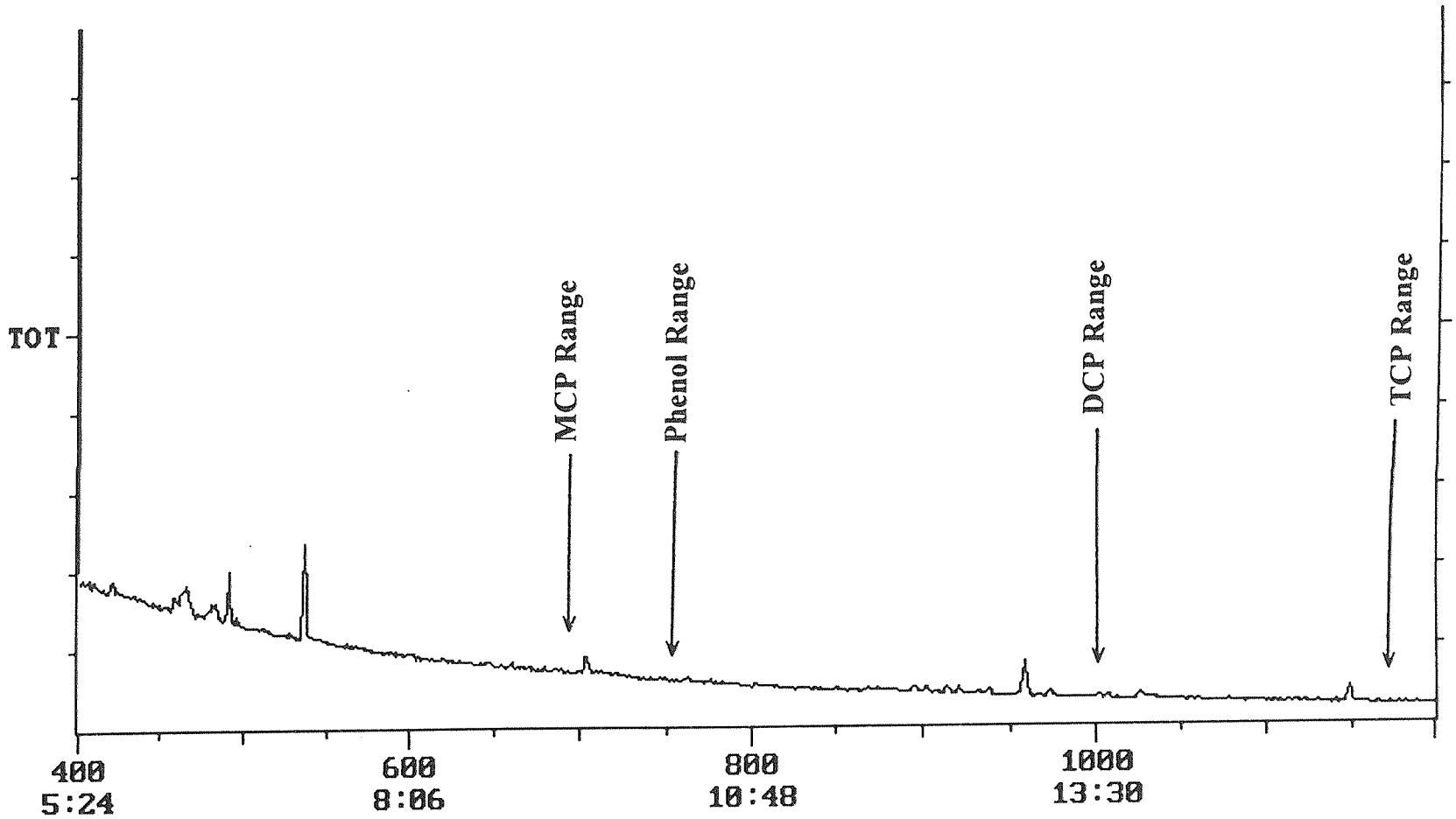


Figure 15 Representative Example of Areas of Influence of TCP, DCP, MCP Phenol

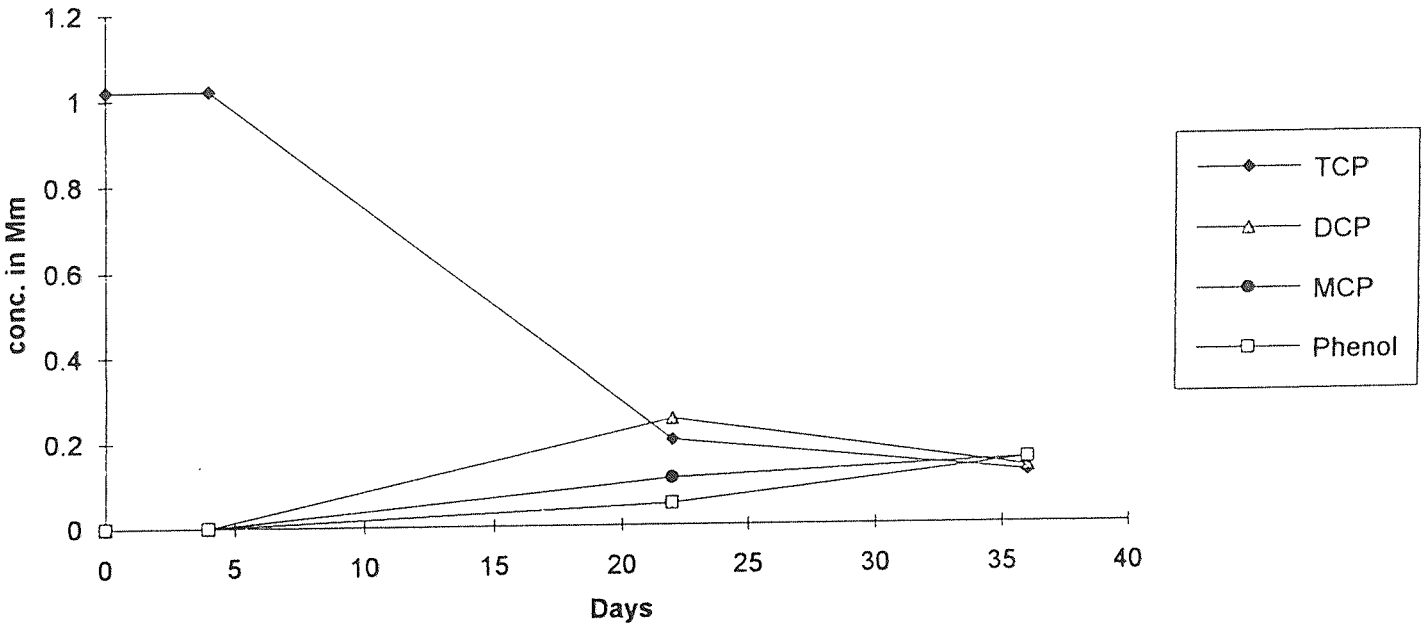


Figure 16 Bottle 102

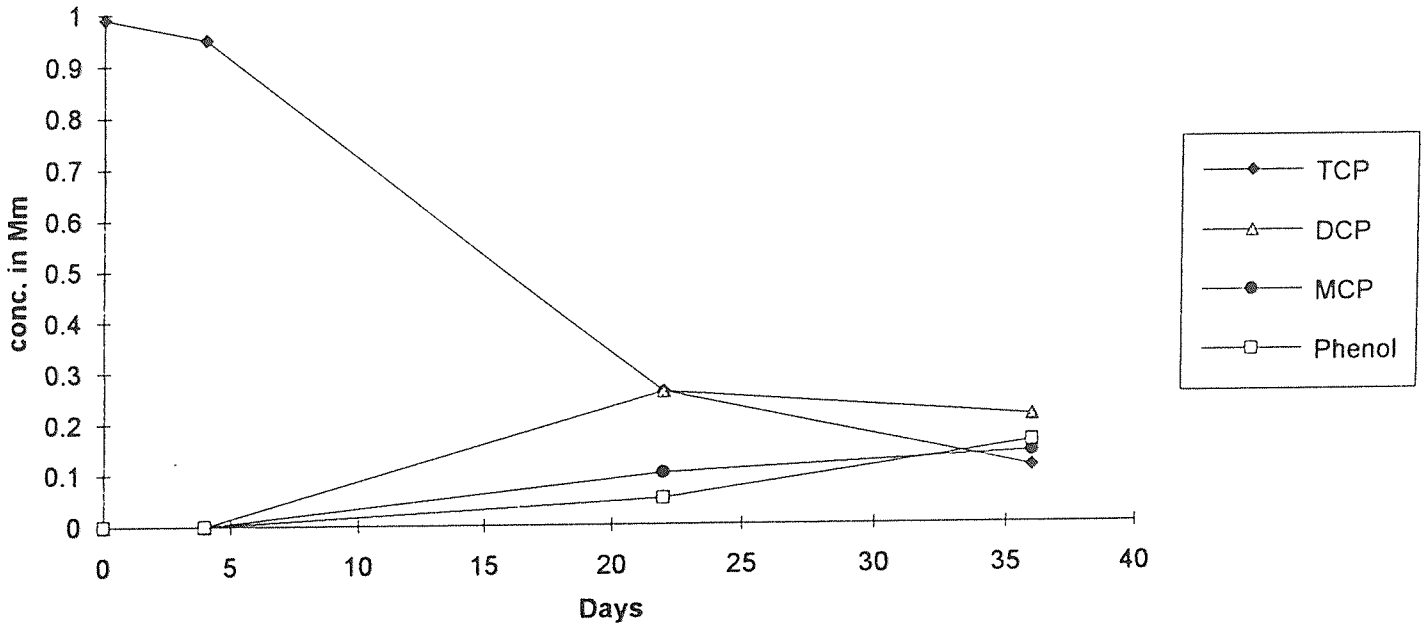


Figure 17 Bottle 202

