Wat. Sci. Tech. Vol. 25, No. 7, pp. 265–273, 1992.
Printed in Great Britain.

0273–1223/92 $15·00

ACCLIMATIZATION OF A METHANOGENIC CONSORTIUM TO POLYCHLORINATED COMPOUNDS IN A FIXED FILM STATIONARY BED REACTOR

P. Modesto Filho, P. Amerlynck, E.-J. Nyns and H. P. Naveau

Unit of Bioengineering, Catholic University of Louvain, Place Croix du Sud, 2/19, B-1348 Louvain-la-Neuve, Belgium

ABSTRACT

With the objective of destruction of organic toxic or recalcitrant compounds by a microbial anaerobic mixed population, a new concept has been devised : the Destox concept. It has been applied here to the destruction of a toxic mixture of about 30 polychlorinated aliphatic compounds (PAC-MIX 1), including 51 % of hexachloro-1,3-butadiene. The basic step for initiating the degradation is thought to be reductive dechlorination by microbial interspecies hydrogen transfer in a system using at all times a non-toxic co-substrate as major source of carbon and energy. In an upflow laboratory-scale reactor with a fixed-film stationary-bed, fed with a co-substrate, amounts of 48 mg/litre working volume per day of PAC-MIX 1 have been added during intermittent periods of time. This paper presents, over a period of 421 days, the evolution from a situation of complete inhibition of all microbial activity, from fermentative to methanogenic, into a situation of partial acclimatization, the fermentative and hydrogenotrophic methanogenic bacteria having become resistant to the action of the toxic mixture while acetogenic bacteria seem in the process of acclimatization. The methane production rate was maintained at 30% of the reference value, together with continuous accumulation of mainly acetate and to a lesser extent propionate.

KEYWORDS

polychlorinated compounds; anaerobic; toxicity; acclimatization; methanogenesis; biodegradation; dehalogenation; fixed-film; waste treatment.

INTRODUCTION

Polychlorinated organic compounds are known to be toxic for living organisms (Jolley *et al.*, 1978; Keith and Telliard, 1979). This reputation is, almost by definition, the reason why biology is so rarely used for destruction of these substances (Derick, 1988). Because of their persistence in natural environments, these compounds accumulate in soils and sediments and contaminate ground and surface waters. Organic toxic compounds enter the environment from a variety of sources (Kringstad and Lindstrom, 1984; Westrick *et al.*, 1984), in general released directly from industrial processes. It is however well established that some of these toxic substances are susceptible to biological degradation, anaerobically as well as aerobically (Rozzi, 1983; Holliger *et al.*, 1988).

Anaerobic strict conditions have been found that resulted in significant dehalogenation of haloaromatics (Hill and McCarty, 1967; Boyd and Shelton, 1984; Tiedje *et al.*, 1987) and halogenated aliphatic organic compounds (Bouwer *et al.*, 1981; Vogel *et al.*, 1985). A knowledge of the predominant ecological conditions and metabolic versatility is essential : haloaromatics are biodegraded in a methanogenic consortium but not under sulfate-reducing conditions, whilst the degradation of cresol isomers occurs in both types of incubations, (Suflita *et al.*, 1988). The basic step for initiating the degradation is a reductive dehalogenation (Guenzi and Beard, 1967; Kobayashi and Rittmann, 1982; Suflita *et al.*, 1982).

Considering the potential antagonism between the toxicity of some compounds for micro-organisms (Lin Chou *et al.*, 1978; Johnson and Young, 1983) and the ability of these micro-organisms to degrade these substances, the conventional biological reactors are not the most appropriate and the best mastered to handle such a process of biodegradation of toxic substances (Parkin and Speece, 1983). This idea has been achieved in a limited number of works (Suidan *et al.*, 1980; Hakulinen and Salkinoja-Salonen, 1982).

With the objective of destruction of organic toxic or recalcitrant compounds by a microbial anaerobic mixed population, a new concept has been devised : the Destox concept. This paper reports the partial acclimatization of a methanogenic consortium to a toxic mixture of polychlorinated aliphatic compounds using the Destox concept.

THE DESTOX CONCEPT

This concept (Dou *et al.*, 1989a, b) aims at the destruction of mixtures of organic toxic or recalcitrant compounds by a microbial anaerobic mixed population. The basic step for initiating the degradation is thought to be reduction, in our case reductive dechlorination, by microbial interspecies hydrogen transfer in a system using at all times a co-substrate as major source of carbon and energy.

