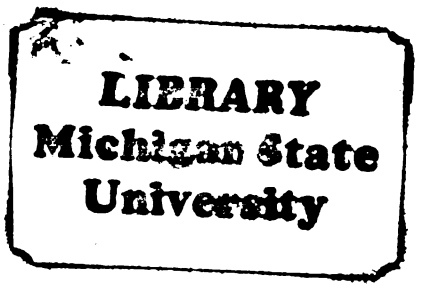


This is to certify that the

dissertation entitled

ANAEROBIC BIODEGRADATION OF CHLORINATED AROMATIC COMPOUNDS WITH SPECIAL EMPHASIS ON THE UNIQUE MICROORGANISMS OF THE DECHLORINATING COMMUNITIES

presented by

DANIEL ROLAND SHELTON

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Crop and Soil Sciences

James M. Tiedje
Major professor

Date Oct. 14, 1983

ANAEROBIC BIODEGRADATION OF CHLORINATED AROMATIC COMPOUNDS WITH SPECIAL EMPHASIS ON THE UNIQUE MICROORGANISMS OF THE DECHLORINATING COMMUNITIES

by

Daniel Roland Shelton

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Crop and Soil Sciences

1983

ABSTRACT

ANAEROBIC BIODEGRADATION OF CHLORINATED AROMATIC COMPOUNDS WITH SPECIAL EMPHASIS ON UNIQUE MICROORGANISMS OF THE DECHLORINATED COMMUNITIES

by

DANIEL ROLAND SHELTON

A methanogenic consortium able to use 3-chlorobenzoate as sole energy and carbon source was enriched from anaerobic sewage sludge. From this enrichment I isolated one dechlorinating bacterium, one benzoate-degrading bacterium, two butyrate-degrading bacteria, two H_2-utilizing methanogens and two sulfate reducers.

The dechlorinating bacterium (DCB-1) is a gram-negative, obligate anaerobe with a unique "collar" surrounding the cell. The only substrate found to support growth of the bacterium was pyruvate. Clarified rumen fluid or a complex protein source were required as a nutritional supplement. Acetate (and presumably CO_2) were the only fermentation products produced from pyruvate. The bacterium grew with a generation time of 4 to 5 days. 3-Chlorobenzoate was dechlorinated stoichiometrically to benzoate which accumulated in the medium. The rate of dechlorination was approximately 0.1 pmoles bacterium^{-1}day^{-1}.

Both butyrate-degrading bacteria are obligate anaerobes which grew only in the presence of H_2-utilizing methanogens on sulfate reducers. One isolate (NSF-1) is gram-negative and does not form spores. The substrates observed to support growth were butyrate, 2-methylbutyrate, valerate, and caproate. The maximum generation time, was 10 h. The second isolate (SF-1) is dissimilar in that it forms heat resistant spores in co-culture with a methanogen and is gram-positive. The substrates observed to support growth were butyrate, isobutyrate,

2-methylbutyrate, valerate and caproate. The maximum time was 9 h. Fermentation products of both strains were acetate and CH_4 (from butyrate and caproate) or acetate, propionate, and CH_4 (from 2-methylbutyrate and valerate) when grown in co-culture with M. hungatii. Nitrate and sulfate were not reduced by either bacterium.

The benzoate-degrading bacterium (BZ-2) is a gram negative, obligate anaerobe which grew only in the presence of a H_2-utilizing methanogen or a sulfate reducer. The cells appear to have a wavy outer membrane. Benzoate was the only substrate observed to support growth and it was fermented to acetate, CH_4 and either CO_2 or formate when grown in co-culture with M. hungatii. Butyrate or other volatile fatty acids were not detected as intermediates during benzoate metabolism.

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. James Tiedje for serving as my major professor, and, in particular, for his patience and continued financial support for the past 5 1/2 years. I would also like to thank the members of my committee: Dr. Lee Jacobs, Dr. John Breznak, and Dr. Matt Zabik.

I owe a special thanks to all of my fellow researchers in the lab who it has been my privilege to know and to work with. I would especially like to thank Joe Robinson, Alan Sexstone, Joe Suflita, Tim Parkin and Daryl Dwyer for just being themselves in their own individual, inemitable style; they made it all bearable.

Last, but not least, I owe a deep debt of gratitude to all the student technicians, without whose help none of this could have been completed. In chronological order: Mary Lou Krumme, Steve Aldrich, Susan Frazier, Marilyn Boucher, Wally Smolenski, and Terri Aust. It has, indeed, been my good fortune to have had the opprotunity to share a small part of my life with each of them, and I wish them all only the best.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

BIODEGRADATION OF CHLORINATED COMPOUNDS IN ANAEROBIC HABITATS

The fate of pollutants (or xenobiotics) in the environment has become a major concern since the realization that many of these compounds adversely affect the flora and fauna, including human health. As a result, numerous studies have been conducted on the degradation or persistence of a wide variety of xenobiotics in soils, natural waters, and in the presence of microbial cultures, but almost always under aerobic conditions. By comparison, few studies have examined the fate of pollutants in anaerobic habitats. The reasons for this lack of interest are probably two fold: 1)most habitats receiving pollutants are considered to be primarily aerobic,i.e., agricultural soils, river and lake waters, and groundwaters; and 2) the metabolic diversity of anaerobic microorganisms (or facultative organisms growing under anaerobic conditions) is considered to be severely limited, particularly in their ability to degrade or transform most xenobiotics. Although both of these reasons argue strongly for a need to understand the fate of pollutants in aerobic habitats they are not adequate justification for ignoring the fate of these compounds in anaerobic habitats.

While most receiving habitats are predominantly aerobic, most also experience transient periods of anaerobiosis; this is particularly true for poorly drained soils and sediments of polluted waters where the rate of oxygen consumption can easily exceed the rate of oxygen diffusion. Transformations which could occur during periods of anaerobiosis may

render some compounds more susceptible to subsequent aerobic transformation. The emphasis on receiving habitat also ignores the fact that many pollutants may be transported to anaerobic sites such as anaerobic sewage digestors (where treatment of essentially all hydrophobic materials entering sewage treatment plants occurs), or through biomagnification to the gastrointestinal tract of higher animals, including man, or eventually to freshwater or estuarine sediments, the final recipient site of many pollutants.

The belief that the metabolic diversity of anaerobes is severely limited is based on the assumption that molecular oxygen is required for degradation of compounds not directly related to intermediary metabolism. Without question, molecular oxygen in the activated state is extremely reactive and facilitates breaking a variety of bond types. Many aerobes have evolved the capacity to harness the reactivity of molecular oxygen (via mono-and di-oxygenases) such that the safe and controlled oxidation of numerous compounds is possible. This allows a variety of natural and synthetic compounds to be funneled into intermediary metabolism for energy and cell carbon.

The importance of molecular oxygen in aerobic metabolism does not preclude the evolution of essentially different metabolic strategies in anaerobes which do not require molecular oxygen. This is precisely what has occurred for aromatic compounds. Definitive evidence for the anaerobic mineralization of benzoic acid by a methanogenic consortium (50), a denitrifier (67), and a photosynthetic bacterium (16) was presented in the late 60's. Since then a variety of substituted aromatic compounds have been shown to be susceptible to degradation by methanogenic consortia (11, 12, 28, 29, 33, 61), denitrifying consortia

(4), and by pure cultures (37, 54, 62, 68). The pathway of benzoate catabolism has been studied in several laboratories and the general pathway appears to follow the scheme: i) reduction of the aromatic ring, ii) beta oxidation of the ring to form 2-oxocyclohexane carboxylate, iii) cleavage of the ring by hydrolysis, and iv) metabolism of the resultant volatile fatty acids via beta-oxidation (18). Hence a reductive pathway, with oxygen arising exclusively from water, accomplishes complete aromatic degradation. This is in contrast to aerobic pathways where incorporation of hydroxyl groups into the ring and subsequent ring cleavage are accomplished with molecular oxygen (14). The author is unaware of any reports of the anaerobic mineralization of aromatic compounds not having at least one carboxyl or hydroxyl substituent, although Hooper (32) has shown that the oxidation of the methyl moiety of p-cresol can be accomplished with water by a pseudomonad growing aerobically. If anaerobes possess similiar capabilities, they remain to be discovered.

The transformation of numerous xenobiotic compounds in a variety of anaerobic habitats, such as flooded soils, digested sludge, anoxic sediments,rumen fluid, and the gastrointestinal tract of mammals has been reported. The types of transformations observed include: hydrolysis of phosphate esters, hydrolysis of aliphatic esters, dealkylation of O-alkyl and N-alkyl compounds, deamination, reduction of nitro groups, and dehalogenation. Several excellent reviews are available which catalogue the majority of known anaerobic transformations (17, 42, 57, 58, 72). The majority of these transformations, however, are not particularly unique to anaerobic microorganisms, but appear to be fairly universal in all habitats. One

very important exception is reductive dehalogenation, which appears to be relatively facile and unique to anaerobic habitats.

In the absence of oxygen, electron acceptors must be found since catabolism requires the constant regeneration of intracellular electron carriers. Anaerobic bacteria have developed the ability to utilize a variety of inorganic electron acceptors such as NO_3^- (denitrifiers, dissimilatory nitrate reducers), SO_4^{2-} (sulfate reducers), or CO_2 (methanogens, acetogens), and organic compounds such as pyruvate, fumarate, or protons (collectively termed fermenters). Depending on the anaerobic habitat, different groups of anaerobic bacteria are likely to predominate: denitrifiers, facultative anaerobic fermenters, and spore-forming fermenters in flooded and anaerobic soils; obligate anaerobic fermenters and methanogens in digested sludge and rumen fluid; facultative and obligate anaerobic fermenters, sulfate reducers and/or methanogens in anoxic sediments; and facultative and obligate anaerobic fermenters in the gastrointestinal tract. As a result of the wide variety of anaerobic habitats and the microorganisms which predominate in them, generalizations are difficult to make regarding anaerobic transformations or the significance of individual groups of anaerobes involved. From the standpoint of reductive dehalogenation, however, the most important class of anaerobic bacteria probably are the fermenting bacteria (obligate and facultative). The reason for this is that reductive dehalogenation appears to be essentially co-metabolic in nature. Just as many aerobes possess oxygenase enzymes which are relatively nondescriminating, fermenting anaerobes also appear to be relatively nondescriminating with respect to where they will dump electrons. Those anaerobic bacteria which use inorganic molecules as

electron acceptors, although important with respect to other anaerobic transformations, appear to be less frequently implicated as the causal agents of reductive dehalogenation. The physical manifestation of an active population of fermenting bacteria is a low redox potential, due to the abundance of electrons. As a result, reductive reactions such as dehalogenation are particularly facile under anaerobic conditions and are relatively unique to such habitats.

The most widely studied halogenated compound in anaerobic habitats has been DDT [1,1,1-tricloro-2,2-bis(p-chlorophenyl)ethane]. DDT has been observed to be dechlorinated to DDD [1,1-dichloro-2,2-bis(p-chlorophenyl)ethane] in anaerobic soils (22, 23, 24, 25, 41, 51, 52), lake water (48), anaerobic sewage sludge (30, 74), rumen fluid (20, 48), the gastrointestinal tract of rats (5, 13, 47), and in pure culture by numerous facultative and obligate anaerobes (5, 38, 47, 55, 63, 70, 71). In almost all of these habitats DDD accumulated as the primary product; however Wedemeyer (71), working with cell free extracts of Aerobacter aerogenes (no standing in nomenclature), has reported the formation of other dechlorinated products: DDMU [1-cloro-2,2-bis(p-chlorophenyl)ethylene] and DDNU [unsym-bis(p-chlorophenyl)ethylene]. DDT is considered to be fairly recalcitrant under aerobic conditions, although degradation of DDT by a species of Hydrogenomonas isolated from sewage sludge has been reported (19).

Insight into the mechanism of dechlorination of DDT was provided by Miskus et.al. (48) with their demonstration that DDT could be reductively dechlorinated to DDD in the presence of hemoglobin (an electron carrier) and sodium dithionite (a reducing agent). Parr and

Smith (52) and Glass (22), who monitored Eh in their soils, observed that the rate of dechlorination increased with decreasing Eh, such that an Eh of -250 mv was required for significant rates of transformation. This finding is consistent with the observations of several workers who showed that autoclaving causes a loss of activity while addition of carbon accelerates the rate of dechlorination. Any treatment which would accelerate the rate and extent of the Eh drop would accelerate dechlorination while treatments which inhibited its reduction (aeration, sterilization) would slow or inhibit dechlorination.

The most definitive work on the mechanism of DDT degradation was performed by Zoro et.al. (74). They demonstrated that anaerobic sewage sludge rapidly dechlorinated DDT to DDD; if sludge was aerated or autoclaved activity was lost. However, if dithionite was added to either the aerated or autoclaved sludge, activity was restored. Similiar experiments were performed with pure cultures of ten different genera. Cells grown to stationary phase were active in dechlorination only if dithionite had been added to the culture; the extent of dechlorination was increased if the cells had been heat killed prior to the incubation. Zoro et al. also demonstrated the ability of iron porphyrins to reductively dechlorinate DDT in the presence of sodium dithionite. This conclusively demonstrated the chemical nature of the reductive dechlorination of DDT. This data is consistent with numerous observations that biological activity is required in order for dechlorination to occur, since the required reduced electron carriers would be generated as a result of the activity of the fermenting bacteria.

Another insecticide which is rapidly dechlorinated under anaerobic

conditions is lindane (hexachlorocyclohexane---the commercial formulations contain various isomers of hexachlorocyclohexane, however the predominant isomer and the one with the highest insecticidal activity is the γisomer). Lindane has been observed to be degraded in anaerobic soils (25, 44, 45, 46, 56, 60, 69, 73), anaerobic sewage sludge (30), and in mixed and pure cultures (6, 26, 35, 43, 57). By comparison, lindane is considered to be extremely persistent under aerobic conditions. As with DDT, researchers have observed that degradation of lindane is inhibited by sterilization (43, 44, 45, 58) and stimulated by increased organic matter (60, 73). Siddaramappa and Sethunathan (60) observed that insignificant losses of lindane occured at positive Eh's; only after the Eh had decreased to below 0 mv did significant degradation occur.

The most enlightening experiments have been performed with pure cultures. Workers in several laboratories have demonstrated the ability of pure cultures to dechlorinate lindane. MacRae et.al (43) and Sethunathan et al. (58) both isolated Clostridia sp. from soil capable of dechlorinating lindane. Work by Jagnow et.al. (35) has demonstrated the ability of several bacteria to degrade lindane, the most active being Clostridium butyricum, C. pasteurianum, and Citrobacter freundii (in stationary phase). Based on Cl^- recoveries in the medium, the molecule was almost completely dechlorinated. Near 100% recovery of chlorine along with the observation that the ring was not protonated would suggest benzene as the probable product; $^{14}CO_2$ was not detected. This data is consistent with the work of Beland et.al. (7) who observed benzene as a product of lindane degradation in anaerobic sewage sludge. Jagnow et. al. (35) also observed that significant dechlorination only

occurred with those organisms known to produce hydrogen as a fermentation product. The possibility that chemical dechlorination may occur as a result of electron flow through the iron sulfur proteins involved in proton reduction is intriguing. However, in the absence of definitive evidence, as with DDT, it can only be postulated.

Other compounds for which a reductive mechanism has been observed, and where a possible chemical mechanism may exist include mirex (1), toxaphene (15, 53), and hexachlorocyclopentadiene (36). Aldrin, endrin, and heptachlor have also been observed to be degraded in anaerobic soil (25) or digested sludge (30); however, since products were not identified, it can only be speculated that reductive dechlorination occurred.

The mechanism of chemical reductive dechlorination has been the focus of research of several investigators (6, 7, 31, 39). Khalifa et.al. (39) and Holmstead (31) have studied reductive dechlorination of toxaphene and mirex, respectively, in an aqueous solution containing dithionite (as reductant), hematin (electron carrier), and ethanol (solubilizer). In each case, rapid dechlorination occurred with approximately half of the chlorine atoms cleaved from toxaphene, and one to four chlorine atoms lost from mirex. In each case there was no reductive dechlorination in the absence of dithionite or hematin. Beland et.al.(7) studied the electrochemical reduction of lindane at a mercury coated platinum electrode, and the degradation of lindane in anaerobic sewage and soil. They observed qualitatively similiar results in that BTC (γ3,4,5,6-tetrachlorocyclohexene) and benzene were observed as products in both systems. Geer (21) tested the hypothesis that anaerobic degradation could be correlated with redox potential of

chlorinated hydrocarbons relative to an SCE electrode. Although his data did not entirely support this hypothesis, he did conclude that reduced iron porphyrins mediated certain dechlorination reactions, such as of mirex and methoxychlor. His work is particularly useful in that initial redox potentials by interrupted sweep voltammetry were determined for a number of chlorinated organic compounds. Based on the data of Geer and others regarding which chlorinated compounds are known to be degraded, it may be possible to establish a relative ranking of compounds which are likely to be dechlorinated vs. those that are not. Geer (21) points out that care should be taken in interpreting data from model studies, since the effective Eh may be more negative than in biological systems (e.g., the Eh of active anaerobic sludge is approximately -500 mv).

Another group of pollutants which have recently come under investigation are the one and two carbon halogenated hydrocarbons. Bouwer et.al. (8, 9, 10) have studied the degradation of chloroform, carbon tetrachloride, various bromo- and chlorobromomethanes, 1,2-dichloroethane, 1,1,1-trichloroethane, trichloroethylene, 1,1,2,2-tetrachloroethane, and tetrachloroethylene in anaerobic sewage sludge and sewage sludge plus NO_3^- (to select for a population of denitrifying bacteria). In anaerobic sewage sludge, they observed the reductive dechlorination of 1,1,2,2-tetrachloroethane to 1,1,2-trichloroethane and tetrachloroethylene to trichloroethylene; chloroform, carbon tetrachloride, and 1,2-dichloroethane were mineralized to CO_2. The bacteria responsible for these reactions are not known. The various bromo and chlorobromomethanes were observed to lose bromine atoms in the sterile controls, indicating a purely chemical

mechanism for debromination. Particularly intriguing is the observation that chloroform, carbon tetrachloride, and 1,2-dichloroethane were oxidized almost completely to CO_2; CH_4 was not a product. Metabolism of these chlorinated compounds appears to be unique to methanogenic habitats. When incubations were done with sewage sludge plus NO_3^-, only carbon tetrachloride was degraded; chloroform was observed as an intermediate.

The majority of studies in which reductive dechlorination has been observed have focused on chlorinated saturated hydrocarbons. Considerably less is known concerning the fate of halogenated aromatic compounds. Ide et.al. (34) were the first investigators to report the reductive dechlorination of an aromatic compound, pentachlorophenol (PCP) in anaerobic paddy soil. They observed the accumulation of several isomers of tetra-, tri-, and di-chlorophenols, and m-chlorophenol. Murthy et.al. (49) also observed the accumulation of tetra- and tri-chlorophenols from PCP in anaerobic soil. Hakulinen and Salkinoja-Salonen (28) have reported the degradation of several chlorinated phenols (including PCP), catechols, and guaiacols in an anaerobic fluidized bed, however, based on more recent work, it is not clear that this was a strictly anaerobic process (personal communication).

Recently, Boyd et.al. (11) and Boyd and Shelton (12) have investigated the fate of isomers of mono- and di-chlorophenols in anaerobic sewage sludge. They observed the rapid loss of the ortho chlorine from o-chlorophenol in a very active sludge (diluted to 10% with an anaerobic mineral salts medium), yielding phenol and presumably HCl. Meta and p-chlorophenol were also degraded but at a significantly

slower rate with lag times > 6 weeks. Experiments with fresh undiluted sludge were consistent with previous results; o-chorophenol was degraded without a lag while m- and p-chlorophenol were degraded only after a lag of 3 weeks. Also, the ortho chlorine of dichlorophenol isomers (2,3-, 2,4-, 2,5-, 2,6-) was lost preferentially, leaving the corresponding monochlorophenols which were degraded after a lag of > 4 weeks. However, in aged sludge acclimated to the degradation of individual monochlorophenols, the pattern of degradation was significantly different. When sludges acclimated to o-, m-, or p-chlorophenol were fed dichlorophenols with chlorines in the 2 or 6 position, loss of the ortho chlorine occurred slowly, if at all. Apparently the ability to dechlorinate at the ortho position was lost with time. The only exception was sludge acclimated to 2,4,6-trichlorophenol (the substrate was consistently introduced in ethanol solution as opposed to neat) where the ortho dechlorinating activity was maintained. Boyd and Shelton interpreted this as suggestive evidence that dechlorination at the ortho position may be essentially chemical in nature while dechlorination at the meta and para positions appears to be enzymatic in nature.