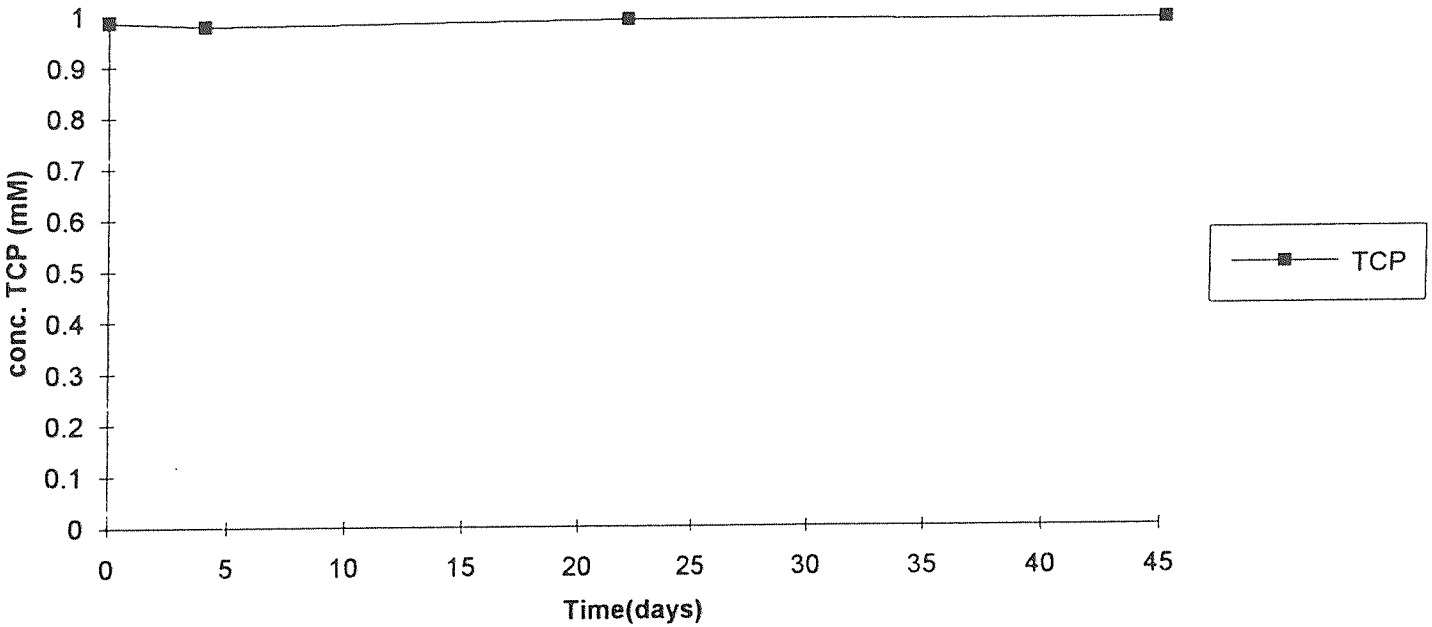


Figure 18 Bottle 103

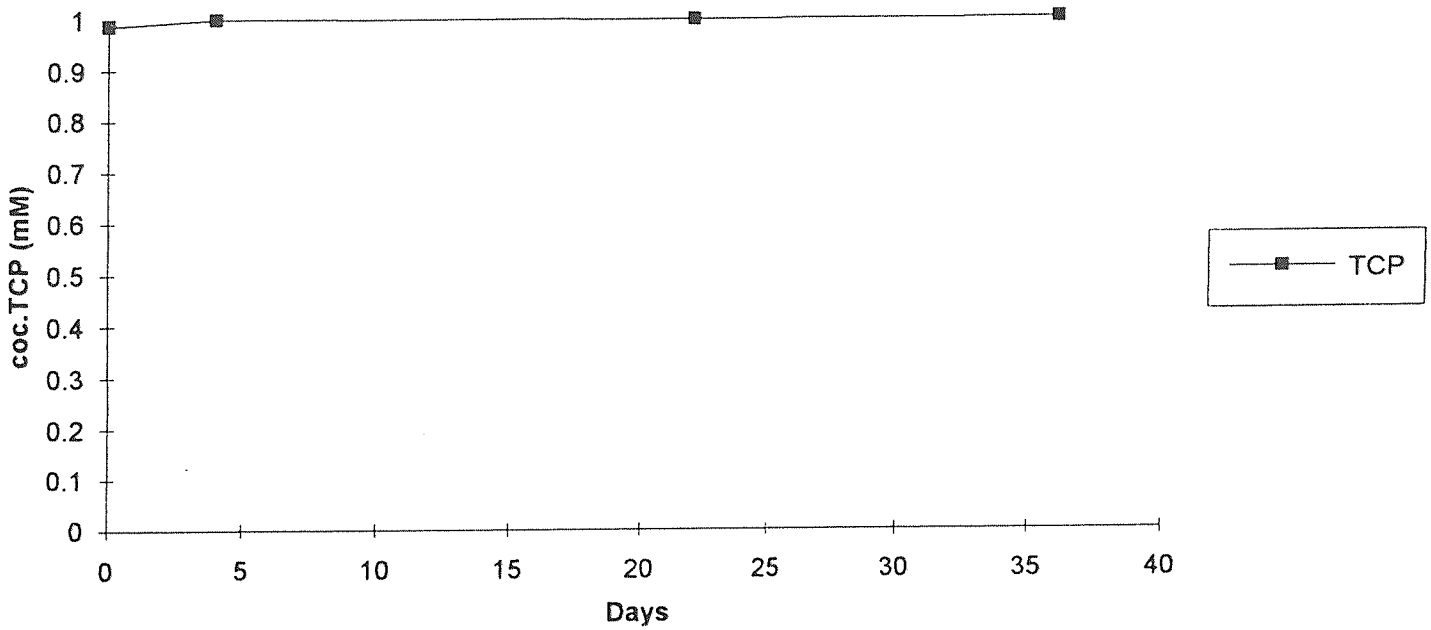


Figure 19 Bottle 203

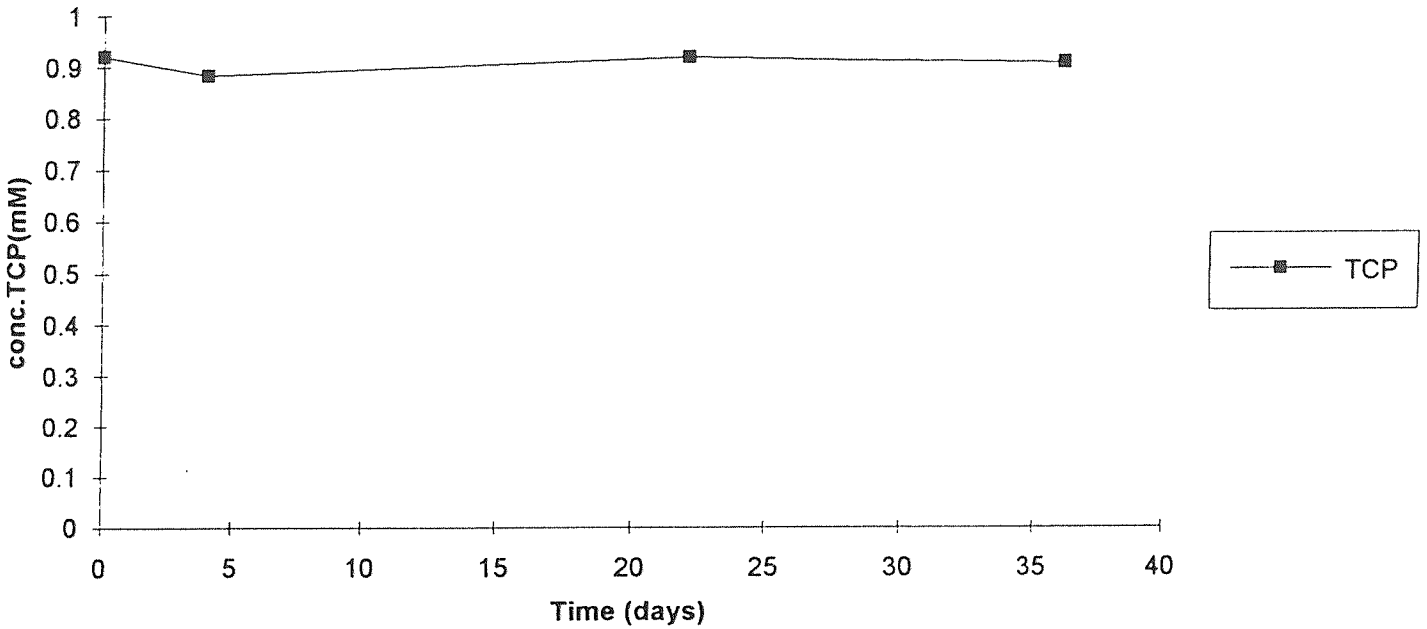


Figure 20 Bottle 104

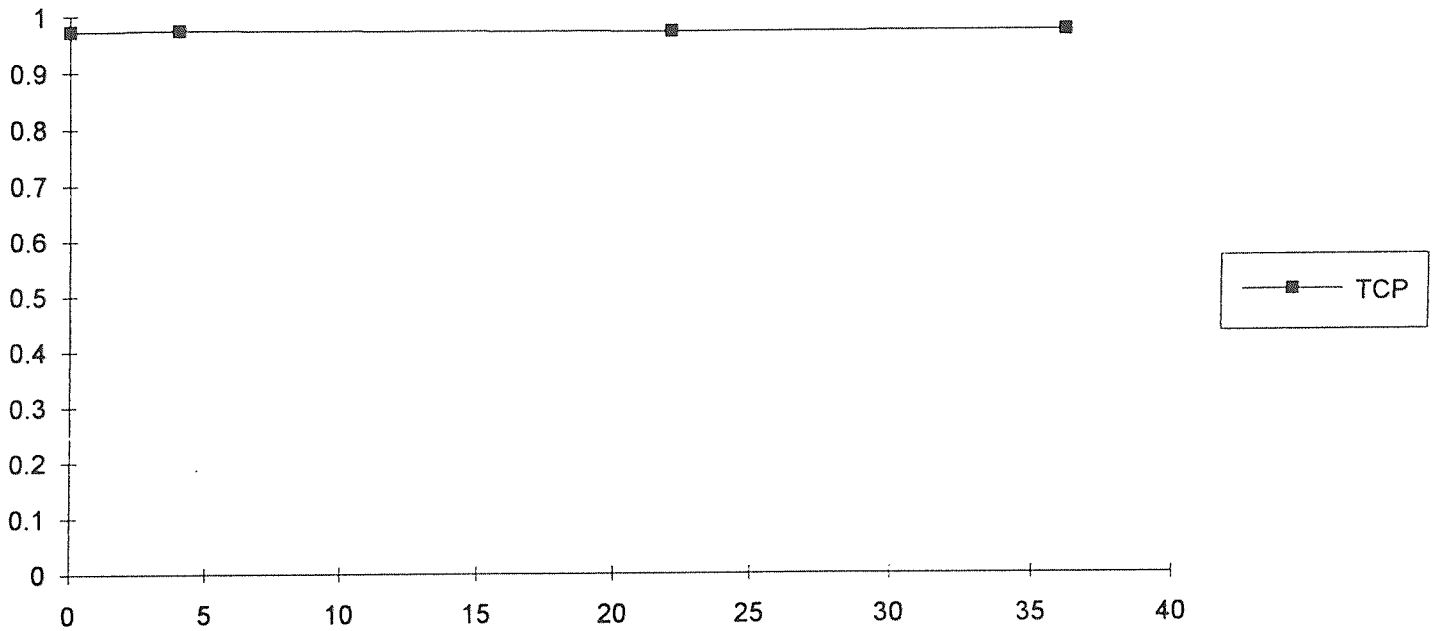