Fermentation of the co-substrate supplies the necessary hydrogen for reduction and maintains the physico-chemical conditions, such as redox potential, needed for methanogenesis.

The Destox concept believes that enhanced biodegradation capabilities can be elicited in a biotechnological system in which the bioreactor itself, its design and its running conditions play a major role in the biodegradation. The reactor design is a fixed-film stationary-bed. The purpose of the carrier is to provide, at the same time, a surface for immobilization of microorganisms and an adsorption volume minimizing contact of microorganisms with excessive concentration of toxic compounds, thus supplying microorganisms with an adequate microenvironment. This "buffering" effect of the carrier may considerably increase the stability of the process and allow substantial amounts of toxic compounds to be treated per unit time in a small volume of reactor. Immobilization of microorganisms to ensure their long mean residence time is important for their acclimatization and allows a greater tolerance to quantitative and qualitative fluctuations of the mixtures of toxic organic compounds.

MATERIALS AND METHODS

Substrate

The non-toxic co-substrate selected is the residual spent liquor from citric acid fermentation; it is diluted to 26.25 g COD/l and contains sodium bicarbonate at 47 mM concentration. The toxic substrate named PAC-MIX 1 is a mixture of about 30 polychlorinated aliphatic compounds and some chlorobenzenes, including about 51 % of hexachloro-1,3-butadiène, 15 % of hexachloroethane, 7 % of perchlorethylene and 3 % of pentachlorobenzene.

Reactor Configuration

The experiments have been performed using two identical fixed-film stationary-bed filters (Fig. 1), built out of a cylindrical elongated glass tube with an internal diameter of 78 mm. One reactor was used for the tests using the polychlorinated compounds and the other as a control, without the polychlorinated compounds. They were operated at 35 °C. For each, the total volume is 6.1 litres, the total bed volume 4.7 litres and the liquid volume of the bed, named the working volume, is 3.5 litres. This volume is the total bed volume less the volume occupied by the carrier. The carrier is a polyurethane foam doped with 50 % by weight activated carbon (PUR BAYER) and 1140 g have been used for each reactor. The volumetric mass of this carrier is 0.98 g/ml. The bed porosity is 75 %. This heterogeneous macro-support is presented as foam granules of rectangular form whose sides are 10 to 15 millimetres.

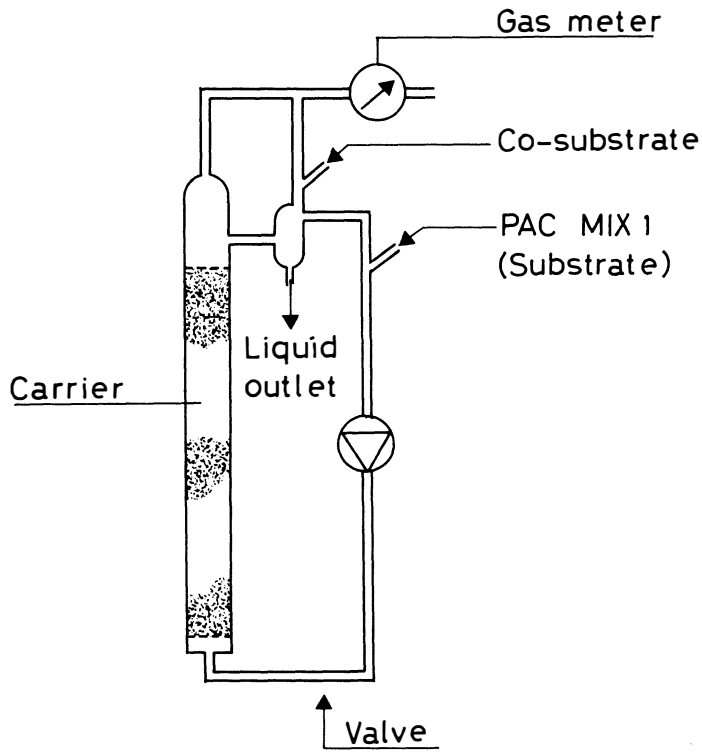

Fig. 1. Scheme of the experimental set-up.