Another class of aromatic compounds, the halosubstituted benzoates have also recently come under investigation. The reductive dehalogenation of several substituted benzoates has been reported by Horowitz et al. (33) and Suflita et al. (64). It was initially observed that 4-amino,3,5-dichlorobenzoate was dechlorinated to 4-amino,3-chlorobenzoate in anoxic sediments of a hypereutrophic lake after a lag of 30 weeks. Subsequently, other substituted benzoates were also observed to be degraded: 3-iodo-, 2-,3-, and 4-bromo-, 3-chloro-,

3-chloro-4-hydroxy-, 3,5-dichloro-, 3,4-dichloro-, 2,3,6-trichloro-, and 3-amino,2,5-dichlorobenzoate; lag times varied from < 1 week to 30 weeks. In general, sediments appear capable of removing bromine at any position while chlorine is removed from only the meta position; fluoro substituted benzoates were not degraded. Sterilized controls with sediment acclimated to the degradation of 4-amino,3,5-dichlorobenzoate indicated that this activity had a biological component, and that titanium citrate (a reductant) could not restore activity to sterilized cultures.

Shelton and Tiedje (61) observed the mineralization of 3-chlorobenzoate in 10% sewage sludge. This activity was enriched for over two years and a stable methanogenic consortium was obtained. The enriched consortium was observed to have activities identical to sediment with the exception that 4-amino-3,5-dichlorobenzoate was degraded to 4-aminobenzoate.

Based on experiments with acclimated sediment and the enriched consortium, the dechlorination reaction appears to be enzymatic. The fact that considerable lag times are observed before degradation occurs (in the presence of active methanogenesis), the observation of saturation kinetics (65), the observation that 3,5-diclorobenzoate inhibits dechlorination of 3-chlorobenzoate (65), and the specificity of dechlorination for the meta position, all argue strongly for an enzymatic mechanism.

Taylor et al. (66) have reported the defluoronation of benzoate by a pseudomonad growing anaerobically with nitrate. Cell suspensions grown with p-hydroxybenzoate were able to degrade o- and p-fluorobenzoate under anaerobic (but not aerobic) conditions;

m-fluorobenzoate or monochlorobenzoates were not degraded. Based on their data it is not possible to conclude at which point in the pathway of benzoate catabolism defluoronation occurred. However, based on experiments with chloramphenicol, the activity appeared to be enzymatic.

Reports of dechlorination of other aromatic compounds are known. Attaway et al. (2, 3) have observed the dechlorination of diuron in pond sediments previously treated with this herbicide. In this case, the chlorine in the para position was cleaved. Suflita has observed dechlorination of the para chlorine of 2,4,5-T by the 3-chlorobenzoate degrading enrichment (personal communication). Kirkpatrick et al. (40) have reported thereductive dehalogenation of techlofthalam to two or more monodehalogenated products in flooded rice paddy soil.

In conclusion, as a result of the reducing conditions (low Eh) found in anaerobic habitats, certain significant transformations appear to be more facile in and unique to anaerobic environments. The most important of these is reductive dehalogenation, particularly of the chlorinated saturated hydrocarbons. Similar to aerobic dehalogenation, anaerobic dehalogenation also appears to be essentially co-metabolic in nature. In several cases an essentially chemical mechanism appears responsible for the dehalogenation. Some compounds are spontaneously dehalogenated (DDT) in the presence of reduced electron carriers (i.e. iron porphyrins) with the electrons provided by an active population of fermenting bacteria. Halogens may be replaced with hydrogen atoms or by electrons; specifically, iron porphyrins appear to mediate displacement of halogens with hydrogen, while iron sulfur proteins mediate displacement with electrons. The reasons for this are not entirely clear since both function as electron carriers, as opposed to hydrogen

carriers, in electron transport. Other dehalogenation reactions do not occur spontaneously, (e.g., 3-chlorobenzoate) but appear to require an enzyme catalyst.

It has been hypothesized that electrochemical reduction potentials for halogenated compounds can be used to predict their fate in anaerobic environments; specifically, their propensity to be reductively dehalogenated. In certain cases (e.g., DDT and lindane), theoretical predictions have been consistent with experimental results, although, in other instances, results have not been consistent. Geer (21) observed that compounds which were predicted to be susceptible to reductive dechlorination were not degraded in anaerobic sewage sludge. Conversely, compounds which were predicted to be recalcitrant, such as chlorinated phenols and benzoates, have been observed to be reductively dechlorinated. As a result, the relationship between measured electrochemical potential and the thermodynamics of these transformations in anaerobic environments is not clear.

A potential application for reductive dehalogenation may be in the anaerobic pretreatment of certain recalcitrant molecules to make them more susceptible to aerobic mineralization, as has been described by Hakulinen and Salkinoja-Salonen (27) for the treatment of chlorophenols. They describe a process in which bleaching effluent from kraft pulping (containing high concentrations of chlorophenols) are treated first in an anaerobic fluidized bed reactor and then in an aerobic trickling filter. With this combined treatment essentially all of the toxic chlorophenols from the final effluent were removed. Another potential application is the flooding and/or application of digested sludge to polluted sites contaminated with compounds susceptible to reductive

dehalogenation. Most of all, further research is needed in order to expand the range of compounds degraded and to increase our understanding of the mechanism(s) by which these processes occur.

In order to study the mechanism(s) by which reductive dechlorination occurs, it is necessary to have pure cultures of those organisms responsible. This is particularly true for the chlorinated aromatic compounds, since these dechlorinations appear to be enzymatic. The primary goal of my research was the isolation and characterization of the bacterium(ia) responsible for the degradation and mineralization of 3-chlorobenzoate, and to characterize the bacterial community in the food chain responsible for mineralizing the substrate.

LITERATURE CITED

1. Andrade Jr., P., W.B. Wheeler, and D. A. Carlson. 1975. Identification of a Mirex metabolite. Bull. Environ. Contam. Toxicol. 14:473-479.

2. Attaway, H.H., N.D. Camper, and M.J.B. Paynter. 1982. Anaerobic microbial degradation of diuron by pond sediment. Pest. Biochem. Physiol. 17:96-101.

3. Attaway, H.H., M.J.B. Paynter, and H.D. Camper. 1982. Degradation of selected phenylurea herbicides by anaerobic pond sediment. J. Environ. Sci. Health. B17:683-699.

4. Bakker, G. 1977. Anaerobic degradation of aromatic compounds in the presence of nitrate. FEMS Lett. 1:103-108.

5. Barker, P.S., F.O. Morrison, and R.S. Whitaker. 1965. Conversion of DDT to DDD by Proteus vulgaris, a bacterium isolated from the intestinal flora of a mouse. Nature. 205:621-622.

6. Beland, F.A., S.O. Farwell, and R.D. Geer. 1974. Anaerobic degradation of 1,1,1,2-tetrachloro-2,2-bis(p-chlorophenyl)-ethane (DTE). J. Agr. Food Chem. 22:1148-1149.

7. Beland, F.A., S. O. Farwell, A. E. Robocker, and R. D. Geer. 1976 Electrochemical reduction and anaerobic degradation of lindane. J. Agr. Food Chem. 24:753-756.

8. Bouwer, E.J., B.E. Rittman, and P.L. McCarty. 1981. Anaerobic degradation of halogenated 1-and 2-carbon compounds. Environ. Sci. Technol. 15:596-598.

9. Bouwer, E.J. and P.L. McCarty. 1983. Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Appl. Environ. Microbiol. 45:1286-1294.

10. Bouwer, E.J. and P.L. McCarty. 1983. Transformation of halogenated organic compounds under denitrification conditions. Appl. Environ. Microbiol. 45:1295-1299.

11. Boyd, S.A., D.R. Shelton, D. Berry, and J.M. Tiedje. 1983. Anaerobic biodegradation of phenolic compounds in digested sludge. Appl. Environ. Microbiol. 46:50-54.

12. Boyd, S.A. and D.R. Shelton. Anaerobic biodegradation of chlorophenols in digested sludge. (submitted to Appl. Environ. Microbiol.).

13. Braunberg, R.C. and V. Bech. 1968. Interaction of DDT and the gastrointestinal microflora of the rat. J. Agr. Food Chem. 16:451-453.

14. Chapman, P.J. 1979. Degradation mechanisms: from Proceeding of the workshop: Microbial degradation of pollutants in marine environments. ed. A.W. Bourquin and P.H. Prithcard. EPA-60019-79-012. p. 28-66.

15. Clark, J.M. and F. Matsumura. 1979. Metabolism of toxaphene by aquatic sediment and a camphor degrading pseudomonad. Arch. Environ. Contam. Toxicol. 8:285-298.

16. Dutton, P.L., and W.C. Evans. 1969. Metabolism of aromatic compounds by Rhodopseudomonas palustis. Biochem. J. 113:525-536.

17. Esaac, E.G. and F. Matsumura. 1980. Metabolism of insecticides by reductive systems. Pharmac. Ther. 9:1-26.

18. Evans, W.C. 1977. Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Nature (London) 270:17-22.

19. Focht, D.D. and M. Alexander. 1970. DDT metabolite and analogues: ring fission by Hydrogenomonas. Science. 170:91-92.

20. Fries, G.F., G.C. Marrow, and C.H. Gordon. 1969. Metabolism of o,p'- and p,p'-DDT by rumen microorganisms. J. Agr. Food Chem. 17:860-862.

21. Geer, R.D. 1978. Completion Report: Predicting the anaerobic degradation of organic chemical pollutants in waste water treatment plants from their electrochemical reduction behavior. U.S. Dept. of Commerce. National Technical Information Service. PB-289 224. 61 pages.

22. Glass, B.L. 1972. Relation between the degradation of DDT and the iron redox system in soils. J. Agr. Food Chem. 20:324-327.

23. Guenzi, W.D. and W.E. Beard. 1967. Anaerobic biodegradation of DDT to DDD in soil. Science. 156:1116-1117.

24. Guenzi, W.D. and W.E. Beard. 1968. Anaerobic conversion of DDT to DDD and aerobic stability of DDT in soil. Soil Sci. Soc. Amer. Proc. 32:522-524.

25. Guenzi, W.D., W.E. Beard, and F.G. Viets, Jr. 1971. Influence of soil treatment on persistence of six chlorinated hydrocarbon insecticides in the field. Soil Sci. Soc. Amer. Proc. 35:910-913.

26. Haider, K. and G. Jagnow. 1975. Abbau von ^{14}C-, ^{3}H- und ^{36}Cl-markietrtem v-hexachlorocyclohexan durch anaerobe Bodenmikroorganismen. Arch. Microbiol. 104:113-121.

27. Hakulinen, R. and M. Salkinoza-Salonen. 1982. Treatment of pulp and paper industry wastewaters in an anaerobic fluidised bed reactor. Process Biochem. March-April: 18-22.

28. Healy, Jr., J.B. and L.Y. Young. 1978. Catechol and phenol degradation by a methanogenic population of bacteria. Appl. Environ. Microbiol. 35:216-218.

29. Healy, J.B. and L.Y. Young. 1979. Anaerobic biodegradation of eleven aromatic compounds to methane. Appl. Environ. Microbiol. 35:216-218.

30. Hill, D.W. and P.L. McCarty. 1967. Anaerobic degradation of selected chlorinated hydrocarbon pesticides. J. Water Poll. Con. Fed. 39:1259-1277.

31. Holmstead, R.L. 1976. Studies of the degradation of myrex with an iron (II) porphyrin model system. J. Agr. Food Chem. 24:620-624.

32. Hooper, D.J. 1978. Incorporation of [^{18}O] water in the formation of p-hydroxybenzyl alcohol by the p-cresol methylhydroxylase from Pseudomonas putida. Biochem. J. 175:345-347.

33. Horowitz, A., J.M. Suflita, and J.M. Tiedje. 1983. Reductive dehalogenations of halobenzoates by anaerobic lake sediment microorganisms. Appl. Environ. Microbiol. 45:1459-1465.

34. Ide, A., Y. Niki, F. Sakamoto, I. Watanabe, and H. Watanabe. 1972. Decomposition of pentachlorophenol in paddy soil. Agr. Biol. Chem. 36:1937-1944.

35. Jagnow, G., K. Haider, and P. Ellwardt. 1977. Anaerobic dechlorination and degradation of hexachlorocyclohexane isomers by anaerobic and facultative anaerobic bacteria. Arch. Microbiol. 115:285x-292.

36. Johnson, L.D. and J.C. Young. Inhibition of anaerobic digestion by organic priority pollutants. Proc. Pes. Symp., 54th Annu. Water Pollut. Control Fed. Conf., Detroit, Mich., Oct. 4-9, 1981.

37. Kaiser, J.P. and K.W. Hanselmann. 1982. Fermentative metabolism of substituted monoaromatic compounds by a bacterial community from anaerobic sediments. Arch. Microbiol. 133:185-194.

38. Kallman, B.J. and A.K. Andrews. 1963. Reductive dechlorination of DDT to DDD by yeast. Science. 141:1050-1051.

39. Khalifa, S., R.L. Holmstead, and J.E. Casida. 1976. Toxaphene degradation by iron (II) protoporphyrin systems. J. Agr. Food Chem. 24:277-282.

40. Kirkpatrick, D., S.R. Biggs, B. Conway, C.M. Finn, D.R. Hawkins, T. Honda, M. Ishida, and G.P. Powell. 1981. Metabolism of N-(2,3-dichlorophenyl)-3,4,5,6-tetrachlorophthalamic Acid (Techlofthalam) in paddy soil and rice. J. Agr. Food Chem. 29:1149-1153.

41. Ko, W.H. and J.L. Lockwood. 1968. Conversion of DDT to DDD in soil and the effect of these compounds on soil microorganisms. Can. J. Microbiol. 14:1069-1073.

42. Lal, R. and D.M. Saxena. 1982. Accumulation, metabolism, and effects of organochlorine insecticides on microorganisms. Microbiol. Rev. 46:95-127.

43. MacRae, I.C., K. Raghu, and E.M. Bautista. 1969. Anaerobic degradation of the insecticide lindane by Clostridium sp. Nature. 221:859-860.

44. MacRae, I.C., K. Raghu, and T.F. Castro. 1967. Persistence and biodegradation of four common isomers of benzene hexachloride in submerged soils. J. Agr. Food Chem. 15:911-914.

45. Mathur, S.P. and J.G. Saha. 1975. Microbial degradation of lindane-^{14}C in a flooded sandy loam soil. Soil 120:301-307.

46. Mathur, S.P. and J.G. Saha. 1977. Degradation of lindane-^{14}C in a mineral soil and in an organic soil. Bull. Environ. Contam. Toxicol. 17:424-430.

47. Mendel, J.L. and M.S. Walton. 1966. Conversion of p,p'DDT by intestinal flora of the rat. Science. 151:1527-1528.

48. Miskus, R.P.,D.P. Blair, and J.E. Casida. 1965. Conversion of DDT to DDD by bovine rumen fluid, lake water, and reduced porphyrins. J. Agr. Food Chem. 13:481-483.

49. Murthy, N.B.K., D.D. Kaufman, and G.F. Fries. 1979. Degradation of pentachlorophenol (PCP) in aerobic and anaerobic soil. J. Environ. Sci. Health. B14-1-14.

50. Nottingham, P.M. and R.E. Hungate. 1969. Methanogenic fermentation of benzoate. J. Bacteriol. 98:1170-1172.

51. Parr, J.F., G.H. Willis, and S. Smith. 1970. Soil anaerobiosis: effect of selected environments and energy sources on the degradation of DDT. Soil Sci. 110:306-312.

52. Parr, J.F. and S. Smith. 1974. Degradation of DDT in an everglades muck as affected by lime, ferrous iron, and anaerobiosis. Soil Sci. 118:45-52.

53. Parr, J.G. and S. Smith. 1976. Degradation of toxaphene in selected anaerobic soil environments. Soil Sci. 121:52-57.

54. Patel, T.R., K.G. Jure, and G.A. Jones. 1981. Catabolism of phloroglucinol by the rumen anaerobe Coprococcus. Appl. Environ. Microbiol. 42:1010-1017.

55. Plimmer, J.R., P.C. Kearney, and D.W. Von Endt. 1968. Mechanism of conversion of DDT to DDD by Aerobacter aerogenes. J. Agr. Food Chem. 16:594-597.

56. Raghu, K. and I.C. MacRae. 1966. Biodegradation of the gamma isomer of benzene hexachloride in submerged soils. Science. 154:263-264.

57. Scheline, R. R. 1973. Metabolism of foreign compounds by gastrointestinal microorganisms. Pharmacol. Rev. 31:471-480.

58. Sethunathan, N., E.M. Bautista, and T. Yoshida. 1969. Degradation of benzene hexachloride by a soil bacterium. Can. J. Microbiol. 15:1349-1354.

59. Sethunathan, N. 1973. Microbial degradation of insecticides in flooded soils and in anaerobic cultures. Residue Reviews 47:143-165.

60. Siddaramappa, R. and N. Sethunathan. 1975. Persistence of gamma-BHC and beta-BHC in Indian rice soils under flooded conditions. Pestic. Sci. 6:395-403.

61. Shelton, D.R. and J.M. Tiedje. Development and validation of a test method for determining anaerobic biodegradation potential (submitted to Appl Environ. Microbiol.).

62. Shink, B. and N. Pfennig. 1982. Fermentation of trihydroxybenzenes by Pelobacter acidgallici gen. nov. sp. nov., a new strictly anaerobic, non-sporeforming bacterium. Arch. Microbiol. 133:195-201.

63. Stenerson, J.H.V. 1965. DDT-metabolism in resistant and susceptible stable-flies and in bacteria. Nature 207:660-661.

64. Suflita, J.M., A. Horowitz, D.R. Shelton, and J.M. Tiedje. 1982. Dehalogenation: a novel pathway for the anaerobic biodegradation of haloaromatic compounds. Science. 218:1115-1117.

65. Suflita, J.M., J.A. Robinson, and J.M. Tiedje. 1983. Kinetics of microbial dehalogenation of haloaromatic substrates in methanogenic environments. Appl. Environ. Microbiol. 45:1466-1473.

66. Taylor, B.F., W.L. Hearn, and S. Pincus. 1979. Metabolism of monofluoro- and monochlorobenzoates by a denitrifying bacterium. Arch. Microbiol. 122:301-306.

67. Taylor, B.F., W.L. Campbell, and I. Chinoy. 970. Anaerobic degradation of the benzene nucleus by a facultative anaerobic microorganism. J. Bacteriol. 102:430-437.

68. Tsai, C.G. and G.A. Jones. 1975. Isolation and idenitrification of rumen bacteria capable of anaerobic phloroglucinol degradation. Can. J. Microbiol. 21:794-801.

69. Tsukano, Y. and A. Kobayaski. 1972. Formation of γ-BTC in flooded rice field soils treated with γ-BHC. Agr. Biol. Chem. 36:166-167.

70. Wedemeyer, G. 1966. Dechlorination of DDT by Aerobacter aerogenes. Science. 152:647.

71. Wedemeyer, G. 1967. Dechlorination of 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane by Aerobacter aerogenes. Appl. Microbiol. 15:569-574.

72. Williams, P.P. 1977. Metabolism of synthetic organic pesticides by anaerobic microorganisms. Residue Reviews. 66:63-135.

73. Yoshida, T. and T.F. Castro. 1970. Degradation of gamma-13HC in rice soils. Soil Sci. Soc. Amer. Proc. 34:440-442.

74. Zoro, J.A., J.M. Hunter, G. Eglinton, and G.C. Ware. 1974. Degradation of p,p-DDT in reducing environments. Nature. 247:235-236.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF AN ANAEROBIC BACTERIUM THAT DECHLORINATES 3-CHLOROBENZOIC ACID

INTRODUCTION

Chlorinated aromatic chemicals are pollutants of major concern because they enter the environment in substantial quantities, are toxic and resistant to degradation, and accumulate in sediments and biota. As a result, the potential for degradation of these pollutants in the environment and by microorganisms has been studied extensively, but almost always under aerobic conditions. Little is known of their fate in anoxic habitats, although some chlorophenols, chlorobenzoates and chlorinated pesticides have been shown to be degraded under anaerobic conditions. Pentachlorophenol was the first chloroaromatic compound to be reported to be dechlorinated under anaerobic conditions (5,8). In these studies tetra-, tri-, and di-chlorophenols were observed to accumulate in the anaerobic soils. In recent studies the dechlorination and mineralization of mono- and di-chlorophenols has been observed in anaerobic digested sludge (2,3). The dechlorination of the herbicide diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] has also been reported (1). Recently, our laboratory has shown the reductive dehalogenation of substituted benzoates by anaerobic lake sediments (4,8,10).

A population active in dehalogenation was enriched from sludge by growth on 3-chlorobenzoate (10). Beside dechlorination of meta substituted chlorobenzoates, the enrichment also removed the para chlorine from the herbicide 2,4,5-T (12). Microscopic examination of the consortium revealed the presence of several gram-negative bacteria

as well as two chemolithotrophic methanogens (based on autofluorescense) and the acetate utilizing methanogen, Methanothrix soehgenii (10). The purpose of this paper is to report on the isolation and characterization of the bacterium in the consortium responsible for dehalogenation.