Figure 21 Bottle 204

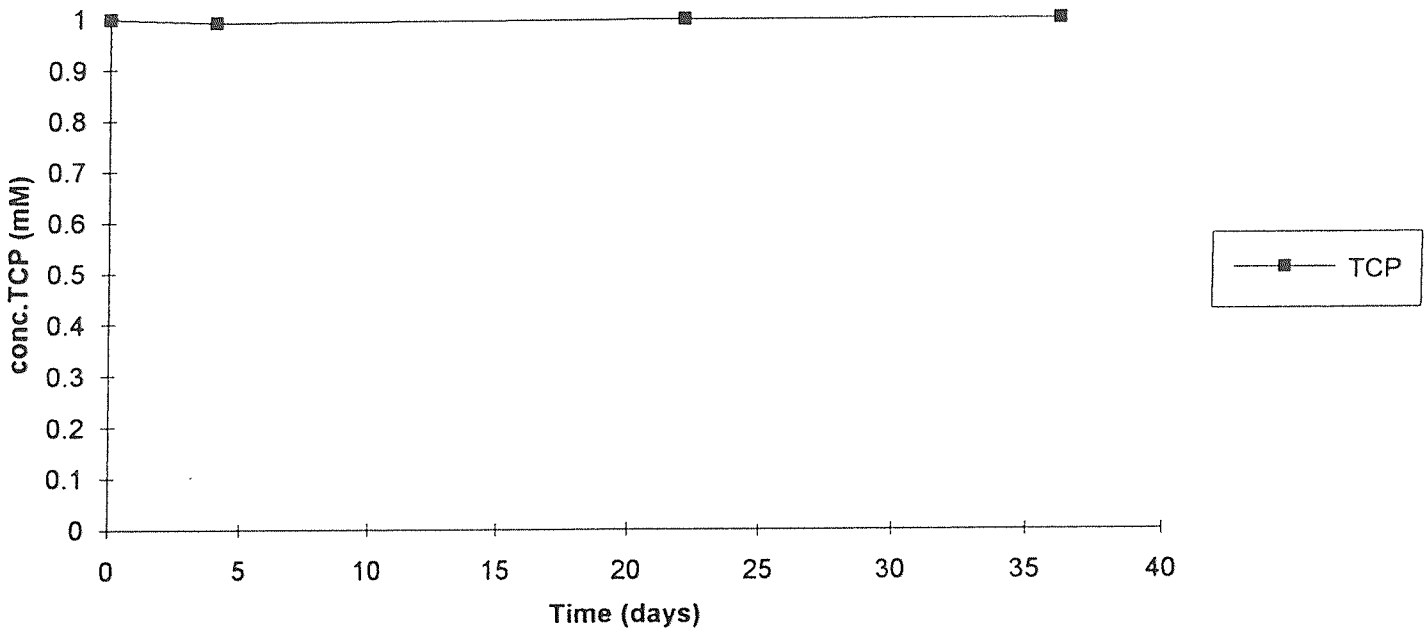


Figure 22 Bottle 105

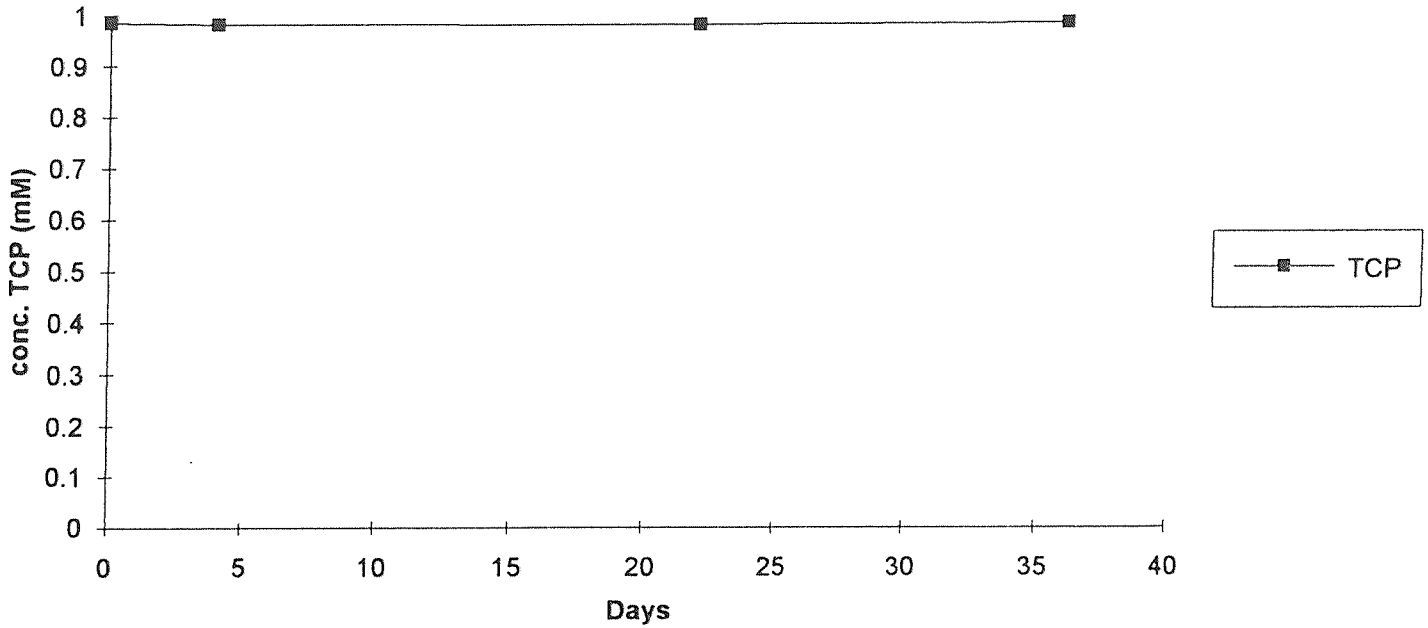


Figure 23 Bottle 205

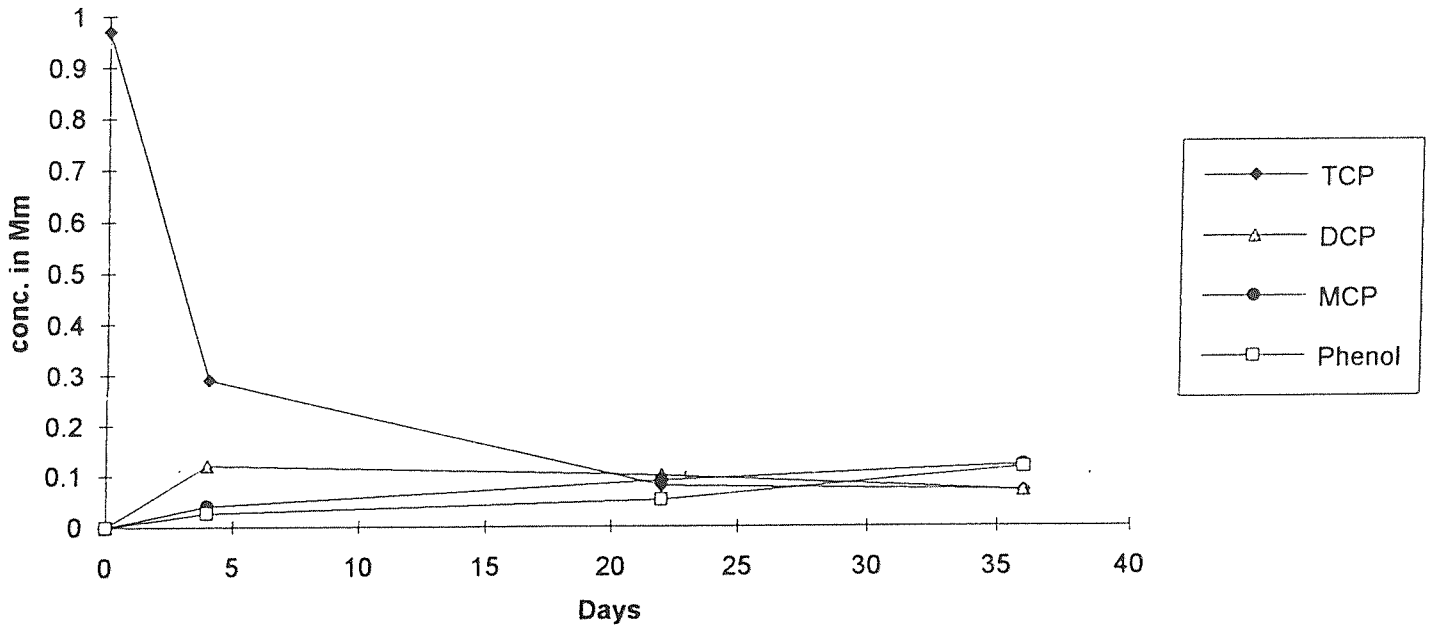


Figure 24 Bottle 106

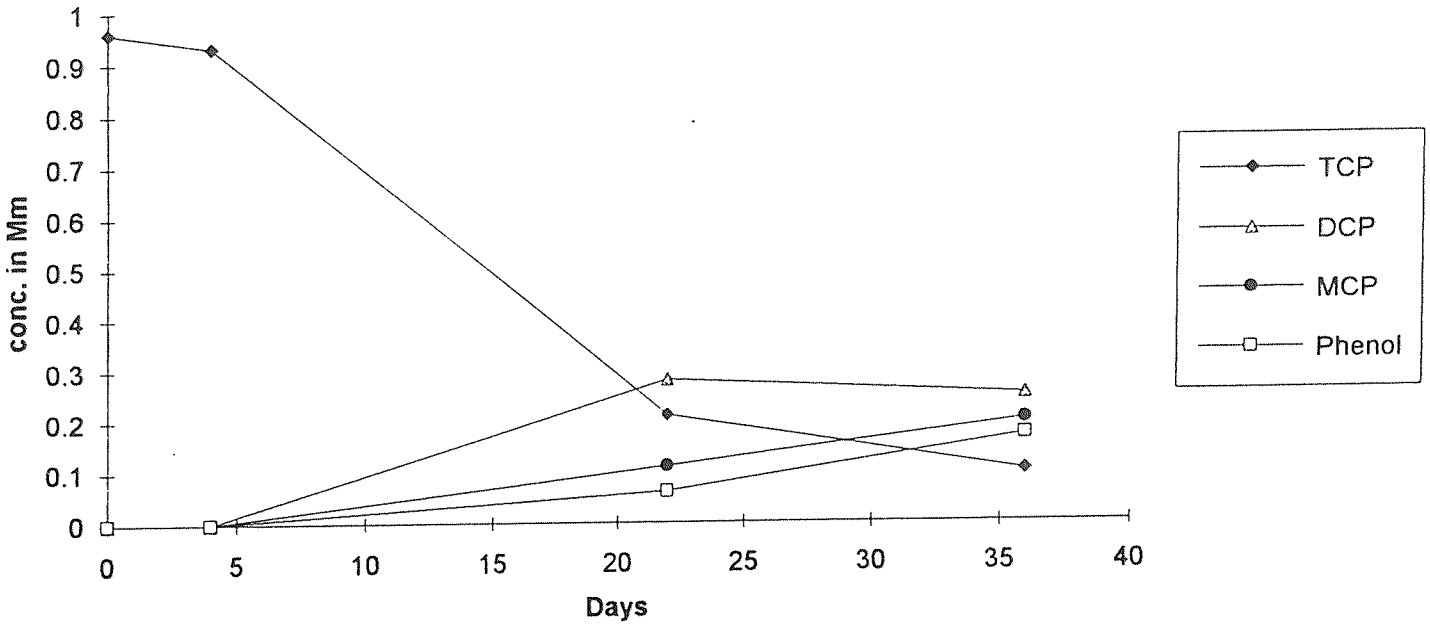


