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Anaerobic Dehalogenation of 2-Chlorophenol in Mixed Bacterial Cultures

Karl G. Themel

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of

MASTER OF SCIENCE

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ANAEROBIC DEHALOGENATION OF 2-CHLOROPHENOL IN MIXED BACTERIAL CULTURES

BY

KARL G. THEMEL

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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List of Abbreviations

3-CB.....3-Chlorobenzoate

OCP.....2-Chlorophenol

PCP.....Pentachlorophenol

OD.....Optical density

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Abstract

This study investigated the effect of 2-chlorophenol (OCP) on mixed anaerobic cultures derived from anaerobic sewage sludge and the degradation of OCP by mixed anaerobic cultures derived from this sludge. There were three objectives: Finding whether microorganisms from anaerobic sewage sludge grow faster with pre-exposure to OCP than without, finding whether the microorganisms degrade OCP, and isolating a degrading organism. OCP slowed bacterial growth, but did not prevent degradation of a complex organic medium, unadapted and pre-exposed cultures grew with similar doubling times. OCP-degradation occurred in the absence of many of the solids and granules commonly found in anaerobic digesters. OCP was degraded in defined anaerobic mineral salts medium at concentrations five times greater than those utilized by known anaerobic chlorophenol and chlorobenzoate degraders. OCP-degradation occurred at concentrations that largely prevented growth of *Methanospirillum hungatei*. Acetate was found to be the end product of OCP-degradation. Carbon dioxide could not shown to be an end product. Sulphate reduction did not have an impact on OCP-degradation. In contrast to the anaerobic OCP-degrader 2-CP1, the culture used here was incubated at 35° C. OCP degrading cultures were viable for extended time periods (1 year) and consisted most likely of endospore formers since OCP-degradation was retained after 1 h incubation at 75° C.

Anaerobic Dehalogenation of 2-Chlorophenol (OCP) by Mixed Bacterial Cultures

1. Introduction

Monochlorophenols are common environmental contaminants, that are difficult to degrade biologically . They occur in sediments contaminated by wood preservatives, in Kraft mill wastewaters, and as breakdown products of chlorophenoxyacetic acid herbicides, (Häggblom, 1990; Reineke and Knackmuss, 1988).

Reductive dehalogenation of chlorophenols is thermodynamically favoured (Dolfing and Harrison, 1992). Chlorophenols are therefore potential electron acceptors in anaerobic environments. Gathering biological information on the chlorophenol-degrading microorganisms is an area of ongoing research. However the anaerobic degradation processes are less well known than aerobic degradations. This study focused on the anaerobic degradation of OCP, because it is a common intermediate of polychlorophenol degradation in anaerobic environments. As well, errors in analysis due to adsorption were minimized by the use of OCP rather than the more highly chlorinated phenols as a laboratory test compound. This study used anaerobic cultures containing a minimum of solid particles to further reduce the potential for absorption of OCP.

Chlorophenol toxicity and degradation is currently an active area of research. The following chapter presents a review of literature on the toxicity and degradation of chlorophenols and related compounds.

Following this, studies on the effect of OCP on cultures degrading a complex organic medium will be described. These were performed to gain insights in the toxicity of OCP on microorganisms found in anaerobic bioreactors for waste treatment. Further, work on OCP-degradation and its byproducts will be presented to gain insight on the conditions required for anaerobic degradation of OCP by a methanogen-free mixed culture derived from biomass of an anaerobic wastewater treatment facility.

2. Toxicity and Degradation of Chlorophenols - State of the Art

Chlorophenols in fresh- or seawater sorb to particulate material (Salkinoja-Salonen et al.,1984; Xie et al.; 1986), once in the sediment they may reach concentrations of >10 mg kg^{-1} of dry sediment. Mohn et al. (1992) studied the sorption of chlorophenols to anaerobic sludge granules. They reported that pentachlorophenol (PCP) was most prone to absorption by anaerobic sludge granules, monochlorophenols were least prone to absorption. Apajalahti's (1987) dissertation indicated that by using adsorption to softwood bark chips, PCP concentration in the medium for *Rhodococcus chlorophenolicus* can be 40 mg/L, rather than 20 mg/L, without incurring toxicity. That has to be taken into account when studying degradation of chlorophenols by anaerobic sludge.

2.1 Bacterial Degraders of Xenobiotic Compounds - Can a Bacterium Degrading a Xenobiotic Compound be Isolated?

a) If a xenobiotic compound can act as a primary carbon and energy source, the microorganism degrading the compound should be isolatable by transferring the inoculum from a complex medium containing the compound of interest to a medium containing only

that compound as carbon and energy source. In this case the culture remains viable when transferred repeatedly into fresh medium of this type (Stolp and Starr, 1981). Final purification may be done by serial dilution or by plating on to solid selective medium.

However a culture capable of degrading a given compound and using it as a carbon and/or energy source may still have a requirement for other growth factors which will need to be added to the medium. When the exact nature of the growth factors is not known yeast extract or trypticase digest (mostly amino acids) can be added in small amounts as a source of some of the growth factors. An example for such a situation is culturing of Tiedje's microorganism that degrades 3-chlorobenzoate, i.e. *Desulfonomile tiedjei* DCB-1. It utilizes 3-chlorobenzoate as an energy source, but not as a carbon source. When grown in pure culture it requires pyruvate as a carbon source (Shelton and Tiedje, 1984; Stevens et al., 1988). *Desulfonomile tiedjei* will grow and dechlorinate in a medium containing 3-chlorobenzoate and pyruvate (Mohn and Tiedje, 1991).

Is the enzyme very specific to the compound but yields energy only when another microorganism removes the degradation product and thus shifts the equilibrium between compound and product away from the compound the culture will also remain a mixed culture. A stable consortium of microorganisms forms in which

one strain degrades the compound and one or more other organisms degrade breakdown products of the compound. The best known example for this situation is the consortium consisting of *D. tiedjei* DCB-1, BZ-2, and *Methanospirillum* PM-1 that jointly degrade 3-chlorobenzoate (Shelton and Tiedje, 1984; Dolfing and Tiedje, 1987; Dolfing, 1990). The full consortium has no requirement for a carbon source other than 3-chlorobenzoate.

There are two reasons why one might see a lag prior to onset of degradation of a xenobiotic compound:

i) A culture which is capable of degrading the compound and uses a very specific enzyme for its initial transformation into a more degradable substance shows a lag time between exposure to the compound and begin of degradation. Cells within the culture will activate the enzyme's gene or genes and to synthesize the enzyme protein before degradation begins. Strain 2-CP1 isolated by Cole et al. (1994) has a lag of more than 6 hours when transferred into medium containing OCP. 2-CP1 may need to synthesize an OCP-degrading enzyme prior to the beginning of degradation.

Degradation of a xenobiotic compound may also require induction: *Desulfonomile tiedjei* can degrade PCP, but 3-chlorobenzoate has to be present for activation of degradation (Mohn and Kennedy, 1992a). 3-chlorobenzoate is an inducer.

ii) A lag time after first exposure has been observed prior to initial degradation to the toxicant of interest e.g. in a mixed culture isolated by Häggblom and Young (1990) from New York's East River that degrade 4-chlorophenol in conjunction with sulphate reduction. This might be due to a different population of microorganisms becoming predominant in the culture.

b) A given substance may be degraded co-metabolically by an enzyme with a different primary substrate. Presence of the compound of interest in the medium might not help in selecting for degrading organisms. Conceivably the enzyme which co-metabolically degrades the compound may be present in different microorganisms, i.e. there could be more than one degrader. An example is 4-chlorophenol degradation by *Pseudomonas putida* PpG4 (Saez and Rittmann, 1991, 1993). Cells have to be grown on an alternate substrate (phenol) before they degrade 4-chlorophenol without undergoing growth. Similarly, Gorlatov and Golovleva (1992) reported that *Rhodococcus erythropolis* 1CP grows on glucose or maltose, but also degrades 50 mg/L 2,4-dichlorophenol. There is no growth when 2,4-dichlorophenol is the only organic substrate.

A co-metabolically degrading culture does not exhibit a lag phase between the exposure to the substrate and the beginning of

degradation (Reineke and Knackmuss, 1988) if the enzyme is present and active in the culture prior to exposure.

Liu et al. (1991) indicated that frequently degradation of xenobiotic compounds is co-metabolic, i.e. a substrate other than the to be degraded compound is required as a carbon or energy source. Generally OCP is degraded faster than 3-chlorophenol and 4-chlorophenol.

2.2. Toxicity of Chlorophenols

2.2.1 Effect of Chlorophenols on Organisms

Chlorophenols are toxic to all pro- and eukaryotic organisms (Kozak et al., 1979). Chlorophenols are lipophilic and therefore accumulate in the food chain (Kobayashi, 1978).

Chlorophenols bioaccumulate in juvenile - and early life stages of pink - and chinook salmon (*Onchorhynchus gorbuscha, O. tshawytscha*) overwintering in the upper Fraser River, British Columbia (Rogers et al, 1988; Servizi et al., 1988).

In studies done with various types of fish at the cellular level, fingerlings of the species *Oncorhynchus tshawytscha* (chinook salmon) exposed to biotreated bleached kraft mill effluent showed

enlarged nuclei in their interrenal cells indicating chronic stress (Servizi et al., 1993). Shigeoka et al. (1988) found hatching inhibition in *Oryzias latipes* when kept in chlorophenol contaminated water. Dixon et al. (1987) reported liver damage in trout (*Salmo gairdneri*) that coincided with an increase in serum sorbitol dehydrogenase activity.

Krijgsheld and van der Gen (1986) indicated in their study a moderate toxicity to mammals for 2,4-dichlorophenol and monochlorophenols. Jorenz and Schepens (1993), Hughes et al. (1993), and Narasimhan et al. (1992) found pentachlorophenol (PCP) to be a metabolic poison. Dermal absorption, inhalation or ingestion led to acute or chronic poisoning. PCP is detectable in urine or serum.

2.2.2 Action of Chlorophenols at the Cellular Level

Why are chlorophenols toxic? Ravanel et al. (1989) found chlorophenols to have a uncoupling effect on *Acer* (maple tree) cells. The best uncoupler was pentachlorophenol (PCP). Jansson and Jansson, 1993 worked with Chinese hamster (V79) cells. The membranes of Chinese hamster cells became more permeable to protons when exposed to chlorophenol. That caused reproductive cell death in V79 hamster cells. The amount of proton permeability was depended on both hydrophobicity of the particular chlorophenol and the electron withdrawing capabilities

of the substituent chlorines. Oenfelt (1987) found spindle disturbances, a disturbed internal pH, and mitochondria with disturbed calcium ion uptake in dividing V79 hamster cells. Izushi et al., (1988) also reported that rat liver mitochondria increased their potassium ion permeability and their potassium ion loss under chlorophenol influence. ATPase activity was also stimulated. Jorenz and Schepens (1994), Hughes et al. (1993), and Narasimhan et al. (1992) indicated that PCP interferes with oxidative phosphorylation. Narasimhan et al. (1992) reported PCP and 4-chlorophenol to be uncouplers. The toxic concentration of PCP is 1.33 μM, that of 4-chlorophenol is 20 μM (Narasimhan et al. 1992). Human erythrocyte membranes have a calcium ion transport ATPase that is inhibited by phenol, 4-chlorophenol, and polychlorophenols (Janik and Wolf, 1992).

2.2.3 Chlorophenols in the Environment

Chlorophenols interfere with photosynthesis and oxygen production and therefore upset the balance between autotrophs and heterotrophs in aquatic ecosystems (Schauerte et al., 1982). As indicated above chlorophenols also accumulate in food chains, because they are lipophilic (Kobayashi, 1978). These compounds may persist for years in the environment, but when chlorophenol degrading microorganisms are present persistence is lower.

2.2.4. Chlorophenols and Cancer in Humans and Mammals

Chlorophenols may increase the cancer risk when drinking water is contaminated, i.e. when there is a long term exposure (Lampi et al., 1991). Cancers that occur more frequently due to long term chlorophenol exposure are non-Hodgkin's lymphomas and soft tissue sarcomas. Smith at al. (1984) indicated an increased cancer risk after 2-chlorophenol exposure. The risk increased by a factor of 1.5 for soft tissue sarcoma, but contrary to Lampi et al. (1991) 24 h of exposure was sufficient to increase the risk of developing soft tissue sarcoma. Also Jaeppinen (1989) reported increased cancer risk for sawmill workers exposed to PCP used as wood preservative.

2.2.5. Limitation of Toxicity

Low OCP doses may have no acute toxic effects. Daniel et al. (1993) reported for example that the exposure of rats to 257 mg/kg bodyweight of OCP over a 10 day period caused no acute toxic effects. Cancer risk to a person exposed to chlorophenols may increase. However Bueno de Mesquita et al. (1993) found no increased cancer risk after a one time exposure of forest industry employees.

2.2.5.1. Relative Toxicity of Different Chlorophenols

Bryant and Schulz (1994) evaluated the effect of PCP and lower chlorophenols on the ciliate *Tetrahymena pyriformis*. They determined that decreasing chlorine substitution led to lower toxicity due to decreasing hydrophobicity and reactivity. Methylation of chlorophenols led to lower toxicity due to lower reactivity while oxidation led to increased toxicity due to higher reactivity (Bryant and Schulz, 1994).

2.2.6. Biosorption

In many published papers concerning anaerobic dehalogenation of chlorophenols mixed cultures or sludges were studied. Anaerobic sludges contain solid matter sludge granules (Mohn et al. 1992). As indicated in Ch. 1 sorption of chlorophenols and their degradation products to solid matter in anaerobic sludges and mixed cultures has not been considered in any work reported to date except Mohn et al. (1992). The biosorption of chlorophenols to anaerobic sludge was greatest for PCP. When developing a sewage treatment system that uses anaerobic sludge it is important to consider whether a chlorophenol is truly degraded by the sludge or whether it is adsorbed to solid matter present in the sludge.

Biosorption of chlorophenols to other organic matter may also

occur. In an aerobic culture containing wood bark chips adsorption may cause false chlorophenol-concentration readings. As indicated in Ch. 1 Apajalahti (1987) found in a culture of *Rhodococcus chlorophenolicus* that adsorption to softwood bark protects the microorganism from otherwise toxic PCP concentrations. Less chlorophenol was in the medium thus causing less PCP-toxicity on *R. chlorophenolicus*. With the bark chips present PCP Concentration in the medium could be doubled to 40 μM and growth of *R. chlorophenolicus* still occurred.

2.2.7. Toxicity of Chlorophenols to Anaerobic Cultures

O'Connor and Young (1989) evaluated the toxicity of 2-chlorophenol on methanogenesis in a culture consisting of 10% sludge from a municipal sludge digester. A concentration of less than 1 mM (100 mg l^{-1}) completely inhibited methanogenesis. Hrudey et al. (1987) in their early study on monochlorophenol degradation noticed inhibition of methane production at above 100 mg/L (0.8 mM) OCP. They also reported inhibition of phenol degradation by 97 mg/L OCP and 30 mg/L 3-chlorophenol or 4-chlorophenol. Kim et al. (1994) found chlorophenols to be most toxic to acetoclastic methanogens and somewhat less toxic to hydrogenotrophic methanogens, but 1 mM of a monochlorophenol caused reductions in methane production. Acetogenic bacteria were also affected by 1 mM of a monochlorophenol. Davies-Venn et al. (1992) also reported inhibition of acetoclastic methanogenesis by chlo-

rophenols. Mohn and Kennedy (1992a) reported PCP to be degraded by Desulfonomile tiedjei, but concentrations greater than 10 μM PCP proved inhibitory. Patel et al. (1991) reported that 2000 mg/L phenol or 76 mg/L pentachlorophenol cause a reduction of methane production by 50% in *Methanospirillum hungatei*.

2.2.8. Toxicity of Chlorophenols to Aerobic and Denitrifying Microorganisms in Pure and Mixed Culture

Chlorophenols inhibit growth of activated sludge standardized according to the Japanese Chemical Inspection and Testing Institute, Tokyo (Shigeoka et al., 1988). Chlorophenols also inhibit growth of the green algae *Selenastrum capricornutum* (Nyholm, 1991) and *Chlorella vulgaris* (Klekner and Kosaric, 1992). The algae in both of the above studies tolerated 1000 mg/L phenol. Klekner and Kosaric (1992) also reported *Chlorella* to degrade 200 mg/L OCP.

Diefenbach et al. (1992) and Heipieper et al. (1992) reported that both phenol and 4-chlorophenol caused an increase of up to 41% in the amount of trans-unsaturated fatty acids compared to cis-unsaturated fatty acids present in cell membranes of *Pseudomonas putida* P8. The change occurred in cells that were growth inhibited by 4-chlorophenol. Cells of *P. putida* P8 that were growing in the presence of 4-chlorophenol increased the proportion of saturated to unsaturated fatty acids, but did not

change the ratio of trans- to cis-unsaturated acids. *P. putida* P8 acts like *E. coli* (Keweloh et al., 1991).

Keweloh et al. (1991) showed that growth of *Escherichia coli* was inhibited when exposed to 4-chlorophenol (1 mM). Chlorophenols are lipophilic and therefore tend to enter membranes. That would disturb the arrangement of the fatty acid residues. Therefore chlorophenols tend to fluidize the membrane. Keweloh et al. (1991) reported increase of rigidity of *E. coli* membranes by the addition of palmitic acid to the culture medium. They showed that palmitic acid when added simultaneously with 4-chlorophenol will lessen its inhibitory effect on growth.

2.3. Degradation of Chlorophenols under Aerobic and Denitrifying Conditions

2.3.1. Dehalogenators Isolated Recently from Activated Sludge and wastewaters

Makinen et al. (1993) aerobically degraded PCP and other polychlorinated phenols in a wastewater treatment type system using a chlorophenol load of 445 mg/L d. No dry weight for the activated sludge was given. Similar results were achieved by Puhakka and Jarvinen (1991), who degraded up to 0.12 mg/L mixed chlorophenols in an actual wastewater using activated sludge.

68% of the chlorophenols were degraded. Singh et al. (1993) isolated *Bacillus* sp., *Pseudomonas* sp., and *Klebsiella* sp. from OCP-degrading activated sludge. The *Bacillus* was reported to be the OCP-degrader. Parker et al. (1993) also found *Bacilli* in PCP degrading activated sludge. Kramer and Kory (1992) isolated two Gram positive coryneform bacteria from an aerobic continuous culture.

Streptomyces rochei 303 (Gololeva et al., 1993) was found capable of degrading polychlorophenols as was also a white rot fungus (*Phanerochaete chrysosporium*) that serves as an OCP-degrader in wastewater treatment systems. White rot fungus degraded 36 mg 2,3,6-trichlorophenol per g carrier. The carrier material was polycaproamide fibre (Gololeva et al., 1993; Lewandowski et al., 1990).

2.3.2. Earlier Isolates of Aerobic Dehalogenators

One of the first examples of a chlorophenol degrader in pure culture was reported by Reiner et al. (1978). They found a saprophytic coryneform pentachlorophenol degrading bacterium designated strain KC-3, while Saber and Crawford (1985) found a PCP degrader that belongs to the genus *Flavobacterium*. Both the *Flavobacterium* and KC-3 degrade pentachlorophenol in the following manner:

Pentachlorophenol

=> 1,4-dihydroxytetrachlorophenol

=> 1,4-dihydroxy-2,3,5-trichlorophenol

=> 1,4-dihydroxy-2,5-dichlorophenol

=> => carbon dioxide

The following section covers an important aerobic dechlorinator:

2.3.3. *Rhodococcus*

Briglia et al. (1994) studied *Rhodococcus chlorophenolicus* PCP-1, which has the potential to be used for PCP biodegradation in contaminated soils. R. chlorophenolicus is the best known aerobic dehalogenator of chlorophenol.

Apajalahti (1987) and Apajalahti and Salkinoja-Salonen (1984) reported that *R. chlorophenolicus* dehalogenated PCP yielding non-chlorinated 1,2,4-trihydroxybenzene that subsequently served as a carbon and energy source. *R. chlorophenolicus* degraded PCP fastest at a concentration of 12 μM (Apajalahti and Salkinoja-Salonen, 1984). *R. chlorophenolicus* is able to dehalogenate in the absence of molecular oxygen with a cytochrome P-450 enzyme utilizing sulphite ion (Uotila et al., 1992).

Apajalahti (1987) reported inhibition of growth of *R. chlorophenolicus* by pentachlorophenol (PCP), 2,3,4,5-tetrachlorophenol

(2,3,4,5-TeCP), and 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP). These compounds were biodegraded by *R. chlorophenolicus*, however growth was reduced by 50% by chlorophenol concentrations of 4 μM (for 2,3,4,5-TeCP) to 20 μM (for PCP).

Degradation of PCP took place in the following manner:

Pentachlorophenol

=> 1,4-dihydroxytetrachlorophenol

=> a dichlorinated 1,2,4-trihydroxyphenol

=> a monochlorinated 1,2,4-trihydroxyphenol

=> 1,2,4-trihydroxyphenol

=> => carbon dioxide

It appeared therefore that *R. chlorophenolicus* did not attack chlorinated ring structures that were not part of its degradation pathways of higher chlorophenols like pentachlorophenol (PCP). There was no evidence that monochlorophenols were degraded by *Rhodococcus*. This would be a disadvantage in a wastewater treatment plant, because any lower chlorinated phenols present would remain undegraded, released to the environment, and possibly cause toxic effects on the treatment plant's sludge. PCP would degrade completely, but there is the potential that a tetrachlorophenol other than 2,3,5,6-tetrachlorophenol would only partially dehalogenate.

2.3.4. Enzymatic Mechanisms of Aerobic Dehalogenations

The following papers present recent examples of dehalogenation processes for which the responsible enzymes are known. Dec and Bollag (1994) observed the release of chloride ions during dehalogenation of chlorinated phenols by oxidoreductive enzymes. Oxidative coupling took place when an unpaired electron was located at a chlorine substituted aromatic carbon (free radical). The process resulted in polymerization of the chlorinated phenols or binding to humic acid. Such a process should produce higher aromatic compounds that may still have some chlorine substitution. Horseradish peroxidase may also produce chlorinated humic substances from OCP and 4-chlorophenol (Lassen et al., 1994; Siddique et al., 1993). A fungal tyrosinase (Wada et al., 1994) could remove phenol and 4-chlorophenol from aqueous solution. *R. chlorophenolicus* PCP-1 uses a cytochrome P-450 enzyme for dechlorination by para-hydroxylation (Uotila et al., 1993).

2.3.5 Denitrifyers Capable of Dehalogenation

Melin et al. (1993) reported that denitrifying bacteria are able to dechlorinate chlorophenol in a sewage treatment prototype system. Removal was between 23 and 82% of 4-chlorophenol. Close to 1.8 mg 4-chlorophenol was degraded per mg nitrate removed. This pointed to a complete oxidation of 4-chlorophenol.