Analyses

The gas composition was determined by gas-liquid chromatography using an Intersmat IGC 120 MB chromatograph equipped with a catharometer and two stainless steel columns of 1/8 ch external diameter. The first, of 3 m length, is filled with PORAPAK Q 50-80 mesh and the second, of 1.52 m length, with 5 A molecular sieve (Zweig and Sherma, 1972). The hydrogen was measured by an Exhaled Hydrogen Monitor (GMI Medical Ltd., Renfrew, Scotland, UK) using a polarographic measure.
Volatile fatty acids concentrations were determined on a sample acidified to less than pH 2 by addition of meta-phosphoric acid, using a gas chromatograph Intersmat IGC 120 DFB with a flame ionisation detector and a 2 m glass column filled with chromosorb WAW (100-120 mesh) (78 % w/w) coated with a mixture (22 % w/w) of 91 % Carbowax 20 M and 9 % phosphoric acid (De Vuyst *et al.*, 1964). The recorder was an Intersmat ICR-1 integrator.
The organochlorine compounds were determined by gas-liquid chromatography (CrC 5890 from Hewlett Packard) using a Ultra 1 column (Crosslinker methyl silicon gum 1 from HP) and coupled to a mass spectrometer (MSD 5970 from Hewlett Packard).

Mass Balance

A mass balance of hexachloro-1,3-butadiene was done after 476 days of operation according to the following procedure. The effluent was collected and analysed at regular intervals for its concentration of hexachloro-1,3-butadiene. At the end of the period of 476 days, portions of the carrier in the bioreactor were taken at different heights in the bed in order to determine the amount of hexachloro-1,3-butadiene adsorbed. They were extracted with methylene chloride and the extracts analysed.

Hexachloro-1,3-butadiene accumulated in dead zones that are situated out of the bed and working volume. These dead zones were washed with methylene chloride and the extracts analysed for hexachloro-1,3-butadiene.

REACTOR START-UP AND RUNNING CONDITIONS

Both reactors were started at the same time. After addition of the carrier, they were filled with a solution of the co-substrate. The inoculum was a mixed liquor taken out of a 60 l CSTR anaerobic digester digesting the co-substrate into methane for 2 years. An amount of inoculum corresponding to 15 % of the reactor volume was added through the "co-substrate feed" entrance (Fig. 1) while the recirculation pump was in action. Both reactors were fed in the same way, but the control without any PAC-MIX 1 addition.

The bioreactor operated in the upflow mode with recirculation using a volumetric pump. The mean hydraulic retention time was 35 days and the recirculation rate was 15 per day (relative to working volume).

Co-substrate volumetric loading rate was 0.75 g COD per litre working volume per day. The test bioreactor was supplied during intermittent periods of time with 167 mg of PAC-MIX 1 per day or 48 mg per litre working volume per day, unless otherwise noted as during the first and second period of addition of toxics (see under "Results"). Co-substrate was fed daily except during periods of recovery of the test reactor, due to loss of activity and/or high concentration of propionate. PAC-MIX 1 was fed for the first time to the test reactor on day 19 ("first period"). The control reactor reached steady state on day 35.

RESULTS

The following results have been obtained with the test column.
The starting period lasted from day 0 to day 18. Increasing amounts of PAC-MIX 1 were added from d19 to d22 (respectively 48, 96, 144 and 192 mg per litre working volume per day). Figure 2 shows the evolution of the concentrations of acetate (VA_2) and propionate (VA_3) and Fig. 3 the production rate of methane during that period of time.

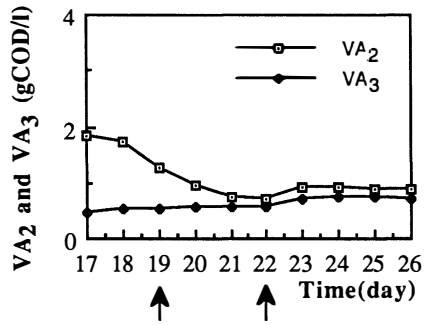

Fig. 2. Acetate (VA_2) and propionate (VA_3) concentration expressed as g COD per l as a function of time around the first test period (days 19 to 22 of operation of the bioreactor). The arrows indicate the first and the last day of introduction of PAC-MIX 1.