Figure 25 Bottle 206

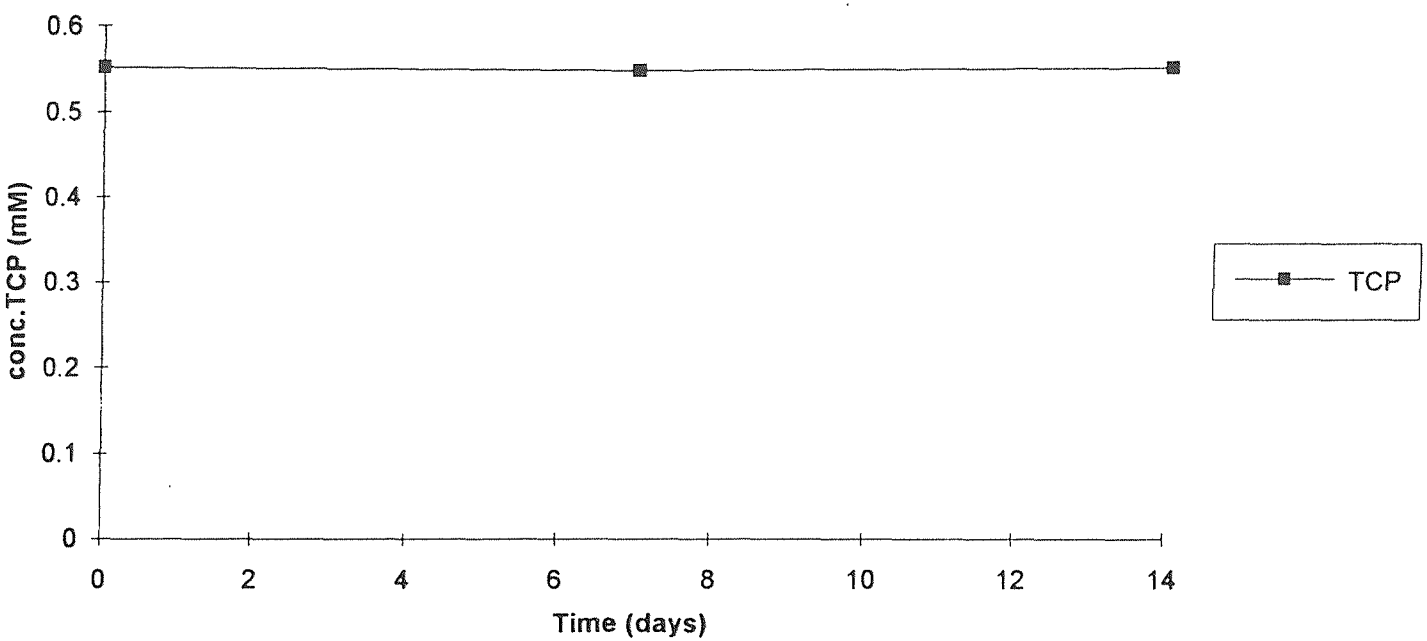


Figure 26 Bottle 107

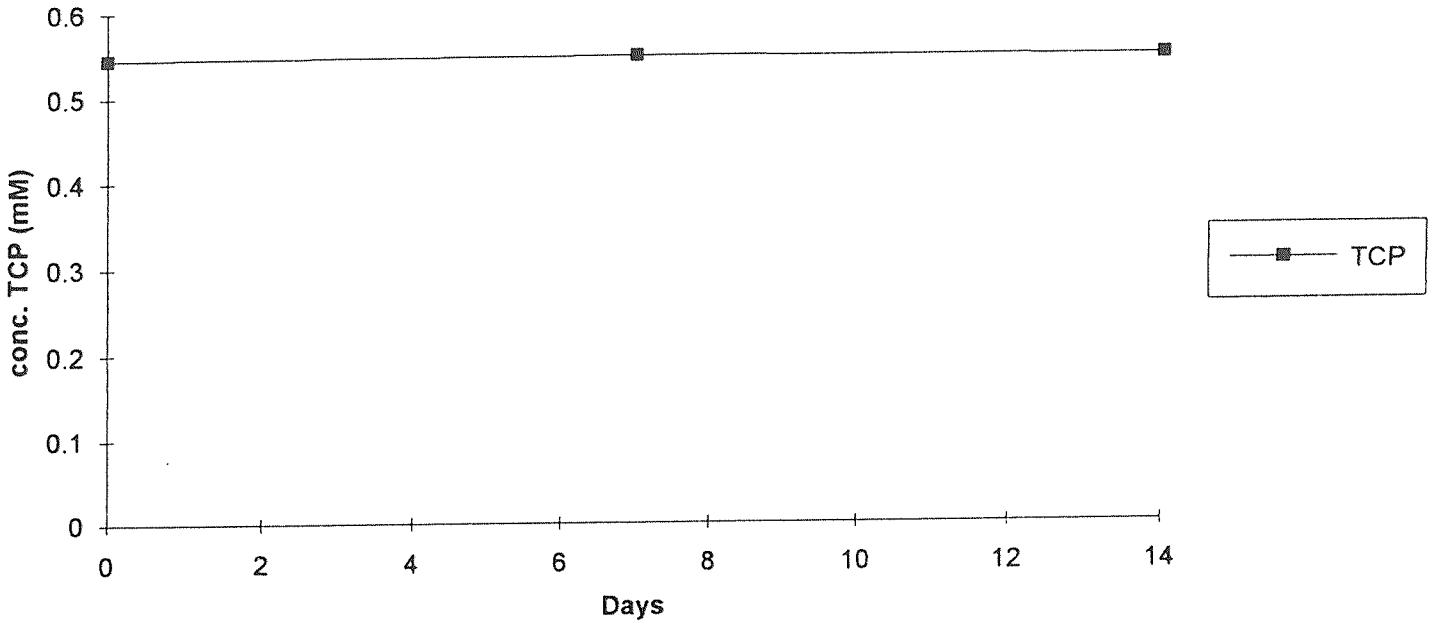


Figure 27 Bottle 207

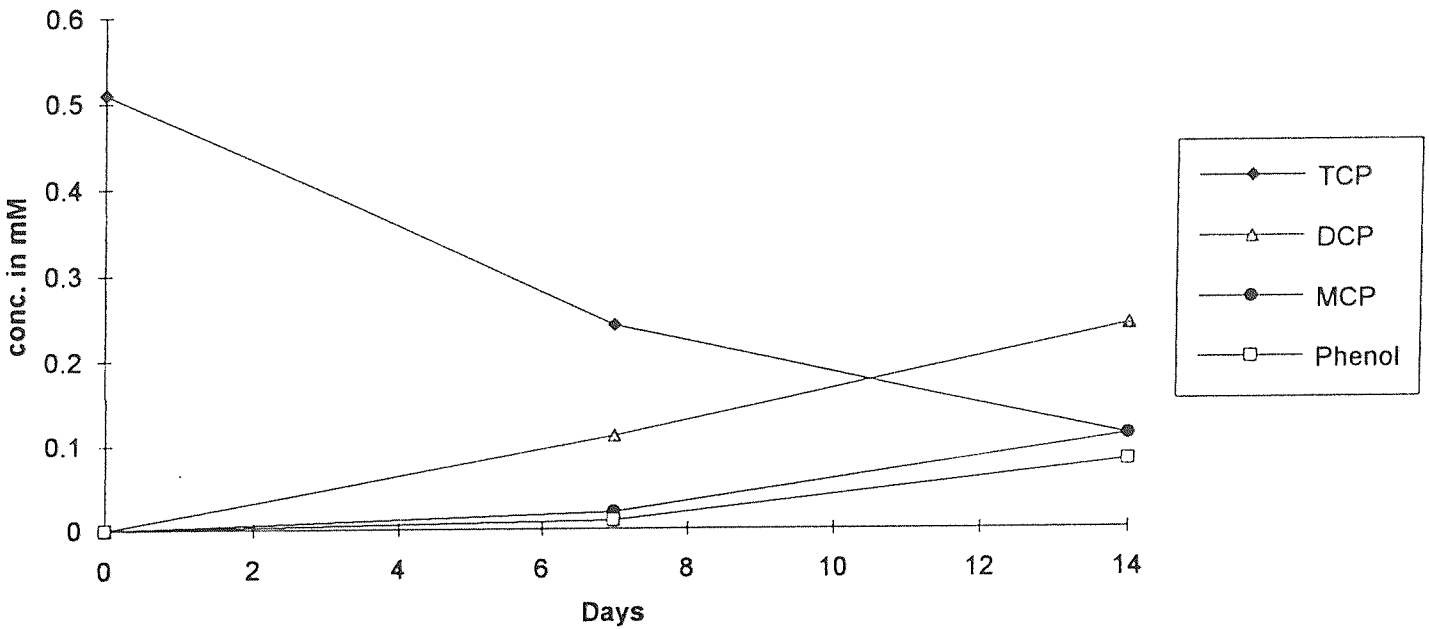


Figure 28 Bottle 108

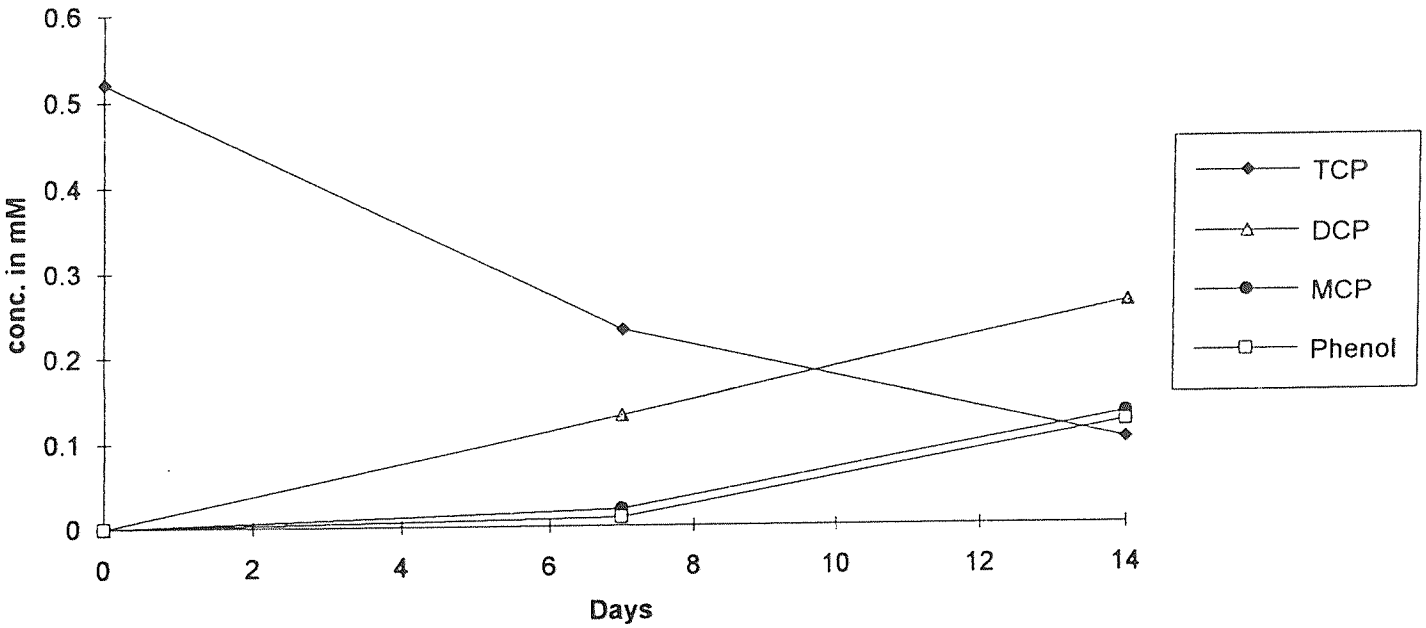