Pseudomonas putida was found to degrade mixtures of monochlorophenols (Dapaah and Hill, 1992; Bestetti et al., 1992; Hinteregger et al., 1991). Saez and Rittmann (1993) reported that *P. putida* PpG4 primarily degraded phenol and 4-chlorophenol as a co-substrate. Both degradation rates were proportional. *P. putida* PpG4 had to be grown on phenol prior to incubation in 4-chlorophenol. *Pseudomonas pickettii* grew on 2,4,6-trichlorophenol as the only carbon and energy source (Kiyohara et al., 1992).

Pseudomonas strain B13 grew on 4-chlorophenol and also metabolized 3-chlorophenol and OCP (Knackmuss and Hellwig, 1978). 3- and 4-chlorophenol were degraded in the following manner:

Monochlorophenol

=> Monochlorocatechol

=> Monochloromuconate

= dechlorination=> 4-carboxymethylenbut-2-en-4-olide

Ring opening occurred via ortho- or meta cleavage. Phenol degradation terminated with a non-chlorinated muconate as a final product (Knackmuss, 1981, 1983; Schmidt and Knackmuss, 1980). In co-culture with *Alcaligenes* strain A7, all monochlorophenols were degraded via ortho-cleavage only. Phenol was degraded via catechol. Madsen and Licht (1992) found an organism designated strain DCB-2, that removed chlorine from di- and trichlorophenol

when growing fermentatively. DCB-2 may also reduce nitrate to ammonia.

2.3.6 Conclusion

Rhodococcus chlorophenolicus (Apajalahti, 1987) did not degrade monochlorophenols. PCP would degrade completely, but there is the potential that a tetrachlorophenol other than 2,3,5,6-tetrachlorophenol would dehalogenate only partially. *Pseudomonas putida* (Saez and Rittmann, 1993) may dehalogenate 4-chlorophenol and thus supplement *Rhodococcus* (Apajalahti, 1987) in a treatment system for a wastewater with halogenated aromatics. Desirable would be a mix of aerobic dehalogenators that collectively can dehalogenate all chlorophenols or one may chose an anaerobic dehalogenating culture for a biological treatment system.

2.4. Degradation of Chlorophenols under Anaerobic non-Sulphate Reducing Conditions

Degradation of chlorophenol occurs both in biomass found in anaerobic wastewater treatment facilities and in natural anaerobic sediment.

a) Anaerobic wastewater treatment is used to treat concentrated and chlorophenol-contaminated waste streams in a process like

that of Flora et al. (1994). The concentrated waste streams for example contain acetic acid and chlorophenol. Flora et al. worked with a synthetic medium containing from 2000 to 5900 mg/L acetic acid, 1000 to 3000 mg/L phenol, 1200 mg/L OCP, 600 mg/L 2,4-dichlorophenol, and 150 mg/L 2,4,6-trichlorophenol. Flora et al. (1994) used an anaerobic fluidized bed activated carbon bioreactor that employed activated carbon replacement. 4-Chlorophenol tended to accumulate unless carbon was replaced regularly.

Another application of anaerobic treatment is digestion of waste activated sludge from an aerobic sewage plant in an anaerobic sludge digester. Anaerobic treatment in a wastewater plant avoids growth of large amounts of biomass and avoids need for aeration. Anaerobic biomass is often able to degrade chlorophenols (Mohn and Kennedy, 1992b; Flora et al., 1994; Nicholson et al., 1992). Degradation of relatively concentrated chlorophenol solutions may be possible (Flora et al., 1994). Mohn and Kennedy (1992b) reported complete degradation of 1.75 mM 2,3,6-trichlorophenol.

b) In natural sediment Hrudey at al. (1987) found a 4-chlorophenol degrading population which could degrade up to 2 mM of this compound. No information was given as to whether any bacterial growth took place. Degradation of chlorinated phenols by anaerobic freshwater and estuarine sediments has been reported repeatedly, Häggblom et al. (1993b) published a recent study.

Wiegel et al. (1990) completely dehalogenated 2,4-dichlorophenol in cultures taken from anaerobic lake sediment.

Chlorophenols including pentachlorophenol (PCP), a wood preservative degrade to lesser chlorinated phenols and mono-chlorophenols when in anaerobic freshwater or marine sediments, (Mikesell and Boyd, 1986; Madsen and Aamand, 1992). There is some earlier work of interest that studied pentachlorophenol (PCP) degradation:

Boyd et al. (1983), Boyd and Shelton (1984) were among the first to show degradation of monochlorophenols and phenol in digested sludge. After acclimation on 3-chlorophenol the sludge was able to degrade pentachlorophenol (PCP):

Pentachlorophenol

=> 3,4,5-trichlorophenol

=> 3,5-dichlorophenol

=> 3-chlorophenol

=> methane + carbon dioxide

If the acclimation used OCP, PCP degradation followed a similar series with different intermediates. The same applied for acclimation with 4-chlorophenol. In order to degrade all chlorophenols sludge had to be separately acclimated with OCP, 3-chlorophenol, and 4-chlorophenol. Mixing the resulting cultures

produced a culture that degraded PCP and all lower chlorinated phenols to non-chlorinated compounds.

Madsen and Aamand (1991) were able to degrade PCP to 3-chlorophenol using an anaerobic mixed culture derived from sewage sludge. This particular culture required methanogenic conditions. Sulphate reduction inhibited dehalogenation. An inhibitor for sulphate reduction restored dehalogenation. As hydrogen depletion also inhibited dehalogenation, sulphate reduction probably used hydrogen needed by the dehalogenator(s).

Anaerobic monochlorophenol and monochlorobenzoate degradation can occur under methanogenic or sulphate reducing conditions (Häggblom et al., 1993a,b) in biomass from estuarine sediments. This means that there are either different types of organisms capable of anaerobic degradation of monochlorinated aromatic compounds or the same organisms are able to degrade the monochlorinated aromatic compounds under both methanogenic and sulphate reducing conditions. Anaerobic chlorophenol degradation was reported to require the presence of other carbon substrates (Parker et al., 1994; Häggblom et al., 1993a). In case of OCP-degradation Cole et al. (1994) reported a requirement for acetate. This means that either chlorophenols are not a source of energy or carbon for the microorganisms and that the degradation is co-metabolic or that additional growth factors are required. Armenante et al. (1993) indicated that a pH of 8.0 to 8.8 is

optimal for degradation of 2,4,6-trichlorophenol. Degradation often stops at the monochlorophenol level (Flora et al., 1994; Armenante et al., 1993; Mohn and Kennedy, 1992b; Nicholson et al., 1992) causing monochlorophenols to accumulate in some mixed cultures. There are also reports where monochlorophenols were degraded (Cole et al., 1994; Häggblom et al., 1993a; Zhang and Wiegel, 1992).

There are more studies that involve like Boyd et al. (1983) anaerobic dehalogenation of chlorophenols under methanogenic conditions. The following papers involve lower chlorinated phenols: Degradation of mono-chlorinated phenols under methanogenic conditions reported Horowitz et al. (1982), Boyd and Shelton (1984), Suflita and Miller (1985), Hrudey et al. (1986), Genthner et al. (1989b), Zhang and Wiegel (1990), Wiegel et al. (1990). Monochlorophenols are dehalogenated to phenol. Phenol in turn is converted to benzoate (Knoll and Winter, 1987, 1989; Béchard et al., 1990; Gallert et al., 1991) when hydrogen is present. Benzoate was detected in dehalogenating enrichments (Genthner et al., 1989a). Wiegel et al. (1990) worked on degradation of 2,4-dichlorophenol and proposed the following degradation pathway:

2,4-dichlorophenol

=> 4-chlorophenol

= dehalogenation => phenol

=> benzoate

According to this pathway reductive dehalogenation is taking place where the chlorine is replaced by a hydrogen leading to formation of dissolved HCl.

Hrudey et al. (1987) found up to 2 mM (285 mg l^{-1}) of OCP to be biodegradable, but indicated that methane was produced in presence 100 mg l^{-1} (0.8 mM) only. O'Connor and Young (1989) reported that anaerobic mixed cultures could produce methane when up to 0.8 mM (100 mg l^{-1}) OCP were present. Three anaerobic organisms in pure culture have been more recently found to attack chlorophenols under certain conditions (Cole et al., 1994; Mohn and Kennedy, 1992a; Madsen and Licht, 1992).

2.4.1. Anaerobic Degradation of Phenol and Benzoate

Products of chlorophenol degradation are benzoate and phenol. It is therefore appropriate to discuss them also:
Fedorak and Hrudey (1987) and Hrudey et al. (1987) reported methane production from batch cultures using phenol as the only carbon source. Phenol concentrations in the cultures were up to 30 mM, p-Cresol in concentrations of up to 20 mM. OCP in concentrations up to 0.8 mM did not prevent methane production, but the period between supplying medium and first gas production increased. Fedorak and Hrudey (1987) suggested that phenol breaks down to volatile fatty acids that in turn degrade to methane and carbon dioxide. A precise breakdown pathway was not deter-

mined. The authors did not define the consortium of organisms in their cultures that came from an anaerobic digester of a municipal wastewater treatment facility.

While the pathway of phenol degradation was not determined, three models have been proposed. Two of the models involve benzoate as an intermediate product. Indeed, as indicated above, benzoate serves as a substrate to anaerobic organisms (Knoll and Winter, 1989). They found benzoate to be an intermediate product of phenol degradation. Knoll and Winter (1989), Gallert et al. (1991) proposed that hydrogen is added to the aromatic ring prior to ring cleavage. There are two possible pathways: The first leads to evolution of two moles of propionate from benzoate. The intermediate product is cyclohexanoic acid. The second possible pathway generates 2-hydroxycyclohexanoic acid, which opens into $HOOC-(CH_3)_5-COOH$. Knoll and Winter (1987) also proposed a third pathway according to which phenol breaks down to cyclohexanol and further to caproic acid without evolution of benzoate. All breakdown products are ultimately converted to acetate. It can be concluded that benzoate, phenol and some related compounds are biodegradable, can be present in an active culture in larger concentration than chlorophenols and are metabolized by one of the following pathways (Knoll and Winter, 1987):

Pathway 1:

Benzoate => Cyclohexanoic acid => Adipic acid => => Acetic acid

Pathway 2:

Benzoate

=> 2-hydroxycyclohexanoic acid

=> $HOOC-(CH_3)_5-COOH$

=> 2 Propionic acid

=> => 3 Acetic acid

Pathway 3:

Phenol => Cyclohexanol => Caproic acid => => 3 Acetic acid

An anaerobic phenol degrader was isolated into pure culture: *Desulfobacterium phenolicum* (Bak and Widdel, 1986). It is a sulphate reducer.

2.4.2 Summary

Anaerobic dehalogenation of chlorophenols has the potential to degrade chlorinated phenols. Some chlorophenols are degradable when present in concentrations larger than 1 mM. So far only a few pure cultures have been identified that degrade a chlorophenol.

2.5. Degradation of Chlorophenols under Anaerobic and Sulphate - Reducing Conditions by Mixed Cultures

Häggblom et al. (1993a,b) found both freshwater and estuarine sediment with anaerobic microorganisms capable of degrading chlo-

rophenols and chlorobenzoates. Degradation occurred under denitrifying -, sulphate reducing -, and methanogenic conditions. Abrahamsson et al. (1991) found dehalogenating anoxic marine sediments that could dehalogenate chlorophenols and bromophenols optimally at temperatures between 6° and 30° C. Häggblom and Young (1990) studied monochlorophenol degradation coupled to sulphate reduction in cultures inoculated with 10% estuarine sediment from the East River, NY. in 0.1 mM of 2-chlorophenol (OCP), 3-chlorophenol, and 4-chlorophenol. After an initial long lag period of 50 to 100 days chlorophenols were degraded completely, after refeeding with fresh monochlorophenol degradation occurred within 10 days at a rate between 8 and 37 μmol $l^{-1}day^{-1}$ while sulphate was consumed. 4-chlorophenol degraded fastest, OCP slowest. Addition of 4-cresol or propionate enhanced monochlorophenol degradation. Addition of 20 mM molybdate prevented degradation. Genthner et al. (1989a) found dehalogenation of 4-chlorophenol by sediment of a marine estuary to be enhanced in presence of sulphate while dehalogenation of the other monochlorophenols was inhibited. Sulphate however was not a requirement for 4-chlorophenol dehalogenation. Both phenol and benzoate appeared in the cultures as the monochlorophenols were degraded.

Kohring et al. (1989) and Wiegel et al. (1990) were able to confirm anaerobic dehalogenation of 0.3 mM 4-chlorophenol under sulphate reducing conditions. They used cultures inoculated with freshwater sediment. Again sulphate was not a requirement for

4-chlorophenol degradation.

A sulphate reducer degrading a related compound is *Desulfonomile tiedjei* DCB-1 (Mohn and Tiedje, 1991). Madsen and Licht (1992) found an organism dehalogenating tri- and dichlorophenols designated DCB-2. DCB-2 is a nitrate reducer, but dehalogenates in absence of nitrate only.

2.6 Degradation of Chlorobenzenes

Holliger et al. (1992) enriched bacteria from the river Rhine that dehalogenated polychlorinated benzenes. Their enrichment cultures were incubated in two phase sealed bottles where a hexadecane phase covered the water phase with the bacteria. Dehalogenation was fastest with a co-substrate like lactate, acetate or pyruvate. The culture medium could be replaced with fresh medium plus chlorobenzene and dehalogenation recurred. The dehalogenation rate increased over the first few cycles of refeeding and incubation until the culture reached a stable population of microorganisms. Hydrogen served as electron donor and chlorobenzene as electron acceptor.

2.7 Dehalogenation of Chlorobenzoates by Mixed Cultures

Häggblom et al. (1993b) found degradation of both chlorophenols

and chlorobenzoates in anaerobic freshwater and estuarine sediment. The sediment bacteria dehalogenated under methanogenic conditions and also with nitrate or sulphate as electron acceptor.

2.8 Dehalogenation of Simple Chlorinated Aromatics by Anaerobic Pure Cultures

In addition to aerobic or denitrifying microorganisms there are few known pure cultures that are able to dehalogenate a chlorophenol anaerobically. *Desulfonomile tiedjei* DCB-1, a sulphate-reducer can be induced to dehalogenate PCP to 2,4,6-trichlorophenol (Mohn and Kennedy, 1992). *Desulfonomile tiedjei* DCB-1 does not grow on PCP or other halogenated compounds as the only carbon and energy source. No sulphate reduction takes place while *D. tiedjei* DCB-1 dehalogenates. PCP-degradation has to be induced using 3-chlorobenzoate.

Mohn and Tiedje (1990b) studied the physiology of *D. tiedjei*. The electron acceptors sulphate and thiosulphate were utilized in conjunction with the electron donors formate or hydrogen yielding sulphide. In absence of an electron donor thiosulphate was disproportioned to sulphate and sulphide. Also supporting growth of *D. tiedjei* were the electron donors carbon monoxide, lactate, pyruvate, butyrate, and 3-methoxybenzoate. In absence of other electron acceptors *D. tiedjei* reduced carbon dioxide

to acetate. *D. tiedjei* coupled the carbon dioxide reduction to oxidation of pyruvate to acetate and carbon dioxide. *D. tiedjei* is therefore a thiosulphate-disproportioning bacterium.

An organism was isolated by Madsen and Licht (1992), that removes a chlorine from a trichlorophenol. This organism is strain DCB-2, it forms endospores and is allied to *Clostridium*. DCB-2 does not reduce sulphate, but can reduce nitrate to ammonium ion. Growth occurs on pyruvate as the sole carbon and energy source, but not on a chlorophenol. Pyruvate is converted to an equimolar amount of acetate. Growth occurs at 37° C. DCB-2 requires pyruvate to dehalogenate tri- and dichlorophenols in an anaerobic environment.

Cole et al. (1994) isolated an OCP-degrader that requires acetate as a co-substrate. This organism is designated strain 2CP-1 and is a member of the delta proteobacteria and related to the myxobacteria. 2CP-1 grows in a defined medium containing both OCP and acetate as carbon and energy sources and produces phenol. 2CP-1 produces 3 g protein per mole dechlorinated OCP with a doubling time of 3.7 days (Cole et al., 1994). Dechlorination activity is inducible, the lag between transfer to 3-chlorobenzoate medium and begin of dechlorination is longer than six hours. Growth occurs at 25° C. The organism grows with fumarate as an electron acceptor when OCP is absent. Onset of OCP-dechlorination after transfer of 2CP-1 from OCP-free fumarate medium

to OCP/acetate medium is delayed more than 6 h.

2.8.1 *Desulfonomile tiedjei* and Degradation of 3-Chlorobenzoate

The best known anaerobic dehalogenation process of a haloaromatic compound is that of a relative to the chlorophenol. Therefore it is worth to study the dehalogenation of 3-chlorobenzoate.

3-chlorobenzoate is degraded by a consortium consisting of *Desulfonomile tiedjei* DCB-1 (DeWeerd et al., 1990), a benzoate degrader known as BZ-2, and a methanogen (Shelton and Tiedje, 1984; Dolfing and Tiedje, 1987a; Stevens et al., 1988). The consortium was able to degrade 3-chlorobenzoate as the only carbon and energy source, produced methane and grew. Early on Dolfing and Tiedje (1987a) showed that 3-Chlorobenzoate caused a biomass increase of 6.8 g protein per mol 3-chlorobenzoate while benzoate produced 4.9 g protein per mol benzoate. Therefore reductive dehalogenation of 3-chlorobenzoate to benzoate was thermodynamically favourable. When a starved culture of the consortium was fed with 3-chlorobenzoate the ATP level was double compared to a starved culture fed with benzoate (Dolfing, 1990). Therefore the electrochemical potential between the electron donating pair H+/molecular hydrogen and the electron accepting pair 3-chlorobenzoate/benzoate is a potential source of energy.

Removing *D. tiedjei* took away the consortium's ability to degrade

3-chlorobenzoate completely. The resulting co-culture used benzoate as a substrate. *D. tiedjei* could not be isolated from the other members of the consortium using 3-chlorobenzoate as the only carbon and energy source. It had been isolated using medium with an alternative carbon and energy source like pyruvate. Dolfing (1990) used acetate and formate as growth substrates. Therefore *D. tiedjei* was dependent on the other members of the consortium when utilizing 3-chlorobenzoate. Removal of the byproduct benzoate by the benzoate degrader made the reductive dehalogenation of 3-chlorobenzoate to benzoate thermodynamically more favourable. The chemical equilibrium shifted towards formation of more benzoate. The benzoate degrader might have also released benzoate degradation byproduct(s) that could serve as a carbon source. 3-chlorobenzoate was not a carbon source for *D. tiedjei*, pyruvate needed to be supplemented. Benzoate degradation could be inhibited (Dolfing and Tiedje, 1987b) using acetate. Therefore acetate was a likely end product of benzoate degradation.

Substrate for the methanogen could be acetate or it could be carbon dioxide and hydrogen if present.

Dolfing and Tiedje (1991) identified the methanogen as a hydrogenotrophic *Methanospirillum* strain PM-1. Therefore the methanogen did not use acetate as a substrate, it used carbon dioxide. Source of the hydrogen in the consortium was the benzoate degrader BZ-2. Hydrogen consumers were both PM-1 and *D. tiedjei*.

Conceivably the two hydrogen consumers could compete for hydrogen. Adding 3-chlorobenzoate to the medium caused *D. tiedjei* to consume hydrogen and lower the hydrogen concentration slightly. *D. tiedjei* also released benzoate which is substrate for the benzoate degrader BZ-2. This stimulated BZ-2 to degrade benzoate faster and in turn produce hydrogen. Signal for increased benzoate degradation was a lower hydrogen concentration. PM-1's methanogenesis was unaffected by this. Therefore the action of the hydrogen consumers stimulated benzoate degradation while at the same time (in case of *D. tiedjei*) providing benzoate from 3-chlorophenol. Therefore all three members of the consortium are vital for the process of degrading 3-chlorobenzoate to non-aromatic compounds. As indicated above (Dolfing and Tiedje, 1987b) acetate acts as an inhibitor to 3-chlorobenzoate degradation and at the same time caused a decrease in hydrogen concentration when added to the medium. If acetate was a substrate for *D. tiedjei* a high acetate concentration might entice *D. tiedjei* to utilize acetate preferentially and thus put out less benzoate. This would explain the lower 3-chlorobenzoate consumption. The lower hydrogen concentration would stimulate benzoate degradation, but there would be less benzoate available to BZ-2 for degradation. Therefore the lower hydrogen concentration had no effect.

How does *D. tiedjei* dehalogenate 3-chlorobenzoate? Mohn and Tiedje (1990a) showed that dehalogenation produces energy for

D. tiedjei. Dehalogenation requires an electron donor which could be elemental iron or formate. When formate was present dehalogenation stimulated its oxidation to carbon dioxide. One Mol formate was oxidized for each Mol 3-chlorobenzoate that was dehalogenated.

Therefore 3-chlorobenzoate was degraded by the consortium as follows:

Step 1:

3-Chlorobenzoate + formate => benzoate + HCl + carbon dioxide

It is likely that dehalogenation can also be coupled to hydrogen:

3-Chlorobenzoate + hydrogen => benzoate + HCl

Step 2:

Benzoate => simple organic carbon cmpds. + hydrogen

The organic carbon from benzoate becomes part of cellular biomass, some will be released as carbon dioxide eventually.

Step 3:

Carbon dioxide + hydrogen => methane

Therefore the consortium converted 3-chlorobenzoate to chloride ion, cellular biomass and methane.

Mohn and Tiedje (1991) investigated the dehalogenation step of 3-chlorobenzoate degradation and came to the following conclusions: The ATP pool of a *D. tiedjei* - culture was replenished with addition of 3-chlorobenzoate. Uncouplers like 2,4-dichlorophenol and PCP reduced the ATP pool relative to the dehalogenation rate. Therefore with addition of a uncoupler efficiency of dehalogenation decreased, more and more 3-chlorobenzoate was consumed to make less and less ATP as the uncoupler concentration increased. PCP had the peculiar effect to stimulate dehalogenation when present at very low concentration. A likely explanation is that dechlorination is coupled to the maintenance of a proton gradient across one of the cell membranes. ATP in turn is generated by an ATPase that makes use of the energy inherent in the proton gradient. The ATPase allows protons to flow downgradient across the membrane in a controlled fashion and harnesses their energy for ATP production. Indeed creating an artificial pH gradient (in absence of substrate and uncoupler) led to ATP production (Mohn and Tiedje, 1991). An uncoupler allows protons to flow downgradient across the membrane while bypassing the

ATPase. The proton gradient becomes smaller. In the case of *D. tiedjei* the dehalogenation rate may actually increase as it is uncoupled from the maintenance of a proton gradient. This is similar to uncoupler action in eukaryotic mitochondria where in place of dehalogenation the oxygen consumption increases as the proton gradient (and therefore ATP production) breaks down (see Stryer, third ed., ch. 17). In contrast to the eukaryotic mitochondria *D. tiedjei* did not completely uncouple dehalogenation from proton gradient maintenance.