MATERIALS AND METHODS

Isolation. A stable methanogenic consortium growing on 3-chlorobenzoate as the sole carbon and energy source was selected from municipal digestor sludge (Adrian, MI) over a period of 2 years. Serial dilutions of this enrichment were inoculated into anaerobic culture tubes (Bellco Glass, Inc. Vineland, N.J.) containing 7 to 8 ml of basal medium plus 5% clarified rumen fluid, 0.1% of either benzoate or butyrate, and 2% agar, rolled onto the tube walls. The basal medium consisted of (per liter): phosphate buffer, 0.27 KH_2PO_4 and 0.35 g K_2HPO_4 (adjusted to pH 7.0): mineral salts, 0.53g NH_4Cl, 15 mg $CaCl_2 \cdot 2H_2O$, 20 mg $MgCl \cdot 6 H_2O$, and 4 mg $FeCl_2 \cdot 4H_2O$: trace metals modified from Zehnder (14), 0.5 mg $MnCl_2 \cdot 4H_2O$, 0.05 mg H_3BO_3, 0.,05 mg $ZnCl_2$, 0.03 mg $CuCl_2$, 0.01 mg $NaMO_4 \cdot 2H_2O$, 0.5 mg $CoCl_2 \cdot 6H_2O$, and 0.05 mg $NiCl_2 \cdot 6H_2O$: the vitamin solution of Wolin (13); 2.4 g $NaHCO_3$: and 1 mg resazurin. The headspace gas was 80% N_2/20% CO_2. Tubes were reduced prior to use with 0.5 mM cysteine-HCl and 0.5 mM Na_2S. Desulfovibrio sp. PS-1 or Methanospirillum hungatii PM-1, previously isolated from the enrichment, were initially added (1 ml of a turbid culture) as the H_2 consumer, since the dechlorinating bacterium was presumed to be a syntroph. Isolates were picked using a sterile Pasteur pipet and transferred to 10 ml of liquid medium. Strict anaerobic techniques were

employed throughout.

Characterization. Experiments to assess substrate use were performed by transferring a 10% inoculum to anaerobic culture tubes containing the basal medium, 20% rumen clarified fluid, and 0.2% of the substrate of interest. All substrates were tested in triplicate. Incubation was at 37°C in the dark. Growth was assessed visually. Where indicated sulfate was added at 20 mM, nitrate was at 5 mM. Experiments to determine fermentation products were conducted using cultures grown in 100 ml of medium in 160 ml capacity serum bottles (Scientific Products, McGraw Park, IL). Subsamples (3 ml) of medium were collected at the beginning and end of incubation and frozen until analysis.

Generation time was determined from optical density measured in anaerobic culture tubes using a Turner spectrophotometer set at 660 nm. Cell numbers were determined using a Petroff-Hauser chamber under a high-dry phase microscope objective.

Whole cell electron micrographs were obtained by floating copper grids (330 mesh), previously coated with Parlodion, on a drop of bacterial suspension for 1 min. Grids were blotted, then stained for 30 s with uranyl acetate. Electron micrographs were taken at 80 KV on a Philips 300 electron microscope.

For thin section electron micrographs, cells were fixed overnight (4°C) in 2% glutaraldehyde and 0.1 M phosphate buffer (pH 7.2). Cells were then embedded in Noble agar and postfixed for 2 h (ambient temperature) in 1% Osmium and Rytel-Kellenberger fixative (pH 6.1). Samples were dehydrated through an ethanol series followed by propylene oxide, then embedded in DER (332-732) plastic resin. Thin sections were

post stained with uranyl acetate and lead citrate and examined on a Philips 300 electron microscope.

Phase photomicrographs were obtained by adding one drop of 1% agarose to one drop of bacterial suspension on a clean slide. A cover slip was pressed firmly onto the slide surface, and the agar-bacterial solution allowed to solidify for approximately 10 min. Photomicrographs were taken on a Zeiss photomicroscope.

Chemical Analyses. 3-Chlorobenzoate and benzoate were quantified using a Varian-3700 high pressure liquid chromatograph (HPLC) coupled to a Hitachi variable wavelength detector at 284 nm. The analytical column was a 250 x 4.6 mm C-18 reverse phase column. The mobile phase consisted of 60:40:5 methanol: water: acetic acid. The flow rate was 1.5 ml/min.

Pyruvate, lactate, succinate, formate, and acetate were also quantified by HPLC with the detector wavelength set at 210 nm. The analytical column was a 300 x 7.8 mm Bio-Rad ion exclusion HP X-87 column for organic acid analysis. The mobile phase consisted of 0.5 mN sulfuric acid; the flow rate was 1 ml/min.

Acetate, propionate, and butyrate were quantified using a PE-900 gas chromatograph (GC) equipped with a flame ionization detector and a 2 m glass column (2 mm ID) packed with Carbopack C/0.3% Carbowax 20 M/0.1% H_3PO_4. The oven temperature was 80°C for 2 min and then increased 16°C/min up to 120°C. The N_2 carrier gas flow rate was 30 to 40 ml/min. Samples were acidified with H_3PO_4 prior to injection.

Hydrogen was quantified using a Carle AGC-111 GC equipped with microthermister detector and two stainless steel columns in series; the first column was Porapak T (1.2m x 3.2 mm ID) and the second was

molecular sieve 5A(2.7 m x 3.2 mm ID). The oven temperature was 35°C. Argon was the carrier gas at a flow rate of 20 ml/min. Injections were made via a 3 ml gas sampling loop.

RESULTS

Our initial isolation strategy was based on the assumption that the dechlorinating bacterium must use benzoate or an intermediate of benzoate metabolism, such as butyrate, as a source of carbon and energy for growth. 3-Chlorobenzoate was not tried as a growth substrate since preliminary experiments had indicated that concentrations of 0.1% were inhibitory to the enrichment. After 8 to 10 weeks small white colonies of up to 0.5 mm diameter appeared in the anaerobic culture tubes. Most of these colonies consisted of large rods typical of one of the dominant morphological types observed in the enrichment. This bacterium was suspected to be involved in dechlorination since this morphological type disappeared when the enrichment was fed only benzoate. Colonies were transferred to liquid culture, and by the eighth week a very slight turbidity was observed. Only cells of the expected morphology were observed by phase microscopy. When these cultures were incubated with 3-chlorobenzoate a slow rate of dechlorination was observed. Analysis of benzoate or butyrate in liquid culture indicated that these substrates were not being consumed. Therefore, we presumed that the bacterium was able to scavenge sufficient carbon and other nutrients from the 5% rumen fluid to support growth in the roll tubes and in liquid culture.

To insure culture purity, cells were successfully isolated two

additional times on roll tubes after growth in liquid culture. After each isolation only the one distinctive cell morphology was observed, and no turbid growth occurred in a complex medium (0.1% glucose-trypticase-yeast extract). The culture obtained after the third isolation was designated strain DCB-1 (for dechlorinating bacterium). For the second and third isolation, benzoate, butyrate, and the syntrophs were omitted from the medium and 0.05% yeast extract was added as a nutritional supplement.

Growth was still meager on the 5% rumen fluid plus yeast extract medium, but was improved by use of 20% clarified rumen fluid. We also found that rumen fluid collected soon after feeding of the fistulated cow gave higher cell densities than rumen fluid collected at a later time. However, cell yields were never high enough to quantify in a spectrophotometer. Initially, the culture was maintained on the basal medium plus 20% rumen fluid.

The isolate is a straight rod, 3 to 6.5 um in length and 0.5-0.8 um in width (Fig. 1). The cells stained gram negative and appeared to have a gram negative envelope as observed in the thin sections (Figs. 2-4). A unique feature was the "collar" that surrounds the cell (Fig. 5). The traverse thin sections show that cytoplasm extends into this collar (Fig. 23). Cross sections show the concentric ring expected for sections cut through the collar (Fig. 4). Almost all of the cells in the culture show the collar.

Twenty-eight chemicals were examined as substrates (Table 1), but only pyruvate plus rumen fluid supported growth. The rumen fluid could be replaced with a complex protein source (yeast extract, trypticase, casamino acids, proteose peptone, or tryptic soy broth), but pyruvate

Fig. 1. Photomicrograph of the dechlorinating bacterium, DCB-1. Bar represents 0.5 um.

Fig. 2. Electron micrograph of the dechlorinating bacterium DCB-1 showing "collar" in relationship to the rest of the cell. Bar represents 0.5 um.

Fig. 3. Electron micrograph of the dechlorinating bacterium DCB-1 showing the collar at high magnification. Bar represents 0.1 um.

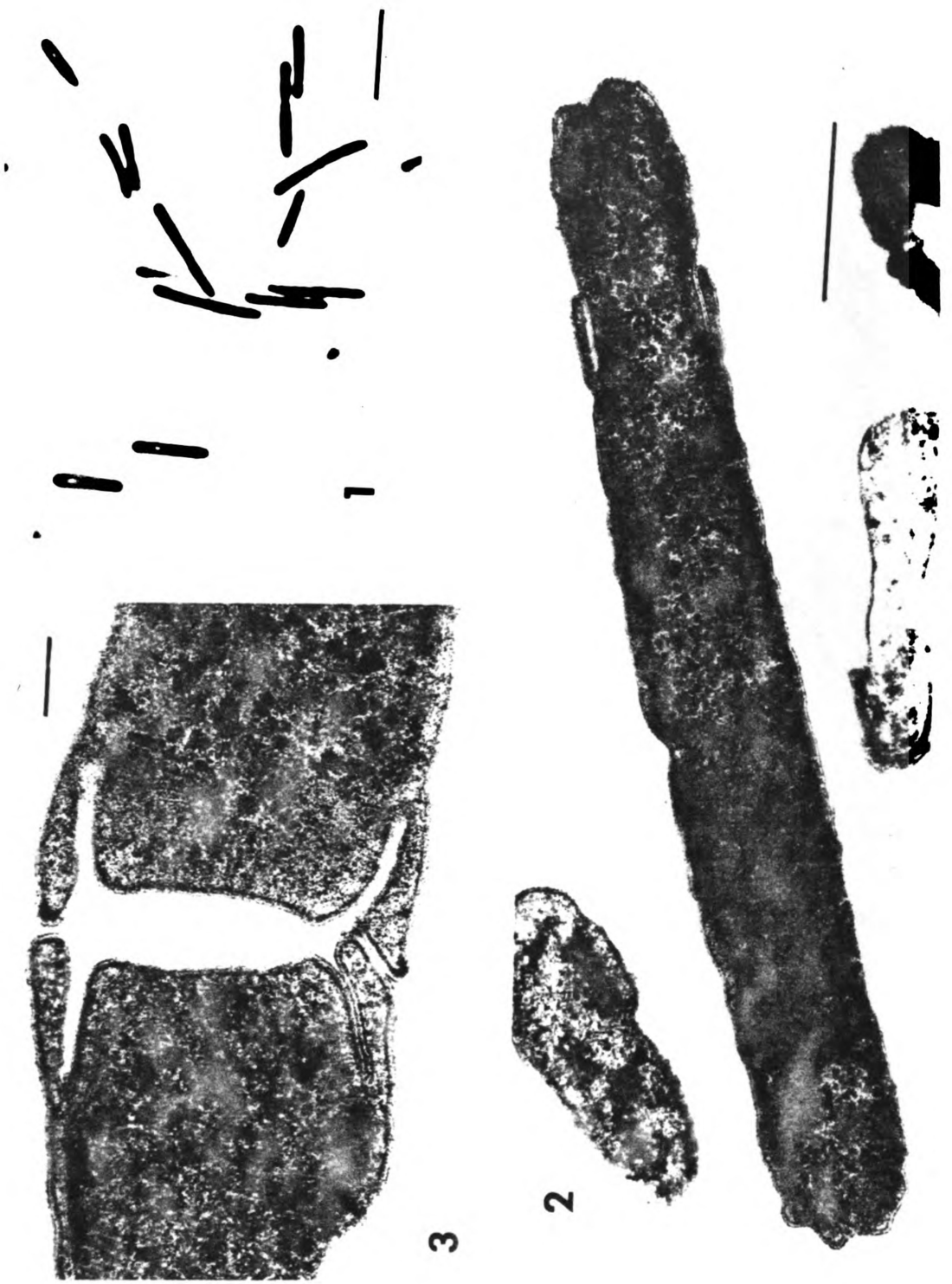
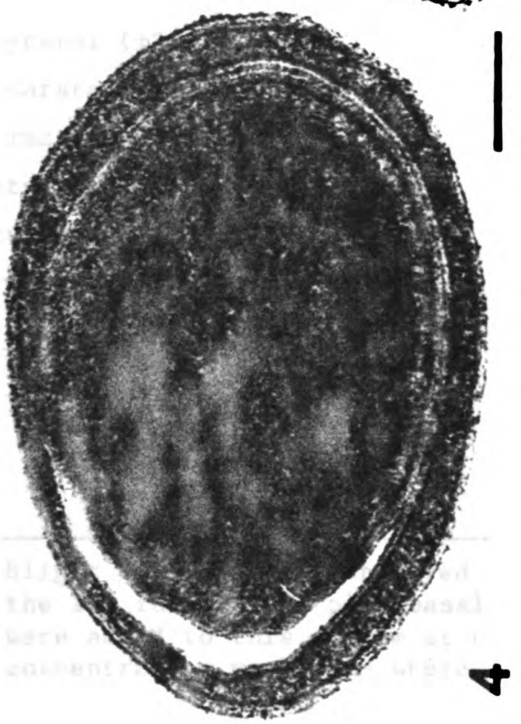

1
2
3

Fig. 4. Electron micrograph of the dechlorinating bacterium DCB-1 showing "collar" in cross-section. Note the concentric rings. Bar represents 0.1 um.

Fig. 5. Electron micrograph of the dechlorinating bacterium DCB-1 positively stained with uranyl acetate showing the outer appearance of the collar. Bar represents 0.5 um.



4

5

Table 1. Substrates and electron acceptors which did not support growth of the dechlorinating bacterium, strain DCB-1.[a]

Glucose ($\pm SO_4$)	Yeast extract ($\pm SO_4$)
Fructose	Trypticase ($\pm SO_4$)
Galactose	Proteose peptone ($\pm SO_4$)
Ribose ($\pm SO_4$)	Casamino acids ($\pm SO_4$)
Xylose	Tryptic soy broth
Lactate ($\pm SO_4$)	Alanine
Glycerol ($\pm SO_4$)	Valine
Fumarate	Glycine
Formate	Aspartate
Butyrate[b]	Glutamate
Benzoate[b]	Lysine
	Arginine
	Serine
	Threonine
	Proline
	Histidine

[a] Slight turbidity was observed in bottles as a result of growth with the 20% rumen fluid plus basal medium. The above carbon sources were added to this medium at a concentration of 0.2%; the sulfate concentration was 20 mM where indicated.

[b] Methanospirillum hungatii PM-1 was added as a H_2 consumer.

alone did not support growth of the bacterium. The bacterium did not respond to the addition of sulfate or to the presence of a H_2 consuming bacterium. Growth did not occur in the presence of oxygen. We also tested combinations of amino acids known to support growth of Clostridia species that carry out a Stickland reaction; no combination of amino acids supported growth. Nitrate was reduced to nitrite. The bacterium was routinely grown at 37°C and grew poorly or not at all at 25°C and 45°C.

The growth curve of isolate DCB-1 on pyruvate plus 20% rumen fluid in the basal medium is shown in Figure 6. The maximum generation time was 4 to 5 days. The low maximum cell yield of 0.05 to 0.06 OD may indicate a nutrient limitation (Fig. 6), although, increased concentrations of pyruvate and trypticase in the medium did not result in increased cell yields.

The effect of increasing concentrations of inorganic nutrients and/or vitamins on cell yields were not determined.

Greater than 90% of the pyruvate carbon was recovered as acetate when cells were grown on 0.2% pyruvate and 0.1% trypticase; presumably CO_2 was also produced. Other possible fermentation products such as lactate, succinate, formate, propionate, and butyrate were not detected. Hydrogen was not detected (detection limit 10 ppm) in the headspace gases. Furthermore when grown in the presence of a methanogen, no methane was detected. When 3-chlorobenzoate was added to the pyruvate medium it was dechlorinated stoichiometrically to benzoate (Fig. 7). 3-Chlorobenzoate was inhibitory to growth as the generation time approximately doubled in the presence of 1 mM chlorobenzoate. Cultures did not dechlorinate if they had reached stationary phase. The rate of

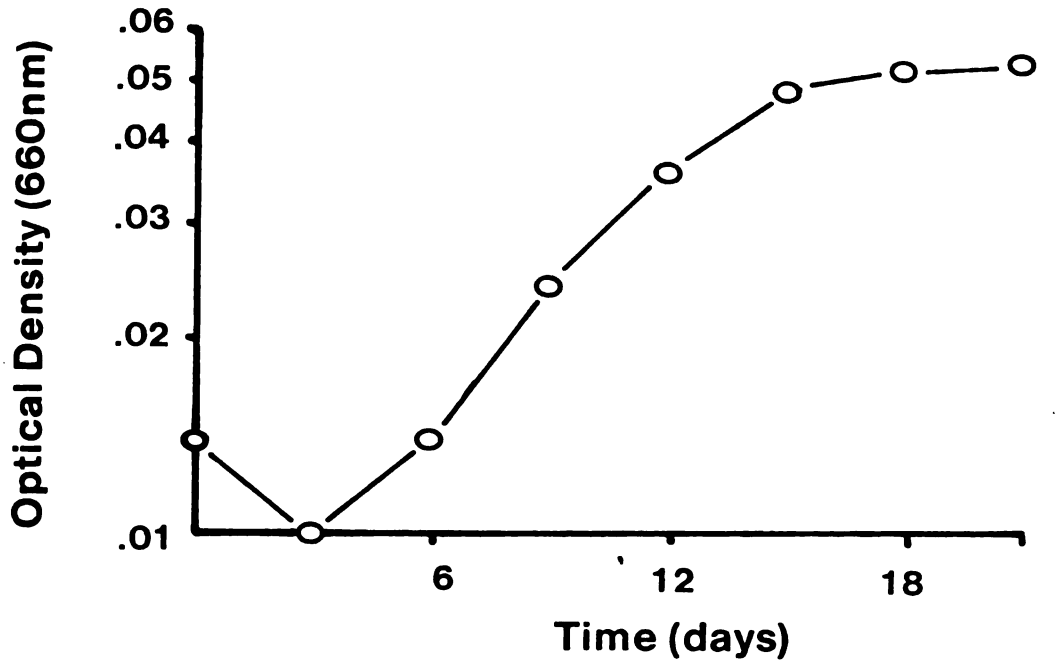


Fig. 6. Growth of the dechlorinating bacterium on 0.2% pyruvate plus 20% rumen fluid.

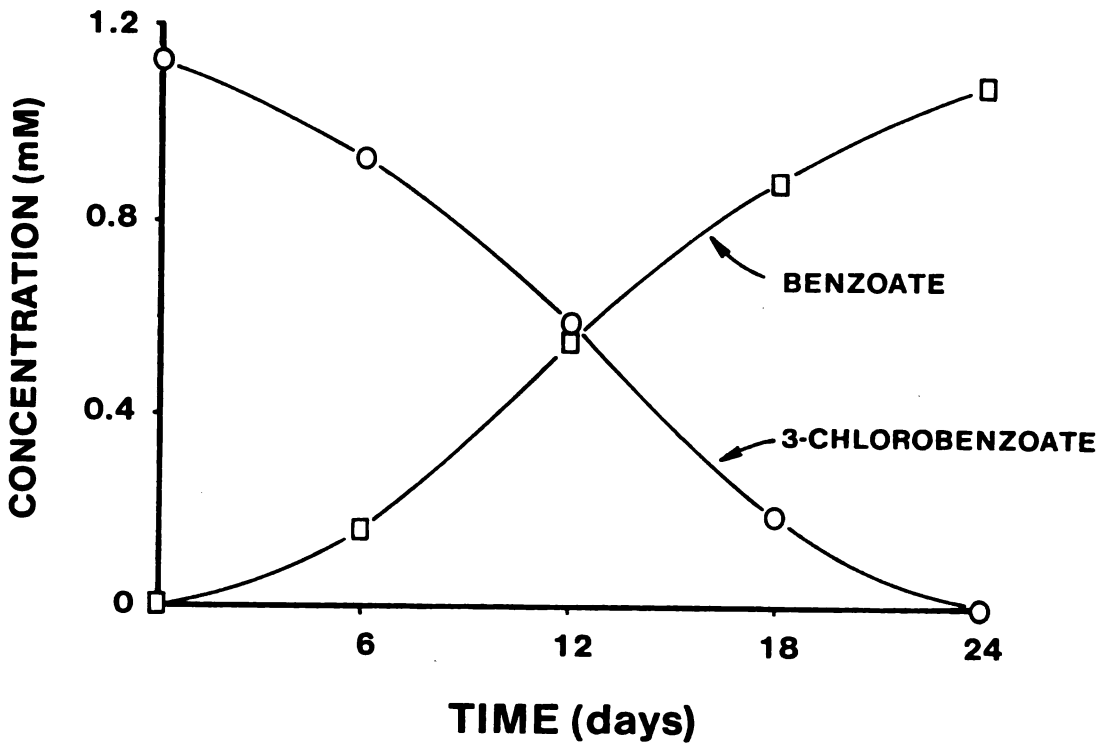


Fig. 7. Dechlorination of 3-chlorobenzoate by the dechlorinating bacterium after inoculation into fresh medium.

dechlorination was approximately 0.1 pmole bacterium^{-1}day^{-1}.