Figure 29 Bottle 208

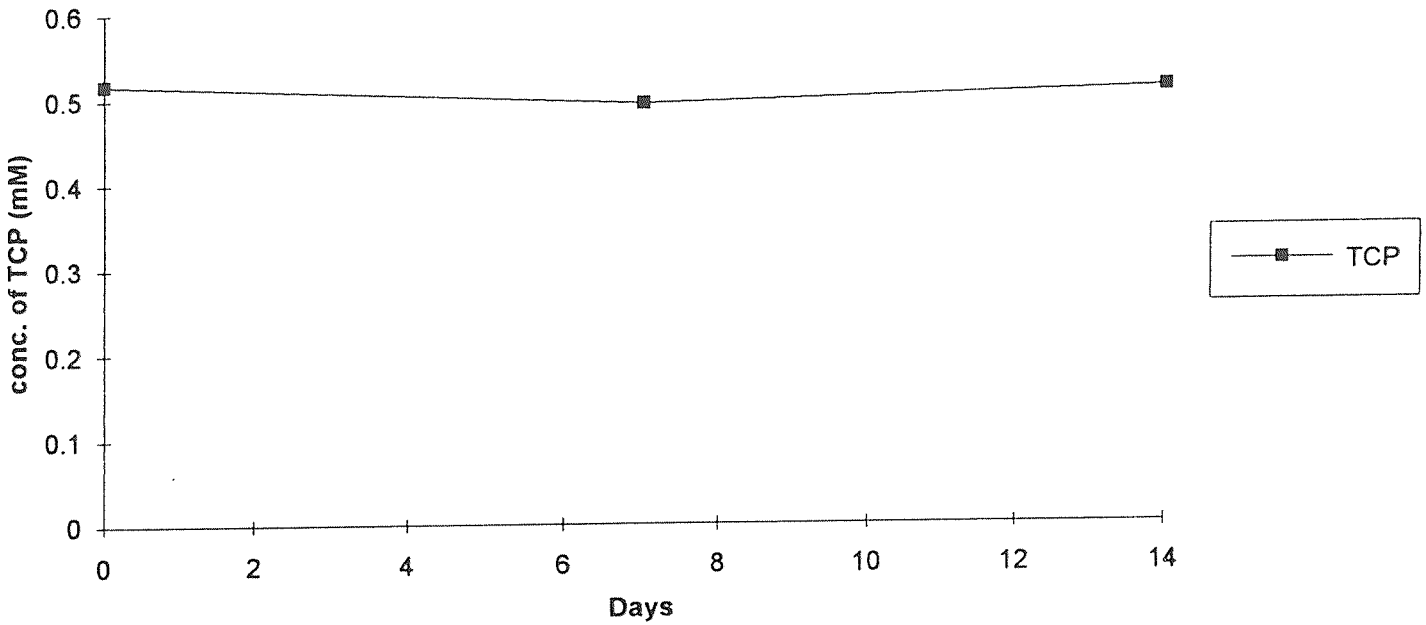
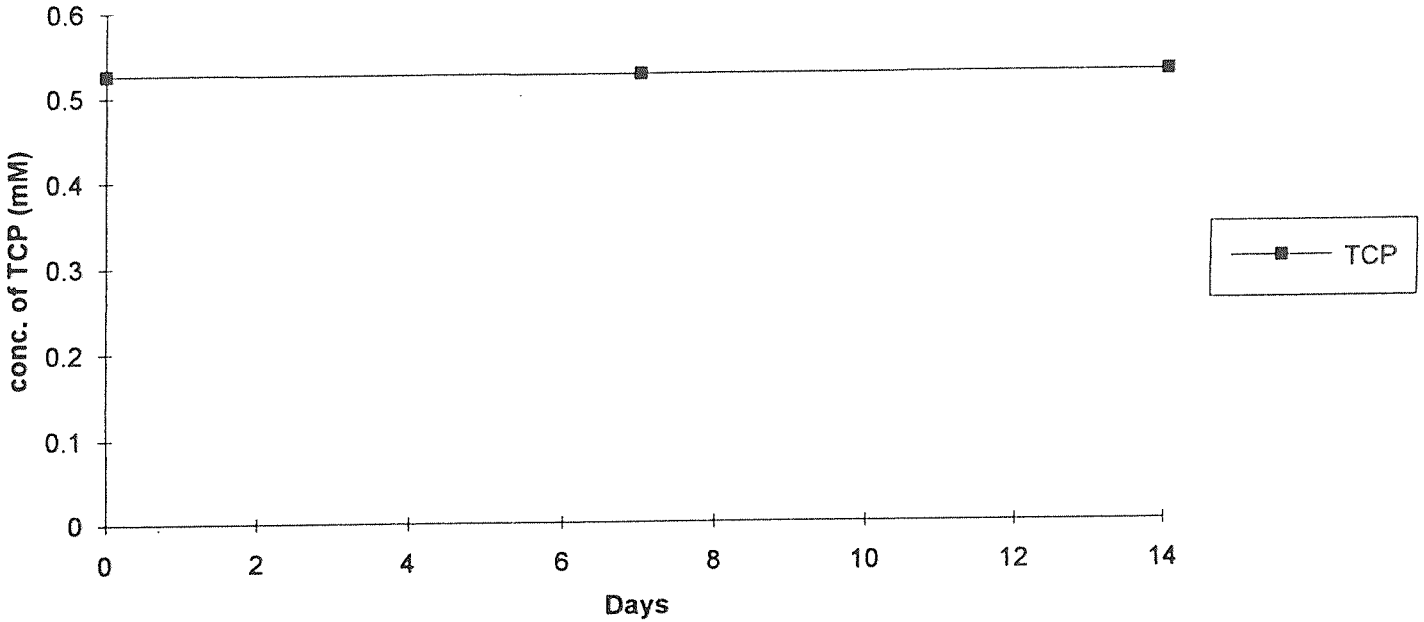


Figure 30 Bottle 109



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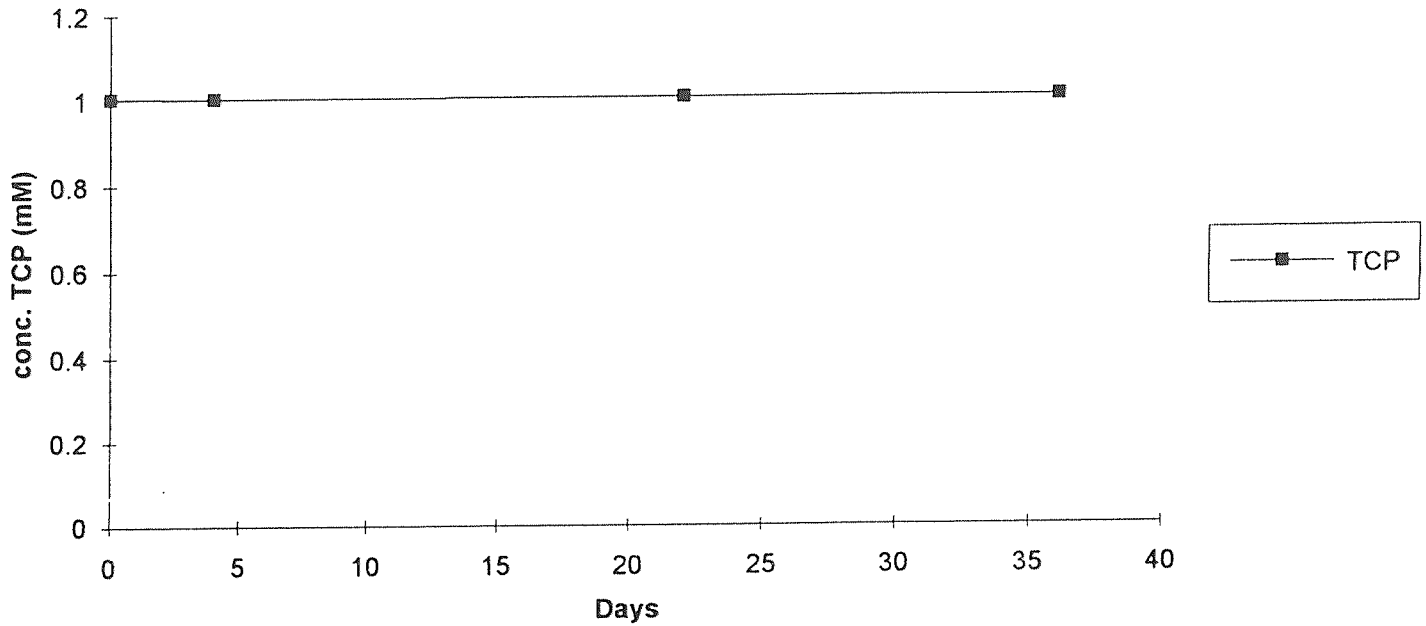