2.9 Conclusion

If the above information is confirmed and dehalogenation of 3-chlorobenzoate by *D. tiedjei* and dehalogenation of OCP by 2-CP1 (Cole et al., 1994) is typical of anaerobic dehalogenation of monochlorinated monocyclic aromatic compounds one could conclude that consortia of microorganisms are required for complete biodegradation degradations. There is circumstantial evidence for a negative effect of sulphate (Zhang and Wiegel, 1990; Wiegel et al., 1989; Madsen and Aamand, 1991, and others). Dehalogenations could also be poisoned by uncouplers which include higher chlorinated phenols, hence the dosage of chlorophenols is important when dealing with chlorophenol degrading cultures.

3. Objectives

Monochlorophenol degradation is difficult in biological treatment of wastewater, both in aerobic - and in anaerobic systems (Nicholson et al., 1992). The pathways of degradation of these compounds in anaerobic systems are still uncertain and are being actively researched. A better knowledge of biological monochlorophenol degradation under anaerobic conditions will allow for improvement of treatment of all potential chlorophenols in wastewater facilities.

This study had the following three objectives:

i. Effects of previous exposure (or "acclimation") of mixed anaerobic cultures of microorganisms from anaerobic sewage sludge on growth rate in presence of OCP.
To reach this objective the following questions were addressed:

- How does OCP affect mixed anaerobic cultures of microorganisms growing on complex organic substrates?
- What levels of OCP-concentrations affect the bacteria and are there differences between cultures previously exposed to OCP and unexposed cultures?
- Can the effect of OCP on mixed cultures be reduced?

ii. What conditions support OCP-degradation.

The following questions were addressed:

- Can OCP-degradation take place in a mixed anaerobic culture of microorganisms that is a bacterial suspension free of most of the non-living matter found in sludge and sediment and can this degradation take place in a defined medium?
- What levels of OCP can be degraded by a mixed anaerobic culture of microorganisms?
- Is concurrent methanogenesis a requirement for OCP-dehalogenation in mixed anaerobic cultures of microorganisms?
- Does presence of sulphate reduction have an influence on OCP-degradation in mixed anaerobic cultures of microorganisms?

iii. Finding information about pathway and end-products of OCP-dehalogenation by mixed anaerobic cultures of microorganisms.

The following questions and problems were addressed:

- Are short-chained fatty acids end-products of OCP-dehalogenation?
- Is carbon dioxide the end-product of OCP-dehalogenation?
- Is the dehalogenation co-metabolic?
- Can the dehalogenating mixed anaerobic culture of microorganisms maintain its viability over extended (1 year) time periods?
- What type of bacteria are the dehalogenators?

4. Materials and Methods

4.1 Source and Enrichment of Cultures

Mixed batch cultures of anaerobic heterotrophs were isolated from two biomass sources: A municipal anaerobic sludge digester at North End Water Pollution Control Centre, Winnipeg, and anaerobic sediment of a wastewater lagoon treating Kraft bleaching wastewater at Fort Frances, Ont.

Biomass from the municipal sludge digester was the source of the "unadapted" cultures, because it presumably was not exposed to chlorinated phenols prior to the experiments. The unadapted cultures were prepared as follows:

Approximately 0.1 ml sludge from the municipal sludge digester was diluted with 10 ml anaerobic mineral medium. The resulting diluted biomass (x1000) was inoculated into 10 ml aliquots of anaerobic nutrient medium contained in sealed Balch-Wolfe culture tubes (Bellco, Vineland, N.J.) and incubated at 35° C for 24 h. Resultant growth caused an optical density at 600 nm of 1.2 to 1.4. After incubation the cultures served as inocula for subcultures in anaerobic nutrient medium amended with OCP-concentrations ranging between 0 and 10 mM.

Biomass from the lagoon at Fort Frances was pre-enriched in an anaerobic bioreactor for 1 year. The reactor consisted of a sealed vessel containing 4 L biomass. The contents were stirred magnetically just enough to keep solids from settling. During pre-enrichment the biomass was fed an organic medium containing both phenol and OCP. Phenol and OCP were each added to achieve final concentrations of 0.1 mM in the reactor and dosage was increased during the year until 2 mM of both compounds were reached. The organic component consisted of whey powder (0.5% w/v). All materials were dissolved in tap water and no mineral salt or vitamin solutions were used. The pH in the reactor was monitored twice a week and kept near 7.0 using sodium bicarbonate whenever required. This biomass served as the adapted inoculum and was treated like the biomass of the municipal sludge digester.

4.2. Medium Composition

The basic broth contained 1% (w/v) yeast extract (Difco Laboratories, Detroit. MI.), 1% (w/v) trypticase (BBL Div. Becton, Dickinson & Co., Cockeysville, MD.), Mineral Elixir (10 ml/L; Daniels et al., 1984; Belay et al., 1984), Vitamin supplement (10 ml/L; Wolin et al., 1963), 0.5 mg/l ammonium chloride, 0.3 mg/l dibasic sodium phosphate, 2000 mg/l sodium bicarbonate and several drops of resazurin (redox indicator). A poorer version of this medium contained no yeast extract. The pH of the medium

was between 7.1 and 7.2 in a 80% nitrogen and 20% carbon dioxide atmosphere. This was checked while sparging the medium with the mixture of 80% nitrogen and 20% carbon dioxide. Subsequently 10 ml of the medium was dispensed to approx. 25 ml Balch-Wolfe culture tubes (Bellco, Vineland, N.J.). The tubes were sealed using black butyl rubber stoppers and aluminium crimp seals. After sealing the headspaces were evacuated for 3 min. and re-filled with a gas mixture of 80% nitrogen and 20% carbon dioxide. Evacuating and refilling was done with a syringe connected to a manifold. Evacuating and refilling was repeated three times. The tubes were subsequently kept under positive pressure (20 psi) of the nitrogen/ carbon dioxide mix. Using a syringe, 200 mM sodium sulphide solution was added to create a concentration of approximately 2 mM sulphide in the medium. The tubes were finally sterilized by autoclaving for 15 min. at 121° C. At time of inoculation undiluted OCP (Sigma Chemical Corp.) was injected into the tubes using a microsyringe. This resulted in a rich medium amended with a range of 1 to 10 mM OCP.

4.3 Degradation of Complex Organic Substrate in Presence of OCP

In addition to chlorophenols wastewater may contain readily degradable organic matter. In sewage treatment organic matter is to be removed. Therefore it is of interest to determine whether chlorophenols interfere with growth of bacteria as found in anaerobic digesters. Can resistance to chlorophenols in a

mixed culture increase with continued chlorophenol exposure? This study evaluated monochlorophenols and therefore OCP is used as an example for a monochlorophenol.

4.3.1 OCP in Cultures of the Adapted and Unadapted Bacteria

In order to compare growth of the exposed population to that of the unexposed, two sets of cultures were prepared. The first set (four replicates) contained 0 to 10 mM OCP and was inoculated from the exposed culture. The second set of cultures (four replicates) was similar to the first set, but derived from municipal anaerobic sludge. To inoculate a culture 0.1 ml inoculum was injected into a sealed culture tube with 10 ml medium using a syringe. This experiment was repeated and one of the two sets of similar results was reported. The growth rate was recorded as the logarithm of the optical density versus time. Time between inoculation and onset of growth in the subcultures was also determined. Onset of growth was considered to be an increase in optical density at 600 nm past 0.1.

4.3.2 Demonstration of the Effect of Palmitic Acid on Cultures Containing OCP

As indicated earlier, chlorophenols were found toxic, because they can uncouple ion movement across a membrane from ATP-production (Ravanal et al., 1989). The monochlorophenols are

weaker uncouplers than polychlorophenols. However chlorophenols including monochlorophenols interfere with membrane fluidity. Monochlorophenols are hydrophobic and tend to enter cell membranes. The following experiment aimed at reducing OCP's alteration of cell membrane fluidity.

Palmitic acid is a C_{16} fatty acid that is fully saturated. Keweloh et al. (1991) indicated that when present in the culture medium palmitic acid is incorporated into cell membranes. They indicated further that palmitic acid packs tightly in the phospholipid bilayer and therefore increases membrane rigidity (Keweloh et al., 1991). Contrary to palmitic acid chlorophenols disturb packing of the fatty acid residues and cause the membrane to become more fluid (Keweloh et al, 1991).

This experiment aimed to demonstrate an effect of palmitic acid analogous to that reported by Keweloh et al. (1991). Two different sets of cultures were used. Both sets, one with and the other without palmitic acid, were derived from the municipal anaerobic digester. There were two replicates for each set of cultures.

Petroleum ether (10 ml/L) was added to the nutrient broth prior to dispensing into culture tubes. The ether was added to aid in the solubilization of palmitic acid. Subsequently the medium was divided into two portions, one of the portions received 10

mg/L palmitic acid (Sigma Chemical Corp.). Both portions were stirred for 30 min, then dispensed and treated as described above. Each set of culture tubes contained a range of 0 to 10 mM OCP, as indicated above there were two cultures per OCP-concentration.

Growth was determined measuring the optical density at 600 nm. Growth was then related to the OCP dose used to amend the culture medium. All cultures were incubated at 35° C.

4.4 Degradation of OCP

The following experiments evaluated biodegradability of OCP.

4.4.1 OCP-Degrading Cultures

Cultures enriched for OCP-degraders were incubated in anaerobic nutrient (1% yeast extract plus 1% trypticase) broth - containing 25 mL culture tubes with 10 mL culture. The tubes were sealed with butyl rubber stoppers and aluminium crimp seals. OCP and phenol concentration was measured periodically. After OCP-degradation and phenol production occurred the cultures were centrifuged in their tubes using a Sorvall Instruments GLC-2 centrifuge, the old medium was withdrawn using a syringe, new broth added, and the pellet resuspended by shaking. Therefore the cul-

ture was not removed from the tube.

Replacement of the medium in a sealed culture tube required to centrifuge for one hour using a Sorvall Instruments GLC-2 with a rotor of approximately 30 cm diameter and a rotation speed of 3000 - 4000 rpm. A solid pellet formed surrounded by fine white material. Care had to be taken not to suspend the white material. The medium could now be withdrawn using a syringe while insuring that no medium was left behind between stopper and glass wall of the tube. Fresh medium and OCP was injected into the tubes with a syringe. The culture was now incubated for another cycle of OCP-degradation. Cultures assessed for OCP-degradation were obtained from the bleaching plant biomass which was kept in the bioreactor.

4.4.2 End product of OCP-Degradation using ^{14}C-OCP?

An OCP-degrading culture as described under 4.4.1 was inoculated to serve a source of inoculum for more cultures and incubated at 35° C. After 4 days of incubation (i.e. after the end of growth) the subcultures were inoculated.

Each subculture consisted of 10 mL of anaerobic nutrient medium contained in a Balch-Wolfe culture tube sealed with a butyl rubber stopper and an aluminium crimp seal. 2 μCi ^{14}C-OCP (Amersham, Oakville, Ont.) was injected into each culture with a micro-

syringe. The labelled OCP was dissolved in dichloromethane such that 1 μL solution contained 1 μCi of OCP. Additionally 3 μL unlabelled OCP were added. The resulting OCP dose was very close to just adding 3 μL as the labelled OCP was very dilute. The cultures were incubated for 21 days at 35° C. Controls were treated like the cultures and contained water instead of bacterial suspension.

The following two procedures were done according to Zehnder et al. (1979):

a) After incubation the cultures were acidified, 3 ml gas were withdrawn from the headspace and injected into 1.5 ml of 0.2N KOH. The KOH-solution was contained in a 25 ml vial. After one hour of shaking and 20 s sparging with nitrogen under a fume hood, 10 ml biodegradable counting scintillant (Amersham, Oakville, Ont.) was added. Radioactivity was determined using a Beckman liquid scintillation counter. Counting time was 10 min. per sample.

b) Culture fluid was prepared for counting by dissolving 2 ml of the fluid in 10 ml biodegradable counting scintillant (Amersham, Oakville, Ont.). Radioactivity was determined in a Beckman liquid scintillation counter. Counting time was 10 min. per sample.

4.4.3 OCP in Concentrated Cell Suspensions Derived From Unadapted Inoculum Provided by a Municipal Sludge Digester

Cultures (250 ml) were centrifuged in a Sorvall Instruments RC-5 Superspeed centrifuge and resuspended in one-tenth of the original volume of mineral medium. The resulting concentrate contained OCP and short chain volatile organic acids carried over from the original medium through centrifuging whenever the withdrawal of the old medium left a drop behind. Resuspending in mineral medium for a second time and repeating centrifugation decreased this carryover. This method was used only to concentrate a culture and transfer to mineral medium plus OCP which minimizes growth of contaminant organisms. Inoculum from the municipal sludge digester and OCP-adapted inoculum was used to prepare the first concentrated cultures. Subsequently the use of the OCP-adapted inoculum was abandoned for preparation of concentrated cultures. The unadapted inoculum produced cultures that were able to dehalogenate OCP in mineral medium just as the adapted cultures. The results from concentrated cultures reported below are based on unadapted inoculum from the municipal sludge digester. After dehalogenation stopped the cultures were centrifuged in their culture tubes, the medium withdrawn and replaced with fresh mineral medium. Centrifugation and replacement of mineral medium was repeated and pure OCP injected to begin the second dehalogenation cycle. The repeat experiment used a different protocol in that prior to each resuspension

in new mineral medium plus OCP the cultures were incubated in anaerobic nutrient medium for two days. This way more than two refeeding cycles could be observed. After concluding the experiments the cultures were examined visually with a phase contrast microscope (Nikon).

4.4.4 pH in Cultures in Nutrient Medium Plus OCP

There is the potential for a pH-shift in a culture incubating in nutrient broth because organic acids form as products. Several of the OCP exposed cultures were incubated for a prolonged time period of up to 4 months. After incubation pH of the culture was measured in order to determine whether pH remains stable during incubation.

4.4.5 Long Term OCP-Degrading Cultures

Long term maintenance (one year) of OCP-degrading cultures required a nutrient plus OCP reservoir that slowly releases new material to the bacteria as they consume their medium. Similarly there should be a sink to take up byproducts of bacterial metabolism. The procedures used here were from Krieg and Gerhardt (1981). The culture vessel was either a 160 or 100 mL Wheaton serum bottle sealed with a butyl rubber stopper and an aluminium crimp seal or a pressure resistant 1 L Pyrex reagent bottle sealed with a rubber stopper secured with wire.

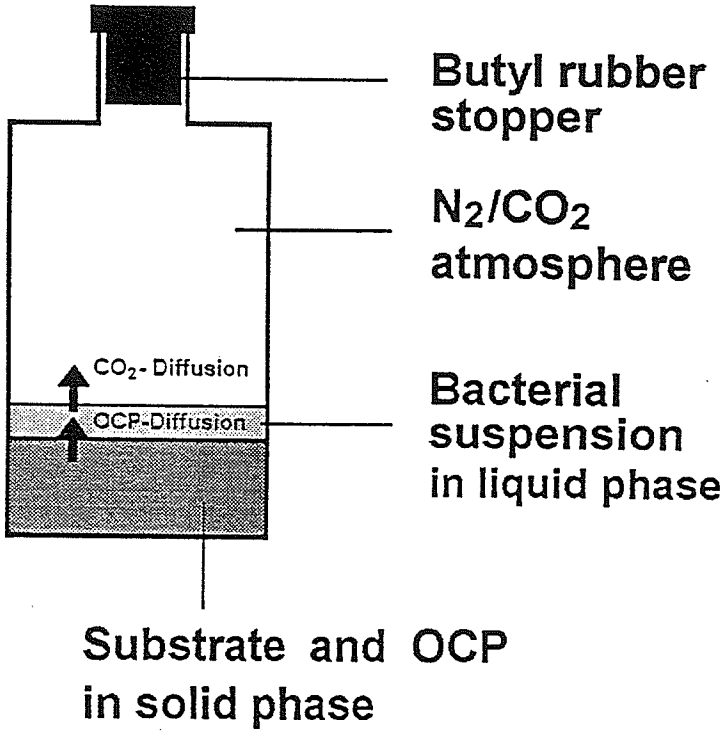

Fig. 1
Serum bottle with OCP amended nutrient agar as the solid phase. When a bacterial suspension forms in the liquid phase, substrate and OCP diffuse in from the solid phase thus resupplying the culture.

In place of anaerobic nutrient broth the bottle's medium contained 3% agar (nutrient agar) to form a solid mass that retained its integrity even when it became detached from the glass of the bottle. OCP (pure reagent, Sigma Chemical Corp.) was added to the agar separately prior to solidification. Gentle swirling allowed the OCP to mix into the agar. A one to two cm thick layer of liquid nutrient medium was added to the top of the agar mass after solidification. A very concentrated culture grew in the liquid phase. Fig.1 on p.48 is a diagramme of a long term culture bottle.

4.4.6 Addition of Sulphate

Sulphate could have interfered with dehalogenation. Sulphate reduction may provide an alternative method of energy generation for the OCP-degrading cultures. The following experiment designed to detect changes in dehalogenation due to sulphate.

10 ml of mixed anaerobic dehalogenating culture of microorganisms (from OCP-preexposed stock) was transferred to sealed 25 ml Balch -Wolfe culture tubes. After centrifugation and removal of the culture medium as in 4.4.1, the cultures were resuspended in nutrient broth with 20 mM sodium sulphate, 1% (w/v) yeast extract (Difco Laboratories, Detroit, MI.) and 1% (w/v) trypticase (BBL Div. Becton, Dickinson & Co., Cockeysville, MD.). OCP (pure reagent, Sigma Chemical Co., St. Louis, MO.) was added. Incubation

was at 35° C. in the dark. Control cultures incubated in similar broth without sodium sulphate.

4.4.7 *Desulfobacterium phenolicum* and OCP

Chlorophenol degradation under sulphate reducing conditions is possible according to Häggblom and Young, 1990. A sulphate reducer, *Desulfobacterium phenolicum,* DSM 3384 (Bak and Widdel, 1986) is known to degrade phenol and was purchased from DSM, Braunschweig, Germany. The following experiment tested whether the sulphate reducing phenol degrader *D. phenolicum* is also an OCP-degrader.

The medium used to grow *D. phenolicum* (DSM, Braunschweig, Germany; Medium 383 as amended for DSM 3384 - *D. phenolicum*) was removed and replaced by fresh similar medium containing 1 mM OCP.

A mixed adapted culture in nutrient medium plus OCP and sodium sulphate was incubated at 25 °C. *D. phenolicum* was added.

4.5 OCP as a Possible Growth Substrate

This experiment tested for indications that OCP can serve as a carbon and energy source. At the same time the experiment

assumed that a co-substrate is required. Possible co-substrates included trypticase broth and yeast extract. Growth was assessed when the optical density at 600 nm (see also 4.7) reached 1.0. Growth that occurred only in presence of the maximum co-substrate concentration plus OCP was interpreted as a negative result. In this case OCP would not need to be a carbon source to the culture.

A set of cultures provided OCP in mineral medium plus 0.02%, 0.1%, and 1.0% yeast extract (w/v). A similar set provided OCP in mineral medium plus 0.005%, 0.05%, and 0.5% trypticase (w/v). A 10% inoculum started the cultures. OCP and phenol concentration was measured periodically over a 10 day period. This told whether OCP might be a growth substrate and also gave an indication about possible additional substrates OCP-degradation may require. Had 0.005% yeast extract or trypticase medium produced dehalogenation, a repeat of the experiment would have included 0% trypticase cultures.

4.6 *Methanospirillum hungatei* and OCP

Methanogens occur in anaerobic wastewater treatment facilities. This experiment tested *Methanospirillum hungatei* (obtained from the Department of Microbiology, University of Manitoba) for its ability to grow in presence of OCP. *M. hungatei* is common in

anaerobic digesters.

Three sets of nine cultures each of *M. hungatei* were prepared using a medium according to Daniels (1986). The medium was amended with 0 mM, 0.5 mM, and 1.0 mM OCP. After five days of incubation at 35° C cultures were checked for clouding as indication of growth. Growth was assessed when the optical density at 600 nm reached 0.4.

4.7 Instrumental Analysis

- Optical density

Optical density was measured at 600 nm using an LKB spectrophotometer. An intact culture tube was inserted into the light path and absorbance measured and compared to a blank containing sterile medium.

- HPLC analysis

Samples were centrifuged using an Eppendorf Microcentrifuge to remove solids. The supernatant was injected into a Millipore-Waters HPLC system operating in isocratic mode. The HPLC used a Millipore-Waters Lambda Max 481 LC Spectrophotometer measuring at a wavelength of 254 nm. The HPLC mobile phase consisted of 2% acetonitrile (1:1) and 98% sodium triphosphate buffer at a flow rate of 2 ml min^{-1}. This method allowed

separation of OCP, phenol, and benzoate.

- Gas chromatography

After OCP/phenol-analysis the samples were acidified with concentrated phosphoric acid for determination of volatile fatty acid (VFA) concentration. A Hewlett Packard HP 5890A gas chromatograph equipped with a flame ionization detector was used. The column was a capillary column, injector temperature was 150° C, detector temperature was 170° C, and the temperature programme raised temperature in two steps from 85° C to 153° C. HPLC - and gas chromatography methods were developed at the laboratory of the Environmental Engineering Division, Department of Civil and Geological Engineering, University of Manitoba.

Methane was measured by injecting a controlled quantity of a culture's headspace gas into a Varian Aerograph Series 2100 gas chromatograph equipped with a flame ionisation detector.

- pH

pH Measurements were done with a Fisher Scientific Accumet 950 pH/Ion Meter equipped with a pH probe of approximately 5 mm diameter. The pH-electrode was held into the liquid culture immediately after opening the culture bottle.

- Radioactivity

^{14}C - Radioactivity was measured in a Beckman scintillation

counter

- Centrifuges

A Sorvall Instruments RC-5 Superspeed Centrifuge and a Sorvall GLC-2 centrifuge were used for centrifuging mixed anaerobic cultures of microorganisms.

- Light Microscopy

A Nikon microscope with 100x phase contrast objective lens, phase contrast condenser, and 10x eye pieces was used for the microscopic observations.

5. Results

5.1 Information on the Source of Adapted Cultures

Headspace gas from the reactor with biomass from the Kraft mill lagoon contained approximately 5% methane when incubated with complex organic medium. Methanogenesis stopped once phenol and OCP-concentrations each exceeded 1 mM in the reactor used to adapt the biomass to OCP and phenol; subcultures from the bio-reactor contained less than 5% methane in the headspace after incubation in nutrient medium. Methane readings were compared to those of a standard composed of pure methane and expressed as a percentage of the standard. Pressure was that of the ambient atmosphere. This was in accordance with Hrudey et al. (1987) who indicated that methanogenesis did not occur when more than 0.8 mM of a monochlorophenol (here 4-chlorophenol) was present. It is not known whether the actual wastewater lagoon in Fort Frances, Ont. produced methane. This reactor provided inocula that had previous exposure to OCP. Inocula for cultures without previous exposure to OCP were diluted directly from biomass of the municipal sludge digester.