DISCUSSION

The dechlorinating bacterium appears to be unique among anaerobes. The presence of a "collar", the extremely restricted substrate range, the slow growth rate, and the ability to dechlorinate aromatic compounds, all are uncommon traits. The function of the "collar" is unknown; perhaps it may be associated with cell division, as suggested by some of the electron micrographs.

The only substrate which we observed to support growth of the bacterium was pyruvate in conjunction with rumen fluid or some complex protein source. The pyruvate was fermented to acetate and presumably CO_2, but the fate of the electrons is not known. The growth substrate(s) found in clarified rumen fluid are not known, but since rumen fluid is considered to be low in soluble substrates, the bacterium appears to be very efficient in scavenging carbon for growth. The basal medium used for routine culturing may not have been optimized considering the low cell densities obtained. Since increasing the concentration of carbon substrates had no effect on cell yields, if a limitation does exist, it is probably due to insufficient minerals or vitamins in the basal medium. We have had no difficulty, however, in growing other anaerobes to high densities using this medium.

The bacterium dechlorinates 3-chlorobenzoate stoichiometrically to benzoate during the growth phase of the culture. Based on previous work (4,11) and our observation that cultures grown in the absence of 3-chlorobenzoate for several doublings dechlorinated only after a lag of 3 to 6 the dechlorination reaction appears to be enzymatic in nature.

Since the bacterium derives no apparent energy from this reaction, it would appear to be a case of co-metabolism or fortuitous metabolism.

Since enzymes for the metabolism of chlorinated aromatic compounds in aerobes are frequently plasmid encoded, we attempted to determine if this bacterium contained plasmid(s). Using protocols for lysis of gram negative (6) and gram positive bacteria (7) were unable to lyse this bacterium, even when cell suspensions were heat shocked (90°C followed by ice-water) during the treatment. Controls of representative gram positive (Streptococcus) and gram negative (Pseudomonas) cells were always successfully lysed. This may indicate some unique feature of the cell wall of this organism.

The growth substrate of the dechlorinating organism in the enriched consortium is not clear. This organism appears to be responsible for the first step in the metabolism of 3-chlorobenzoate (i.e., the dechlorination) but it cannot metabolize benzoate. Benzoate is catabolized by another population of bacteria in the enrichment (9). Presumably one or more of the other bacteria in the enrichment are cross-feeding the dechlorinating bacterium. The cross-feeding of the dechlorinating bacterium may account for the variety of other bacteria isolated from the enrichment that do not appear to be directly associated with the 3-chlorobenzoate food chain. These include two butyrate oxidizing bacteria (butyrate or other volatile fatty acids could theoretically come from benzoate metabolism, although this has not been demonstrated, or as a fermentation product of other substrates); two sulfate reducers (sulfate was never added to the enrichment, however, both isolates can ferment pyruvate); and several isolates of fermenting bacteria (isolated on glucose-trypticase-yeast extract). We do not feel that the presence of these bacteria can be attributed to

contamination alone, considering the high degree of dilution of the original inoculum (over 2 years), that 3-chlorobenzoate was the only carbon and energy source added to the enrichment, and that all isolates are obligate anaerobes.

Further research is needed in order to elucidate the physiology and taxonomic standing of this bacterium, to understand the mechanism of dechlorination, and to unravel the symbiotic interactions of the consortium members as they cooperate to degrade this xenobiotic chemical.

Acknowledgements

We thank Terry Aust for technical assistance and Stuart Pankratz and Walter Smolenski for electron and photomicrographs.

LITERATURE CITED

1. Attaway, H.H., N.D. Camper, and M.J.B. Paynter. 1982. Anaerobic microbial degradation of diuron by pond sediment. Pest. Biochem. Physiol. 17, 96-101.

2. Boyd, S.A. and D.R. Shelton. Anaerobic biodegradation of chlorinated phenols in fresh and acclimated sludge. Appl. Environ. Microbiol. (submitted).

3. Boyd, S.A., D.R. Shelton, D. Berry, and J.M. Tiedje. 1983. Anaerobic biodegradation of phenolic compounds in digested sludge. Appl. Environ. Microbiol. 46:50-54.

4. Horowitz, A., J.M. Suflita, and J.M. Tiedje. 1983. Reductive dehalogenations of halobenzoates by anaerobic lake sediment microorganisms. Appl. Environ. Microbiol. 45:1459-1465.

5. Ide, A., Y. Niki, F. Sakamoto, I. Watanabe, and H. Watanabe. 1972. Decomposition of pentachlorophenol in paddy soil. Agric. Biol. Chem. 36:1937-1944.

6. Kado, C.I. and S.-T. Tui. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.

7. Leblanc, D.J. and L.M. Lee. 1979. Rapid screening procedure for detection of plasmids in Streptococci. J. Bacteriol. 140:1112-1115.

8. Murthy, N.B.K., D.D. Kaufman, and G.F. Fries. 1979. Degradation of pentachlorophenol (PCP) in aerobic and anaerobic soil. J. Environ. Sci. Health. B14:1-14.

9. Shelton, D.R. and J.M. Tiedje. Isolation and characterization of syntrophic anaerobic bacterium that oxidizes benzoate. Appl. Environ. Microbiol. (submitted).

10. Suflita, J.M., A. Horowitz, D.R. Shelton, and J.M. Tiedje. 1982. Dehalogenation: a novel pathway for the anaerobic biodegradation of haloaromatic compounds. Science. 218:1115-1117.

11. Suflita, J.M., J.A. Robinson, and J.M. Tiedje. 1983. Kinetics of microbial dehalogenation of haloaromatic substrates in methanogenic environments. Appl. Environ. Microbiol. 45:1466-1473.

12. Suflita, J.M., J. Stout, and J.M. Tiedje. Dechlorination of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) by anaerobic microorganisms. J. Agric. Food Chem. (in press).

13. Wolin, E. A., M. J. Wolin, and R. S. Wolfe. 1963. Formation of methane by bacterial extracts. J. Biol. Chem. 238:2882-2886.

14. Zehnder, A.J.B. and K. Wuhrmann. 1977. Physiology of a Methanobacterium strain AZ. Arch. Microbiol. 111:199-205.

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF TWO SYNTROPHIC ANAEROBIC BACTERIA THAT OXIDIZE BUTYRATE AND HIGHER VOLATILE FATTY ACIDS

INTRODUCTION

The production of volatile fatty acids (VFA's) as intermediates in the metabolism of carbohydrates, proteins, and lipids in methanogenic habitats long has been recognized. Although little was known about the bacteria responsible for VFA metabolism, it was originally believed that methanogenic species were responsible. Bryant et al. (2), however, showed that the fermentation of ethanol by Methanobacillus omelianskii was carried out by a syntrophic association of two bacteria. This suggested that another group of bacteria, distinct from methanogens, was responsible for the metabolism of VFA's to acetate and H_2, but which required the presence of H_2-utilizing bacteria in order for the energy yield of the first reaction to be favorable. An anaerobic bacterium which degraded butyrate and higher VFA's in syntrophic association with methanogens or sulfate reducers was subsequently isolated by McInerney, et al. (3) and soon afterwards a bacterium able to degrade propionate in syntrophic association was also isolated (1). Both organisms were unique in that their substrate ranges were limited to VFA's, and that they could be cultured only in the presence of a H_2-utilizing organism.

We were able to isolate two butyrate degrading bacteria from an enrichment which degraded 3-chlorobenzoate to $CH_4 + CO_2$. We report here the isolation and characterization of these bacteria.

MATERIALS AND METHODS

The isolation techniques used were essentially those described by McInerney et al. for the isolation of VFA degrading bacteria (3). Samples of the 3-chlorobenzoate enrichment described previously (7) were fed 0.1% butyrate. After a lag of 2 to 3 days, the butyrate was rapidly consumed. Serial dilutions of the butyrate acclimated enrichment were rolled out in anaerobic culture tubes containing 7 to 8 ml of the basal medium described previously (6), plus 5% rumen fluid, 0.1% butyrate, and 2% agar. The headspace gas was O_2-free 80% N_2/20% CO_2. Tubes were reduced prior to use with 0.5 mM cysteine-HCl and 0.5 mM Na_2S. Desulfovibrio sp. strain PS-1 or Methanospirillum hungatii strain PM-1, previously isolated from the 3-chlorobenzoate degrading enrichment, were added (1 ml of a turbid culture) as the H_2 consumers. These organisms were isolated on 80% H_2/20% CO_2 (PM-1) or 80% H_2/20% CO_2 + 20 mM SO_4 (PS-1); 0.1% acetate was added for cell carbon. These organisms were selected as H_2 consumers from our culture collection because they possessed the lowest H_2 Km values for a methanogen and sulfate reducer: 2.0 uM for M. hungatii PM-1, 0.7 uM for Desulfovibrio PS-1 (5).

Colonies were picked using a sterile pasteur pipet with a bent tip and transferred to 10 ml of liquid medium. Three milliliters of a turbid culture of Desulfovibrio PS-1 or M hungatii PM-1 was added to the fresh liquid medium to ensure a low partial presssure of H_2 in the bottle.

Characterization. Experiments to assess substrate use were performed by transferring 10% of a culture to anaerobic culture tubes

containing the basal medium, 5% clarified rumen fluid, plus 0.2% of the substrate of interest. Incubation was at 37°C in the dark. Growth was assessed visually. All substrates were tested in triplicate. Nitrate reduction was tested by adding 5 mM NO_3^- to the basal medium.

Fermentation balances were performed with propionate, butyrate, iso-butyrate, 2-methylbutyrate, valerate, isovalerate. Five milliliters of turbid culture was transferred to 50 ml of medium with 0.15% substrate in 160 ml capacity serum bottles. Subsamples (1 ml) were collected at the beginning and end of incubation and frozen until analysis. Bottles were incubated statically in the dark for 3 to 4 weeks at 37°C.

Growth rates were determined by plotting log of substrate consumed vs. time; doubling time was calculated graphically based on the length of time required for the amount of substrate consumed to double. This assumes a constant Y (growth yield) over the period of time used for the growth determination. Experiments were conducted as previously described for the fermentation balance except that bottles were shaken on a rotary shaker at 120 rpm.

Phase photomicrographs and thin section electron micrographs were prepared from stationary phase cells grown on butyrate using the microscopy methods previously described (6). Organic acids and H_2 were quantified by gas chromatography using methods previously described (6). Methane was quantified by injecting 0.2 ml of headspace gas into a Carle GC equipped with microthermister detector and a 2 m column packed with Porapak QS.

RESULTS

After 2 to 3 weeks small white colonies up to 1 mm in diameter appeared in co-culture with M. hungatii or Desulfovibrio. The morphology of these bacteria was not consistent with any of the predominant morphological types observed in the enrichment. Colonies were transferred to liquid culture and by the second week turbidity was observed. After the initial isolation, two bacteria were observed in liquid culture: a non-spore forming bacterium with a morphology consistent with the butyrate degrading bacterium isolated by McInernery et al. (3) and a spore forming bacterium. To insure purity of the non-spore forming isolate, cells were successfully isolated two additional times on anaerobic roll tubes following growth in liquid culture. After each isolation only one distinctive cell morphology was observed and no turbid growth occurred in a complex medium (0.1% glucose-trypticase-yeast extract).

To insure purity of the spore forming bacterium, the culture was pasteurized three times at 80°C for 30 min. Before the final heat treatment, the culture was sparged with air until the O_2 indicator (resazurin) had turned pink. After each pasteurization, 5 ml of the treated culture and 5 ml of a turbid culture of Desulfovibrio or M. hungatii were added to 50 ml of liquid medium plus 0.15% butyrate. After spore germination only the one distinctive cell morphology was observed and no turbid growth occurred in a complex medium (0.1% glucose-trypticase-yeast extract).

The non-spore forming butyrate degrading bacterium was designated

strain NSF-2 (for non-spore forming). The spore forming butyrate degrading bacterium was designated SF-1 (for spore forming).

The non-spore forming isolate is a slightly curved rod with tapered ends 2 to 4 um in length and 0.2 to 0.3 um in width (Fig. 1). The cells stained gram negative. We have occasionally observed what appears to be a twitching type of motility, although, it is difficult to distinguish from Brownian motion. The only substrates observed to support growth were butyrate, 2-methylbutyrate, valerate, and caproate (higher VFA's were not tested); those not supporting growth are given in Table 1. Fermentation products were acetate and CH_4 (from the butyrate and caproate) or acetate, propionate, and CH_4 (from 2-methylbutyrate and valerate) when grown in co-culture with M. hungatii (Table 2). Cultures fed caproate accumulated equimolar quantities of butyrate and acetate until the fourth day after inoculation, after which caproate and butyrate were catabolized simultaneously (Fig. 2). Nitrate was not reduced. When sulfate was added to bottles with M. hungatii as the H_2-consuming syntroph, CH_4 production from butyrate was not affected. Growth did not occur in the presence of oxygen.

The spore-forming isolate is a slightly curved rod 3 to 5 um in length and 0.4 to 0.6 um in width. The cells appeared to have a gram positive cell wall as observed in the thin section (Fig. 3). The culture produced heat and oxygen resistant spores (Fig. 4). The bacterium did not produce spores when grown in co-culture with Desulfovibrio, but did so with M. hungatii. The only substrates observed to support growth were butyrate, iso-butyrate, 2-methylbutyrate, valerate, and caproate (higher VFA's were not tested) (Table 1). Fermentation products were acetate and CH_4 (from butyrate,

Fig. 1. Photomicrograph of the non-spore forming butyrate oxidizing bacterium NSF-2 in co-culture with M. hungatii PM-1. Bar represents 5 uM.

Fig. 3. Electron micrograph of the spore forming butyrate oxidizing bacterium SF-1 showing incipient spore and gram positive cell wall. Bar represents 0.5 um.

Fig. 4. Photomicrograph of spores from the spore forming butyrate oxidizing bacterium. Note occasional intact cells with incomplete spores (arrow). Bar represents 0.5 um.

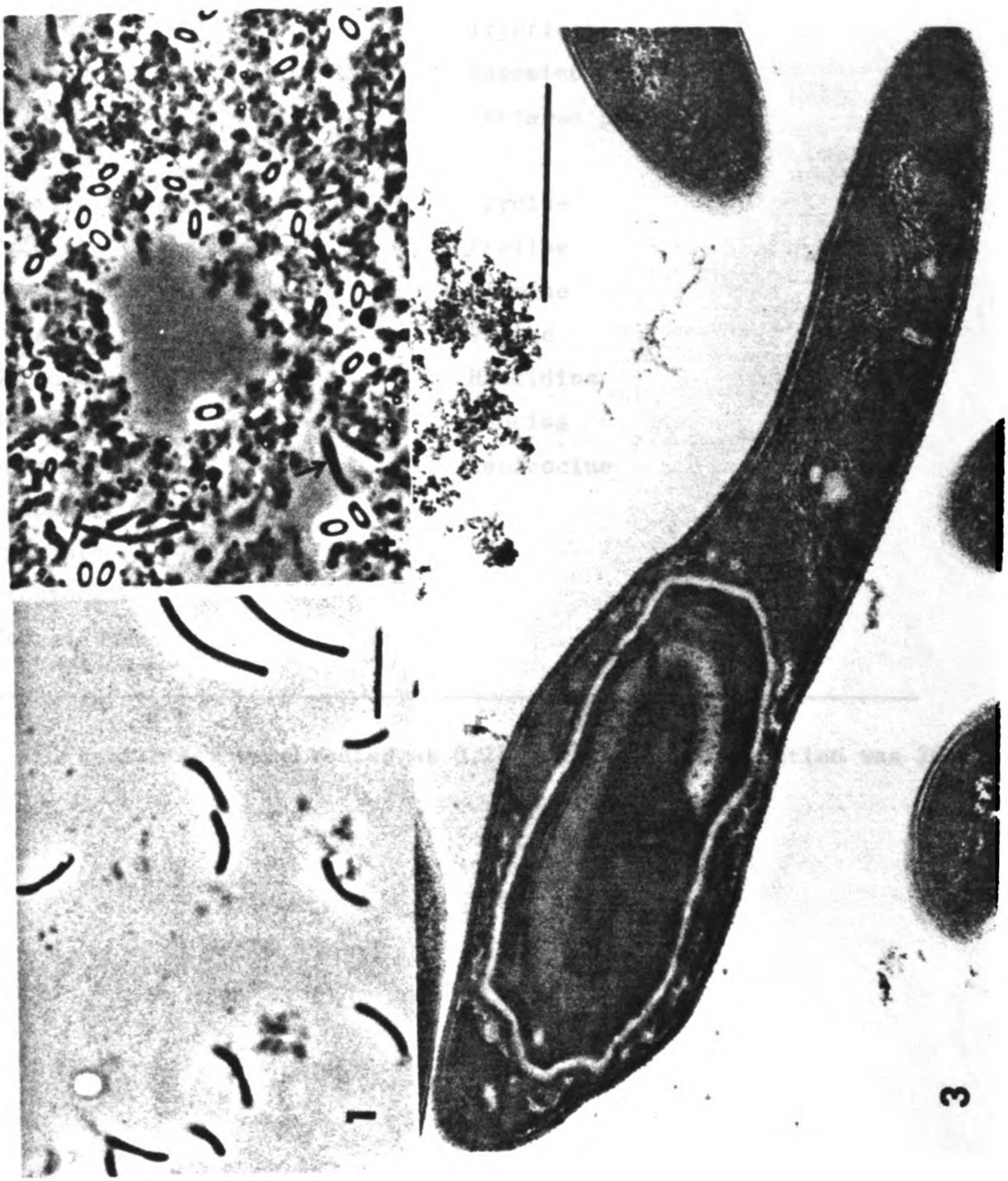

1
3

Table 1. Substrates which did not support growth of either butyrate degrading bacterium in co-culture with *M. hungatii* PM-1.[a]

Glucose	Yeast extract
Fructose	Trypticase
Ribose	Casamino acids
Xylose	Proteose peptone
Lactate ($\pm SO_4$)	Glycine
Pyruvate ($\pm SO_4$)	Proline
Glycerol	Alanine
Fumarate	Valine
Ethanol	Histidine
Butanol	Leucine
Benzoate	Isoleucine
Adipate	
Pimelate	
Propionate	
Isovalerate	

[a] all substrates were tested at 0.2%, sulfate concentration was 20 mM.

Table 2. Fermentation products formed from volatile fatty acids by the non-spore forming isolate NSF-2 in co-culture with M. hungatii PM-1.

Substrate	Substrate consumed (mmoles)	Products (mmoles)			% Recovered	
		Acetate	Propionate	CH_4	C	H
Butyrate	0.78	1.53	0	0.39	98	100[a]
2-Methyl-butyrate	0.71	0.65	0.58	0.31	87	87[a]
Valerate	0.63	0.65	0.58	0.35	98	90[a]
Caproate	0.53	1.42	0	0.55	90	104[b]

[a] Based on 0.5 moles of CH_4 produced per mole of substrate consumed.
[b] Based on 1.0 mole of CH_4 produced per mole of substrate consumed.