Figure 32 Bottle 301

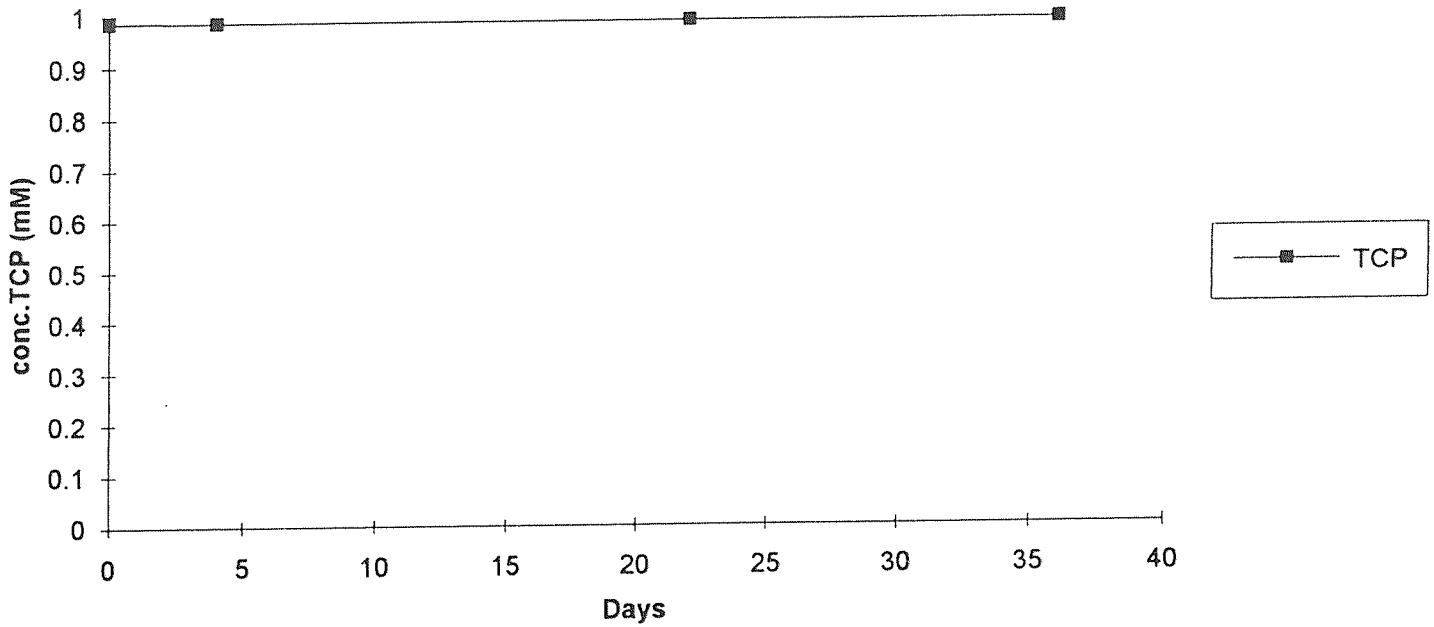
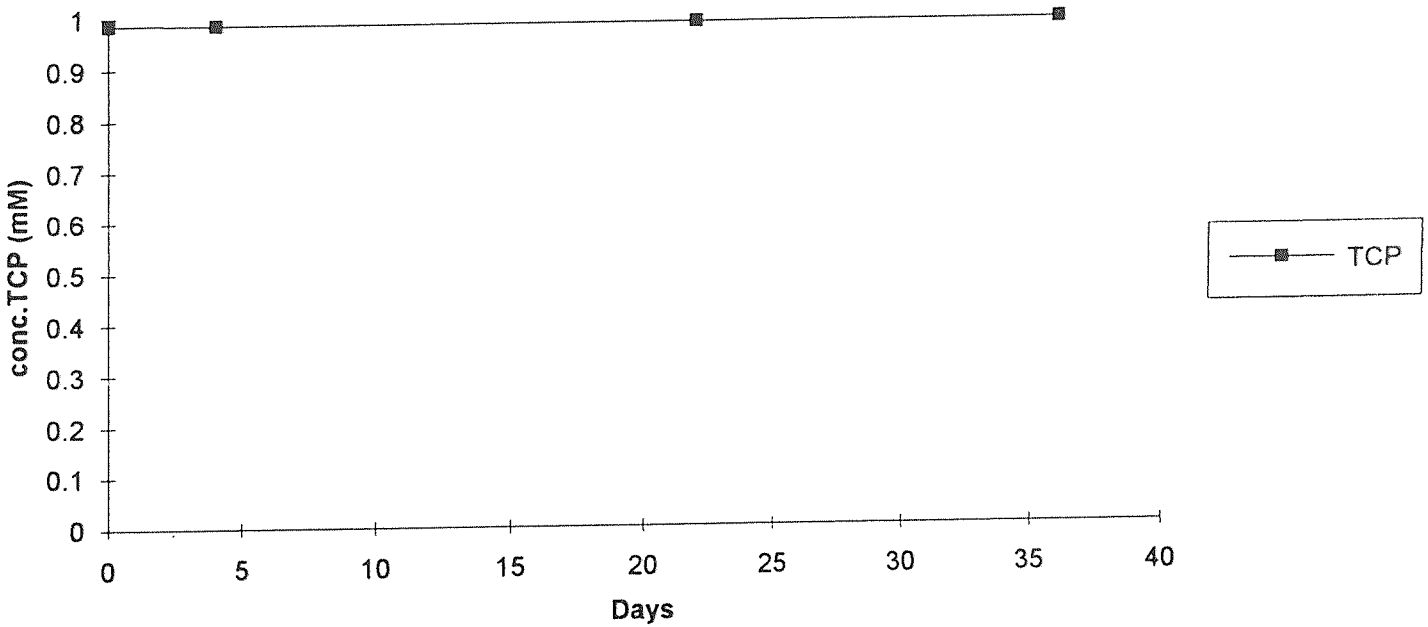


Figure 33 Bottle 302



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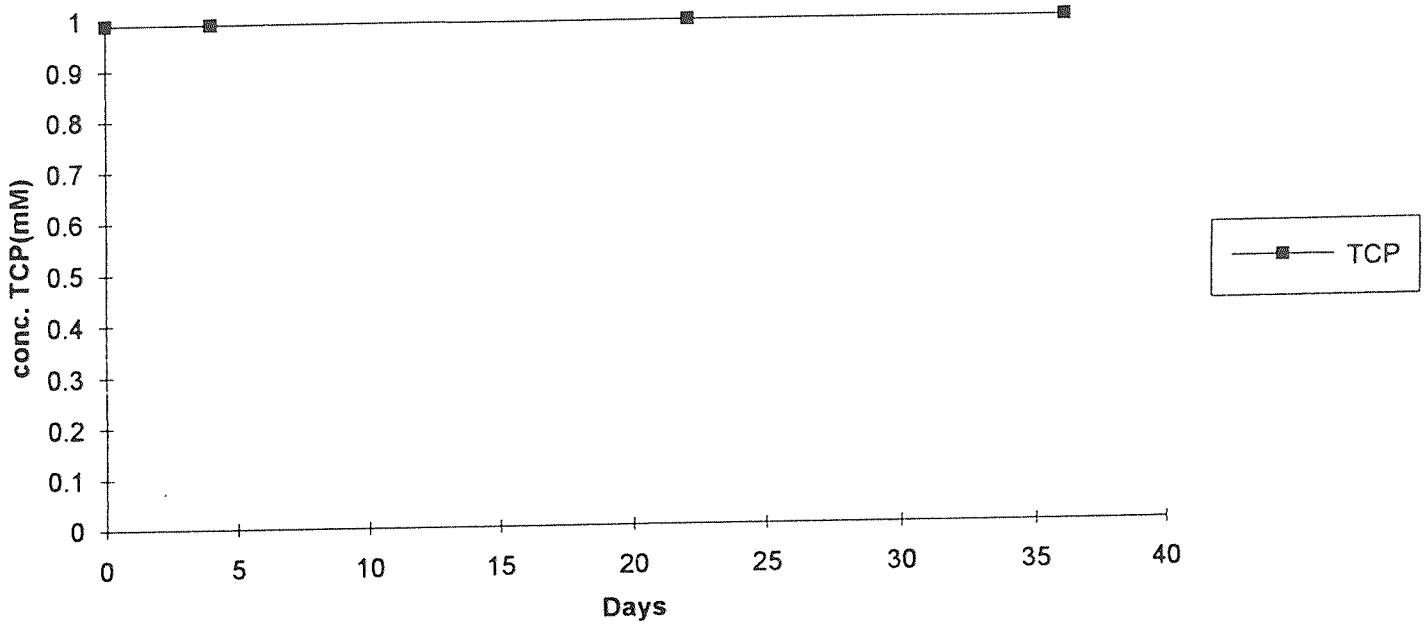