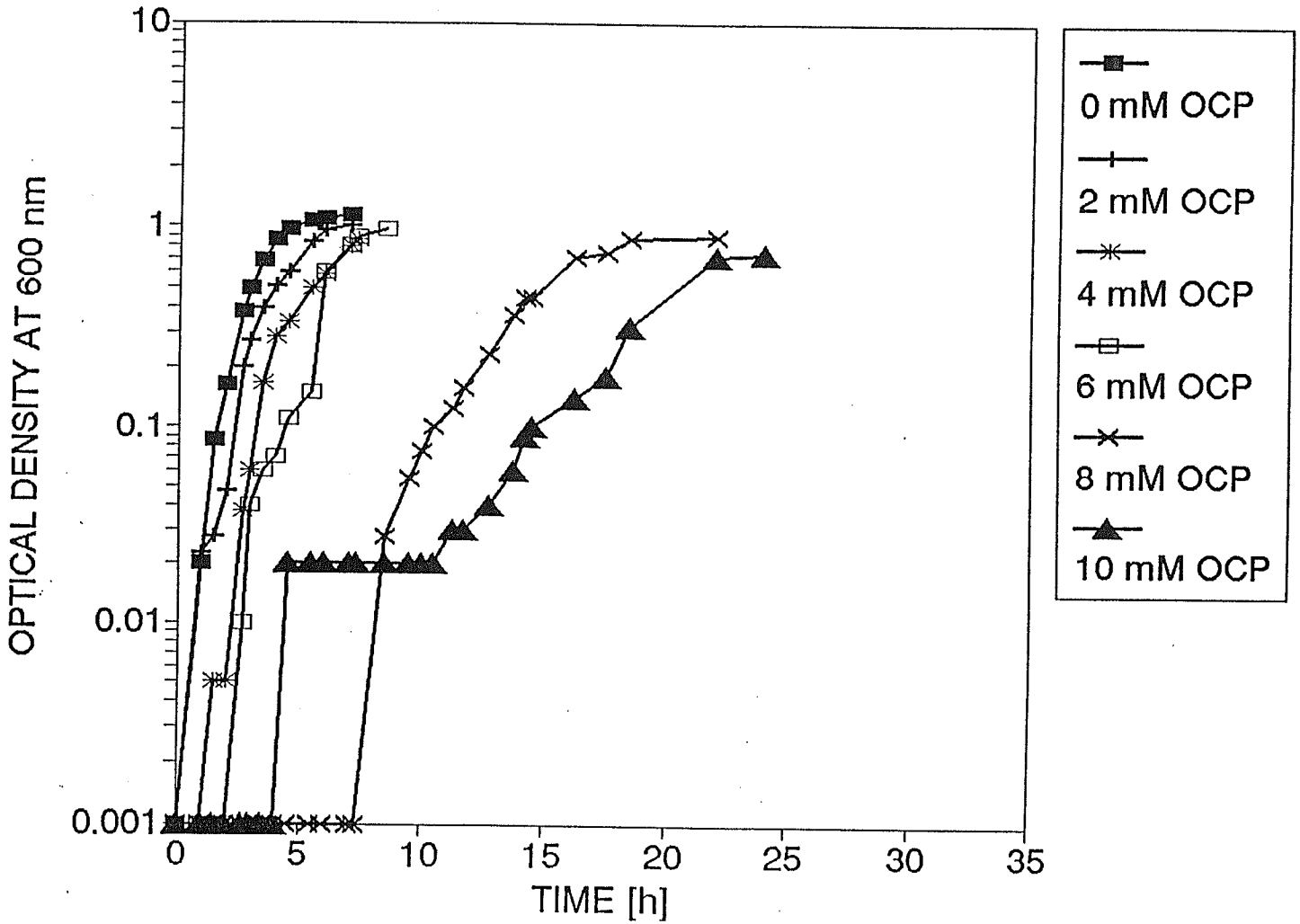

Fig. 2. The effect of various concentrations of OCP on the growth of OCP – adapted cultures. Growth was recorded as the increase in optical density at 600 nm. The cultures grew on anaerobic nutrient medium plus OCP. Solid rectangle: 0 mM; plus: 2 mM; asterisk: 4 mM; open rectangle: 6 mM; cross: 8 mM; solid triangle: 10 mM.

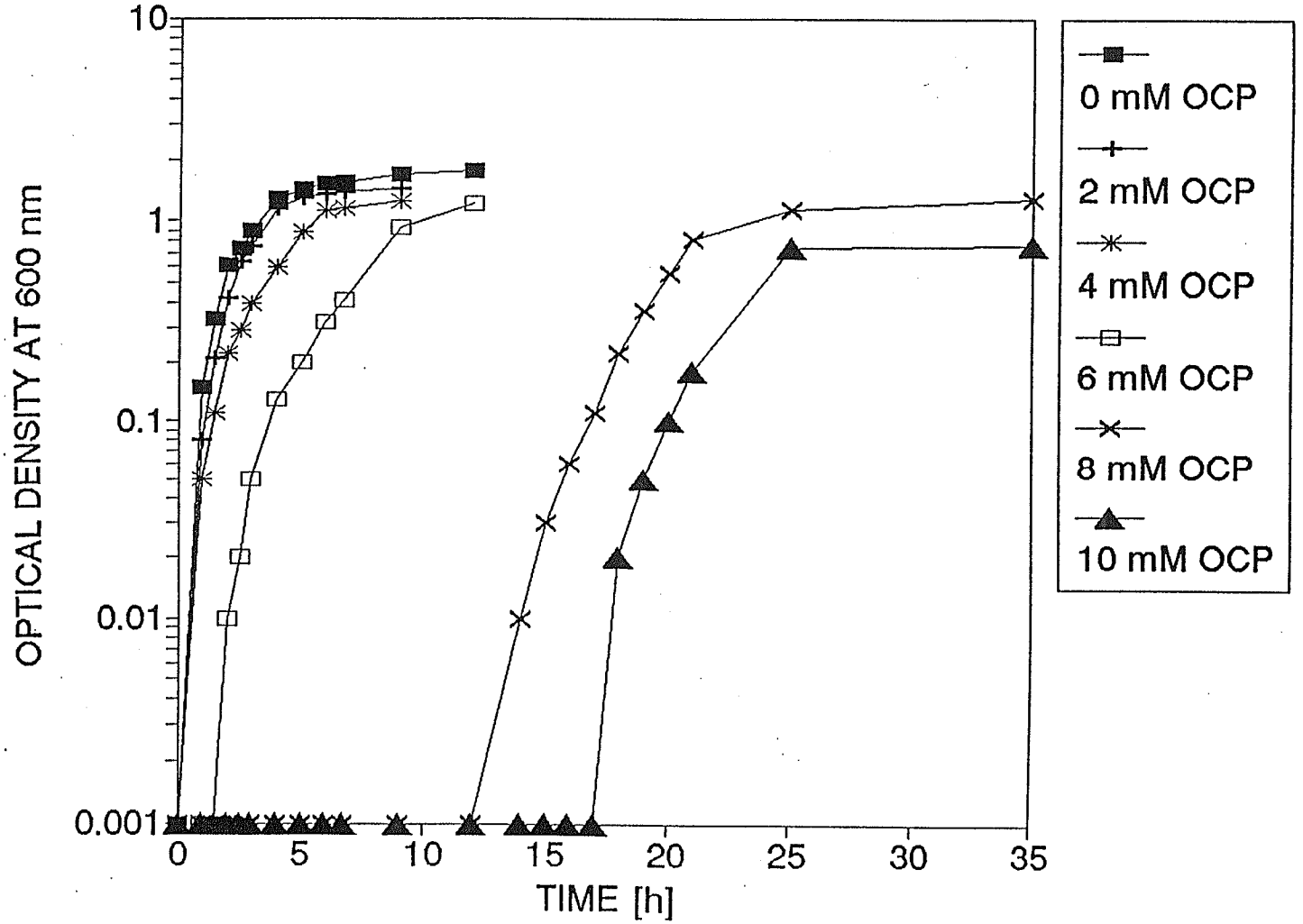

Fig. 3. The effect of various concentrations of OCP on the growth of OCP-unadapted cultures. Growth was recorded as the increase in optical density at 600 nm . The cultures grew on nutrient medium plus OCP. Solid rectangle: 0 mM; plus: 2 mM; asterisk: 4 mM; open rectangle: 6 mM; cross: 8 mM; solid triangle: 10 mM.

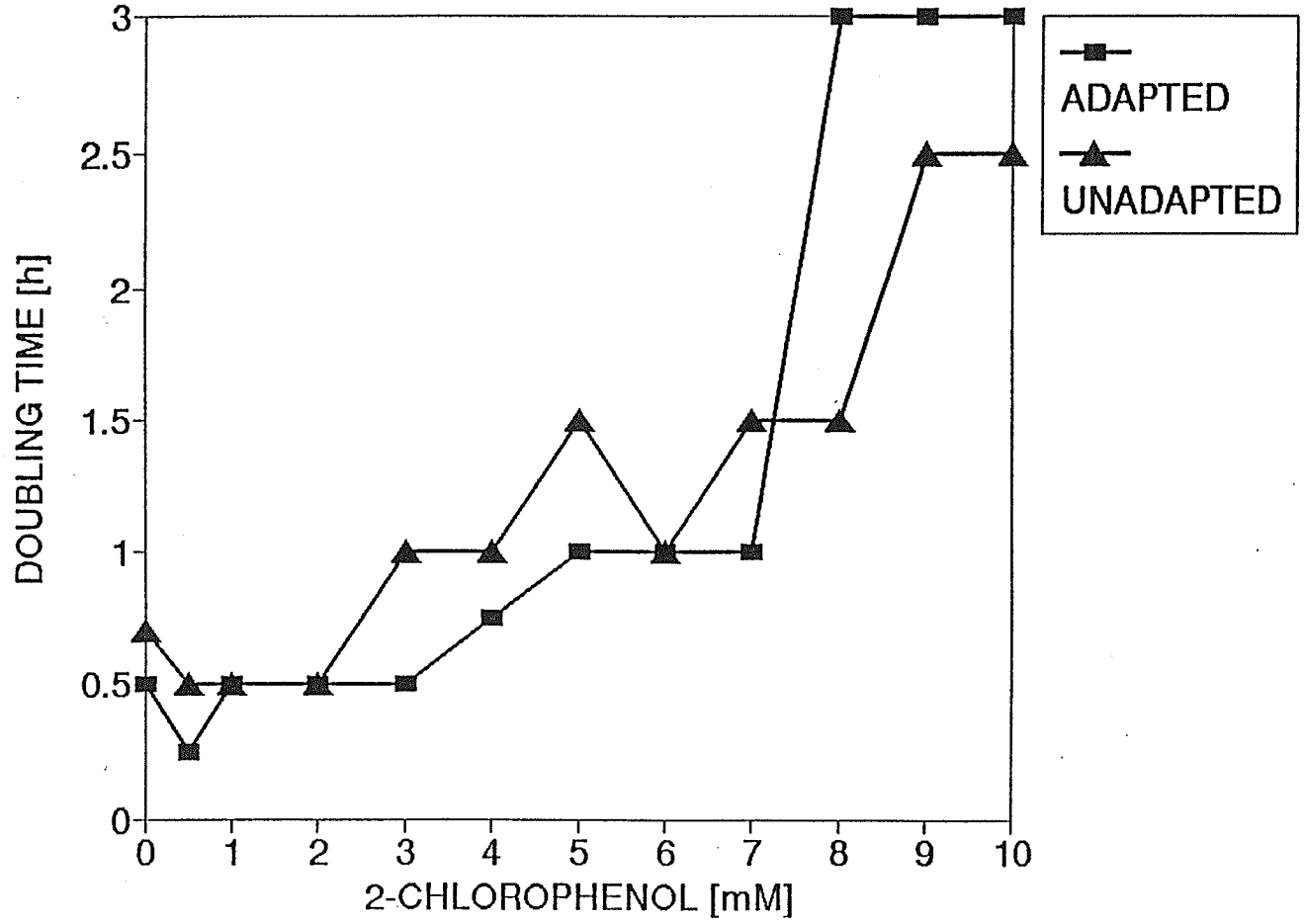

Fig. 4. The effect of increasing concentrations of OCP on doubling time of adapted and unadapted cultures. The cultures grew on anaerobic nutrient medium plus OCP. Solid rectangle: adapted; solid triangle: non-adapted.

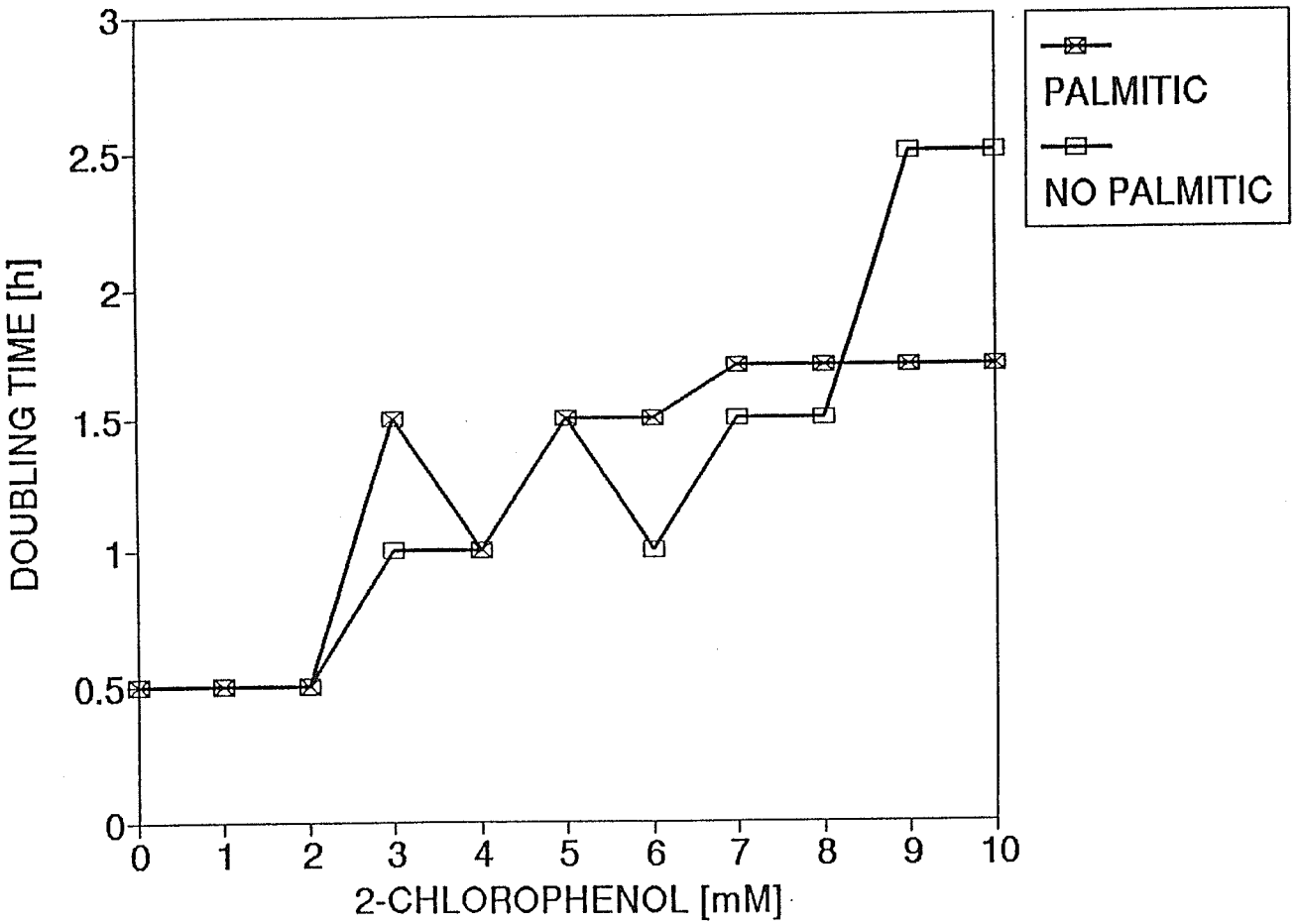

Fig. 5. The effect of increasing concentrations of OCP on doubling time of unadapted cultures in presence and absence of palmitic acid. The cultures grew on anaerobic nutrient medium plus OCP. Solid rectangle: with palmitic acid; solid triangle: without palmitic acid.

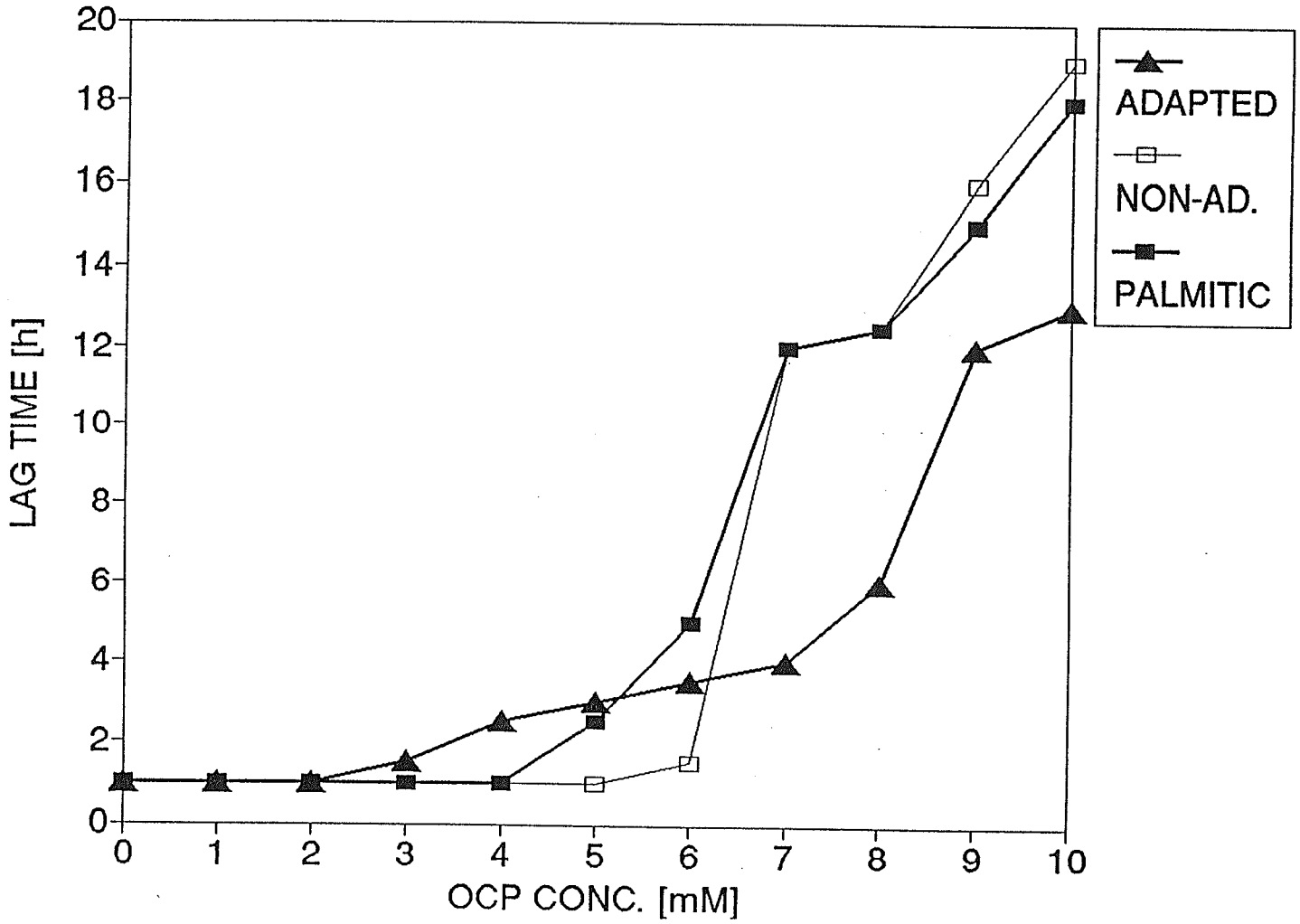

Fig. 6. Lag time with respect to OCP concentration for adapted and unadapted cultures. Solid triangle: adapted culture without palmitic acid; open rectangle: non-adapted culture without palmitic acid; solid rectangle: non-adapted culture with palmitic acid.

5.2 *Methanospirillum hungatei* and OCP

An OCP-concentration of 1 mM OCP prevented growth (Table 1). A culture was recorded as having showed growth when its optical density at 600 nm increased to above 0.3.

Table 1. Growth of *M. hungatei* in Presence of OCP

Culture type	No. of Replicate Cultures	Replicate Cultures with Growth
0 mM OCP	9	9
0.5 mM OCP	9	3
1.0 mM OCP	9	0

Incubation had been 5 days. OCP could be bacteriostatic. To prove a bactericidal effect, one would have to repeat the experiment and after incubation transfer cultures without growth to non-OCP medium and check for any subsequent growth. The OCP - concentrations used for dehalogenation tests of the mixed anaerobic cultures contained 3 - 5 mM OCP. *M. hungatei* is not

capable of growing under such conditions and the mixed anaerobic dehalogenating cultures of microorganisms were therefore non-methanogenic and it was not possible to show methane production in the cultures.

5.3 Mixed Cultures Degrading a Complex Organic Substrate in Presence of OCP

Cultures growing in nutrient medium in the presence of >2 mM OCP grew slower than the controls (cultures growing without OCP, see Fig.2 and 3). Fig.2 and 3 plot growth as optical density at 600 nm vs. time for a range of OCP-concentrations from 0 mM to 10 mM OCP.

The linear portion of each growth curve was used to assess doubling time. Fig.4 and 5 plot the doubling times for cultures incubating in presence of a range of OCP-concentrations from 0 mM to 10 mM OCP. The doubling times and the lag between the beginning of incubation and the beginning of OD-increase lengthened as the OCP-concentration increased (Fig.4,5,6). Doubling times of adapted and unadapted cultures were similar. Interestingly both sets of cultures in Fig.4 showed slightly shorter doubling times at OCP-concentrations of 0.5 and 1 mM than at 0 mM (control). The lag phases of the adapted cultures were shorter than those of the unadapted cultures, when the OCP-concentration was greater than 6 mM (Fig.6).

The next experiment served to show whether OCP could be prevented from slowing growth of the mixed culture:

This experiment provided information on doubling time with respect to OCP-concentration for a set of unadapted cultures with palmitic acid and a control set of unadapted cultures without palmitic acid. There were similar doubling times between the two sets (Fig.5). Therefore palmitic acid was not able to increase growth rate of a non-adapted culture when growing in presence of OCP. There was no difference in the lag time periods between palmitic-acid-amended and - unamended cultures (Fig.6).