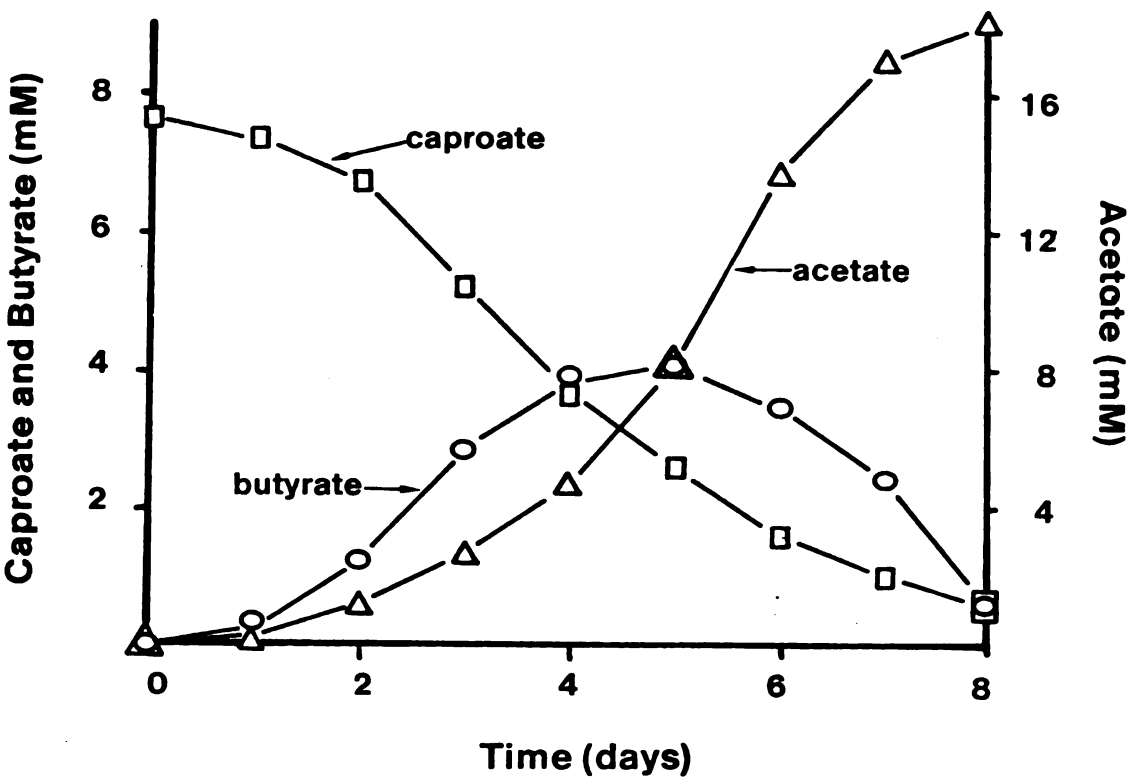


Fig. 2. Production and consumption of butyrate as an intermediate of caproate metabolism by the non-spore forming bacterium NSF-2 .

iso-butyrate, and caproate) or acetate, propionate, and CH_4 (from 2-methylbutyrate and valerate) when grown in co-culture with M. hungatii (Table 3). Butyrate was produced as an intermediate of caproate catabolism. Nitrate was not reduced. When sulfate was added to bottles with M. hungatii as the H_2-consuming syntroph, CH_4 production from butyrate was not affected. Growth did not occur in the presence of oxygen.

Growth rates of the non-spore former (NSF-2) were determined by monitoring substrate disappearance in bottles in co-culture with the methanogens, the sulfate reducer, or with extra methanogens added; the latter was done to insure that hydrogen consumption would not be rate limiting. An exponential increase in butyrate consumption occurred through the first 51 h of incubation (Fig. 5, first five data points), representing from 17% to 26% of the added substrate. During the next 37 to 61 h the rate of butyrate consumption appeared to be linear (zero-order), and then finally first-order as substrate was depleted.

Based on plots of the log of butyrate consumed vs. time over the first 51 h of incubation, the estimated doubling time for the non-spore forming bacterium was 10 h (Fig. 5, inset). Whether the H_2 consumer was a methanogen or a sulfate reducer did not appear to affect the doubling time of either butyrate degrading bacterium. The estimated doubling time for the spore forming bacterium, determined by the same procedure, was 9 h.

DISCUSSION

We report here the isolation of two butyrate catabolizing bacteria.

Table 3. Fermentation products formed from volatile fatty acids by the spore forming isolate SF-1 in co-culture with *M. hungatii* PM-1.

Substrate	Substrate consumed (mmoles)	Products (mmoles)				% Recovered	
		Acetate	Propionate	Butyrate	CH_4	C	H
Butyrate	0.72	1.59	0	0	0.35	111	97[a]
Isobutyrate	0.28	0.51	0	0	0.16	91	114[a]
2-Methyl-butyrate	0.30	0.28	0.30	0	0.14	97	93[a]
Valerate	0.49	0.46	0.53	0	0.23	101	94[a]
Caproate	0.54	1.20	0	0.17	0.45	100	98[b]

[a] Based on 0.5 moles of CH_4 produced per mole of substrate consumed.
[b] Based on 1.0 mole of CH_4 produced per mole of caproate consumed minus 0.5 mole of CH_4 per mole of butyrate produced.

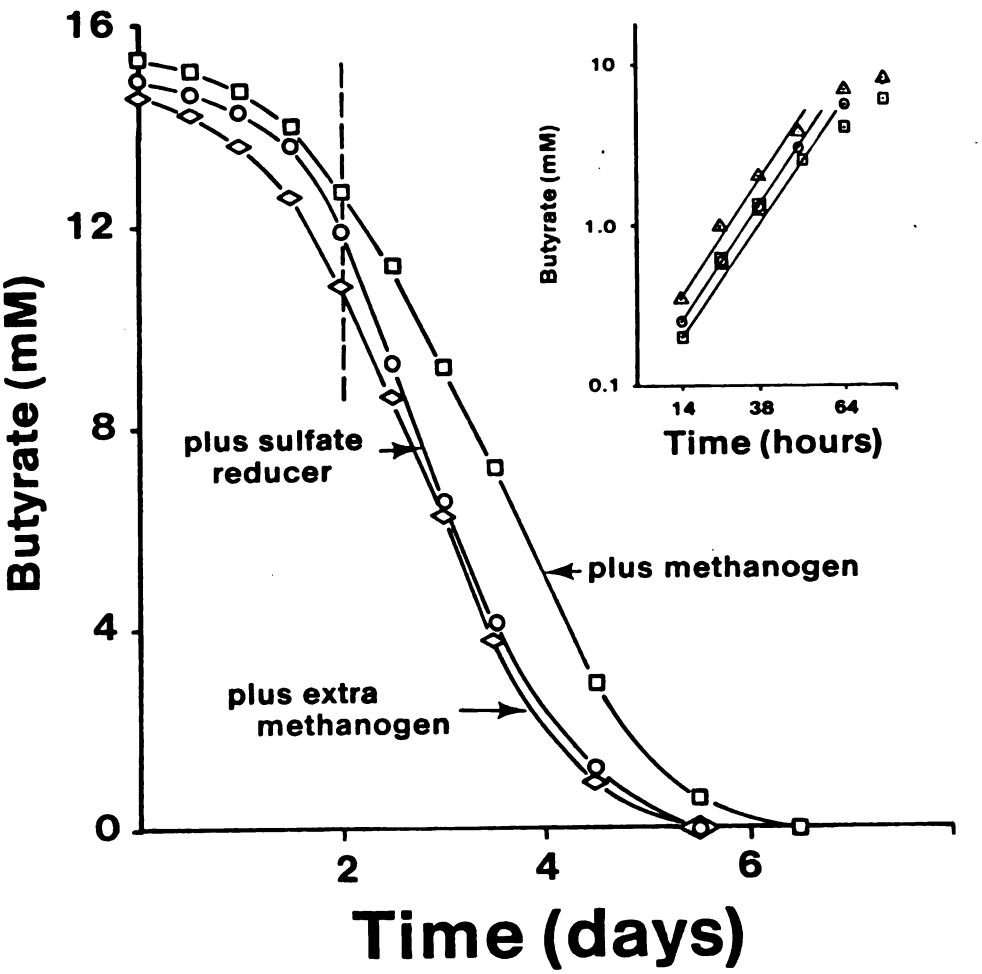


Fig. 5. Butyrate consumption by the non-spore forming bacterium NSF-2 growing with a methanogen or with additional methanogen added, or a sulfate reducer. Inset: Log of butyrate consumption vs. time with the three H_2 consuming conditions above.

The non-spore forming bacterium (NSF-2) is morphologically and physiologically consistent with S. bryantii isolated and characterized by McInerney, et al. (3) with the exceptions that our isolate catabolized 2-methylbutyrate and appeared to have a faster growth rate.

The isolation of spore forming, butyrate-catabolizing bacterium has to our knowledge not been previously reported. The spore forming bacterium did not produce spores when grown in co-culture with Desulfovibrio. The most likely explanation is the accumulation of toxic sulfide, although this was not pursued. The spores were both heat and oxygen resistant. The organism is also unique in that it is the first gram-positive, butyrate-oxidizing organism reported. In the vegetative state, the two bacteria are morphologically and physiologically similar with the exception that the spore former can catabolize isobutyrate.

We are intrigued by the significantly faster apparent growth rates exhibited by our isolates in relation to those previously described. We also did not observe any discrepancy between doubling times when the isolates were co-cultured with a methanogen vs a sulfate reducer, as was reported by McInerney et al. (3) for S. bryantii. Even with the addition of extra M. hungatii PM-1 to bottles already in co-culture with the methanogen we did not observe an increased growth rate. As a result, we feel that the estimated doubling times of 10 h and 9 h for NSF-2 and SF-1, respectively, are the true maximum growth rates. This assumes a constant Y (growth yield) during the first 51 h of incubation during which time an exponential increase in butyrate consumption was observed. Since population densities would have been relatively low during this time and thus little chance for nutrient limitations, we feel that this is a reasonable assumption.

As shown in Fig. 5 butyrate consumption became linear with time after only 25% of the added substrate had been metabolized. The reasons for this were not clear; however, a consideration of the thermodynamics of butyrate catabolism may offer some insight. The bacterium likely performs beta-oxidation of butyrate to form acetate and H_2:

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \quad \Delta G^{o'} = 11.5 \text{ kcal/mole} \quad (8)$$

The $\Delta G^{o'}$ of this reaction is $^+11.5$ kcal/mole making it unfavorable under standard conditions. However, standard conditions do not exist in an actively growing culture; butyrate and acetate are changing with time while the the concentration of H_2 is maintained at a low level by an active population of H_2-utilizing methanogens. In preliminary experiments, we measured a steady state partial pressure of 5.0×10^{-5} atm H_2 in cultures actively metabolizing butyrate. Using Henry's Law constant for 35°C and assuming equilibrium conditions between headspace gas and gas dissolved in pure water, 5.0×10^{-5} atm H_2 yields 0.035 uM H_2 in the aqueous phase. This value is consistent with the findings of Robinson and Tiedje who observed a steady state endogenous concentration of approximately 0.05 uM H_2 in Holt sludge and 0.01 uM H_2 in Wintergreen sediment (4).

In growth experiments initial concentrations of butyrate was 15 mM and concentration of acetate was approximately 5 mM (from the 5% rumen fluid and carry over from inoculation). Therefore:

$$\Delta G^o = \Delta G^{o'} + RT\ln \frac{[CH_3COO]^2\,[H_2]^2}{[CH_3CH_2CH_2COO^-]}$$

at zero time $\Delta G^{o} = +11.5 + 1.42 \log \frac{[5.0]^2\ [3.5 \times 10^{-5}]^2}{[15]}$

Calculations of G^{o}'s at zero time and for concentrations of butyrate and acetate as butyrate is consumed are presented in Table 4. After only approximately 2 mM of butyrate is consumed the ΔG^{o} for butyrate consumption becomes zero. The overall ΔG^{o} for CH_4 production is still favorable, so that butyrate should continue to be consumed. We hypothesize that growth of the butyrate degrading bacterium can only occur during the time period when the ΔG^{o} for butyrate catabolism is negative, i.e. exponential rates of butyrate consumption during early phases of incubation. After the ΔG^{o} for butyrate catabolism becomes positive, growth of the butyrate oxidizer can no longer occur, but the methanogens or sulfate reducers continue to consume H_2 such that the overall reaction ($CH_3CH_2CH_2^{-}COO^{-} \rightarrow CH_4$ or $S^{=}$) remains thermodynamically favorable, i.e. a linear or zero-order rate of butyrate consumption occurs. Eventually the rate will drop to first order as the butyrate concentration becomes rate limiting. Experiments are currently being conducted in order to obtain more accurate determinations of these thermodynamic relationships during the course of substrate consumption.

ACKNOWLEDGEMENTS

We thank Terry Aust for technical assistance, Stuart Pankratz and Walter Smolenski for electron and photomicrographs, and Daryl Dwyer for helpful insights and discussions.

Table 4. Calculated $\Delta G^{o'}$s at different concentrations of butyrate and acetate assuming a steady-state H_2 concentration of 0.035 uM.

ΔGo(kcal/mole)	Butyrate (mM)	Acetate (mM)
-0.84	15	5
-0.38	14	7
-0.03	13	9
+0.27	12	11

LITERATURE CITED

1. Boone, D.R. and M. P. Bryant. 1980. Propionate degrading bacterium, Syntrophobacter wolinii sp. nov. gen nov. from methanogenic ecosystems. Appl. Environ. Microbiol. 40:626-632.

2. Bryant, M. P., E. A. Wolin, M. J. Wolin, and R. S. Wolfe. 1967. Methanobacillus omelianskii, a symbiotic association of two species of bacteria. Arch. Microbiol. 59:20-31.

3. McInerney, M.J., M.P. Bryant, and N. Pfennig. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol. 122, 129-135 (1979).

4. Robinson, J.A., and J. M. Tiedje. 1982. Kinetics of hydrogen consumption by rumen fluid anaerobic digestor sludge and sediment. Appl. Environ. Microbiol. 44:1374-1384.

5. Robinson, J.A. and J.M. Tiedje. Competition between sulfate-reducing and methanogenic bacteria for H_2 under resting and growing conditions. Arch. Microbiol. (Accepted).

6. Shelton, D. R. and J. M. Tiedje. Isolation and characterization of an anaerobic bacterium that dechlorinates 3-chlorobenzoate. Appl. Environ. Microbiol. (submitted).

7. Suflita, J. M., A. Horowitz, D. R. Shelton, and J. M. Tiedje. 1982. Dehalogenation: a novel pathway for the anaerobic biodegradation of haloaromatic compounds. Science. 218:1115-1117.

8. Thauer, R.K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41:100-180.

CHAPTER 4

ISOLATION AND CHARACTERIZATION OF A SYNTROPHIC ANAEROBIC BACTERIUM THAT OXIDIZES BENZOATE

NOTE

Anaerobic oxidation of benzoate has been known since 1934 when Tarvin and Buswell (5) reported methane production from benzoate by anaerobic sewage sludge. More recently, have been shown that denitrifiers and phototrophs, as well as methanogenic consortia, oxidize benzoate anaerobically (1). The pathway involves reduction of the aromatic ring, hydrolysis of the ring, and beta-oxidation of the resulting volatile fatty acids (1).

Several denitrifying and phototrophic benzoate-oxidizing bacteria have been isolated in pure culture, but despite repeated attempts, isolation of the fermentative benzoate oxidizers has proved fruitless until the recent report by Mountfort and Bryant (2). The reason for this is that the fermentation of benzoate is not thermodynamically favorable under standard conditions:

$C_7H_5O_2^- + 7H_2O \rightarrow 3H_2 + HCO_3^- + 3CH_3COO^- + 3H^+ \rightarrow \Delta G^{o'} = + 21.5$ kcal/mole

Therefore, this reaction can proceed only if the products (particularly H_2) are rapidly removed from the culture medium. This is conventionally done by co-culturing the isolate with an H_2-utilizing organism. Mountfort and Bryant used this procedure to isolate an anaerobic syntrophic benzoate-degrading bacterium from sewage sludge. We provide a second report of the successful isolation of a anaerobic syntrophic benzoate oxidizing bacterium in co-culture with a sulfate reducer.

We were able to enrich from anaerobic sewage sludge a methanogenic

consortium which used 3-chlorobenzoate as the sole energy and carbon source. The enriched consortium also metabolized benzoate without a lag and at a faster rate than it metabolized 3-chlorobenzoate. Serial dilutions of the 3-chlorobenzoate degrading enrichment were inoculated into anaerobic roll tubes as previously described (4) except that benzoate (0.1%) was the growth substrate. Methanospirillum hungatii PM-1 and Desulfovibrio sp. PS-1 were added to separate tubes as the H_2 consumers.

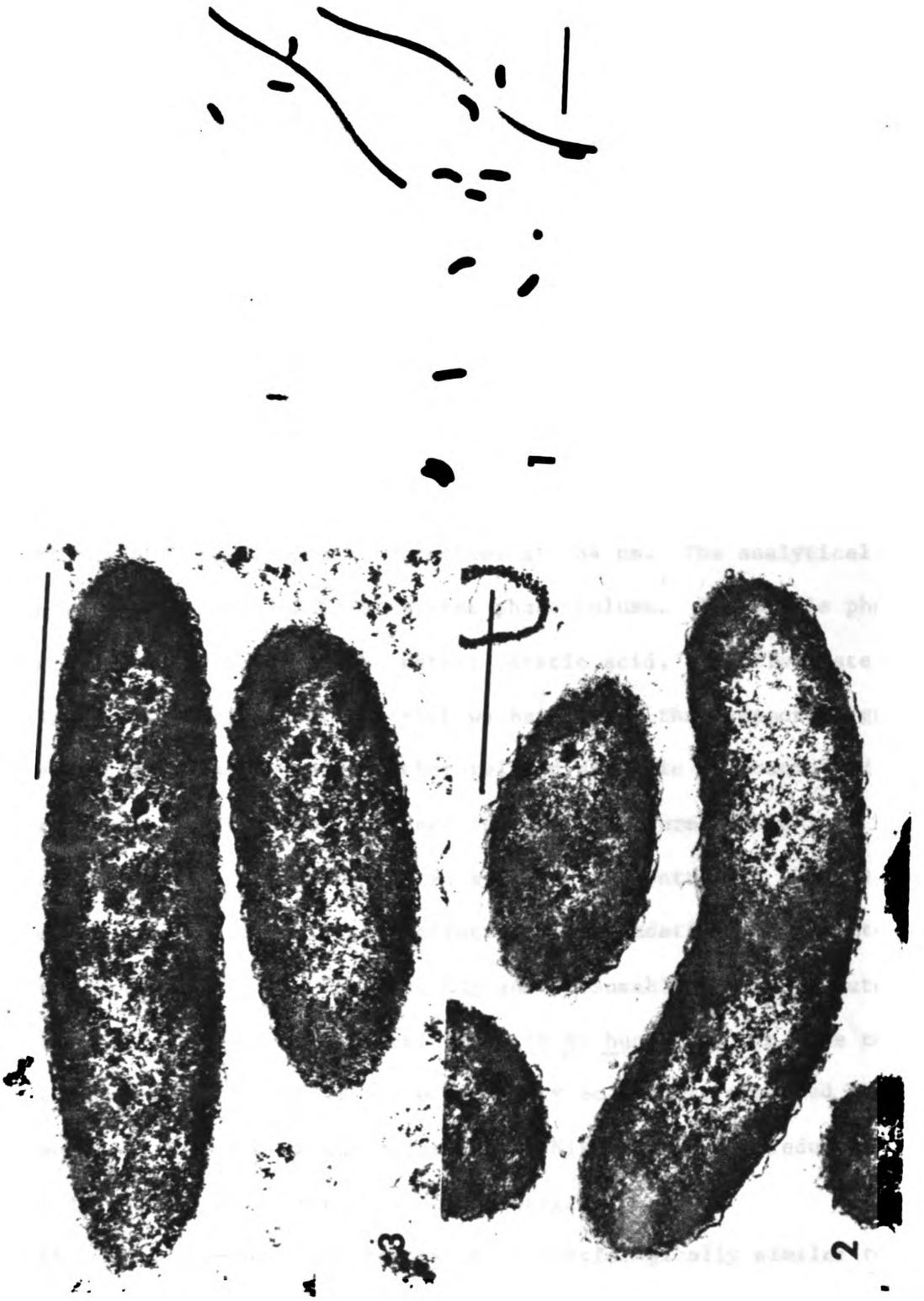
After 8 to 9 weeks, pinpoint colonies of approximately 0.1 mm diameter appeared in anaerobic culture tubes in co-culture with both the methanogen and the sulfate reducer. When colonies were transferred to liquid culture, however, only those incubated with Desulfovibrio sp. grew. Turbidity was evident after 8 to 9 weeks of incubation; the maximum density observed was 0.1 OD. Serial dilutions of this initial culture were again inoculated into anaerobic cultures tubes and colonies isolated, to assure purity. We have had no difficulty in shifting the benzoate oxidizing bacterium from co-culture with Desulfovibrio to M. hungatii as the H_2 consumer. However, even after several transfers of a 10% inoculum, low numbers of Desulfovibrio persisted in the co-culture with M. hungatii. Presumably, Desulfovibrio was maintaining itself on the low levels of fermentable substrates produced by the benzoate degrader or on the 5% clarified rumen fluid in the medium.