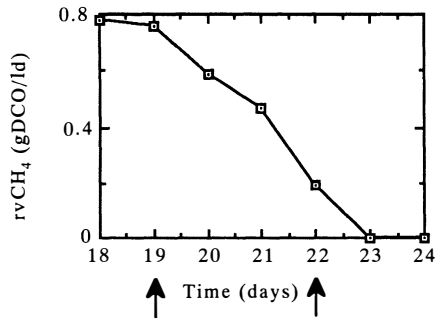

Fig. 3. Methane production rate expressed as g COD per l working volume per day as a function of time during the first test period (days 19 to 22). Arrows : see Fig. 2.

Feeding of the reactor was stopped on day 23 for substrate PAC-MIX 1 as well as co-substrate and then resumed on d33 for the co-substrate. For the second period, PAC-MIX 1 was added at the level of 48 mg per litre working volume per day on day 35 and 96 mg/l.d daily from days 36 to 39. The methane production rate dropped from 0,28 g COD (0,11 l CH_4) per liter working volume per day on day 35 to 0,17 g COD (0,07 l CH_4)/l.d on day 40 and zero on day 42. The acetate concentration increased from 1.24 g COD/l on day 35 to 2.04 g COD/l on day 40 and the propionate concentration increased also, during the same period of time, from 0.81 to 1.23 g COD/l.

Later, three periods,with additions of 48 mg of the PAC-MIX 1 mixture per liter of working volume and per day, totalling 21 days, alternated with periods without any addition of the PAC-MIX 1 mixture, until day 195. The periods without addition of the toxics were intended for the recovery of gas production and for the consumption of accumulated volatile fatty acids produced during the periods of addition of toxics. During the sixth period of PAC-MIX 1 addition at 48 mg/l.d, (days 196 to 212 of operation of the bioreactor), the methane production rate decreased abruptly from 0.74 g COD (0.29 l CH_4) per litre working volume per day on day 196 to about 35-40 % of this value, and remained constant throughout the period (0.27 g COD or 0.11-0.12 l CH_4 per litre working volume per day). Acetate concentration increased from 0.48 to 4.60 g COD/l and propionate concentration increased from 0.0 to 0.71 g COD/l between days 196 and 212.

During the seventh period (days 368 to 421 of operation of the bioreactor or a 54 day period), the methane production rate, equal to 100 % of the control value on day 368, declined continuously during the first forty days to reach approximately 30 % of the control value, that is from 0.77 g COD (0.30 l CH_4) to 0.23 g COD (0.09 l CH_4) per litre working volume per day. This methane production rate of 30 % of the control value was persistent during the rest of the seventh period (Fig. 4). One observed an increase in the acetate and propionate concentrations, concomitant with the fall of methane production (Fig. 5). Some hydrogen determinations in the gas phase have been performed. Concentrations of 11 to 25 ppm have been found in the test reactor and of 5 to 10 ppm in the control reactor.

A mass balance has been done around the hexachloro-1,3 butadiene in the reactor, as this compound is the most abundant in the mixture PAC-MIX 1. The mass balance has been achieved after 476 days of operation. There has been no injection of substrate PAC-MIX 1 after day 421. The result is presented in Fig. 6.

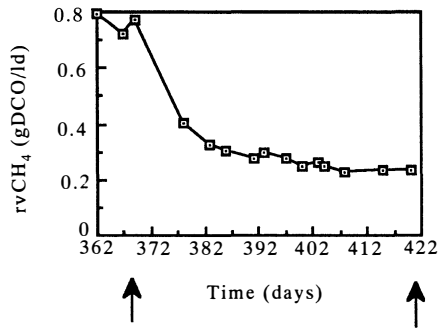

Fig. 4. Methane production rate expressed as g COD per litre working volume per day as a function of time during the seventh period (days 368 to 421). Arrows : see Fig. 2.

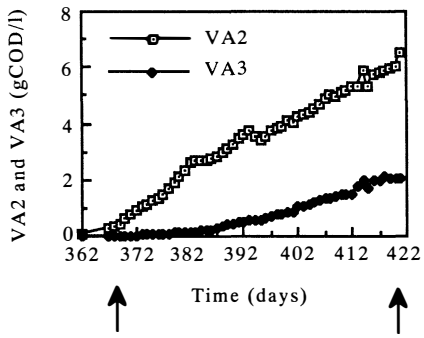

Fig. 5. Acetate (VA_2) and propionate (VA_3) concentrations expressed as g COD per litre as a function of time during the seventh period (days 368 to 421). Arrows : see Fig. 2.