The following section presents the results of OCP-biodegradation tests:

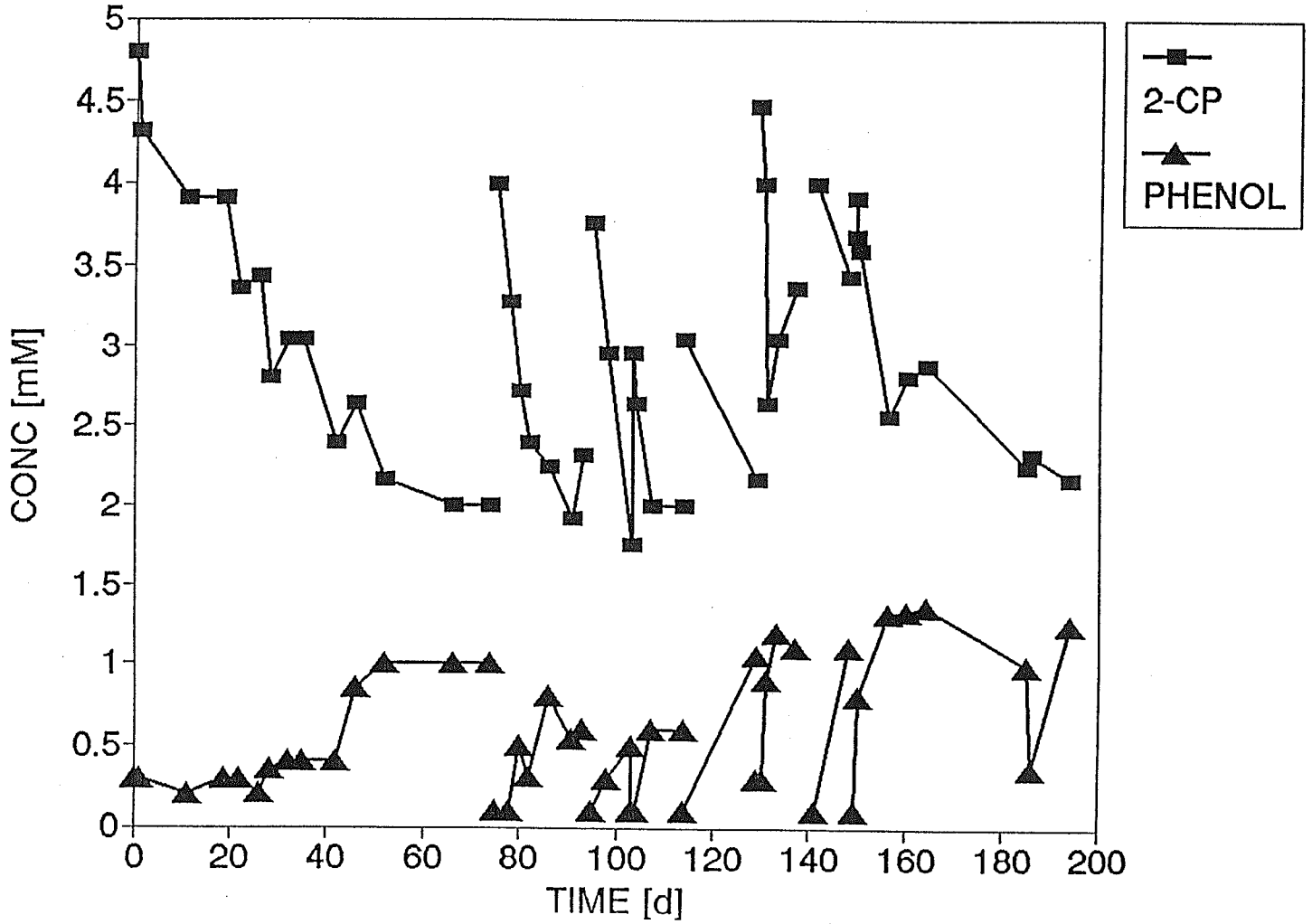

Fig. 7. Phenol and OCP in a dehalogenating mixed culture incubating in anaerobic nutrient medium plus OCP. Each peak in OCP concentration and each low in phenol concentration are when culture was centrifuged and medium replaced. Solid rectangle: OCP; solid triangle: phenol.

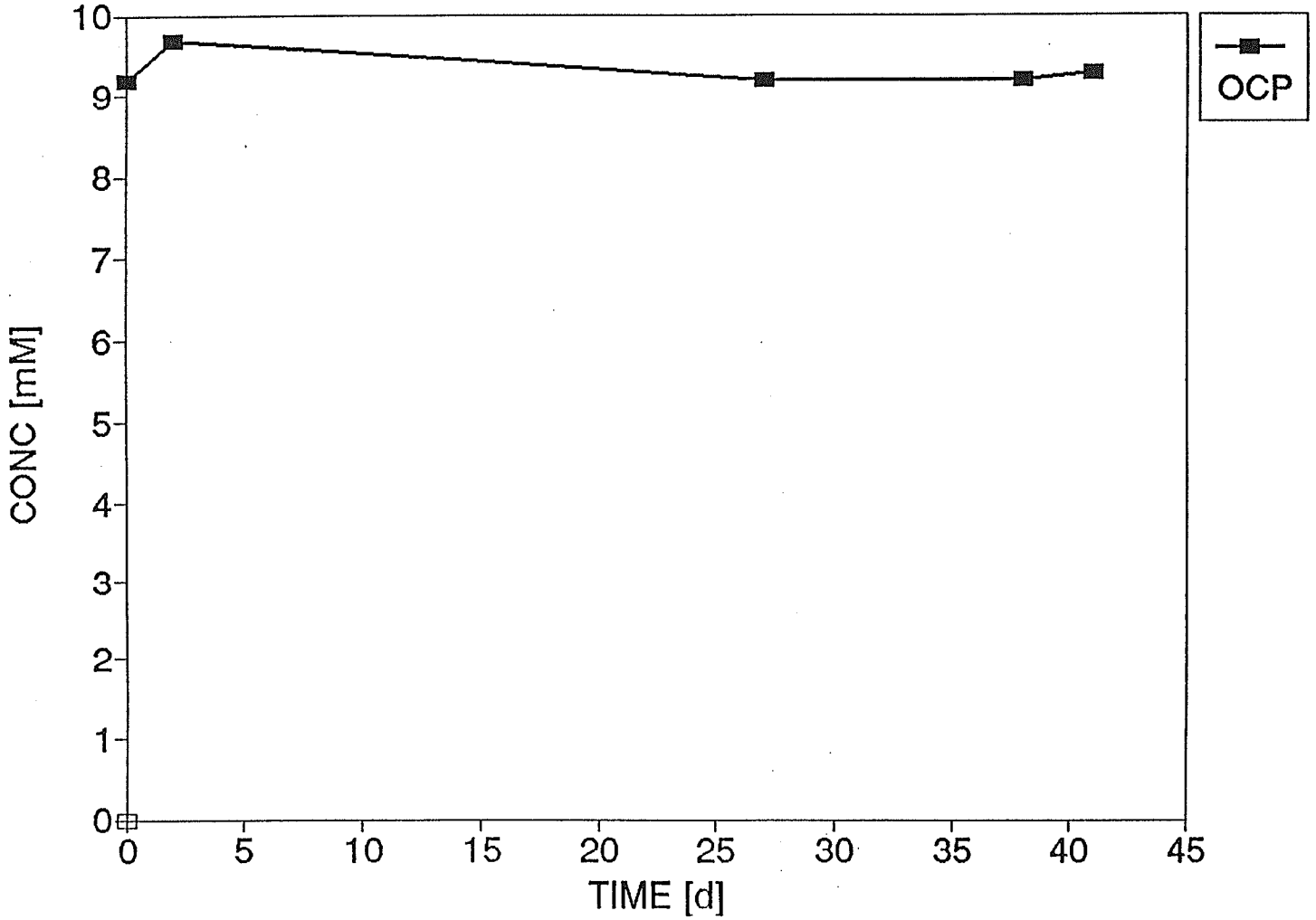

Fig. 8. Autoclaved culture incubated with OCP. Solid rectangle: OCP.

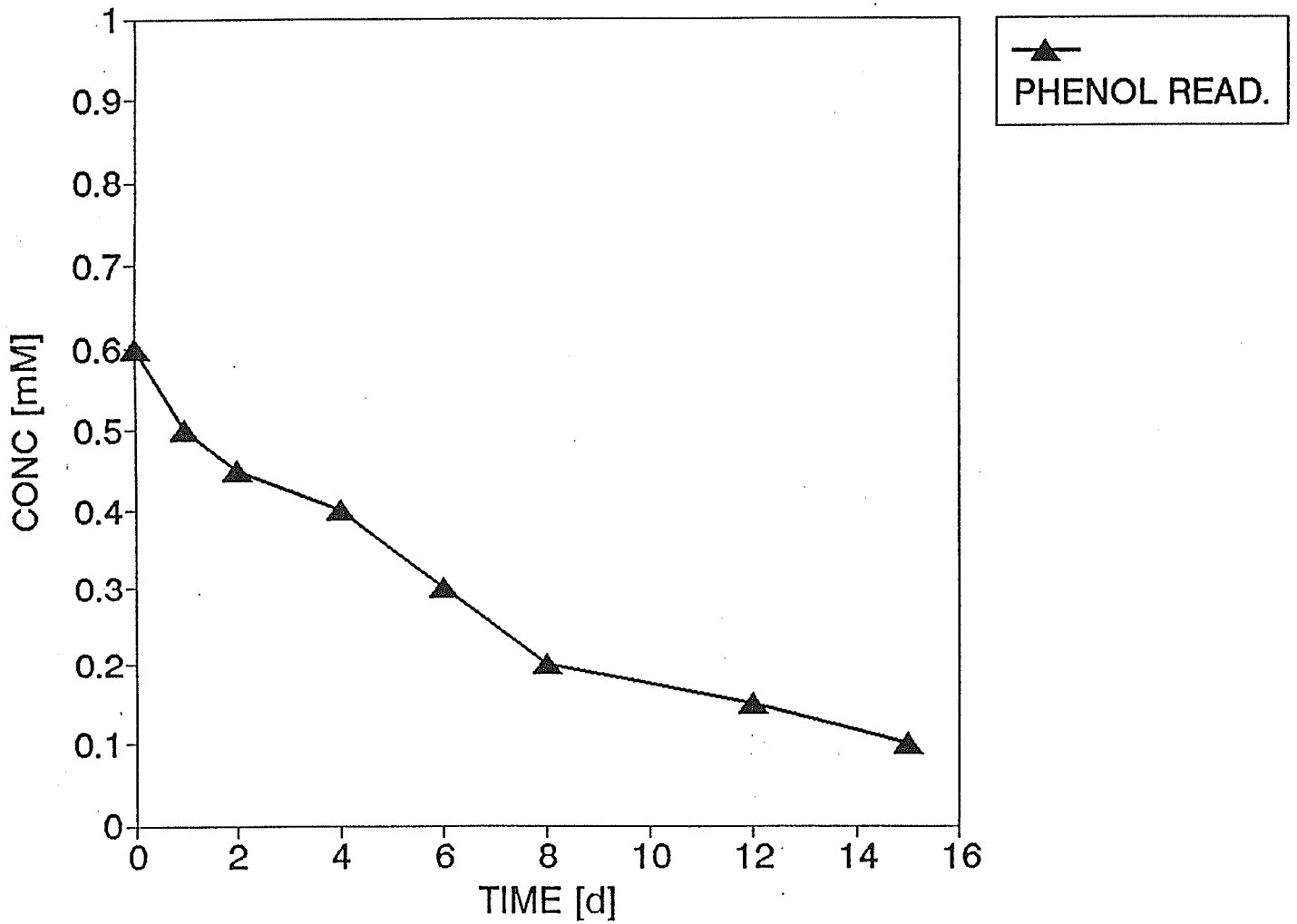

Fig. 9. Interference of nutrient medium to phenol analysis. Culture grew in nutrient medium without OCP and phenol. Solid triangle: false phenol reading.

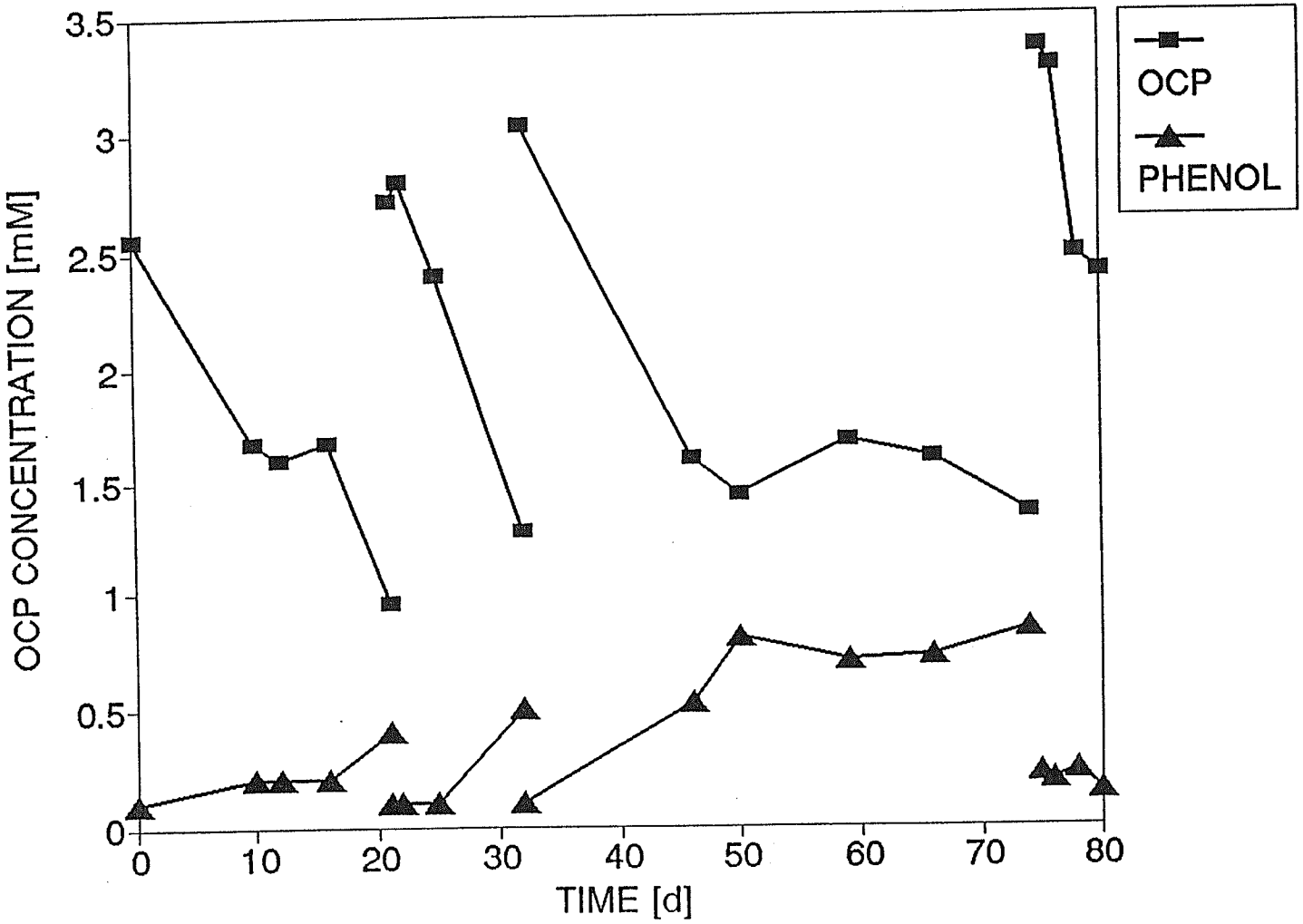

Fig. 10. Phenol and OCP in a dehalogenating mixed culture incubating in anaerobic trypticase medium plus OCP. Each peak in OCP concentration and each low in phenol concentration are when culture was centrifuged and medium replaced. Solid rectangle: OCP; solid triangle: phenol.

5.4 OCP-Degradation

5.4.1 OCP-Degradation in Complex Medium

Cultures showed a decrease in OCP and an appearance of phenol over time. The presence of phenol is an indication of OCP dehalogenation (Fig.7). The drop in OCP (OCP-loss to 50% of the initial concentration) taken on its own makes a loss due to leakage or abiotic removal unlikely. An autoclaved culture that was incubated at 35° C with 8 mM OCP did not lose its OCP (Fig.8) during a time period of 40 days. Therefore an abiotic OCP-loss was very unlikely.

Medium containing trypticase and yeast extract interfered with phenol analysis. A culture freshly transferred into anaerobic nutrient broth showed false phenol readings that decreased with time (Fig.9) and were significantly lower than the phenol concentrations in dehalogenating cultures incubating in nutrient medium. A correction was made in the data in Fig.7 to subtract the interference reading. Dehalogenation did not proceed to completion and phenol accumulation stopped eventually. After transferring the culture to fresh medium with OCP dehalogenation and phenol production resumed. Refeeding with new medium and OCP resulted in the sawtooth-like graphs where each OCP peak represented a transfer into fresh medium followed by OCP dehalo-

genation. Note that an increase in rate of OCP-degradation and increase in phenol production rate were observed upon successive refeeding. Holliger et al. (1992) reported results as in Fig.7 using a mixed culture that dehalogenated 1,2,3-trichlorobenzoate to 1,3-dichlorobenzoate and was repeatedly refed with new 1,2,2-trichlorobenzoate.

As shown in Fig.7 OCP-dehalogenation occurred in cultures derived from the bleaching plant wastewater lagoon that were incubated in nutrient broth containing 1% yeast extract and 1% trypticase digest plus OCP. OCP-Dehalogenation also occurred in cultures that were incubated in 0.5% trypticase medium plus OCP (Fig.10). OCP-dehalogenation reduced OCP-concentration to approximately 50% of the initial value. In addition to phenol, benzoate was detectable. Benzoate concentration followed a curve similar to that of phenol.

The pH in the nutrient broth might change over time due to volatile fatty acid accumulation. Initially the pH was 7.0 to 7.2. However after four months of incubation in anaerobic nutrient medium pH was found to be still above 6.8.

Agar bottles (Fig.1, p.50) provided mixed anaerobic dehalogenating inoculum that was viable for over a year. The agar bottles were used to maintain stock of mixed anaerobic cultures of microorganisms and they provided inoculum for several experiments.

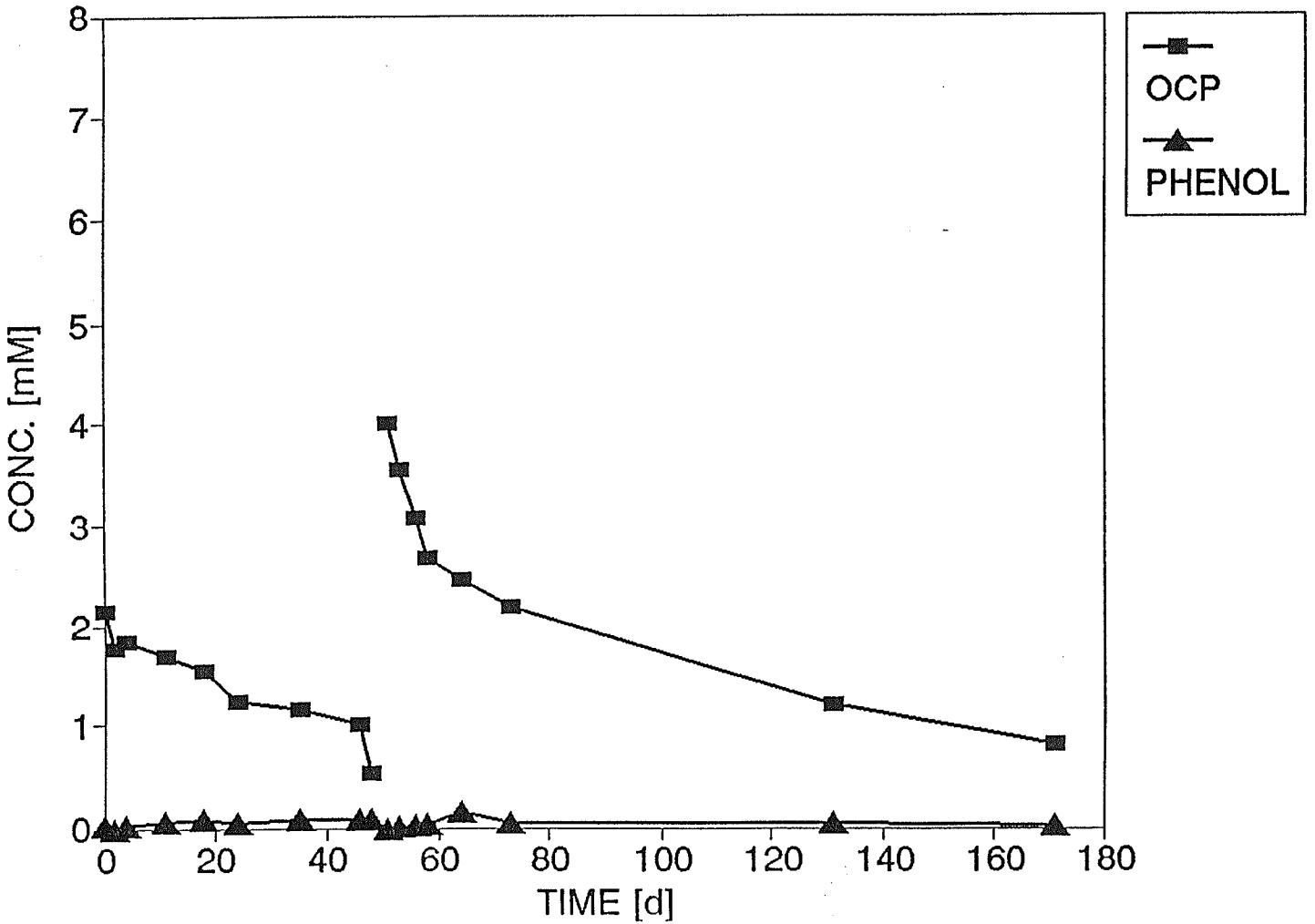

Fig. 11. OCP and phenol in a culture that had been concentrated tenfold and incubated in mineral medium plus OCP. The culture had been washed after concentration to remove residual acetate and phenol. Each peak in OCP concentration and each low in phenol concentration are when culture was centrifuged and medium replaced. Solid rectangle: OCP; solid triangle: phenol.