Both straight and curved rod shaped cells are seen. The cells are 1 to 2 um in length and 0.2 to 0.4 um in width (Fig. 1). We have occasionally observed what appears to be a twitching type of motility, however, it is difficult to distinguish from Brownian motion. The cells stained gram negative and appeared to have a gram negative envelope as

Fig. 1. Photomicrograph of the benzoate-oxidizing bacterium BZ-2 in co-culture with M. hungatii PM-1 showing curved and straight cells. Bar represents 5 um.

Fig. 2. Electron micrograph of the benzoate oxidizing bacterium BZ-2 showing curved cells. Bar represents 0.5 um.

Fig. 3. Electron micrograph of the benzoate oxidizing bacterium BZ-2 showing straight cells. Bar represents 0.5 um.



observed in the thin sections (Figs. 2-3). The wavy outer membrane of both cell types (Figs. 2-3) appears to be a distinctive feature of these organisms. Whether the curved and straight bacteria are distinct strains, or simply represent variable morphology is not clear. We were able to isolate two subcultures in which the curved (Fig. 2) and the straight rod (Fig. 3) morphologies were predominant (but not purely one type), however, we were not able to demonstrate any physiological differences between these two cultures. Thus, we cannot conclude that there are two different strains.

Aromatic substrates (benzoate, o-, m-, and p-hydroxybenzoate, phenylacetate, phenylpropionate, transcinnamate, and phthalate) fed to strain BZ-1 were quantified using a Varian 3700 HPLC coupled to a Hitachi variable wavelength detector set at 284 nm. The analytical column was a 250 x 4.6 mm C-18 reverse phase column. The mobile phase consisted of 60:40:5 methanol: water: acetic acid. The flow rate was 1.5 ml/min. The only substrate that we have found that supported growth of the organism was benzoate. Other related aromatic compounds did not support growth and were not consumed in liquid culture (Table 1). Even when aromatic substrates were tested at a low concentration (0.05%) to insure the absence of inhibitory effects, no degradation was detected. Benzoate was fermented to acetate, CH_4 and presumably either formate or CO_2 (Table 2) when grown in co-culture with M. hungatii. In time course experiments neither butyrate nor other fatty acids were observed as intermediates during benzoate metabolism. Nitrate was not reduced. Growth did not occur in the presence of oxygen.

This benzoate-degrading bacterium is physiologically similar to the bacterium isolated by Mountfort and Bryant (2). Both isolates use only

Table 1. Substrates which did not support growth of the benzoate degrading bacterium BZ-2 in culture with *M. hungatii* PM-1.

Glucose	Yeast extract
Fructose	Trypticase
Galactose	Casamino acids
Ribose	
Xylose	*o*-Hydroxybenzoate
	m-Hydroxybenzoate
Fumarate	*p*-Hydroxybenzoate
Glycerol	Phenylacetate
Ethanol	Phenylpropionate
Adipate	Trans-cinnamate
Pimelate	Phthalate
Butyrate	

Table 2. Fermentation products formed from benzoate by the benzoate degrading isolate BZ-2 in co-culture with M. hungatii PM-1.

Substrate	Substrate consumed (mmoles)	Products (mmoles)		% Recovered	
		Acetate	CH_4	C	H
Benzoate	0.27	0.82	0.20	101	99[a]

[a] Based on 0.75 mole of CH_4 produced per mole of benzoate metabolized.

benzoate as a growth substrate and benzoate is fermented to acetate, H_2, and either formate or CO_2. Neither bacterium appears to produce higher volatile fatty acids as fermentation products of benzoate catabolism. There are several differences, however. The isolate of Mountfort and Bryant did not produce any curved cells. Also, we were unable to achieve the high cell yields reported by Mountfort and Bryant.

The role this bacterium plays in the enrichment growing on 3-chlorobenzoate is apparently to metabolize the benzoate produced from the original substrate by the dechlorinating bacterium (3). The benzoate-degrading bacterium is consistent with the morphology of one of the dominant organisms in the enrichment, although this does not exclude the presence of other benzoate degrading organisms. In fact, we have observed colonies growing in co-culture with Desulfovibrio or M. hungatii in benzoate containing anaerobic culture tubes with distinctly different morphologies. However, we have not succeeded in isolating them.

ACKNOWLEDGEMENTS

We thank Terry Aust for technical assistance and Stuart Pankratz and Walter Smolenski for electron and photomicrographs.

LITERATURE CITED

1. Evans, W. C. 1977. Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Nature 270:17-22.

2. Mountfort, D. O. and M. P. Bryant. 1982. Isolation and characterization of an anaerobic syntrophic benzoate-degrading bacterium from sewage sludge. Arch. Microbiol. 133:249-256.

3. Shelton, D. R. and J. M. Tiedje. Isolation and characterization of an anaerobic bacterium that dechlorinates 3-chlorobenzoate. Appl. Environ. Microbiol. (submitted).

4. Shelton, D. R. and J. M. Tiedje. Isolation and characterization of two syntrophic anaerobic bacteria that oxidize butyrate. Appl. Environ. Microbiol. (submitted).

5. Tarvin, D. and A. M. Buswell. 1934. The methane fermentation of organic acids and carbohydrates. J. Amer. Chem. Soc. 56: 1751-1755.

CHAPTER 5

A GENERAL METHOD FOR DETERMINING ANAEROBIC BIODEGRADATION POTENTIAL

INTRODUCTION

Most manufactured chemicals will pass through anoxic environments and, in some cases they will reside in these habitats for long periods of time. These anoxic habitats include sediments of all types, anaerobic waste treatment systems, gastrointestinal tracts, poorly drained or flooded soils, and some landfills and groundwaters. In order to make an environmental risk assessment for a chemical, it may be important to determine its susceptibility to anaerobic biodegradation. Furthermore, information on anaerobic degradability is also requested under EPA guidelines implementing the U.S. Toxic Substances Control Act and is of interest to the OECD (Organization for Economic Cooperation and Development). To obtain this information a simple, general screening method for assessing anaerobic biodegradability is needed.

Owen et al. (10) provided the first description of such a test method drawing on previous gas measurement (9) and incubation bottle (8) methods. They based their method on measurement of the excess gas volume (CH_4 + CO_2) produced after addition of a test chemical to an anaerobic seed incubated in sealed bottles. The gas volume was measured from the displacement of the piston in a glass syringe whose needle had been inserted into the bottle. Subsequently Gledhill improved the method with the goal of defining a simple protocol that could be established by ASTM (American Society for Testing Materials) as a standard method (4). He introduced the use of a pressure transducer to

measure the gas pressure, recommended 50 mg carbon/l to be the test chemical concentration and used 10% anaerobic sludge as described by Healy and Young (5). He did not, however, evaluate each aspect of the method to determine if it was optimum, evaluate the variability and reproducability, and evaluate the method against other methods, all of which are necessary to validate a standard method. In this paper we report on this evaluation and describe the further refinements which we feel are beneficial. Furthermore, we report on chemicals which we found to be degraded anaerobically, on an improved anaerobic medium, and on the differences among municipal anaerobic sludges in their degradation capacities.

MATERIALS AND METHODS

Source and characteristic of anaerobic sludge. Sludge samples were collected from primary or secondary anaerobic digesters in 1 or 2 liter jars, tightly capped, and stored at 4°C until use. Sludges were from waste treatment plants in the following mid-Michigan communities: Adrian, Ann Arbor, Chelsea, Holt, Ionia, Jackson, Mason, Portland and St. Johns. Percent organic matter (total solids x volatile solids) varied from 0.89% (Holt) to 1.99% (Jackson) with a median value of 1.53%. Average retention times varied from 17 days (Ionia) to 39 days (Holt) with a median value of 20 days. Inflow varied from 1.6×10^6 1/day (Chelsea) to 68×10^6 1/day (Jackson) with a median value of 4.4×10^6 1/day.

Preparation of test bottles. Serum bottles of 160 ml capacity (described as 125 ml Wheaton serum bottles, Scientific Products, McGraw Park, Ill.) were amended as follows with 50 ppm of carbon of the test

compound: liquids were dispersed via microsyringe; water soluble solids were dissolved in water, then dispensed; water insoluble solids were dissolved in diethylether, dispensed, and the ether allowed to evaporate ($\geq$ 2 h); insoluble polymers were weighed out and added to serum bottles as solids. The revised anaerobic mineral medium (RAMM) developed for this study consisted of (per liter): phosphate buffer, 0.27 g KH_2PO_4 and 0.35 g K_2HPO_4 (adjusted to pH 7.0); mineral salts, 0.53 g NH_4Cl, 75 mg $CaCl_2 \cdot 2H_2O$, 100 mg $MgCl \cdot 6H_2O$, and 20 mg $FeCl_2 \cdot 4H_2O$; and trace metals modified from Zehnder (17), 0.5 mg $MnCl_2 \cdot 4H_2O$, 0.05 mg H_3BO_3, 0.05 mg $ZnCl_2$, 0.03 mg $CuCl_2$, 0.01 mg $NaMO_4 \cdot 2H_2O$, 0.5 mg $CoCl_2 \cdot 6H_2O$, 0.05 mg $NiCl_2 \cdot 6H_2O$, and 0.05 mg Na_2SeO_3. The medium was autoclaved for 5 to 10 min to drive off O_2, then cooled to approximately 35°C while sparging with a 10% CO_2/90% N_2 gas mixture passed through copper fillings at 300°C to remove traces of O_2. After cooling, 1.2g/l $NaHCO_3$ and 0.5g/l $Na_2S \cdot 9H_2O$ (optional) were added to the medium. A 10% diluted sludge was prepared by adding one part of sludge filtered through one layer of cheesecloth to nine parts of cooled mineral medium while stirring. The diluted sludge was dispensed into the serum bottles while sparging with an O_2-free 10% CO_2/90% N_2 gas mixture. Serum bottles were sealed with 1 cm thick butyl rubber stoppers (Bellco Glass, Inc., Vineland, New Jersey) and capped with aluminum crimp seals (Scientific Products, McGraw Park, Illinois). All compounds were tested in triplicate with the exception of the compound survey where compounds were tested in duplicate. Bottles were incubated stationary and in the dark at 35°C.

Measurement of gas production. After the medium had equilibrated to 35°C, the bottles were vented by syringe needle to atmospheric

pressure (generally there was 1 to 3 ml overpressure). Total gas production (CH_4 + CO_2) was measured by a UniMeasure pressure transducer (Grants Pass, Oregon) equipped with a P-8 adapter (bellows) capable of measuring up to 8 psi gas pressure (Fig. 1). The needle was inserted through the stoppers of the serum bottles and the signal (in mohms) from the transducer was quantified using a Fluke multimeter (Mountlake Terrace, Washington). Serum bottles were shaken vigorously before pressure measurements were taken and excess gas pressure was vented afterwards through the 3-way valve to avoid cumulative gas pressures beyond the response range of the P-8 adapter. The mohm response was related to ml of gas produced by a standard curve constructed from adding known quantities of gas to serum bottles by syringe; the R^2 was > 0.999%. Net gas production was calculated by subtracting gas produced in unamended bottles from test bottles. Degradation is expressed as % of theoretical gas production based on the stoichiometry of mineralization to CH_4 + CO_2 and correcting for gas solubilities.

Methane production was quantified by injecting 0.3 ml of headspace gas from serum bottles into a gas chromatograph equipped with a flame ionization detector. Net methane production was calculated by subtracting background methane production in unamended bottles from test bottles. Degradation is expressed as % theoretical methane production based on the stoichiometry of degradation.

Experimental. To determine the effect of length of sludge storage on assay results, primary anaerobic sludge from Jackson, Holt, and Chelsea sewage treatment plants was stored, sealed at 4°C. Incubations were begun by taking new containers of sludge from storage after 0, 1, 2, 3, and 4 weeks. Ethanol, p-cresol, phthalic acid, and

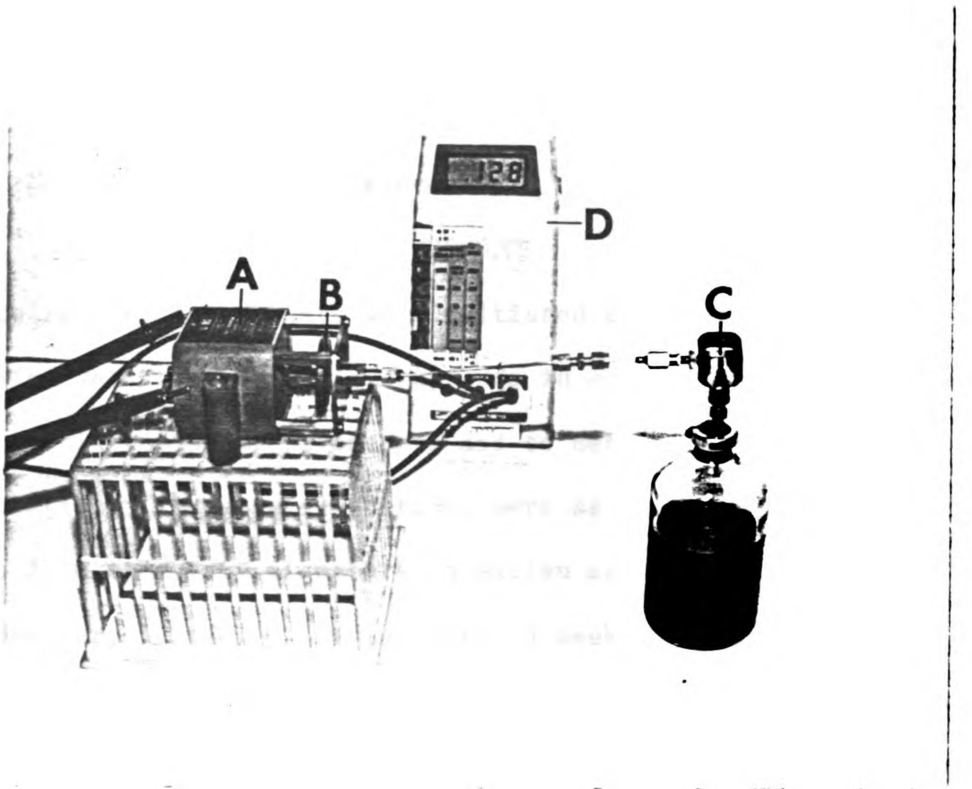


Fig. 1. Gas production was measured with a pressure transducer (A) equipped with a P-8 bellows (B). The transducer was connected via 10 cm of 1/16" stainless steel tubing and a 1/16" Swagelok union to a Hamilton 3-way valve (C) with a 20-gauge needle attached. The signal from the transducer was quantified with a multimeter (D).

di-n-butylphthalate at 50 ppm-C were used as test compounds.

The effect of oxygen intrusion on the assay was investigated by replacing equal volumes of headspace gas with oxygen added by syringe. Oxygen concentrations were quantified using a gas chromatograph equipped with a thermal conductivity detector.

RESULTS

Preliminary experiments were initiated to obtain degradation data for a wide variety of organic compounds in order to select compounds for future testing of assay parameters and to determine the minimum length of incubation. The assay conditions were as described except that sludges from secondary digesters in Adrian and Jackson were used. Compounds were initially incubated for 4 weeks, however, this proved to be inadequate. Subsequently, all compounds were incubated for 8 weeks, or until net methane production had ceased. Of 94 compounds tested, 27 were mineralized (> 75% of theoretical methane production) in at least one sludge (Table 1); of these, eight had lag times of > 2 weeks. Ten compounds were partially mineralized (> 30% < 75% of theoretical methane production). 5-Chlorosalicyclic acid, p-nitrophenol, m-chlorophenol, p-chlorophenol, pentachlorophenol, chloroform, hexachloropentadiene, trichloroacetic acid, 4-phenoxybutyric acid, and atrazine were inhibitory (p-nitrophenol caused only transient inhibition in 10% primary sludge). For completeness, compounds which we have subsequently observed to be degraded in 10% primary sludge are also listed (Table 1). These include several compounds which were not degraded in the above experiment with secondary sludge.

From this survey we selected compounds that represented a range from easily degraded to those requiring much longer times for degradation, and used these as standard chemicals for further testing of

Table 1. Summary of organic compounds mineralized under anaerobic conditions in 10% sludge.

Compounds mineralized in 10% secondary sludge.[a]

Acetylsalicyclic acid	p-Cresol	1-Octanol
Acrylic acid	Di-n-butylphthlate	Phenol
p-Anisic acid	Di-methylphthalate	Phloroglucinol
o-Anthranilic acid	Ethylacetate	Phthalic acid
Benzoic acid	2-Hexanone	Polyethylene-
Benzyl alcohol	o-Hydroxybenzoic acid	Protocatechuic acid
2,3-Butanediol	p-Hydroxybenzoic acid	Pyrogallol
Catechol	3-Hydroxybutanone	
m-Cresol	3-Methylbutanol	

Compounds partially mineralized in 10% secondary sludge[b]

p-Aminobenzoic acid	Geraniol
Butylbenzylphthalate	4-Hydroxyacetanilide
4-Chloroacetanilide	p-Hydroxybenzyl alcohol
m-Chlorobenzoic acid	2-Octanol
Di-ethylphthalate	Propionanilide

Compounds mineralized in 10% primary sludge[a]

Butylbenzylphthalate	m-Methoxyphenol
m-Chlorobenzoic acid	p-Methoxyphenol
o-Chlorophenol	o-Nitrophenol
Di-ethylphthalate	p-Nitrophenol
o-Methoxyphenol	

[a] Greater than 75% of theoretical methane production.

[b] Between 30% and 75% of theoretical methane production.

the method.

Evaluation of the assay conditions. A typical temporal pattern of degradation as measured weekly by the pressure transducer for some of the selected compounds is shown in Fig. 2. Lag times such as seen for p-cresol and phthalic acid are readily detected by this method.

The optimum concentration of test chemical was assessed by incubating four test chemicals at concentrations of 25, 50, 100, and 200 ppm carbon in 10% Jackson primary sludge (Table 2). There was no significant effect of substrate concentration on lag times or extent of degradation. Theoretical gas production from 50 ppm carbon substrate ranged from 20 to 25% of the background gas production. Standard deviations tended to decrease with increasing substrate concentration.

To evaluate the anaerobic mineral salts medium for the assay, the ion concentrations in 10% sludge from waste treatment plants in eight states (14) were compared with several published anaerobic media (Table 3). With the possible exception of K^+, NH_4^+, and Co, all mineral and metal nutrients should be in ample supply in 10% sludge. Based on this, a supplemental medium consisting of 6 mM phosphate (to improve buffering capacity), 9 mM K^+, 10 mM NH_4^+, and 10 uM Co was evaluated. We also derived a complete mineral salts medium from considering the contents of published media (3,5,6,7,11,13,15,16,17,18), the requirements of the organisms and a desire to avoid excessive precipitation. The medium is termed RAMM (revised anaerobic mineral medium). The supplemental, RAMM and ASTM (4) media were compared for their effect on background gas production, lag times, and extent of degradation in Jackson, Ionia, and Holt primary sludges. An inhibition of background gas production was observed in the ASTM medium (Table 4). Further experimentation indicated that an inverse correlation existed between added sulfide and

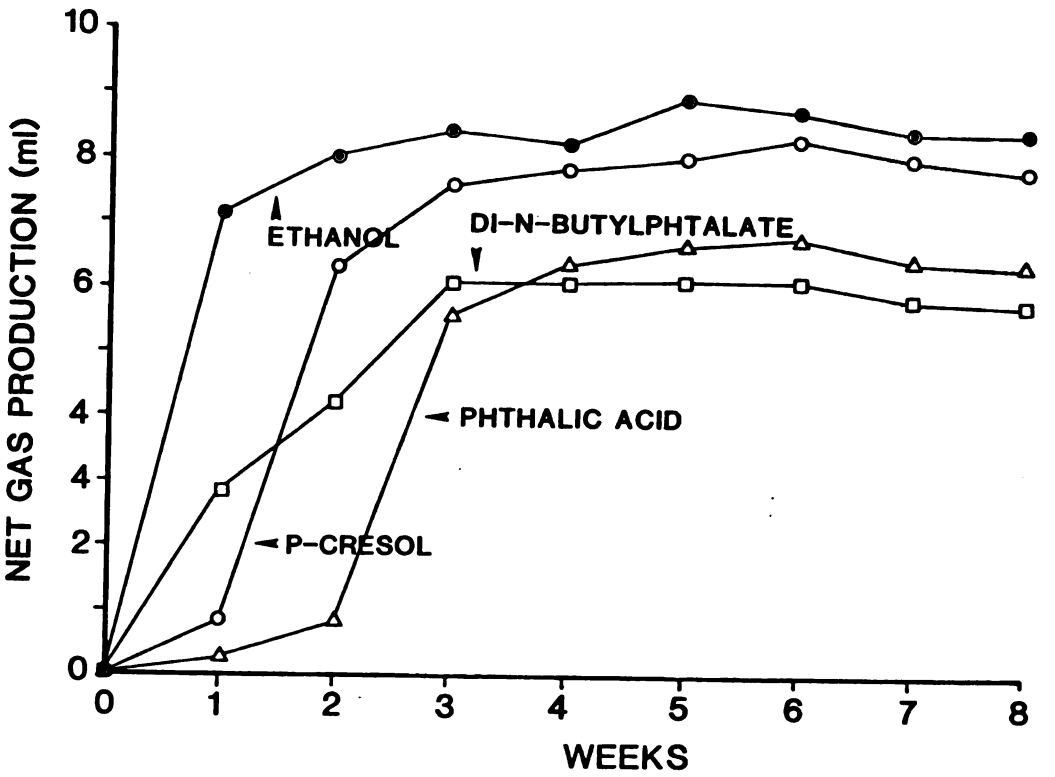


Fig. 2. Pattern of gas production (CH_4 + CO_2) from ethanol, p-cresol, phthalic acid, and di-n-butylphthalate incubated anaerobically with 10% digester sludge.