Figure 35 Bottle 304

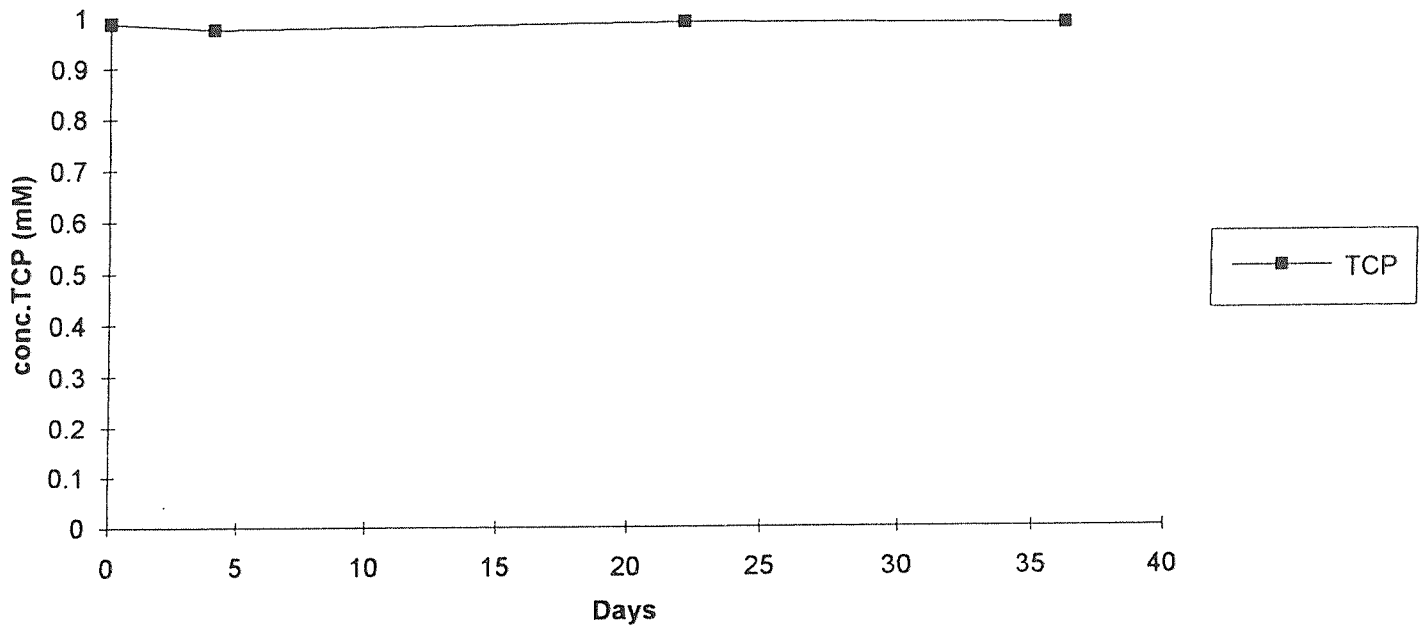

Figure 36 Bottle 305

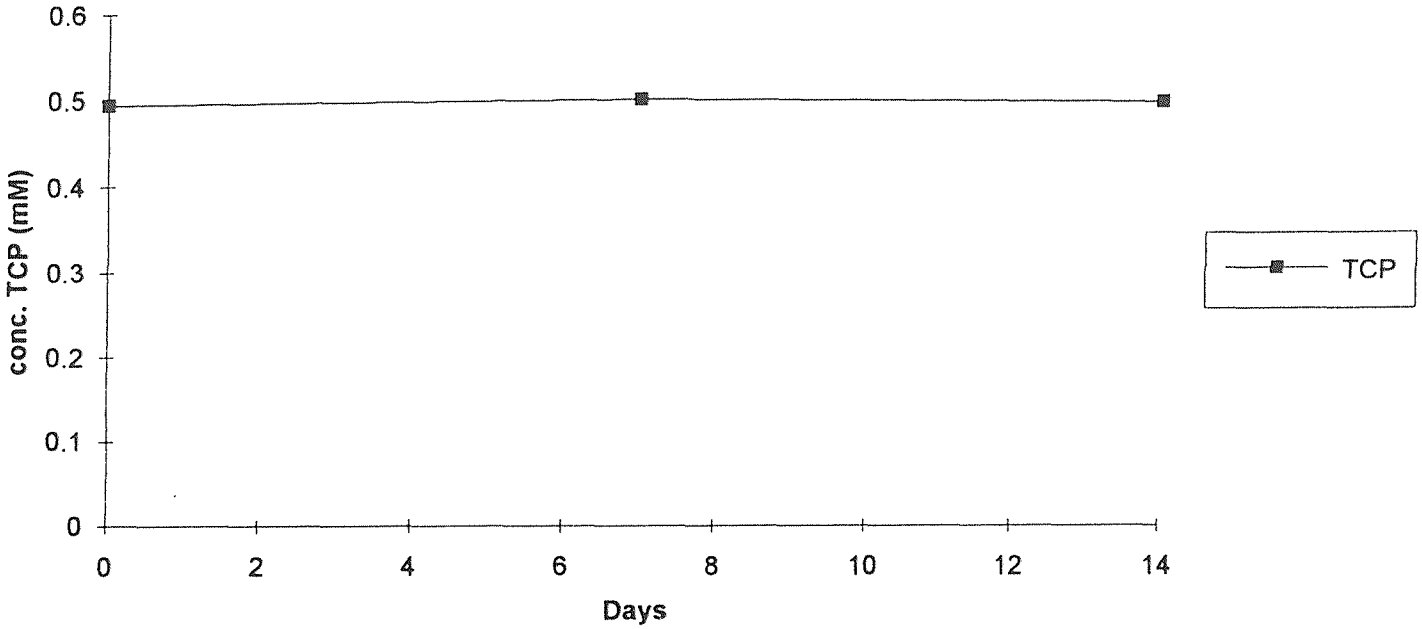

Figure 37 Bottle 306

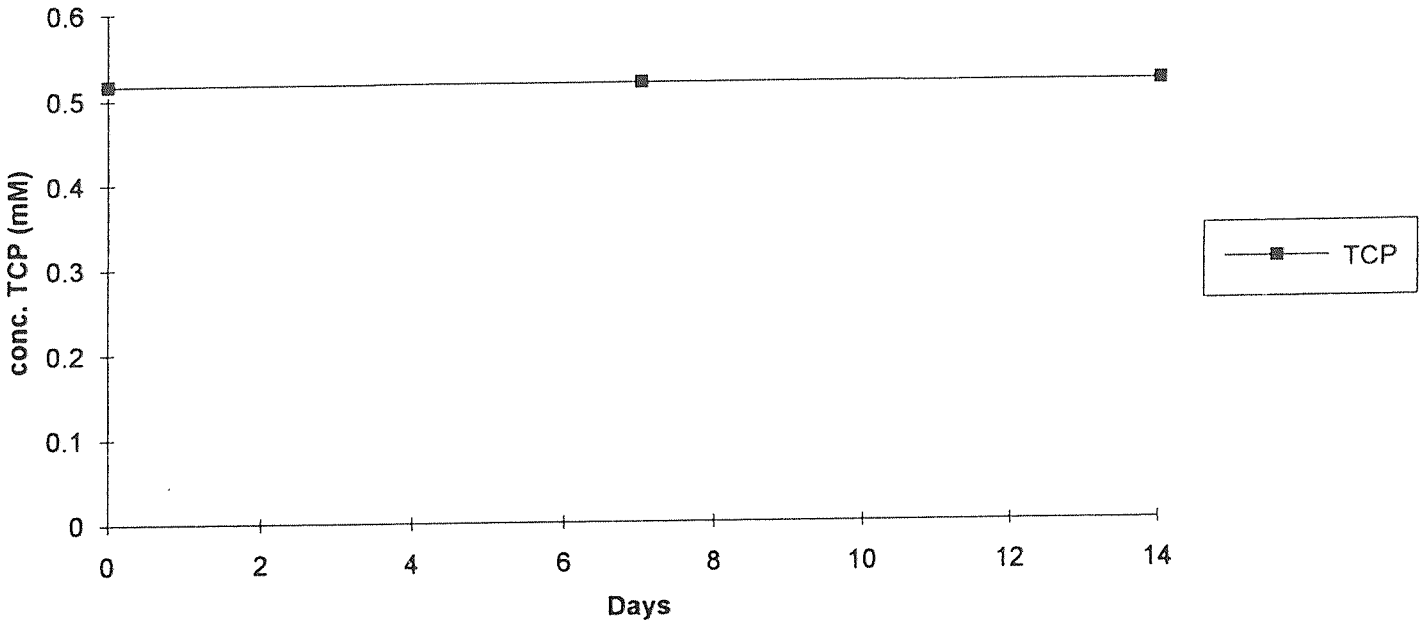

Figure 38 Bottle 307

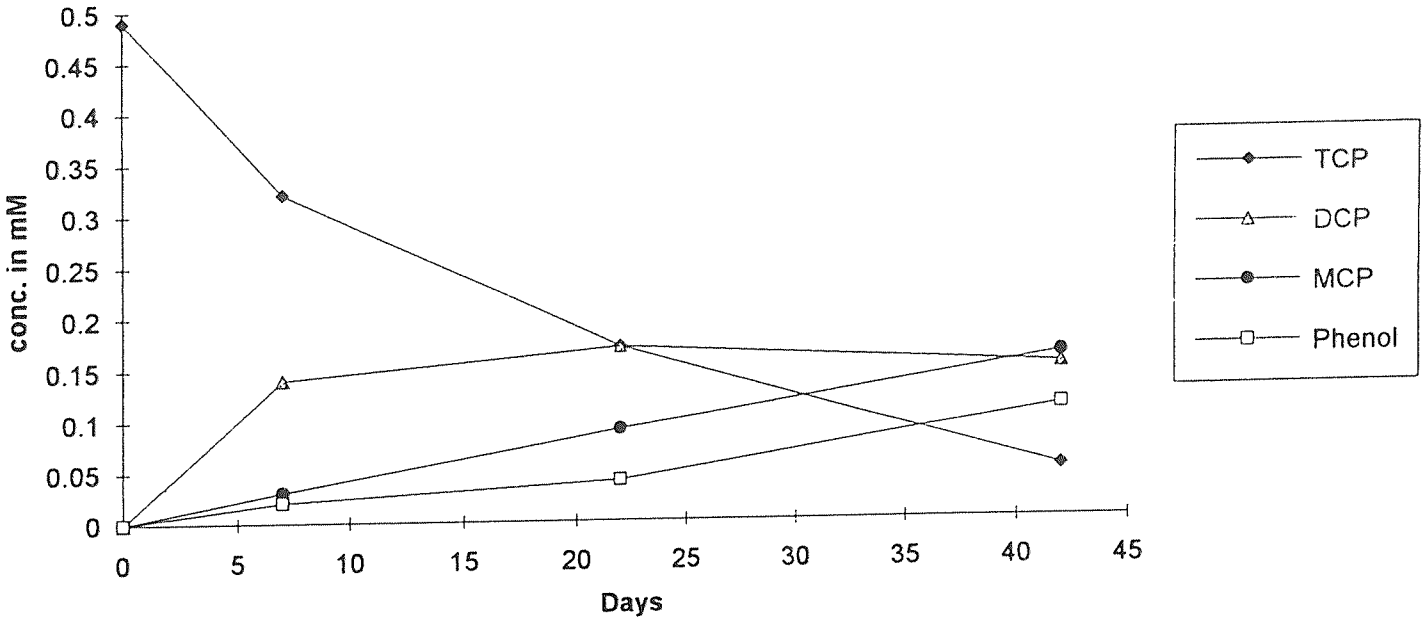

Figure 39 Mass Balance Culture 1

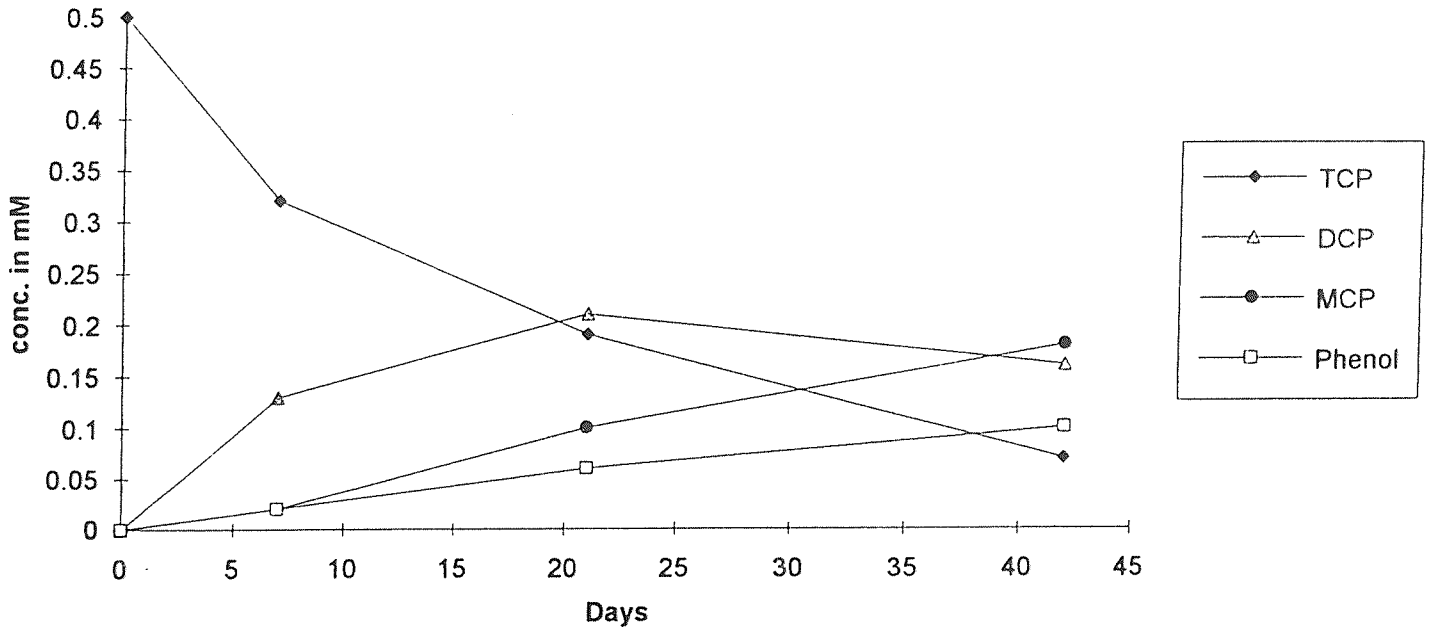