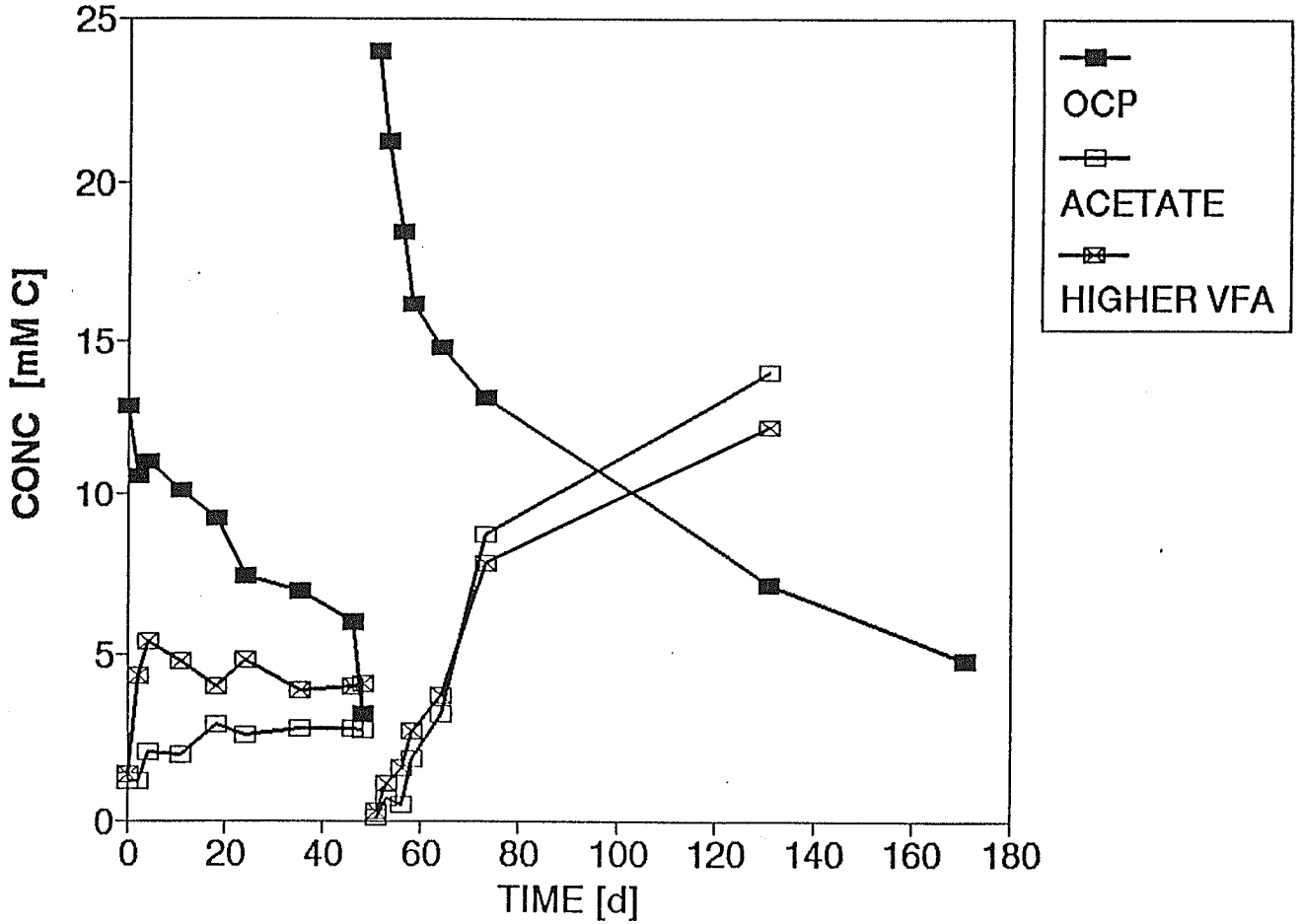

Fig. 12. OCP, higher VFA, and acetate in the culture from Fig. 11, that had been concentrated tenfold and incubated in mineral medium plus OCP. The culture had been washed after concentration to remove residual acetate, VFA, and phenol. Each peak in OCP concentration and each low in phenol concentration are when culture was centrifuged and medium replaced. OCP removal accounts for acetate and VFA appearance. Solid rectangle: OCP [mM C]; open rectangle: acetate [mM C]; crossed rectangle: higher VFA [mM C].

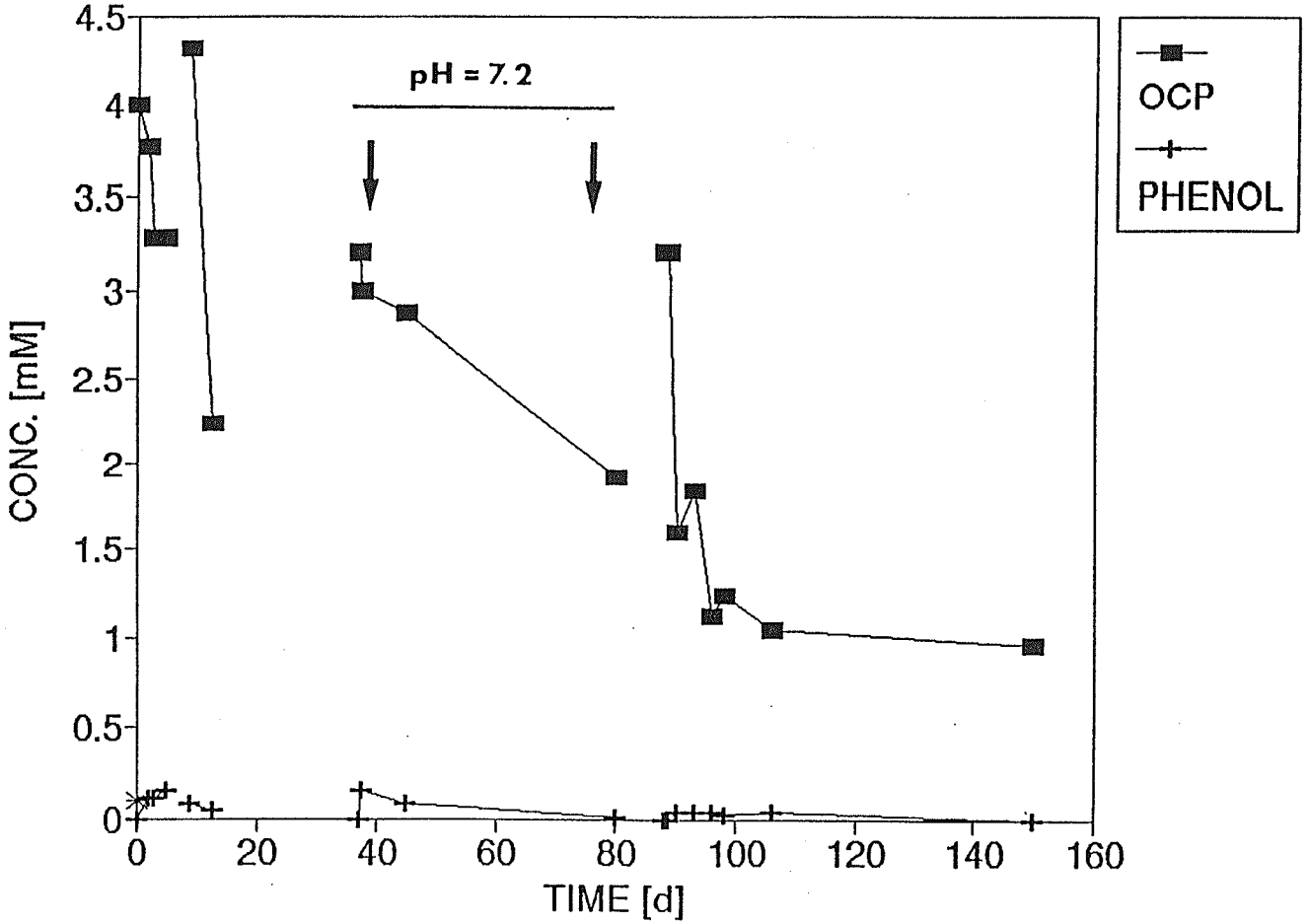

Fig. 13. OCP and phenol in a culture that had been concentrated tenfold and incubated in mineral medium plus OCP (Repeat experiment to Fig. 11). The culture had been washed after concentration to remove residual acetate and phenol. Each peak in OCP concentration and each low in phenol concentration are when culture was centrifuged and medium replaced. pH in medium was 7.5 exept between day 35 and day 80 (arrows). Solid rectangle: OCP; solid triangle: phenol.

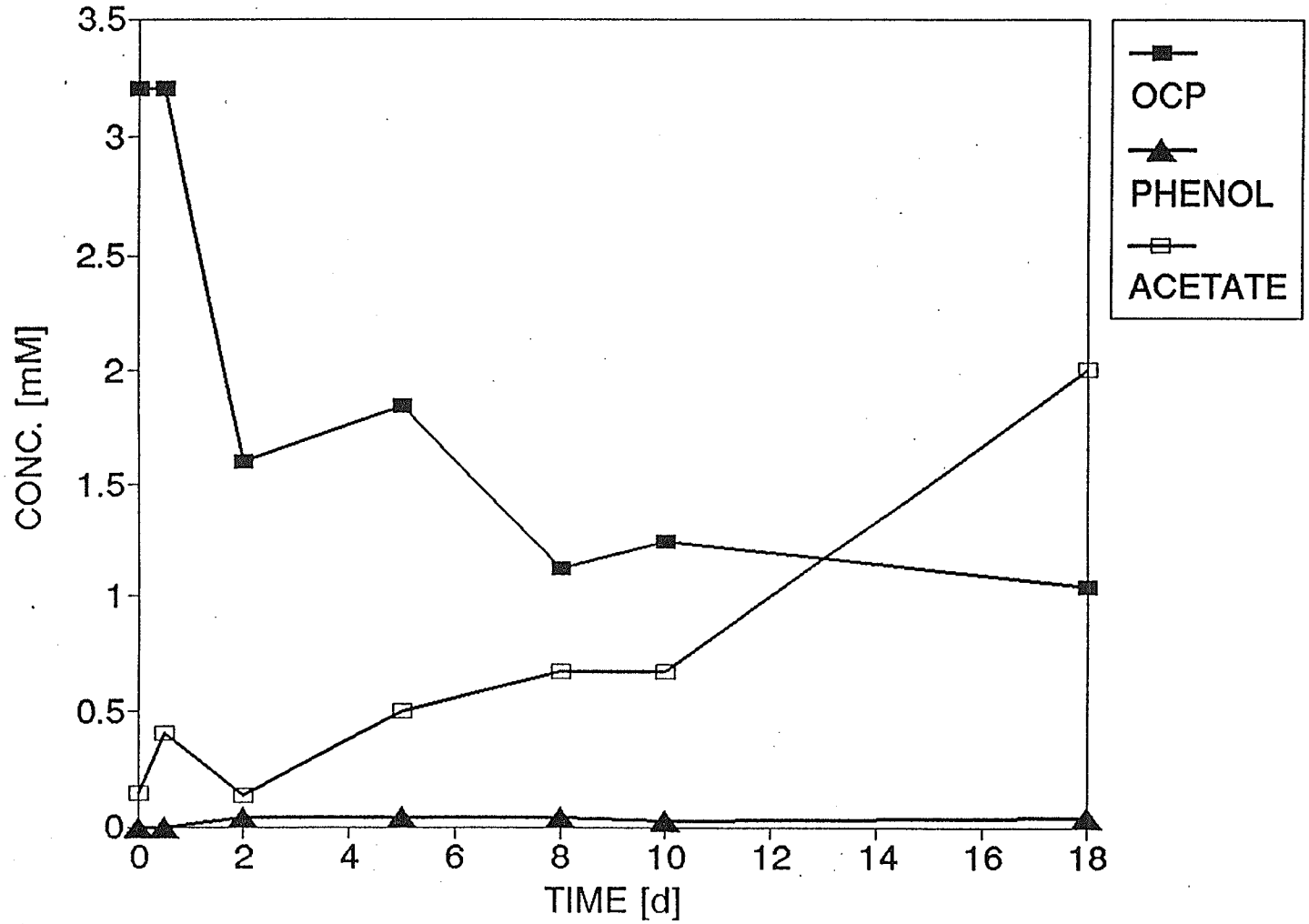

Fig. 14. OCP, phenol and acetate in a culture that had been concentrated tenfold and incubated in mineral medium plus OCP. The culture had been washed after concentration to remove residual acetate and phenol. Solid rectangle: OCP; solid triangle: phenol; open rectangle: acetate.

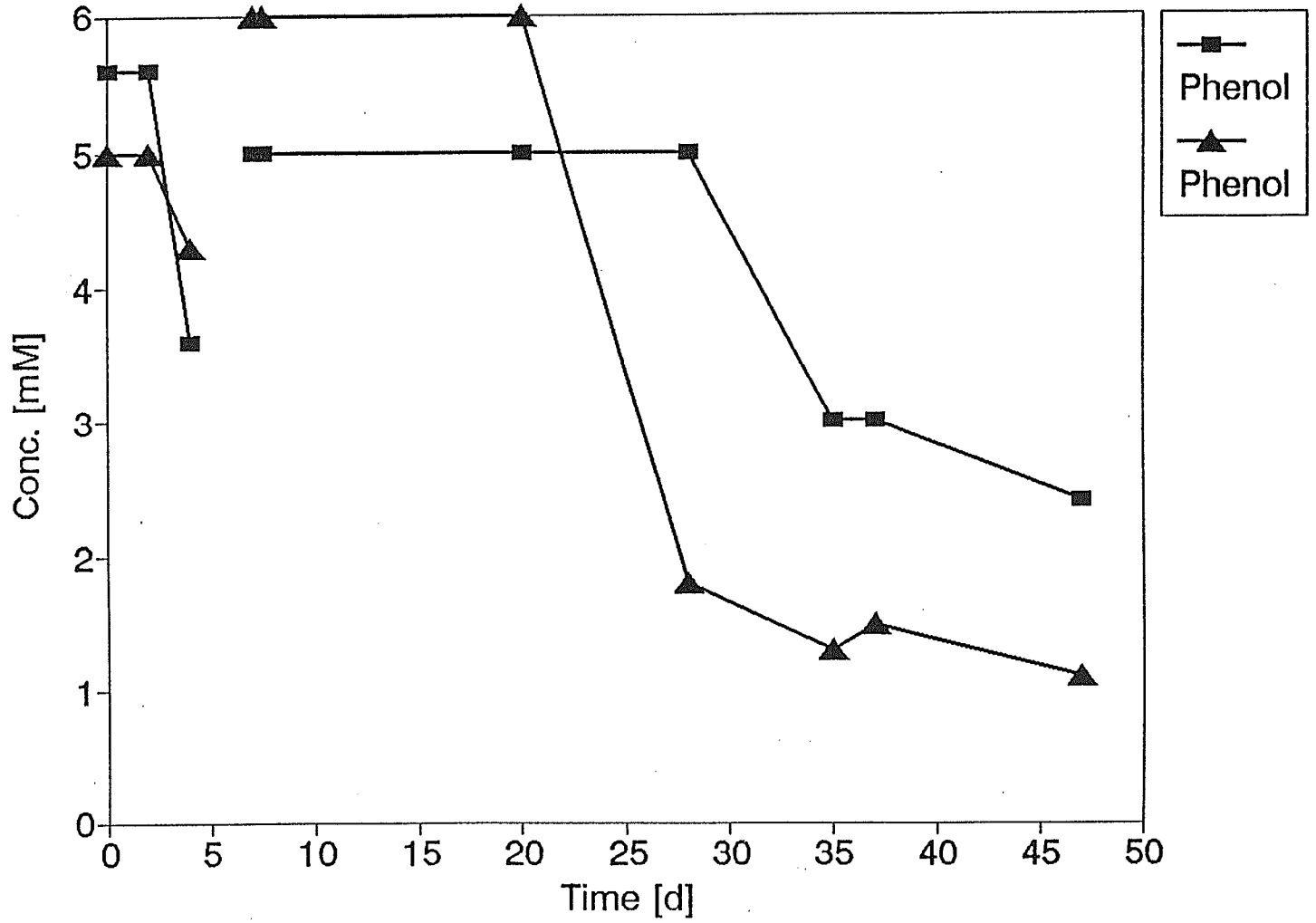

Fig. 15. Phenol in duplicate cultures that had been concentrated tenfold and incubated in mineral medium plus phenol. The cultures had been washed after concentration to remove residual acetate and phenol. Each peak in phenol concentration are when culture was centrifuged and medium replaced. Solid rectangle: phenol of first culture; solid triangle: phenol of second culture.

5.4.2 OCP-degradation in Mineral Medium by Concentrated Cell Suspensions

OCP dehalogenation to 30% to 40% of the initial OCP-concentration was reached with cultures growing in mineral medium plus OCP. The cultures were derived from the municipal anaerobic sludge. After a tenfold concentration the cells were incubated in mineral medium plus OCP (Fig.11 and 12). In trypticase medium OCP dehalogenation down to 50% of the initial OCP-concentration was reached. OCP - and phenol concentrations of concentrated cultures in mineral medium are presented in Fig.11, OCP -, higher volatile fatty acids (VFA) -, and acetate concentrations in Fig.12. In order to compare OCP and phenol with acetate, concentrations are given in [mM C] in Fig.12. Similar cultures from OCP-adapted inoculum produced slower OCP-degradation and were not used in the following experiments. Concentrated cultures in mineral medium plus OCP contained nearly 0.6 mM C as acetate (20 mg/L acetate) as a carryover from the previous nutrient broth. In the course of incubation in mineral medium the acetate concentration increased for 10 days over threefold to about 2 mM C as acetate (60 mg/L acetate). 3 mM C as higher volatile fatty acids and trace concentrations of benzoate (<10 mg/L or <0.7 mM C as benzoate) formed also.

After washing and resuspending in new mineral medium plus OCP the culture dehalogenated 24 mM C as OCP (3 mM OCP) and released

14 mM C as acetate (7 mM acetate) and 12 mM C of other volatile fatty acids (VFA), mostly propionate, n-butyrate, and n-valerate. Therefore OCP removal was roughly stoichiometric to VFA-release. Armenante et al. (1993) recommended a pH up to 8.8 for degradation of trichlorophenols. A repeat experiment using medium with a pH of 7.5 rather than 7.2 (Fig.13) was performed. Again acetate was produced, Fig.14 plots acetate, phenol, and OCP during the last cycle of feeding. The higher pH caused an improvement in dehalogenation performance, because the third feeding cycle from 37 to 80 days used OCP-medium with a pH of 7.2 and dehalogenation was poor compared with that of the first and second feeding cycles. Return to a pH of 7.5 in the fourth feeding cycle produced dehalogenation rates similar to those of the first and second cycle. Acetate could be a byproduct of a dehalogenating organism or consortium. An example is in Dolfing and Tiedje (1987b) and Dolfing and Tiedje (1991). Madsen and Licht (1992) reported that the trichlorophenol dehalogenator strain DCB-2 releases acetate, although acetate was a product of pyruvate rather than chlorophenol - or phenol degradation. Acetate acted as an inhibitor to 3-chlorobenzoate degradation by *D. tiedjei* DCB-1 (Dolfing and Tiedje, 1987b). Acetate also could be a substrate for an OCP-dehalogenator (Cole et al., 1994). Therefore acetate was an important compound in this mixed culture. OCP-dehalogenation proceeding concurrently with acetate release points to acetate as an end product of OCP-degradation. Acetate can inhibit OCP-degradation as reported by Dolfing and

Tiedje (1987b) for *D. tiedjei*. Conceivably presence of an end product can inhibit production of more end product.

A confirmation of phenol-degradation were cultures similar to the above OCP-degrading concentrated cultures, but their mineral medium was amended with phenol rather than OCP. Phenol removal occurred (Fig.15).

After concluding the observations of OCP-degradation, the mixed anaerobic dehalogenating cultures of microorganisms consisted primarily of two types of short rods that frequently occurred in chains of two or four organisms and were capable of forming endospores.

5.4.3 Is Carbon Dioxide the End product of OCP-Degradation?

Boyd et al. (1983) indicated carbon dioxide to be the end product of chlorophenol degradation. However Boyd's cultures were methanogenic and were able to generate carbon dioxide from acetate. The following experiment was an attempt to find proof of OCP-degradation to carbon dioxide:
The incubation of dehalogenating cultures and water with ^{14}C-OCP produced the following results (Table 2), radioactivity was measured after 21 days of incubation. Both the headspace gas and the culture fluid (Table 2) were analyzed with the exception of two cultures and one control tube:

Table 2

Radioactivity in Headspace Gas and Culture Fluid after Incubation with ^{14}C-OCP.

Sample	Counts per min. after 21 d of incubation	
	Headspace	Culture Fluid
Atmosphere background	30	
Water (no label)		30
Water 1	1160	38000
Water 2	1450	36320
Water 3		21440
Replicate 1		28090
Replicate 2		29740
Replicate 3	830	36320
Replicate 4	520	17190
Replicate 5	1160	45620
Replicate 6	1070	20460
Replicate 7	1860	28650
Replicate 8	1340	37630
Replicate 9	1780	33560

Water and cultures showed similar headspace radioactivity. Therefore no ^{14}C - carbon dioxide was released by the cultures.

Any OCP-degradation that took place must have produced products that remained in the culture fluid and therefore no difference between blanks and cultures could be observed. This is consistent with the recovery of carbon from OCP as acetate and volatile fatty acids in Fig.12.

Cultures had been allowed to dehalogenate 20% of the present OCP prior to addition of ^{14}C-OCP. Phenol was produced.

5.4.4. OCP-Degradation, Sulphate, and *Desulfobacterium phenolicum*

The addition of *Desulfobacterium phenolicum* plus 20 mM sulphate to a mixed dehalogenating culture at 25 °C did not result in dehalogenation. The OCP-concentration decreased from 2.5 to 2.3 mM within the first 24 hours, thereafter remained constant at 2.3 mM. A 2.5 mM OCP solution without organisms fluctuated up +/- 0.1 mM. Therefore no proof of OCP-degradation was achieved.

Adding 20 mM sulphate to a mixed dehalogenating culture and incubating at 35° C without *D. phenolicum* (which does not grow at 35° C) did not affect dehalogenation, presumably sulphate reduction had no effect on dehalogenation if present in the municipal anaerobic sludge (Fig.17). The different initial OCP-concentrations were a result of OCP-injection at the beginning of the experiment. Dehalogenation took place during the first five days and lowered chlorophenol concentration by 0.5 mM.