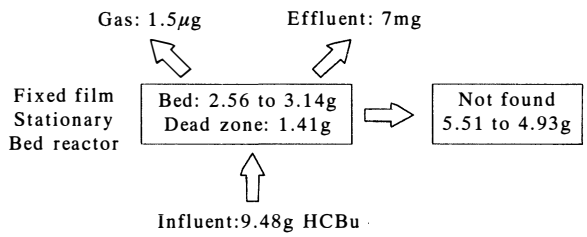

Fig. 6. Mass balance around the hexachloro-1,3-butadiene in the bioreactor

DISCUSSION

When the PAC-MIX 1 mixture was applied during the first period (days 19 to 22 of operation of the bioreactor), the methane production went down from 0.78 g COD/l.d or 0.31 l CH_4/l.d to zero within five days (Fig. 3). This resulted from a complete inhibition of the methanogenic communities. During the same period, the results showed no accumulation of acetate and propionate which suggest that the fermentative communities were also completely inhibited.

During the second period (days 35 to 39) the concomitant loss of all methane production and the increase in volatile fatty acids concentration show a continued complete inhibition of the methanogenic communities and, at the same time, an acclimatization of fermentative bacteria producing the volatile fatty acids.

The decrease of methane production to ± 35 % of its reference value during periods VI and VII (respectively days 196 to 212 and 368 to 421) suggests that the hydrogenotrophic methanogenic community is acclimatized, since it is known that, in a typical anaerobic digester, approximately 30 % of the methane produced originates from the reduction of CO_2 by H_2 (McCarty, 1964). The low hydrogen concentration found in the gas phase (11-25 ppm) supports this assumption.

On the other hand, the aceticlastic methanogenic and acetogenic communities remained inhibited. Indeed, one observes in both periods an increase in the acetate and propionate concentrations, concomitant with the fall of methane production. The seventh period lasted 54 days. The addition of the PAC-MIX 1 mixture had to be interrupted because the accumulation of acetate and particularly propionate would become inhibitory (McCarty and McKinney, 1961) since a propionate concentration of 3 g per litre (4.5 g COD/l) appears to be an upper limit (Asinari di San Marzano *et al.*, 1981).

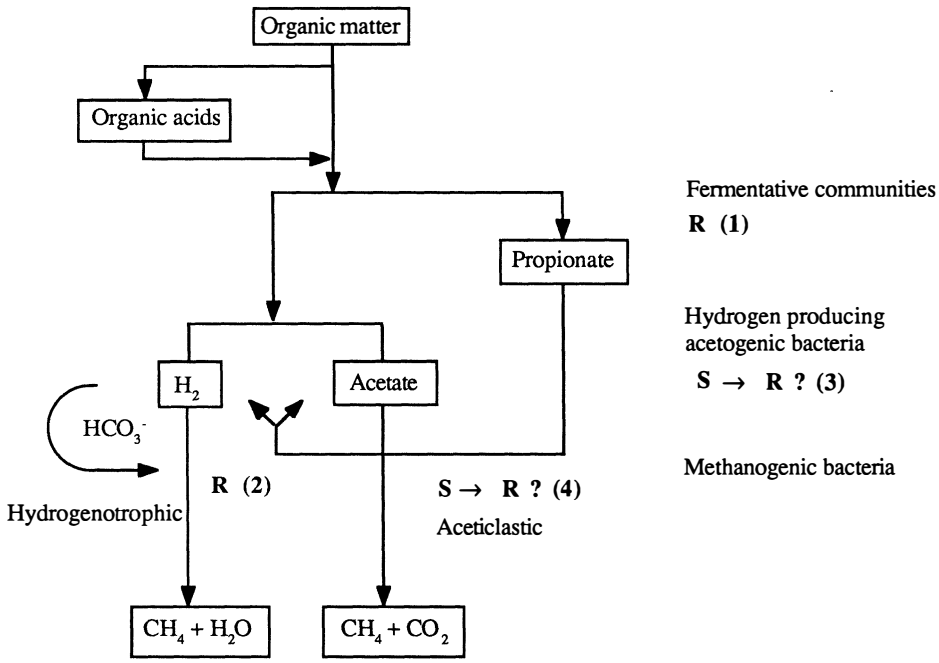

Fig. 7. Scheme of organisation of the groups of bacteria in methanogenic consortium with indication of the likely relative timing (1 → 4) of successive acclimatization to PAC-MIX 1 (R = resistant, S = sensitive to PAC-MIX 1)