Figure 40 Mass Balance Culture 2

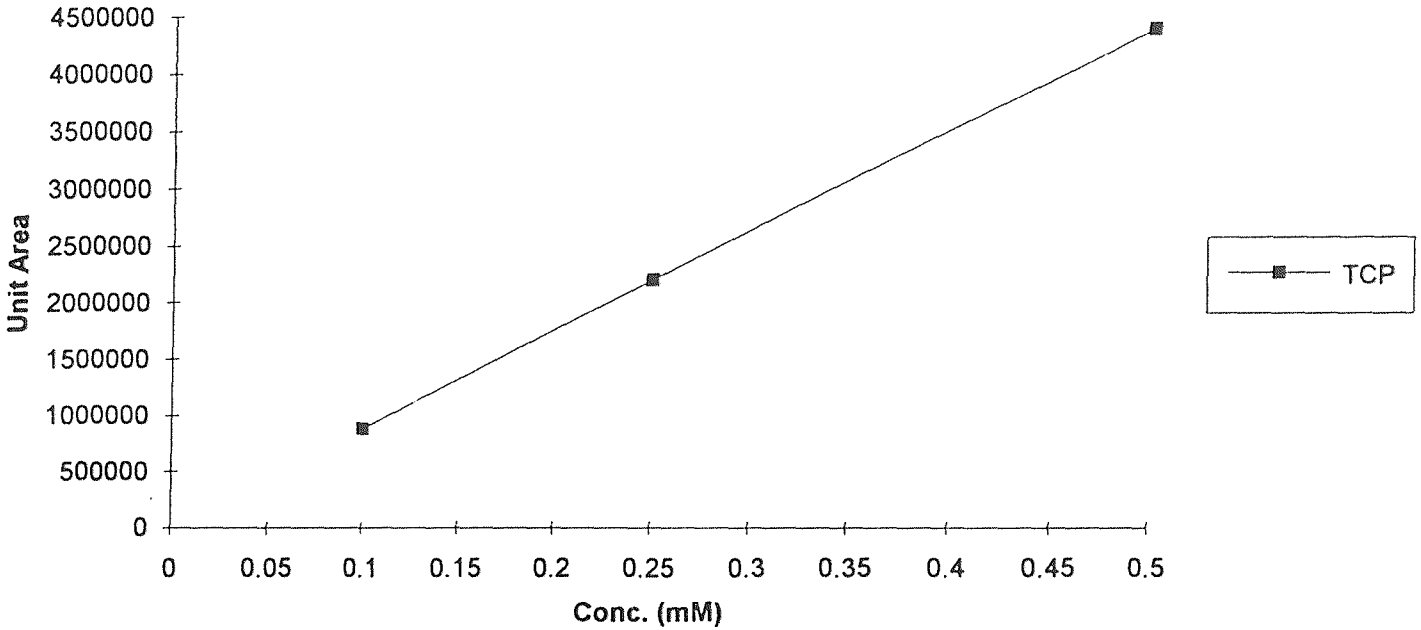

Figure 41 TCP Standards

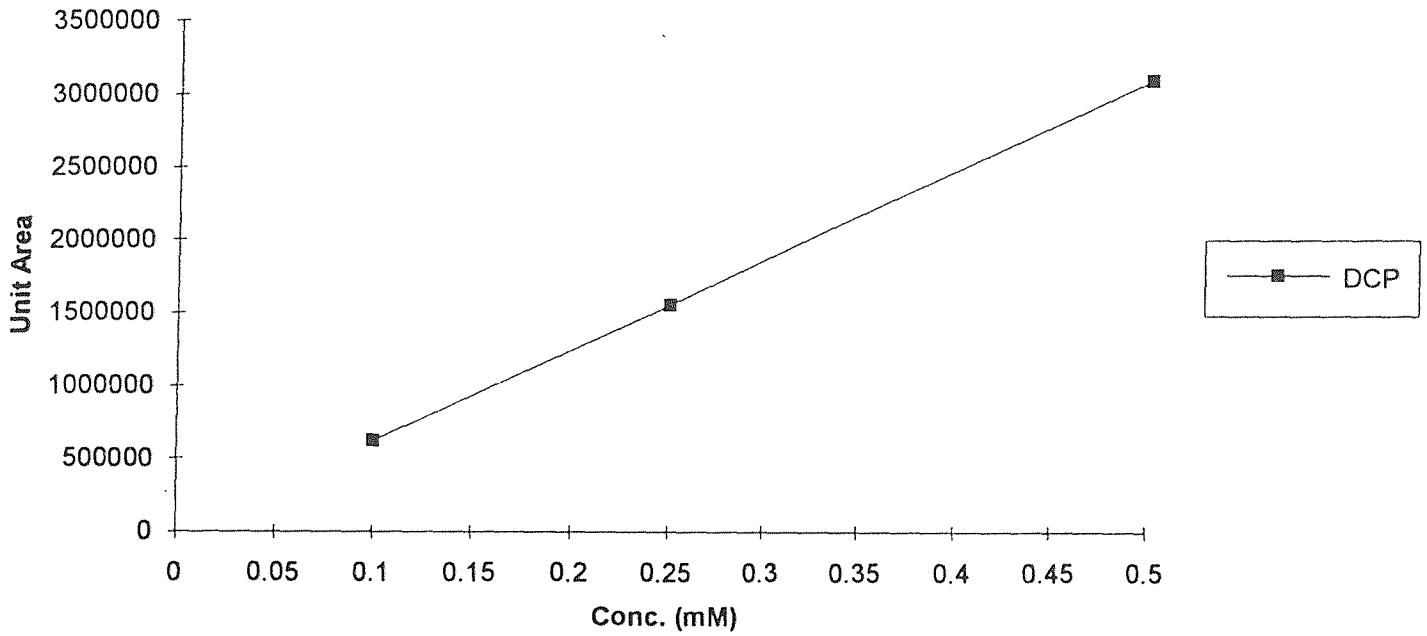

Figure 42 DCP Standards

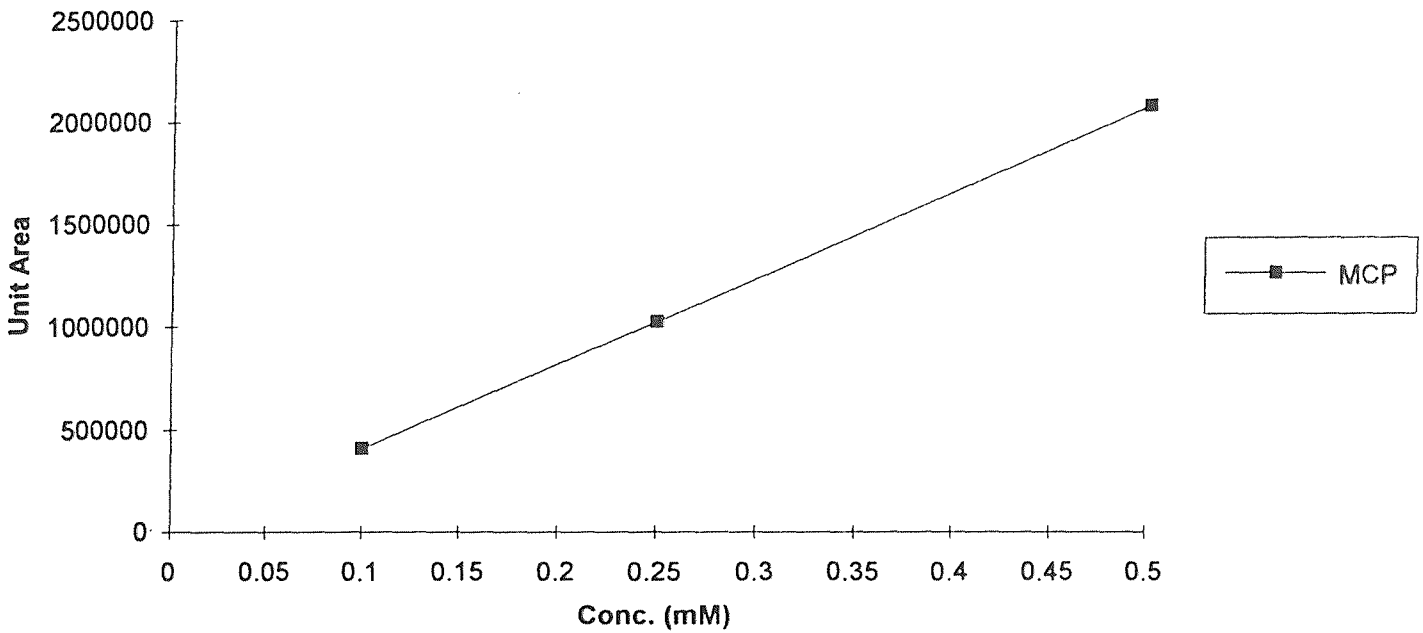

Figure 43 MCP Standards

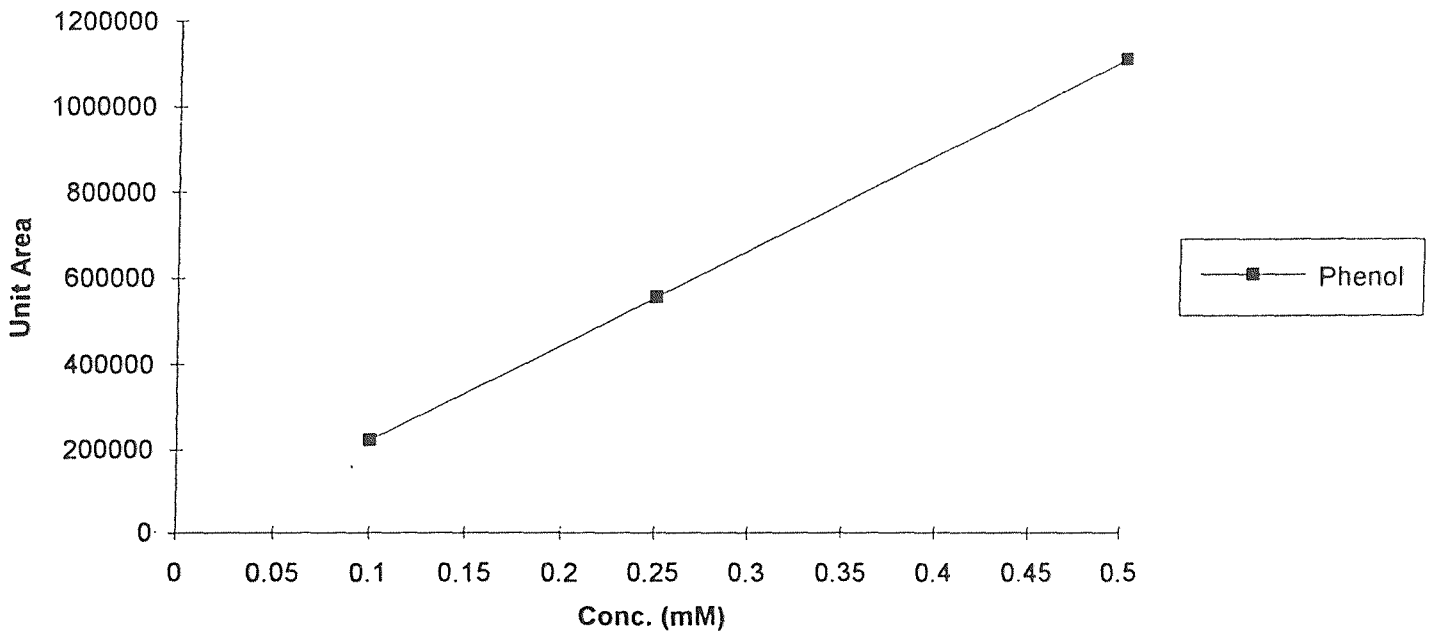

Figure 44 Phenol Standards

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