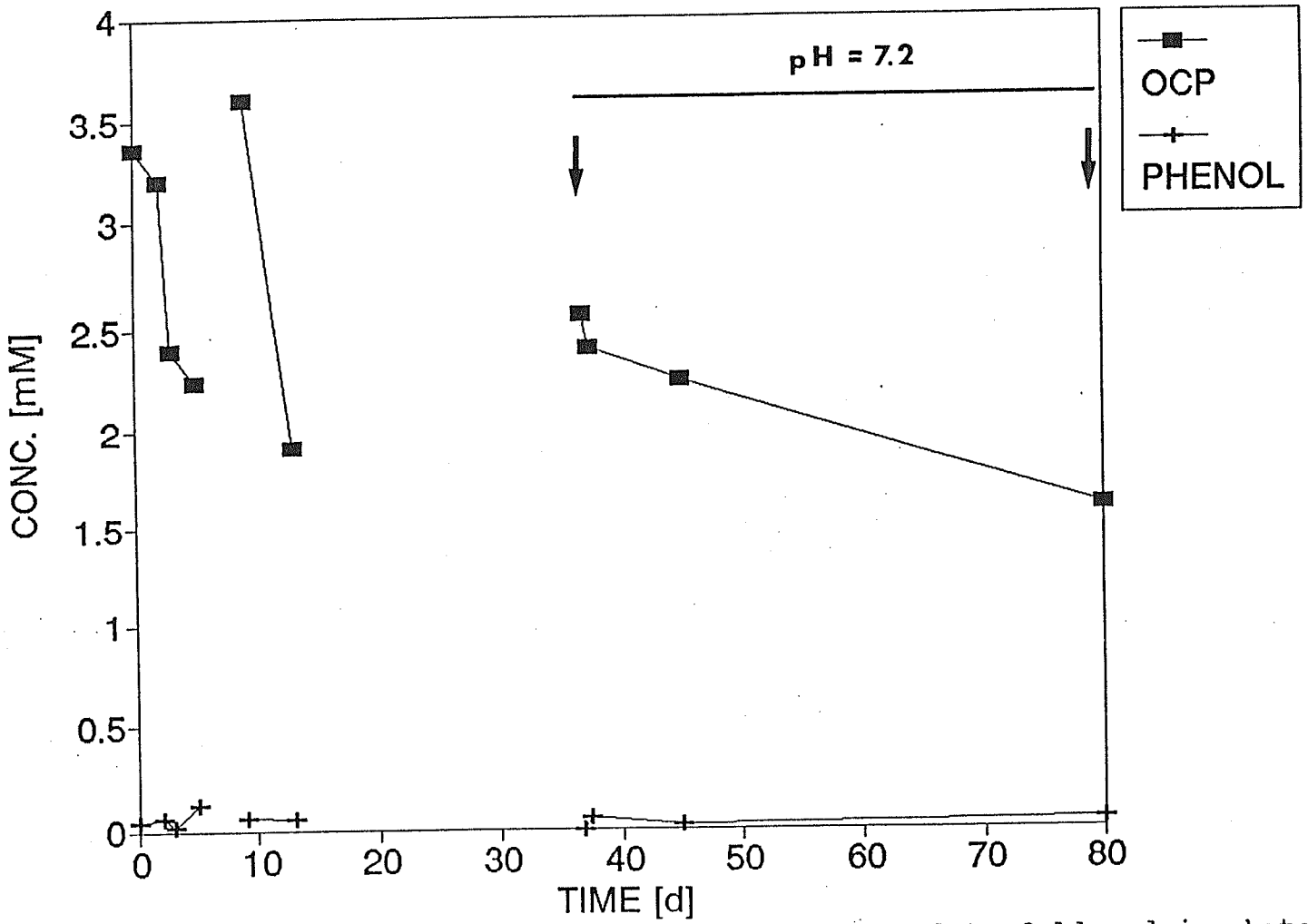

Fig. 16. OCP and phenol in a culture that had been concentrated tenfold and incubated in mineral medium plus OCP after heat treatment for 1 h. at 75° C. The culture had been washed after concentration to remove residual acetate and phenol. Each peak in OCP concentration and each low in phenol concentration are when culture was centrifuged and medium replaced. pH between day 35 and day 80 (arrows) was 7.2 instead of 7.5 and degradation was slower. Solid rectangle: OCP; plus: phenol.

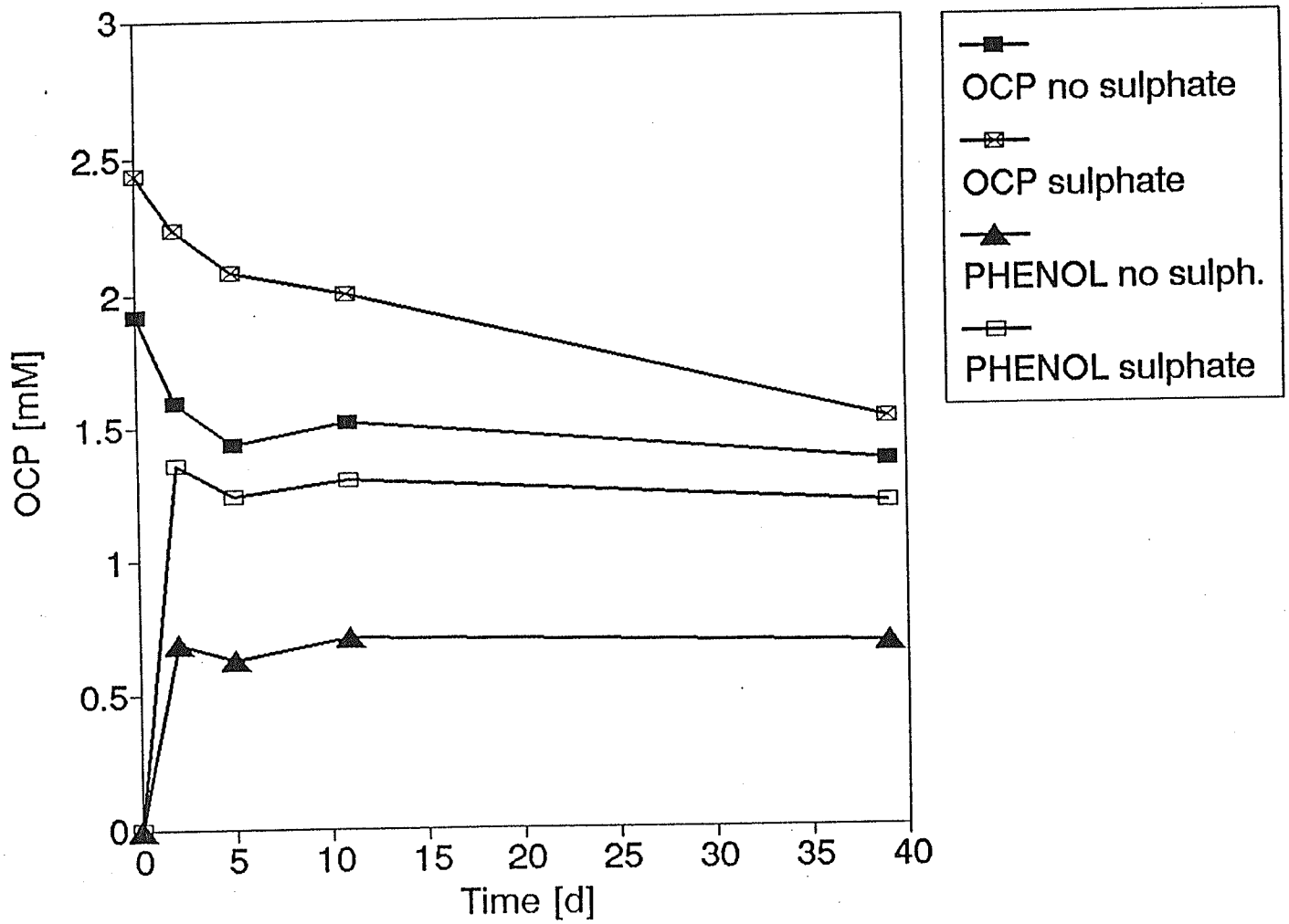

Fig. 17. Phenol and OCP in dehalogenating mixed cultures incubating in anaerobic nutrient medium plus OCP in presence and absence of 20 mM Na-sulphate. The medium is complex yeast extract/trypticase medium and therefore phenol accumulates. Solid rectangle: OCP, no sulphate-medium; crossed rectangle: OCP, sulphate-medium; solid triangle: phenol, no sulphate-medium; open rectangle: phenol, sulphate-medium.

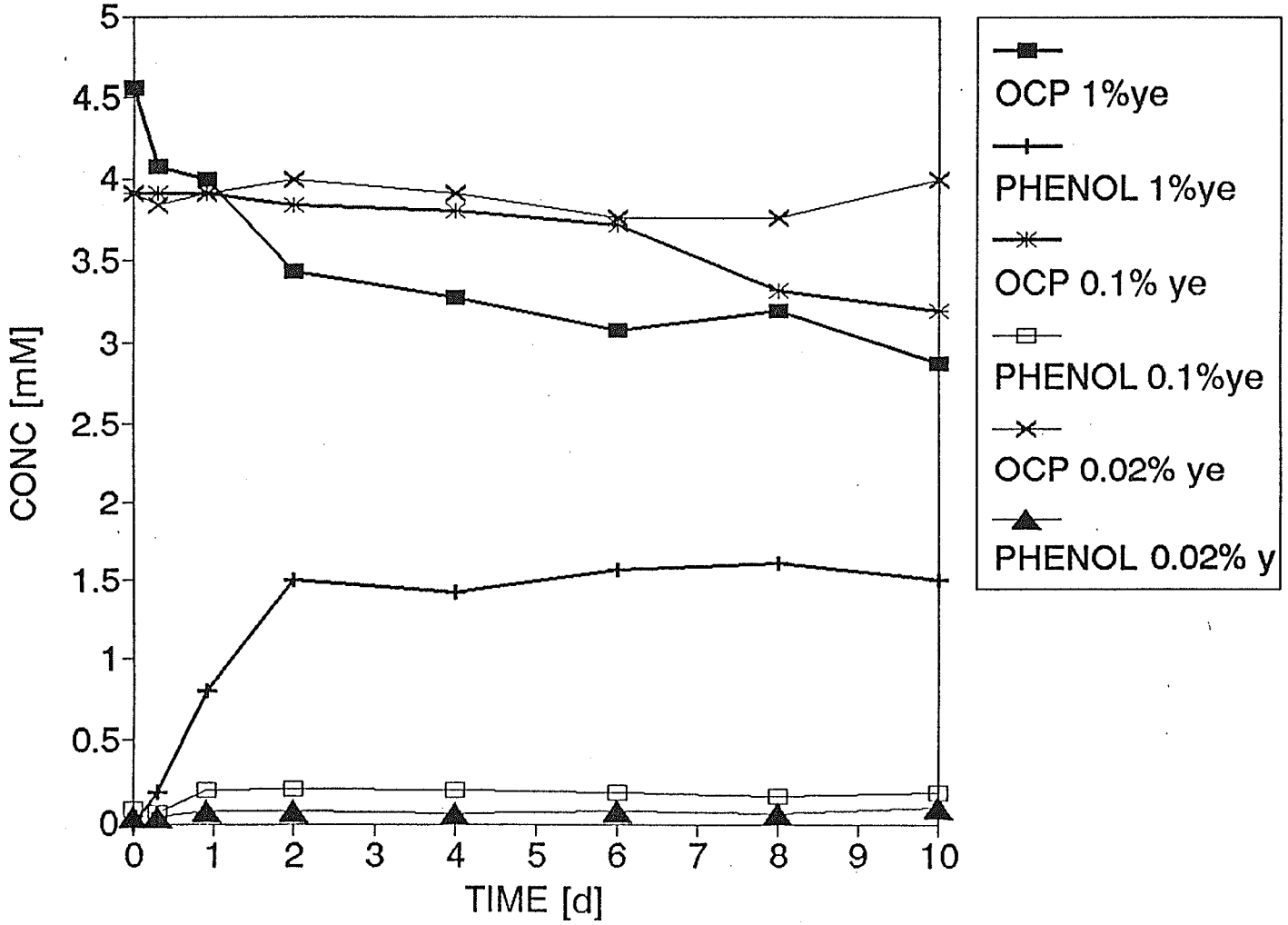

Fig. 18. Phenol and OCP in mixed cultures incubating in anaerobic medium plus OCP and a range of yeast extract - and trypticase concentrations. Yeast extract - and trypticase concentrations were 1%, 0.1%, and 0.02% (w/v) respectively. Cultures had been grown up from 10% inoculum. Solid rectangle: OCP, 1% YE/trypt.; plus: phenol, 1% YE/trypt.; asterisk: OCP, 0.1% YE/trypt.; open rectangle: phenol, 0.1% YE/trypt.; cross: OCP, 0.02% YE/trypt.; solid triangle: phenol, 0.02% YE/trypt.

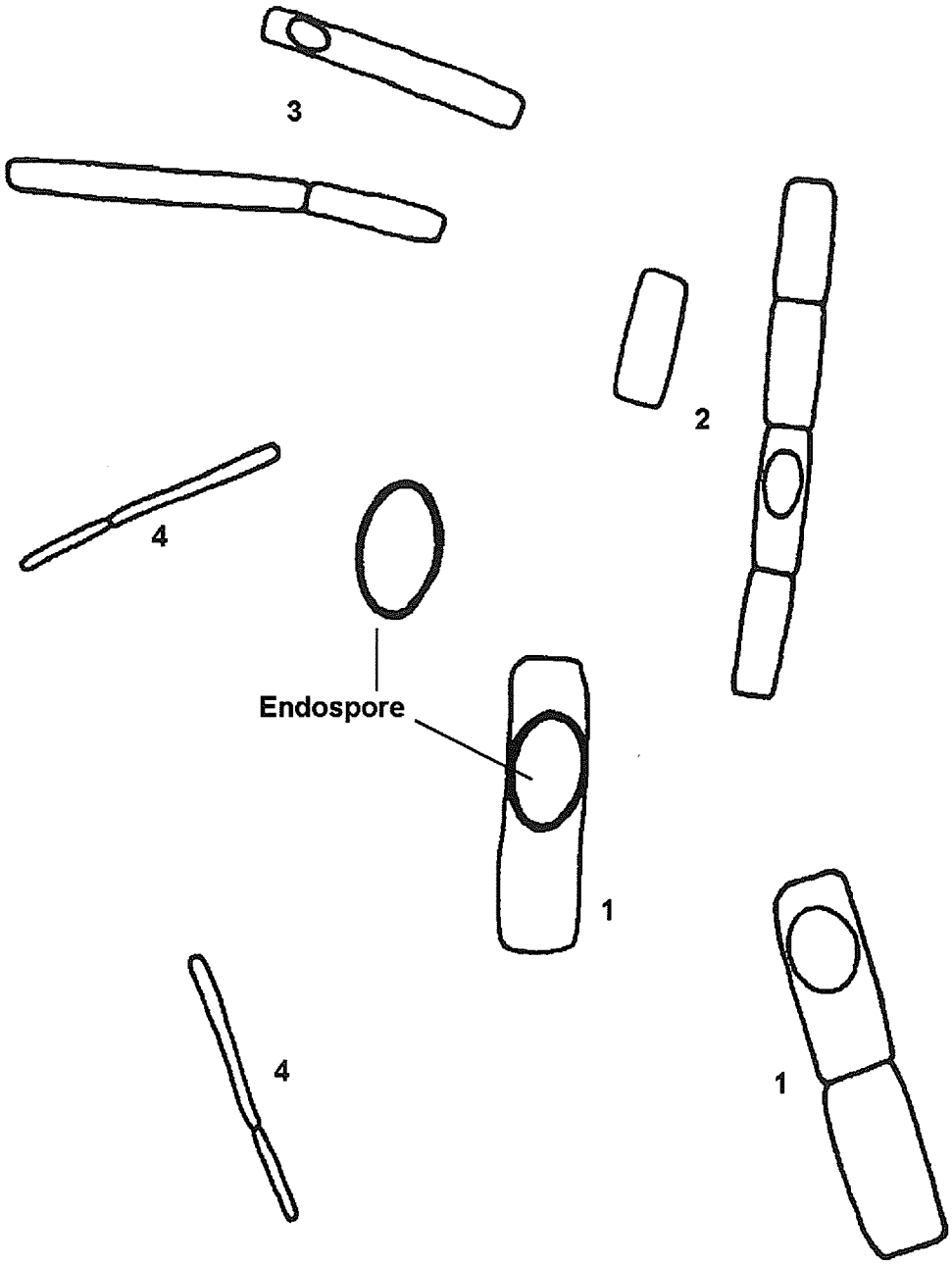

Fig.19

Organisms found in mixed anaerobic dehalogenating cultures of microorganisms after incubation in mineral medium plus OCP. The cultures were kept in the dark at 35° C. The two types of large short rods (1 and 2) were the most common organisms. The larger of the short rods (1) became less abundant as the cultures grew older. The least abundant organism was (4).

Therefore dehalogenation was not assisted by adding sulphate nor did sulphate appear to harm dehalogenation.

Desulfobacterium phenolicum incubating on its own at 25° C in pure culture could be shown to not remove OCP. The OCP-concentration decreased from 1 mM to 0.8 mM in the first 24 hours after setting up the culture, then the OCP-concentration remained constant. A 1.0 mM OCP solution without organisms fluctuated up +/- 0.1 mM. Therefore no proof of OCP-degradation was achieved.

5.4.5 OCP-Degradation by Heat-Treated Cultures

Heat treatment of the OCP-dehalogenating cultures (1 h incubation at 75° C) did not reduce the ability to dehalogenate. Organism(s) in the mixed cultures were therefore endospore formers or other heat-resistant organisms (Fig.16). The mixed anaerobic dehalogenating cultures were therefore similar to those of Madsen and Licht (1992). Madsen and Licht found di- and trichlorophenol degradation after incubation at 80° C. They were able to isolate strain DCB-2 that is a di- and trichlorophenol dehalogenator capable of forming endospores. The mineral medium was adjusted to a pH of 7.5 between 0 and 35 days while pH was 7.2 from 37 to 80 days and OCP-dehalogenation was slower in the medium with the latter pH.

5.4.6 Morphology of Organisms in Mixed Anaerobic Dehalogenating Cultures

After conclusion of the observations of OCP-degradation, cultures incubating in mineral medium plus OCP contained primarily two types of short rods that were capable of forming endospores. There were also two types of long rods one of which also formed endospores (Fig.19). The larger of the two types of short rods decreased in abundance as the cultures became older. Fig.18 was drawn from a sample of the same culture that provided data for Fig.13 and Fig.14 using a Nikon microscope equipped for phase contrast. A magnification of 1000x was used.

5.4.7 OCP-Degradation in Subcultures in Media with Different Concentrations of Yeast Extract and Trypticase

Subcultures inoculated into media with different (i.e. 0.02%, 0.1%, and 1% w/v) yeast extract and trypticase medium amended with OCP degraded OCP only in 1% yeast extract and trypticase medium (Fig.18) at 35° C. Growth was ascertained by measuring optical density at 600 nm. Table 3 shows optical density at 600 nm with respect to time for the mixed anaerobic dehalogenating cultures of microorganisms that grew in a range of yeast extract and trypticase concentration and in presence of OCP. Dehaloge-

nation started within 24 hours of inoculation only in medium that had 1% yeast extract plus 1% trypticase. Therefore OCP was not a carbon source for the mixed anaerobic dehalogenating cultures of microorganisms.

Table 3

Growth of mixed anaerobic dehalogenating cultures of microorganisms in media with a range of yeast extract and trypticase concentrations [%] and OCP.

Time [h]	OD_{600} at 0.02% yeast extract and trypticase	OD_{600} at 0.1% yeast extract and trypticase	OD_{600} at 1% yeast extract and trypticase
	Culture 1	Culture 2	Culture 3
0	0.03	0.03	0.06
6	0.03	0.08	0.26
8	0.04	0.10	0.96
11	0.09	0.19	1.10
23	0.12	0.27	1.12

6. Discussion

6.1 Degradation of a Xenobiotic Compound by Bacteria

Xenobiotic compounds when released into the environment affect microorganisms by either stimulating or slowing growth of some or all of the microorganisms present. Such compounds including chlorophenols can be sufficiently toxic to prevent all growth even if all required nutrients and carbon and energy sources are present. In the latter case such a compound would prevent bacterial degradation of several or all organic materials present. This would have grave consequences, for example if the toxic effect occurred in a biological wastewater treatment plant since the release of organic material with the effluent would increase greatly and transformation of the organic material to carbon dioxide would stop. In a milder case there could be shifts in the microbial population. For example chlorophenols and chloroanilines reduce the ability of acetoclastic methanogenes to produce methane (Davies-Venn et al., 1992). A sufficiently long exposure to an inhibitory concentration of a toxicant could eliminate methanogens from the system.

This study indicated that OCP also slows growth of mixed anaerobic cultures of microorganisms. OCP does not prevent bacterial growth, but is prevents methanogenic activity.

A toxicant may be either degraded by a specific enzyme (Anderson, 1986) whose gene is activated in presence of the toxicant; or a pre-existing enzyme that degrades primarily a different compound may also attack the toxicant resulting in a co-metabolic process. This study supports co-metabolism as most likely responsible for OCP-degradation.

6.2 Discussion of Results

When cultures derived from both a municipal anaerobic sludge and a from a wastewater lagoon of a pulp bleaching plant were incubated in a complex organic medium with OCP as shown in Fig.2 and 3, OCP did not completely inhibit bacterial growth even in a concentration of 10 mM. Indeed growth was observed at up to 8 to 10 mM OCP at a slower rate (factor of 5 to 6 compared to control). The fact that these cultures could tolerate over 5 mM OCP is consistent with the fact that Hrudey et al. (1987) were able to find OCP-degradation in anaerobic sludge containing 2 mM OCP and that this study showed OCP-degradation of up to 5 mM OCP. Growth rates of the mixed anaerobic cultures of microorganisms are similar at all OCP-concentrations. Therefore OCP is able to slow growth of the mixed bacteria. What might be the reasons for the faster growth in the cultures with 0.5 and 1 mM OCP in Fig.4? A similar stimulation was observed by Poggi (unpublished data) in a bench scale bioreactor degrading paper

laced with OCP. Adaptation to OCP or selective growth could have occurred. The cultures with previous exposure to OCP tended to have shorter lag times after transfer to OCP medium with concentrations of OCP that are greater than 6 mM. Lower concentrations of OCP still forced a longer lag, but pre-exposure to OCP did not appear to train the cultures to shorten the lags caused by OCP-concentrations under 6 mM.

Keweloh et al. (1991) proposed that chlorophenols enter the cell membranes where they disturb the order of the fatty acid residues of the phospholipids. The chlorophenols are lipophilic and tend to be attracted by the lipophilic environment between the fatty acid residues. When the chlorophenol molecules disturb the arrangement of fatty acid chains the membranes become more fluid. A factor that also affects membrane fluidity is temperature. Keweloh et al. (1991) indicated that microorganisms grown at a high temperature have more fully saturated fatty acid residues like palmitic acid residues than when grown at a lower temperature. Apparently the organisms keep a certain membrane fluidity by adjusting the amount of fatty acid saturation according to temperature. Presence of palmitic acid in the medium of an *E. coli* culture with 4-chlorophenol caused the growth rate to be closer to that of a culture without this toxicant in the medium (Keweloh et al., 1991).

In the present study it was not possible to observe findings

similar to those of Keweloh et al. (1991). Added palmitic acid did not improve the growth rate (Fig.5) nor did it result in a decrease in lag time at OCP-concentrations that were greater than 6 mM (Fig.6).