Table 2. Effect of substrate concentration on gas production in 10% Jackson sludge

Substrate concentration (ppm carbon)	Mean percent of theoretical degradation ± S.D.			
	Phenol	p-Cresol	Benzoic acid	Phthalic acid
25	100 ± 9.9	98	92 ± 18.0	105 ± 4.8
50	104 ± 8.0	86 ± 13.5	92 ± 6.0	104 ± 18.7
100	106 ± 6.3	98 ± 5.5	96 ± 4.7	109 ± 3.7
200	113 ± 3.1	95 ± 4.5	98 ± 3.6	100 ± 1.5
Background gas production (ml)	31.4	31.6	24.5	28.4
Theoretical gas production from 50 ppm carbon (ml)[a]	7.4	7.6	6.5	5.7

[a] Corrected for gas solubilities.

Table 3. Comparison of mineral salts and metals in anaerobic media vs. 10% sludge.

	RAM medium	ASTM medium[a]	Survey of anaerobic media[b]	Survey of sludges[c] Range	Median	Mean
Minerals (mM)						
K	6.0	3.5	2.0–36.2	0.02–2.8	0.3	0.5
NH_4^+	10.0	4.3	3.2–42.4	0.03–15.4	0.4	2.1
PO_4^{2-}	4.0	0.3	0.3–23.6	0.7–18.9[c]	0.9[c]	4.4[d]
Ca	0.5	0.3	0.05–1.0	1.9–20.5	5.0	5.9
Mg	0.5	1.8	0.05–1.8	0.05–2.3	0.8	1.0
Fe	0.1	1.85	0.007–1.85	0.07–11.2	0.9	1.2
S^{2-}	0.5	2.0	—	—	—	—
Metals (uM)						
Mn	2.53	101.0	0.15–101.0	4.0–530	21	30
Zn	0.37	14.7	0.35–15.4	7.0–1,740	120	210
Cu	0.22	17.2	0.06–20.1	5.0–650	60	90
Co	2.10	126.0	0.42–126	0.2–1.2	0.5	0.6
Ni	0.21	—	0.08–0.21	0.1–240	6.0	30
B	0.81	97.1	0.81–97.1	5.0–280	14.0	37
Mo	0.04	41.7	0.04–41.7	1.0–1.3	1.3	1.2
Se	0.29	—	0.29–119.4	—	—	—
$NaHCO_3$	1.2 g/l	2.64 g/l				
Resazurin	1 mg/l	1 mg/l				
CO_2/N_2	10%/90%	30%/70%				

[a] From reference 4.

[b] From references 3, 5, 6, 7, 11, 13, 15, 16, 17, 18.

[c] Calculations based on a median total solids of 4.1%.

[d] Total phosphorous

Table 4. Effect of three mineral salts media on gas production in 10% sludge.

Medium	Mean percent of theoretical gas production (± S.D.)				
	p-Cresol	Phthalic acid	Di-n-butyl-phthalate	m-Chloro-benzoid acid	Background gas production
		Jackson sludge			
ASTM[a] medium	102 ± 3.2	156 ± 0	128 ± 3.9	30 ± 8.6	13.5
RAM medium	108 ± 3.9	130 ± 5.0	101 ± 9.7	0	18.8
Supplemental medium[b]	79 ± 7.9	85 ± 7.7	89 ± 14.8	0	17.9
		Holt sludge			
ASTM medium	106 ± 10.3	145 ± 19.0	59 ± 0	153 ± 6.1	9.6
RAM medium	104 ± 4.5	112 ± 13.7	46 ± 3.2	101 ± 3.5	18.1
Supplemental medium	89 ± 4.6	96 ± 2.9	19 ± 5.4	65 ± 5.2	19.0
		Ionia sludge			
ASTM medium	127 ± 7.1	183 ± 9.5	117 ± 4.7	50 ± 9.4	16.2
RAM medium	115 ± 9.5	118 ± 11.4	72 ± 3.6	15 ± 10.4	20.1
Supplemental medium	99 ± 6.7	104 ± 24.8	77 ± 16.7	0	21.6

[a] Added 3.6 g $NaHCO_3$/liter to be in equilibrium with the 30% CO_2 specified instead of the 2.64 $NaHCO_3$/liter indicated in the reference.

[b] 6 mM $PO_4^{=}$, 9 mM K^+, 10 mM NH_4^+, 10 uM Co, 10% CO_2/90% N_2 headspace.

background gas production (data not shown). There was no significant effect on lag times with any of the media, however the extent of degradation did vary (Table 4). Percent theoretical gas production was consistently higher in the ASTM medium than in the RAM medium, while the supplemental medium generally yielded the lowest % theoretical gas production. The effect was particularly pronounced with phthalic acid and m-chlorobenzoic acid (both amended into sludges as the monoacid) where % theoretical gas production in the ASTM medium exceeded 150%. With m-chlorobenzoic acid virtually all of the excess gas production was observed in the first week of incubation, well before the end of the typical lag period for this compound (Fig. 3).

There was no significant effect of sludge storage on extent of degradation, however, there was an effect on lag times before degradation began for compounds that were degraded more slowly. This is illustrated for p-cresol (Fig. 4) where lag times increased from 4 to 7 weeks for Holt sludge, 2 to 3 weeks for Jackson sludge, and 4 to 4.5 weeks for Chelsea sludge; lag times for ethanol, which is readily degraded, were unaffected (data not shown).

Effect of oxygen on gas production. The rate of oxygen consumption by 10% sludge was investigated since oxygen could inadvertently enter the test bottles and affect test results. Adrian sludge consumed approximately 1 ml of O_2 per day (Fig. 5). The upper portion of the sludge suspension in serum bottles containing a headspace gas mixture of $\geq$ 8% O_2 was lightly pink due to the oxidation of resazurin (Fig. 5).

The effect of O_2 intrusion on gas production was tested in Holt primary sludge. In serum bottles without substrate there was approximately 4 ml less gas production in bottles injected with 4 ml O_2

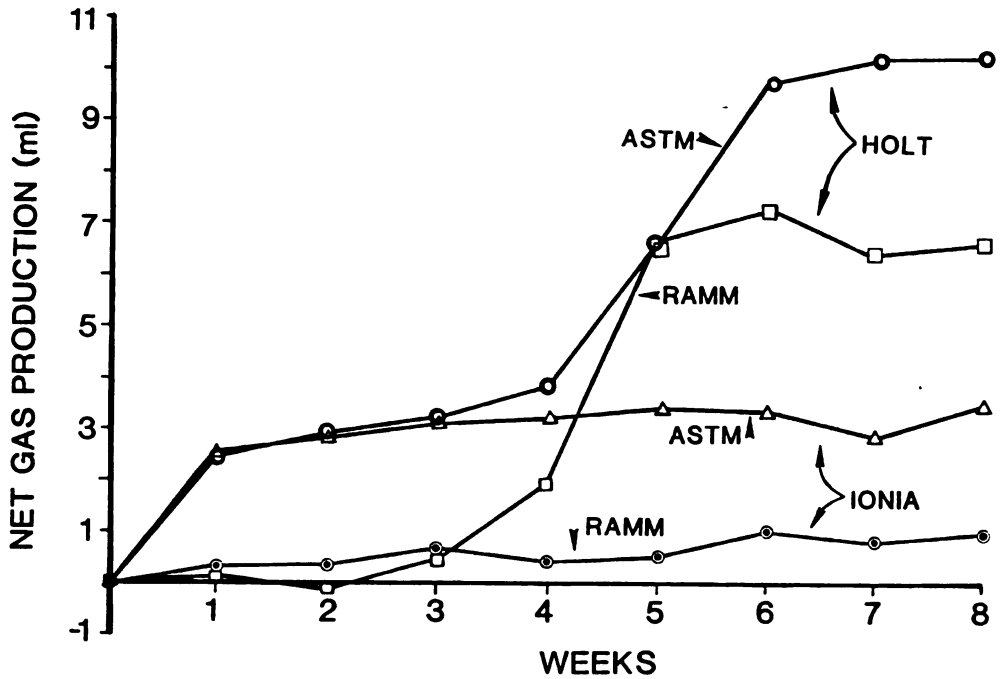


Fig. 3. Comparison of two mineral salts media (RAMM vs ASTM) on gas production from m-chlorobenzoic acid in Holt and Ionia sludges.

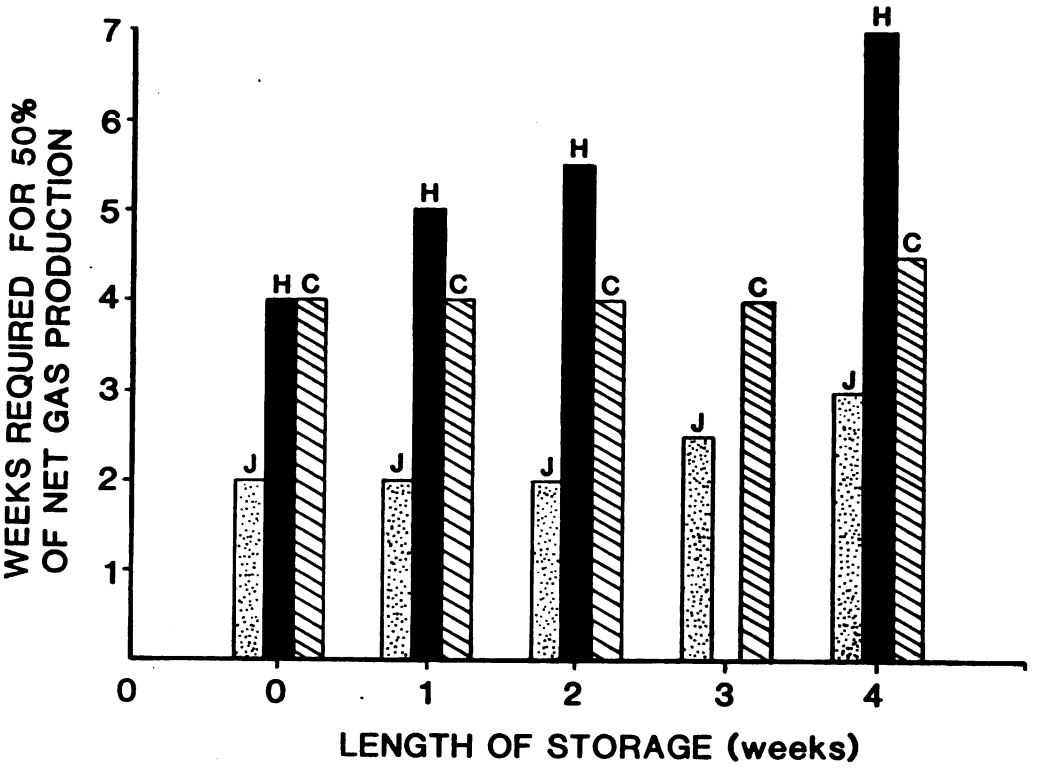


Fig. 4. Weeks required for 50% of net gas production from p-cresol as affected by length of storage of sludges from Jackson (J), Holt (H), and Chelsea (C).

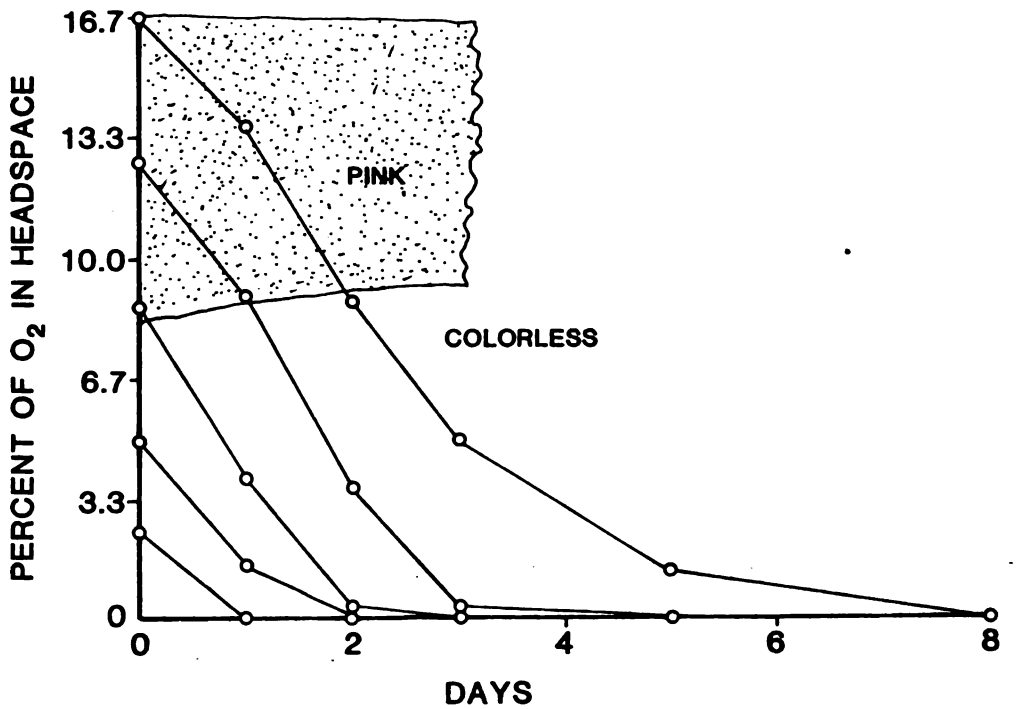


Fig. 5. Rate of consumption of different O_2 concentrations by 10% sludge. The conditions where the resazurin was pink is shown by the stippled area.

(due to O_2 consumption) than in bottles with no O_2 (Fig. 6). In serum bottles containing benzoic acid plus 4 ml O_2 there was approximately 6 ml less gas production. Using serum bottles receiving no benzoic acid as controls, the % theoretical degradation of benzoic acid was 115% in bottles receiving no O_2 and 87% in bottles receiving 4 ml O_2. After 16 h bottles receiving 4 ml of O_2 (7% O_2 in the headspace) were slightly pink at the gas-water interface, however, the pink disappeared if the bottles were slightly agitated; no pinkish color was evident after 40 h.

Capacity of different anaerobic sludges to degrade selected compounds. Primary anaerobic sludges from nine sewage treatment plants were compared for their ability to degrade nine test compounds (Table 5). Ethanol, polyethylene glycol-20,000, p-cresol, and phthalic were degraded in all sludges. m-Cresol and di-n-butylphthalate were degraded in approximately half the sludges, while 2-octanol, m-chlorobenzoic acid, and propionalilide were degraded in three sludges or less. Percent degradation was generally greater than 75% for m-cresol, p-cresol, phthalic acid and m-chlorobenzoic acid. The lower % degradation for polyethylene glycol-20,000 and 2-octanol may be in part due to their slower rates of degradation such that an 8 week incubation was not sufficient to allow for complete methane recovery. We suspect that low methane recoveries for propionanilide was due to metabolism of only the propionate moiety.

DISCUSSION

The only feasible approach to a generalized method to screen organic compounds for anaerobic biodegradation is to measure their common terminal products, $CH_4 + CO_2$. A number of approaches to

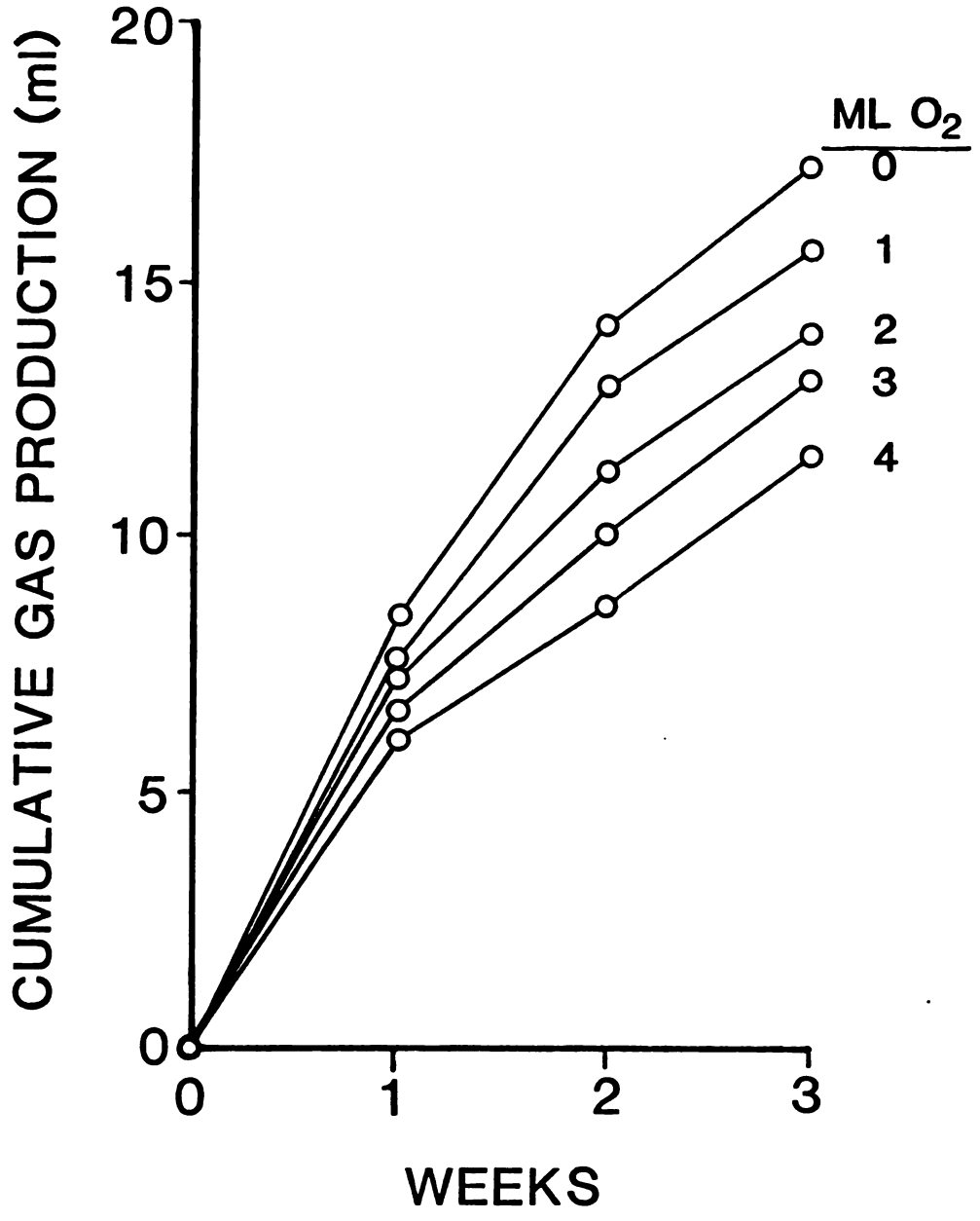


Fig. 6. Effect of different amounts of added oxygen on cumulative gas production in 10% Holt sludge (no substrate added).

Table 5. Percent theoretical methane production from nine substrates by sludges from nine municipalities.[a]

Sludge	Ethanol	Polyethylene glycol-20,000	p-Cresol	Phthalic acid	m-Cresol	Di-n-butyl phthalate	2-Octanol	m-Chlorobenzoic acid	Propionanilide
Adrian	62	61	91	88	82	24	0	85	0
Jackson	78	43	88	80	103	49	22	0	0
Ann Arbor	86	i.d.[b]	101	132	85	91	58	0	36
St. Johns	78	78	79	113	0	37	0	0	33
Ionia	71	51	80	73	0	36	0	0	0
Holt	33	53	88	86	77	i.d.	0	85	23
Mason	94	82	94	96	91	0	87	0	0
Chelsea	54	38	62	60	i.d.	0	0	0	0
Portland	52	98	77	96	0	0	0	0	0

[a] 10% fresh primary sludge incubated 8 weeks after which methane was measured by gas chromatography.