Subcultures of the OCP-adapted biomass did not produce methane. That is in accordance with Hrudey et al. (1987), O'Connor and Young (1989) and Kim et al. (1994) who found OCP-concentrations of less than 1 mM toxic to methanogens and growth of pure cultures of *Methanospirillum hungatei* were completely inhibited by 1 mM OCP.

The presence of phenol was observed in most of the cultures that dehalogenated OCP. Phenol formation is a likely product of OCP-dehalogenation and it occurred in cultures of the of strain 2CP-1 isolated by Cole et al. (1994). Benzoate in the cultures in trypticase - and also mineral medium indicated phenol degradation involving addition of a carbon atom to the ring prior to ring opening (Fig.10) as observed for example by Xang and Wiegel (1990).

Initially dechlorination was slow (see Fig.7 and 10). It increased after refeeding the cultures with fresh medium and OCP. This also occurred in the mixed cultures of Holliger et al. (1992). Presumably the population of microorganisms was not yet stable prior to the first refeeding. The lower phenol concen-

trations in cultures maintained in trypticase and particularly mineral medium were likely a result of phenol being utilized as a carbon and energy source. The utilization was greatest in the mineral medium which contained no other carbon sources. This might also explain the more complete OCP-degradation in the cultures with mineral medium, i.e. without yeast extract or trypticase. Phenol disappearance in cultures incubated with phenol, but without OCP is an indication for phenol degradation (Fig.15). To obtain positive proof of phenol utilization an experiment incubating a dehalogenating culture in trypticase and yeast extract/trypticase medium would be needed. Removing phenol might make OCP-degradation energetically more favourable.

The incomplete removal could conceivably be due to a pH change. As indicated above four months old cultures in complex organic medium contained up to 4 g/L acetate, but the medium was sufficiently well buffered to keep the pH above 6.8 after 4 months of incubation. A low pH could be the reason for incomplete OCP-degradation if OCP can act as an acid (Apajalahti, 1987). When pH falls due to VFA-production dehalogenation may stop, because at a very low pH OCP would not lose a hydrogen ion and it would therefore be more hydrophobic and could penetrate cell membranes more easily. Armenante et al. (1993) recommended a pH of 6.0 to 8.8. Holliger et al. (1992) used a pH of 7.2 for dehalogenating enrichments.

Cultures in mineral medium were set up by concentrating biomass while cells in rich - and trypticase medium were not concentrated. The concentrating will have resulted in more cells per ml and a faster degradation rate compared to cultures that were not concentrated.

OCP could be degraded to phenol, the phenol in turn could be converted to hexanol by saturating the aromatic ring, ring opening would occur now (Knoll and Winter, 1989). That would form caproic acid (a 6 C acid) which then turns to lower fatty acids. However presence of benzoate in a mixed anaerobic dehalogenating culture in medium with OCP as the only carbon source points to a degradation pathway where OCP is converted to phenol which in turn is converted to benzoate. Ring opening should occur to form adipic acid which is than converted to lower fatty acids.

The concentration of acetate in the cultures increased with time in this study (Fig.12). That pointed to acetate as an end product of OCP-degradation. However the total concentration of the higher fatty acids reached a rough plateau within two days which indicated that the cells may have contained a lot of carbon they have accumulated while growing in the nutrient broth. Some of this carbon was than released after transfer into the mineral medium plus OCP during the first OCP-degradation cycle. This makes it difficult to draw conclusions from information on VFA - concentrations over time, however the second refeeding - and

OCP-degradation cycle in Fig.12 was not preceded by a growth phase in nutrient broth eliminating possible carbon uptake from nutrient medium. Acetate and higher volatile fatty acid production during the second cycle (Fig.12) was roughly stoichiometric to OCP-removal thus confirming acetate and higher volatile fatty acids as end products of OCP-degradation.

Acetate was the most abundant acid and accumulated in the cultures: It is likely to be at least one of the end products. Cultures that were incubated in ^{14}C-OCP medium did not produce ^{14}C-carbon dioxide when compared to controls (Table 2). This supported the conclusion that acetate rather than carbon dioxide was the end product of OCP-degradation.

OCP-degradation with heat-treated cultures indicated that endospore formers were most likely involved (Fig.16). Vegetative cells were unlikely to survive for one hour at 75° C (Madsen and Aamand, 1992). Therefore this mixed anaerobic culture was similar to Madsen and Licht's (1992) isolate (strain DCB-2) which grows at 35° C, is an endospore former and produces acetate when growing. This culture did not require methanogenesis to take place concurrently with dehalogenation.

The degradation rate was improved by adjusting pH in the medium to 7.5 rather than 7.2 (Fig.13 and 16). Both values are within the pH-range recommended by Armenante (1993) for anaerobic dehalogenation.

Degradation was partial and occurred at OCP-concentrations of 3 to 5 mM. Most previously reported work quoted concentrations of monochlorophenol of less than 1 mM, but also reported complete degradation. Onset of degradation was less than 24 hours after transfer to OCP-medium (Fig.13). Degradation taking place at high concentration and rapid onset after exposure to the OCP medium pointed to a co-metabolic process of degradation where the enzyme affecting degradation lost efficiency with decreasing OCP-concentration. However K_m might also have been high. It is still conceivable, that the end of OCP-degradation prior to complete removal was due to exhaustion of an essential component of the medium and that an enzyme specific for dehalogenation of OCP was made available to the degrader's metabolism less than 24 hours after exposure. In fact Madsen and Licht (1992), Mohn and Kennedy (1992a), and Cole et al. (1994) reported their dehalogenators to be inducible. Cole et al. (1994) reported a delay, that was longer than 6 h and occurred between transfer of 2-CP1 to OCP-medium and the beginning of dehalogenation.

The start of new mixed anaerobic dehalogenating cultures of microorganisms required growing up a large inoculum in nutrient broth and subsequent transfer of the inoculum to an OCP-medium. Using a small inoculum in OCP-medium did not yield appreciable dehalogenation (Fig.18). Therefore the OCP not prove to be a carbon and energy source able to support growth. It is possible that phenol provided some carbon and energy to the culture.

Phenol was removed by a mixed anaerobic dehalogenating culture of microorganisms (Fig.15). Dehalogenation in a culture inoculated into a medium of OCP with a very low concentration of trypticase or yeast extract would have made OCP a likely carbon and energy source. In such a case the culture could have been transferred to a bioreactor and the degrading organism enriched while keeping the culture in an environment with a comparatively low OCP-concentration of 3 mM. However no enrichment was possible and OCP was not used as a carbon source.

6.3 Future Work

A follow-up experiment to the growth tests should check what the growth rates of cultures are after the high OCP-concentration is removed.

This study involved work with mixed cultures. As a next step the cultures could be developed to a stable consortium with known members that can be identified. It could than be ascertained how much energy OCP-degradation provides for the consortium by determining ATP levels. Following establishment of a stable consortium the dehalogenating strain could be isolated and characterized further. When the requirements for growth in pure culture are known, the dehalogenating enzyme could be purified for further study.

7. Conclusion

Experiments were conducted to evaluate the following objectives:

i. Effects of previous exposure (or "acclimation") of microorganisms from anaerobic sewage sludge on growth rate in presence of OCP.

ii. What conditions support OCP-degradation.

iii. What are pathway and end products of OCP-dehalogenation by mixed cultures derived from anaerobic sewage sludge.

The results can be itemized as follows:

1. OCP slowed the growth of mixed anaerobic cultures of microorganisms derived from anaerobic sewage sludge, however some growth occurred even at the highest OCP-concentration of 10 mM. Effects of OCP on growth rate were noticeable at OCP-concentrations greater than 2 mM. Doubling time of the microorganisms lengthened with increasing OCP-concentration. The lag between inoculation and the beginning of an increase in optical density also lengthened with increasing OCP-concentration. A culture pre-exposed to OCP had shorter lags than an unexposed culture.

2. Palmitic acid which based on current literature was thought to increase the growth rate or shorten the lag time was found

to exert no effect on the mixed anaerobic cultures of microorganisms.

3. A novel technique of obtaining concentrated inoculum of obtaining concentrated inoculum for biodegradation studies using solidified agar with the target compound was successfully adapted to OCP-degradation.

4. OCP-degradation took place in mixed anaerobic cultures of microorganisms that were free of most of the non-living matter found in a sludge or sediment. The degradation also took place in defined medium.

5. OCP was degraded in concentrations up to 5 mM. Higher OCP levels were not tried. 60% of the OCP initially present was completely removed in all samples at all concentrations ranging from 3 to 5 mM.

6. OCP-degradation tests were carried out at OCP-concentrations toxic or inhibitory to methanogenesis.

7. OCP-degradation took place with 20 mM sodium sulphate present in the medium.

8. Acetate was found to be the end product of OCP-degradation. Phenol and benzoate were intermediate products.

9. Carbon dioxide was not found to be the end product of OCP-degradation.

10. Mixed anaerobic dehalogenating cultures developed in this study were found to maintain viability for over a year, when kept at 35° C in the dark under anaerobic conditions.

11. Microscopic studies of the mixed dehalogenating cultures revealed the presence of two types of short rods that occurred in chains of two to four members and that formed endospores. The mixed anaerobic cultures developed in this study differed from the dehalogenating bacteria isolated earlier by others. The cultures developed in this study differed from the pure strain 2-CP1 isolated by Cole et al. (1994) in that they degraded OCP at 35° C. The organism of Cole et al. (1994) degraded OCP at 25° C. The cultures developed here attacked OCP and therefore differed from Madsen and Licht's dehalogenating strain DCB-2 and from *Desulfonomile tiedjei* (Mohn and Kennedy, 1992a) which did not attack monochlorinated phenols. As with strain DCB-2 the dominant organism in the dehalogenating cultures developed in this study was capable of forming endospores. The pure strain of the dehalogenating species in the mixed anaerobic dehalogenating cultures developed in this study has not been isolated.

8. References

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9. Appendix

Raw data of OCP-biodegradation tests:
Data to Fig.7:

DATE	DAY [d]	PHENOL	OCP [mM]
20.07.91			
20.07.91		2	8.8
09.01.92		1.5	1.8
REFEEDING			
16.01.92	0	0.3	4.8
17.01.92	1	0.3	4.32
27.01.92	11	0.2	3.92
04.02.92	19	0.3	3.92
07.02.92	22	0.3	3.36
11.02.92	26	0.21	3.44
13.02.92	28	0.35	2.8
17.02.92	32	0.4	3.04
20.02.92	35	0.4	3.04
27.02.92	42	0.4	2.4
02.04.92	46	0.85	2.64
08.04.92	52	1	2.16
22.4.92	66	1	2
30.4.92	74	1	2
REFEEDING			
1.5.92	75	0.1	4
4.5.92	78	0.1	3.28
6.5.92	80	0.5	2.72
8.5.92	82	0.31	2.4
14.5.92	86	0.8	2.24
19.5.92	91	0.54	1.92
21.5.92	93	0.6	2.32

DATE REFEEDING	TIME [d]	PHENOL [mM]	OCP [mM]
22.5.92	95	0.1	3.76
25.5.92	98	0.3	2.96
30.5.92	103	0.5	1.76
30.5.92	103	0.1	2.96
31.5.92	104	0.1	2.64
3.6.92	107	0.6	2
10.6.92	114	0.6	2
REFEEDING			
10.6.92	114	0.1	3.04
25.6.92	129	0.1	2.16
REFEEDING			
25.6.92	129	0.3	4.48
26.6.92	130	0.3	4
29.06.92	131	0.9	2.64
30.06.92	133	1.2	3.04
03.07.92	137	1.2	3.36
REFEEDING			
07.07.92	141	0.1	4
14.07.92	148	0	3.44
REFEEDING			
15.07.92	149	0.1	3.92
15.07.92	149	0.1	3.68
16.07.92	150	0.8	3.6
22.07.92	156	1.32	2.56
26.07.92	160	1.33	2.8
30.07.92	164	1.36	2.88
10.08.92	185	0.99	2.24
11.08.92	186	0.36	2.32
17.08.92	194	1.25	2.16

Data to Fig.8:

DATE	DAY	OCP [mM]	
16.01.92	0	0	
07.02.92	2	0.016	
10.02.92	27	0.216	
11.02.92	38	0.304	
14.02.92	41	0.328	

Data to Fig.9:

INTERFERENCE DUE TO NUTRIENT BROTH		
PHENOL READ. [mM]		DAY [d]
0.6		0
0.5		1
0.45		2
0.4		4
0.3		6
0.2		8
0.15		12
0.1		15

Data to Fig.10:

CULTURE 8				
DATE	DAY	OCP	PHENOL	BENZOATE
09.05.92	0	2.56	0.1	0.1
19.05.92	10	1.68	0.2	0.19
21.05.92	12	1.6	0.2	0.19
25.05.92	16	1.68	0.2	0.19
30.05.92	21	0.96	0.4	0.41
30.05.92	21	2.27	0.1	0.1
31.05.92	22	2.8	0.1	0.1
03.06.92	25	2.4	0.1	0.2
10.06.92	32	1.28	0.5	0.4
10.06.92	32	3.04	0.1	
25.06.92	46	1.6	0.52	
29.06.92	50	1.44	0.8	
07.07.92	59	1.68	0.7	
14.07.92	66	1.6	0.72	
22.07.92	74	1.36	0.84	
23.07.92	75	3.36	0.22	
24.07.92	76	3.28	0.19	
26.07.92	78	2.48	0.23	
28.07.92	80	2.4	0.15	

Data to Fig.11 and Fig.12:

OCP-DEHALOGENATION IN MINERAL MEDIUM					
		STERILIZED CONTROL CULTURE			
DATE	TIME [d]	OCP [mM]	BENZ. [mM]	PHENOL [mM]	
09.09.93	0	2.4	0	0.01	
07.10.93	28	2.4	0	0.04	
21.10.93	43	2.56	0	0.07	
04.11.93	57	2.56	0	0.07	
18.11.93	70	2.32	0	0.05	
15.12.93	98	2.16	0	0.07	
DATE	TIME [d]	OCP [mM]	BENZ.[mM]	PHENOL [mM]	
25.06.93	0	2.15		0.02	
27.06.93	2	1.77		0	
29.06.93	4	1.85		0.02	
06.07.93	11	1.69		0.06	
13.07.93	18	1.54		0.07	
19.07.93	24	1.23		0.05	
30.07.93	35	1.15		0.07	
11.08.93	46	1		0.07	
13.08.93	48	0.54		0.07	

0.5% TRYPT-BROTH FROM 13.08.93 - 16.08.93					
DATE	TIME	OCP	BENZ.	PHENL	
16.08.93	51	4	0	0	
18.08.93	51	3.54	0	0.01	
21.08.93	53	3.08	0	0.02	
23.08.93	56	2.69	0	0.03	
29.08.93	58	2.46	0	0.05	
07.09.93	64	2.19	0	0.05	
09.09.93	73	2	0.004	0.05	
07.10.93	103	1.76	0.004	0.04	
21.10.93	117	1.76	0	0.03	
04.11.93	131	1.19	0	0.05	
15.12.93	171	0.81	0.008	0.02	
DATE	TIME [d]	ACETIC	PROP.	BUTYRIC	VALERIC [mM]
25.06.93	0	0.61	0.06	0.18	0.1
27.06.93	2	0.61	0.2	0.47	0.4
29.06.93	4	1.05	0.31	0.57	0.44
06.07.93	11	1.02	0.32	0.52	0.35
13.07.93	18	1.3	0.26	0.45	0.3
19.07.93	24	1.41	0.33	0.43	0.42
30.07.93	35	1.41	0.35	0.41	0.27
11.08.93	46	1.4	0.36	0.43	0.27

DATE	TIME	ACETIC	PROP	BUT	VAL
13.08.93	48	1.4	0.36	0.41	0.27
0.5% TRYPT-BROTH FROM 13.08.93 - 16.08.93					
16.08.93	51	0.05	0.04	0.03	0.02
18.08.93	51	0.36	0.08	0.13	0.08
21.08.93	53	0.25	0.08	0.2	0.13
23.08.93	56				
29.08.93	58				
07.09.93	64				
09.09.93	73	1.61	0.23	0.5	0.22
07.10.93	103				
21.10.93	117	7.03	1.03	1.9	1.11
04.11.93	131				
15.12.93	171				

Data to Fig.13 and Fig.14:

DATE	TIME [d]	OCP [mM]	PHENOL [mM]	
09.07.94	0	4	0	
11.07.94	2	3.76	0.11	
12.07.94	3	3.28	0.11	
14.07.94	5	3.28	0.15	
INCUBATION IN NUTRIENT BROTH				
18.07.94	9	4.32	0.08	
22.07.94	13	2.24	0.05	
INCUBATION IN NUTRIENT BROTH				
16.08.94	37	3.2	0	
16.08.94	37.5	3	0.15	
23.08.94	45	2.88	0.08	
29.09.94	80	1.92	0.01	ACETIC [mM]
INCUBATION IN NUTRIENT BROTH				
06.10.94	88	3.2	0	0.15
06.10.94	88.5	3.2	0	0.4
08.10.94	90	1.6	0.04	0.13
11.10.94	93	1.84	0.04	0.5
14.10.94	96	1.12	0.04	0.67
16.10.94	98	1.24	0.03	0.67
24.10.94	106	1.04	0.04	2
07.12.94	150	0.96	0	2.33

Data to Fig.15:

TIME [d]	PHENOL	[mM]
	CULTURE 1	CULTURE 2
0	5.6	5
2	5.6	5
4	3.6	4.3
7	5	6
7.5	5	6
20	5	6
28	5	1.8
35	3	1.3
37	3	1.5
47	2.4	1.1

Data to Fig.16:

DATE	TIME [d]	OCP [mM]	PHENOL [mM]
09.07.94	0	3.36	0.04
11.07.94	2	3.2	0.06
12.07.94	3	2.4	0.02
14.07.94	5	2.24	0.12
INCUBATION IN NUTRIENT BROTH			
18.07.94	9	3.6	0.06
22.07.94	13	1.92	0.05
INCUBATION IN NUTRIENT BROTH			
16.08.94	37	2.56	0
16.08.94	37.5	2.4	0.05
23.08.94	45	2.24	0.02
29.09.94	80	1.6	0.04

Data to Fig.17:

TIME [d]	OCP [mM]		PHENOL [mM]	
	No Sulphate	Sulphate	No Sulphate	Sulphate
0	1.92	2.44	0	0
2	1.6	2.24	0.69	1.36
5	1.44	2.08	0.63	1.24
11	1.52	2	0.71	1.3
39	1.36	1.52	0.68	1.2

Data to 3.4.4 OCP-Degradation, Sulphate, and *Desulfobacterium phenolicum*:

D. phenolicum incubating in OCP-medium at 25° C:

Time [d]	OCP [mM]	Phenol [mM]
0	1.0	0
1	0.8	0
2	0.8	0
4	0.8	0
7	0.9	0
14	0.8	0

D. phenolicum incubating in nutrient broth with anaerobic dehalogenating mixed culture of microorganisms at 25° C:

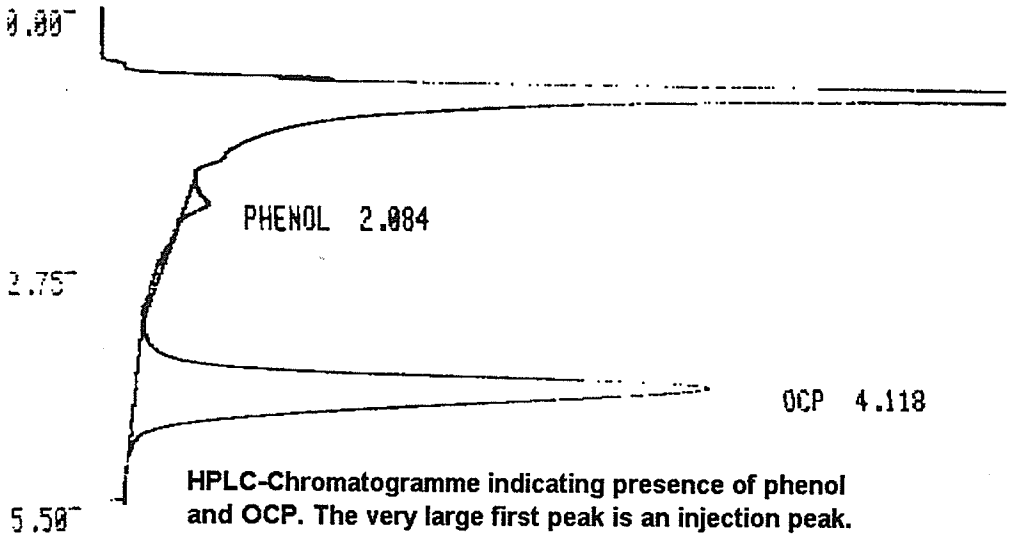
Time [d]	OCP [mM]	Phenol [mM]
0	2.5	.03
1	2.4	.05
2	2.4	.05
5	2.3	.04
8	2.3	.04
14	2.4	.03

Data to Fig.18:

1% YEAST EXTRACT AND TRYPTICASE			
DATE	TIME [d]	OCP [mM]	PHENOL [mM]
10.02.93	0	4.56	0
10.02.93	0.3	4.08	0.18
11.02.93	0.9	4	0.8
12.02.93	2	3.44	1.5
14.02.93	4	3.28	1.42
16.02.93	6	3.08	1.56
18.02.93	8	3.2	1.6
20.02.93	10	2.88	1.5
0.1% YEAST EXTRACT AND TRYPTICASE			
DATE	TIME [d]	OCP [mM]	PHENOL [mM]
10.02.93	0	3.92	0.08
10.02.93	0.3	3.92	0.06
11.02.93	0.9	3.92	0.19
12.02.93	2	3.84	0.2
14.02.93	4	3.8	0.19
16.02.93	6	3.72	0.18
18.02.93	8	3.32	0.16
20.02.93	10	3.2	0.18

0.02% YEAST EXTRACT AND TRYPTICASE			
DATE	TIME [d]	OCP [mM]	PHENOL [mM]
10.02.93	0	3.92	0.04
10.02.93	0.3	3.84	0.04
11.02.93	0.9	3.92	0.07
12.02.93	2	4	0.07
14.02.93	4	3.92	0.06
16.02.93	6	3.76	0.07
18.02.93	8	3.76	0.06
20.02.93	10	4	0.09
A SECOND OF THREE 0.02% YE/TRYPT CULTURES			
THAT WAS NOT PLOTTED			
DATE	TIME [d]	OCP [mM]	PHENOL [mM]
10.02.93	0	4.08	0.09
10.02.93	0.3	3.76	0.05
11.02.93	0.9	3.76	0.08
12.02.93	2	3.76	0.08
14.02.93	4	4.08	0.08
16.02.93	6	3.92	0.08
18.02.93	8	3.52	0.08
20.02.93	10	3.52	0.08

SAMPLE 990



HPLC-Chromatogramme indicating presence of phenol and OCP. The very large first peak is an injection peak. The numbers on the left indicate retention times of the compounds in minutes after injection.