[b] i.d. = insufficient data; generally due to leaky bottles or only one bottle showing evidence for degradation.

measuring these digestion products have been used, but only recently have these evolved towards a routine test method. We began our work by evaluating the Owen et al. (10) and closely related ASTM provisional (4) methods since these seemed to be the most promising of the existing methods.

We found that a 10% sludge inoculum will generally yield from 10 to 40 ml of gas, depending on the retention time and % organic matter of the sludge. A 50 ppm-carbon addition will theoretically yield 10.5 ml of gas, while the actual gas yield (corrected for solubility) may range from 5.5 to 8.5 ml depending on the stoichiometry of mineralization. Concentrations less than 50 ppm-C led to increased variability and perhaps incorrect conclusions on biodegradability, while concentrations greater than 50 ppm-C are not needed for reliability and could lead to more cases of toxicity by the test chemical. Although a pre-incubation of the sludge to reduce background gas production would allow for use of a less dilute inoculum or of concentrations of test compound less than 50 ppm-C, we do not feel that the added expenditure in time and effort are necessary. In our opinion, the presence of some readily degradable organic matter may be helpful after the perturbation caused by mixing and dispensing of the sludge solution.

It is possible that some compounds which are relatively slowly degraded in whole sludge may not be appreciably degraded in 10% sludge. We have compared in undiluted and 10% Jackson sludge the degradation of six phthalic acid esters (12) (di-methyl-, di-ethyl-, di-n-butyl-, butylbenzyl-, di-(2-ethylhexyl)-, and di-n-octylphthalate) and six monosubstituted phenols (1,2) (o-, m-, p- cresol and o-, m-, p-chlorophenol). Of the six phthalic acid esters four were mineralized

in both undiluted and 10% sludge, while two were persistent in both. Of the six substituted phenols five were mineralized in undiluted sludge (o-cresol was persistent) while three were mineralized in 10% sludge; m- and p-chlorophenol were not mineralized in 10% sludge. For compounds which are slowly mineralized and/or are toxic at higher concentrations, e.g. m- and p-chlorophenol, the 10% sludge assay may yield false negatives, however, for many compounds we feel that the 10% sludge assay should successfully reflect the activity of undiluted sludge.

Based on a survey of the literature and our own experience, we do not feel that there is a strong basis for preferring any particular anaerobic medium. In most cases, 10% sludge alone is likely to contain all mineral and metal nutrients in ample supply, with the possible exce ption of K^+, NH_4^+, and Co. However, a more complete medium is probably advisable as insurance. We recommend the use of a 4 mM phosphate buffer in conjunction with a 10% CO_2/90% N_2 headspace and 1.2 g $NaHCO_3$/liter rather than the ASTM medium because of the effect the latter can have on abiological gas production. In test bottles containing the ASTM medium, % theoretical degradation exceeded 100%; this was particularly pronounced for phthalic acid and m-chlorobenzoic acid. We believe that the overpressures were a result of abiological fluxes of CO_2 into the headspace. Since the ASTM medium is buffered only by a 30% CO_2/HCO_3^- system, any introduction of acids into the medium would result in a flux of CO_2 into the headspace. This interpretation is supported by the fact that there was a flush of gas into the headspace of bottles containing the ASTM medium amended with m-chlorobenzoic acid during the first week (Fig. 3), even in the absence of subsequent degradation; this accounted for much or all of the increased gas production. The RAM medium and the

supplemental medium contained a 4 mM and a 6 mM phosphate buffer, respectively, in addition to 10% CO_2/HCO_3^-, so that abiological fluxes of CO_2 would be diminished. We have routinely used the RAM medium with satisfactory results. Actual gas production with this medium has been consistent with theoretical predictions throughout the study.

Sludges may be stored for up to 4 weeks at 4°C in tightly capped containers, however, fresh sludge should be used whenever possible. Attenuation of sludge activity can occur during storage, but, this is more likely to be a factor with sludges having long retention times and/or a % organic matter less than 1.0%. Holt sludge, which was most affected by length of storage, had a 39 day retention time and 0.89% organic matter. We recommend that serum bottles be incubated for a minimum of 8 weeks. Lag times for p-cresol and phthalic acid in fresh sludge varied from 2 to 4 weeks, so that shorter incubation times are not advisable.

Anaerobic methods are always subject to error from O_2 contamination, however, the high O_2 consuming capacity of most sludges, the use of new, thick butyl rubber stoppers and the use of standard anaerobic methods should prevent any serious errors. If O_2 intrusion does occur it will reduce the gas pressure somewhat (due to respiratory activity). We did not find it necessary to add reductant (sulfide) to the anaerobic medium because of the high O_2 scavenging capacity of the sludge. Its use is optional, but it should not be used in concentrations > 1 mM since it may cause some toxicity (Table 4).

We recommend the use of primary anaerobic sludge with a retention time of 15 to 30 days and % organic matter (total solids x volatile solids) of 1.0 to 2.0%. Shorter retention times or higher % organic

matter will result in high background gas production, while longer retention times or a lower % organic matter will result in attenuation of activity. The use of secondary anaerobic sludge, even though it has low background gas production, is not advised due to attenuation of sludge activity. Secondary sludge digesters are unheated and unmixed and often have retention times up to 90 days. An incubation temperature of 35°C is advisable because most anaerobic digesters are operated at this temperature. Based on a comparison of degradation of nine test aompoundq in ppimary anaerobic sludges from nine sewage treatment plants, sludges varied with respect to their ability to degrade particular compounds within an 8 week incubation. We were unable, however, to correlate the range of compounds degraded with any single factor (plant inflow, % industrial input, etc.) which might select a more active and versatile sludge community.

We have monitored mineralization both by measuring CH_4 production and measuring total gas pressure. Although assessment of degradation by measuring gas production is subject to error due to abiological flux of CO_2 between solution and headspace; the speed, accuracy, precision, and cost effectiveness of the gas pressure method makes it the preferred method for a screening level test. We have systematically corrected for gas solubilities to have a more accurate assessment of degradation. These deviations can range up to 45% of theoretical but, because of the complexity of these calculations for a routine test, they may not be necessary once experience is gained with interpreting biodegradation results.

It is advisable to have reference chemicals included with each series of incubations to characterize the activity of the sludge. We

have routinely used p-cresol and phthalic acid throughout our own testing. We feel that they are useful as reference chemicals because both compounds appear to be degraded in all sludges, degradation results for both compounds have been consistently reproducible, and lag times before degradation have varied from 2 to 6 weeks depending on age and source of sludge. Ethanol can be used as a positive control as it is more easily degraded than the above two.

We have found reproducibility among replicate bottles to be good; standard deviations were generally less than 10%. Three test bottles seem sufficient to assess biodegradation. We have had little difficulty in obtaining reproducible results with the same sludge over a 2 year period. When degradation results for test compounds which were tested more than once (in all experiments) are compared, there are nine discrepancies out of a total of 65 comparisons (Table 6) and for eight of the nine, at least, partial mineralization was observed. Results for di-n-butylphthalate exemplify what we feel is likely to be the most significant problem with this test method, particularly for compounds which are difficult to degrade - - that is assessing biodegradation when only partial mineralization is observed. Such results could be due to poor technique, faulty equipment, a relatively inactive sludge, or susceptibility of only a portion of the compound to mineralization. In those instances when only partial mineralization is observed, the safest course is to repeat the incubation using fresh sludge. We have typically considered theoretical gas production > 75%, after correction for gas solubilities, to be indicative of complete mineralization.

Table 6. Reproducibility of results with different batches of sludge for several compounds and sludges.

No. of times degradation observed[a]/ No. of times degradation tested

Sludge	p-Cresol	Phthalic acid	m-Chlorobenzoic acid	Di-n-butyl-phthalate
Jackson	5/5	5/5	0/3	3/4
Adrian	2/3	3/3	1/2	2/3
Holt	3/3	3/3	2/2	0/2
Mason	2/2	2/2	0/2	1/2
Ionia	2/2	1/2	0/2	1/2
Chelsea	1/2	1/2		0/2

[a] Degradation is defined as > 75% of theoretical gas or methane production.

[b] Phenol and benzoic acid were both degraded in Jackson sludge two out of two times.

Summary of recommended protocol for anaerobic biodegradation test.

1) Use primary anaerobic sludge with 15-30 day retention time and total organic solids of approximately 1.0-2.0%. Sludges can be stored for up to 4 weeks at 4°C in tightly capped containers; however, fresh sludge should be used whenever possible. Since sludges vary in their selected populations, it may be useful to use more than one sludge when working with more persistant compounds.

2) We suggest use of our revised anaerobic mineral medium since it has been thoroughly evaluated under the test conditions. If another anaerobic mineral medium is used a 4 mM phosphate buffer and 1.2 g $NaHCO_3$/liter with a 10% CO_2/90% N_2 headspace is recommended. Sulfide can be added to ensure reducing conditions in concentrations not to exceed 1 mM, but it is not necessary.

3) Incubate a 10% homogenous sludge solution with 50 ppm-C if test chemical in 160 ml serum bottles with new butyl rubber stoppers and aluminum crimp seals. Each chemical should be tested in triplicate; a standard deviation of $< 15\%$ is expected.

4) Incubate serum bottles at 35°C for at least 8 weeks or until biodegradation is complete. If gas production is still in progress at the eighth week, then incubations should be continued until gas production is complete.

5) Measure gas production with a pressure transducer and multimeter which is connected to the test bottle with a 3-way valve and 20-gauge needle. Measurements should be made on a weekly basis; excess gas pressure should be vented.

6) Reference chemical(s) should be included with each series of incubations in order to confirm sludge activity. p-Cresol and phthalic

acid are suggested as a more rigorous test of biodegradation capacity. Ethanol, which is easily degraded, may be used as a positive control.

ACKNOWLEDGEMENTS

We thank Mary Lou Krumme, Steve Aldrich, Susan Frasier, and Marilyn Boucher for their excellent technical assistance. This research was supported by EPA Office of Toxic Substances Contract BCL T-6420(7197)-033.

LITERATURE CITED

1. Boyd, S. A., D. R. Shelton, D. Berry, and J. M. Tiedje. 1983. Anaerobic biodegradation of phenolic compounds in digested sludge. Appl. Environ. Microbiol. 46:50-54.

2. Boyd, S. A. and D. R. Shelton. 198 . Anaerobic biodegradation of chlorinated phenols in digested sludge. (submitted to Appl. Environ. Microbiol.

3. Ferry, L. R. and R. S. Wolfe. 1977. Nutritional and biochemical characterization of Methanospirillum hungatii. Appl. Environ. Microbiol. 34:371-376.

4. Gledhill, W. E. 1979. Proposed standard practice for the determination of the anaerobic biodegradability of organic chemicals. Working document. Draft 2. #-35.24, Amer. Soc. Testing Materials.

5. Healy, J. B. and L. Y. Young. 1979. Anaerobic biodegradation of eleven aromatic compounds to methane. Appl. Environ. Microiol. 38:84-89.

6. Khan, A. W., T. M. Trotlier, G. B. Patel, and S. M. Martin. 1979. Nutrient requirement for the degradation of cellulose to methane by a mixed population of anaerobes. J. Gen. Microbiol. 12:365-372.

7. McInerney, M. J., M. P. Bryant, and N. Pfennig. 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol. 122:129-135.

8. Miller, T. L. and M. J. Wolin. 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Microbiol. 27:985-987.

9. Nottingham, P. M. and R. E. Hungate. 1969. Methanogenic fermentation of benzoate. J. Bacteriol. 98:1170-1172.1

10. Owen, W. F., D. C. Stuckey, J. B. Healy Jr., L. Y. Young, and P. L. McCarty. 1979. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Research. 13:485-492

11. Patel, G. B., A. W. Khan, and R. A. Prins. 1978. Methanogenic fermentation of benzoate in an enrichment culture. Microbiol. Ecol 45:347-356.

12. Shelton, D. R., S. A. Boyd, and J. M. Tiedje. 1982. Anaerobic biodegradation of phthalic acid esters in sludge. Environ. Science Technol. (in press, in Nov. issue).

13. Shlomi, E. R., A. Lankhorst, and R. A. Prins. 1978. Methanogenic fermentation of benzoate in an enrichment culture. Microbiol. Ecol. 4:249-261.

14. Sommers, L. E. 1977. Chemical composition of sewage sludges and analysis of their potential use as fertilizers. J. Environ. Qual. 6:225-261.

15. Taylor, G. T. and S. J. Pirt. 1977. Nutrition and factors limiting the growth of methanogenic bacterium (Methanobacterium thermoautotrophicum) Arch. Microbiol. 13:17-22.

16. Toerien, D. F. 1970. Population description of the non-methanogenic phase of anaerobic digestion - 1. Isolation, characterization, and identification of numerically important bacteria. Water Research. 4:129-148.

17. Zehnder, A. J. B. and K. Wuhrmann. 1977. Physiology of Methanobacterium strain AZ. Arch. Microbiol. 111:199-205.

18. Zeikus, J. G., P. J. Weimer, D. R. Nelson, and L. Daniels. 1975. Bacterial methanogenesis: acetate as a methane precursor in pure culture. Arch. Microbiol. 104:129-134.

APPENDIX 1

PARTIAL CHARACTERIZATION OF A PHENOL DEGRADING ENRICHMENT

A methanogenic consortium able to degrade phenol as sole energy and carbon source was enriched from a mixture of anaerobic sewage sludges obtained from Adrian and Jackson, Michigan. The enrichment was maintained by transferring 10% of a turbid culture (approximately every 2 months) to fresh medium for over three years. Between transfers the culture was fed 1 mM phenol every 2 weeks. The medium was described in Chapter 5.

Microscopic examination of the enrichment revealed the presence of three dominant cell types. One cell type was a medium sized rod which autofluoresced; this was presumed to be an H_2-consuming methanogen (probably Methanobacterium). The second type had a morphology identical to Methanothrix soehgenii and thus thought to be the acetate-utilizing methanogen. The third type was a medium-sized, eliptical-shaped coccobacillus. This last organism was presumed to be the phenol-degrading bacterium. I attempted to isolate this organism as a syntroph on roll tubes using 0.05% phenol as substrate in the basal medium described in Chapter 2. All attempts at isolation failed. The most likely reasons for this are: i) the growth rate with phenol was extremely slow; although exact growth rates were not determined we estimate a minimum generation time of 12 days; ii) phenol is mildly toxic at the minimum concentration required as substrate addition (0.05%).

Experiments to assess substrate use of the enrichment were

conducted using serum bottles and measuring methane production (by GC) as described in Chapter 5. The enrichment degraded the following aromatic compounds without a lag (in order of increasing rate): p-cresol < phenol < benzoate < p-hydroxybenzoate < phenylacetate. Other aromatic compounds were observed to be degraded but only after a lag of several weeks; therefore we believe that these compounds were probably degraded by contaminants in the enrichment. Compounds which were not immediately degraded by the phenol-degrading consortium were: o- and m-hydroxybenzoate, 3,4-dihydroxybenzoate, o-, m-, and p-methyoxybenzoate, o-, m-, and p-methylbenzoate, o-, m-, and p-aminobenzoate, o-, m-, and p-chlorobenzoate, phthalate, resorcinol, catechol, o- and m-cresol, and o-, m-, and p-chlorophenol.

It has been suggested that phenol is an intermediate in the metabolism of p-cresol. If true, this would be an instance of oxidation of a methyl group with water rather than oxygen. To the authors' knowledge, this reaction has not been previously reported for an anaerobic bacterium. Preliminary experiments were initiated in which samples of the phenol enrichment culture, acclimated to p-cresol over a six month period, were fed both p-cresol and phenol simultaneously; other samples were fed p-cresol alone as a control (Fig. 1). During the first 24 h, the rate of p-cresol metabolism in bottles, with or without phenol, was the same; phenol was degraded but at a very low rate. During the next 3 days, however, the rate of phenol consumption increased exponentially while the rate of p-cresol consumption decreased. After approximately 85% of the phenol had been consumed, the rate of p-cresol consumption returned to the initial rate. These data suggest that the synthesis of one or more enzymes is required in order

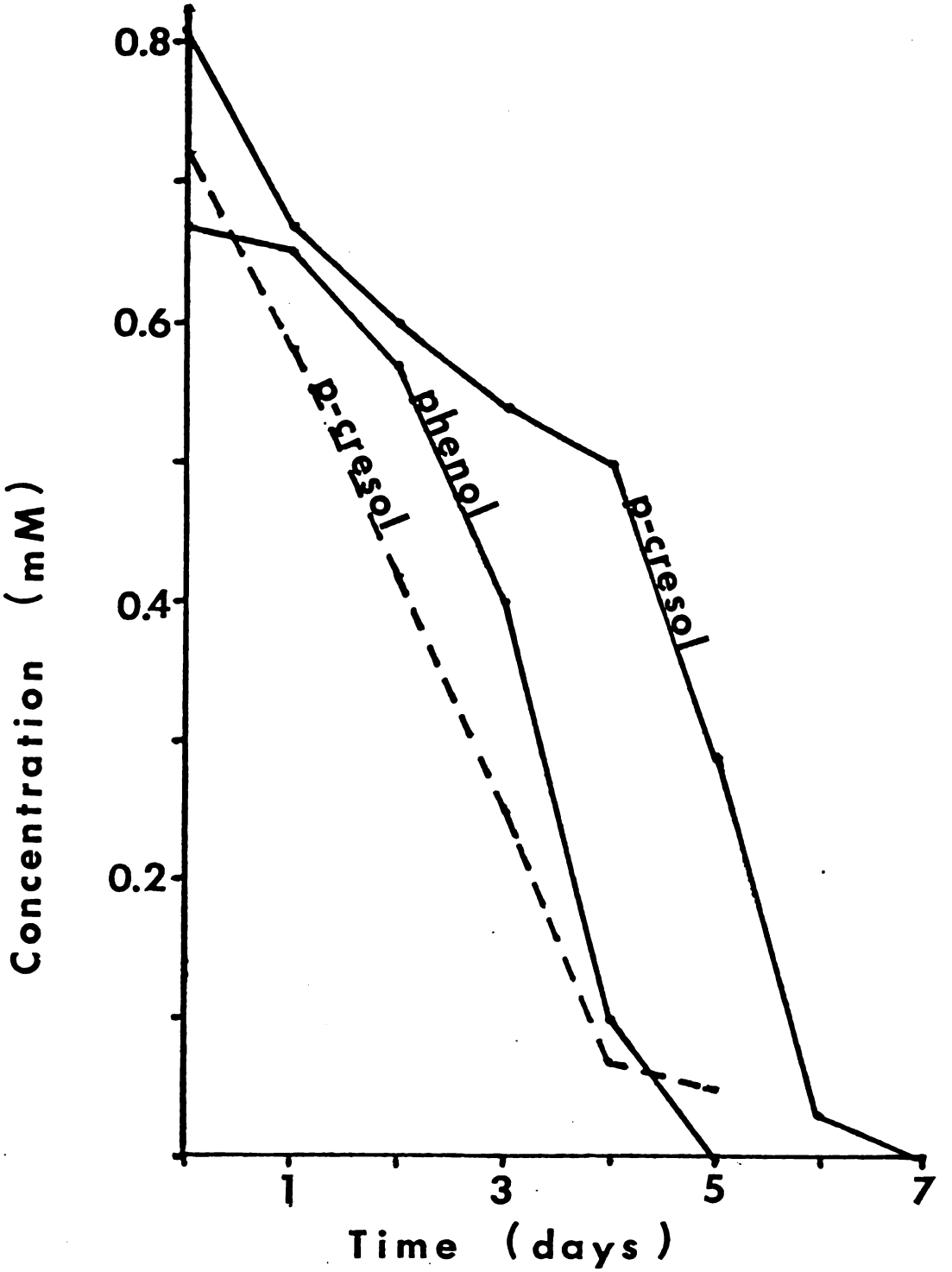


Fig. 1. Metabolism of p-cresol alone (dashed line) or p-cresol and phenol together (solid lines) in the phenol enrichment adapted to p-cresol degradation.

for phenol to be metabolized, since phenol consumption increased exponentially with time. The time course of the experiment was too short for any significant growth to have occurred. The data also suggest that both phenol and p-cresol share, at some point, a common pathway, since the rate of p-cresol metabolism decreased as the rate of phenol metabolism increased. Experiments were also initiated with ^{14}C-p-cresol, in the presence (isotopic dilution experiment) or absence of phenol, to directly test for ^{14}C-phenol as an intermediate in the p-cresol pathway. These samples have not yet been analyzed.

LITERATURE CITED

1. Shelton, D. R. and J. M. Tiedje. A general method for determining anaerobic biodegradation potential. (submitted to Appl. Environ. Microbiol.).

2. Shelton, D. R. and J. M. Tiedje. Isolation and characterization of an anaerobic syntrophic bacterium that oxidizes benzoate. (submitted to Appl. Environ. Microbiol.).