However, the rate of increase of acetate concentration during the sixth period was 0.258 g COD/l.d, more than twice the rate of 0.11 g COD/l.d observed during the seventh period; the rate of increase of the propionate concentration was quite similar during the two periods, that is respectively 0.044 g COD/l.d and 0.039 g COD/l.d. The fact that the drop in methane production rate during the sixth period was abrupt while it was progressive during the seventh period, together with more than halving the rate of accumulation of acetate during period VII as compared to period VI, may be good indication of a beginning acclimatization of the aceticlastic methanogenic community.

The rate of propionate accumulation concentration is lower than that of acetate, and it remains more constant. This suggests that the acetogenic (obligate hydrogen producing acetogens) community may also have become partly acclimatized.

The mass balance results around hexachloro-1,3-butadiene indicates that a negligible amount was eliminated with the effluent and that 52 to 58 % disappeared. This is in favour of a biotransformation mechanism, that still needs to be proved.

CONCLUSION

Figure 7 reproduces the classical scheme of the three groups of bacteria present in a methanogenic consortium, together with their relative timing of acclimatization to PAC-MIX 1. Clearly, there is a different period of time necessary for the acclimatization of the various communities of bacteria. Although we have no indication of the number of species that are acclimatized in each group, the results shown allow the conclusion that there is at least some acclimatization in each community.

The time necessary for acclimatization is very short for the fermentative bacteria -about 15 days at the maximum- but may reach about 200 days or 28 weeks, as in our experiences, for the hydrogenotrophic methanogens and up to one year for the aceticlastic methanogens and the obligate hydrogen producing acetogens.

ACKNOWLEDGEMENTS

This work has been done in collaboration with Solvay & Co, Brussels, Belgium. The authors also thank IRSIA (Institut pour l'encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture, Brussels, Belgium) for financial support.

REFERENCES

Asinari di San Marsano, C.M., Binot, R., Bol, T., Fripiat, J.L., Hutschemackers, J., Melchior, J.L., Perez, I., Naveau, H. and Nyns, E.-J. (1981). Volatile fatty acids, an important state parameter for the control of reliability and the productivities of methane anaerobic digestion. *Biomass, 1*, 45-59.

Bouwer, E.J., Rittmann, B.E. and McCarty, P.L. (1981). Anaerobic degradation of halogenated 1- and 2-carbon organic compounds. *Environ. Sci. Technol., 15,* 596-599.

Boyd, S.A. and Shelton, D.R. (1984). Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge. *Appl. Environ. Microbiol., 47*, 272-277.

Derick, M. (1988). Recherche d'une solution biotechnologique à l'épuration des eaux résiduaires, notamment industrielles et toxiques, en région wallonne. *Mémoire présenté en vue de l'obtention du titre d'Ingénieur Agronome*, Université Catholique de Louvain, Louvain-la-Neuve (Belgique).

De Vuyst, A., Vervack, W., Vanbelle, M., Morelle, A., et Arnould, R. (1964). Comparaison du dosage des acides gras volatils dans les ensilages et le liquide du rumen par la méthode de chromatographie en phase gazeuse et la méthode de Leppe-Flieg. *Agricultura, 12*, 223-226.

Dou, S., Modesto Filho, P., Naveau, H. and Nyns, E.-J. (1989a). Anaerobic biological destruction of toxic and recalcitrant molecules : the Destox concept. Synopsis of the invited contribution Overzicht van het probleemveld, relevatie to.o.v. anaërobe gisting. In Proc. Semin. *Anaërobe Afbreekbaarheid van Recalcitrant Verbindingen*, Veldhoven, The Netherlands, April.

Dou, S., Modesto Filho, P., Naveau, H. and Nyns, E.-J. (1989b). A new concept for the biological destruction of toxicants or recalcitrants.: *Med. Fac. Landbouwwetenschappen. RUG, 54* (4b), 1401-1404.

Guenzi, W.D. and Beard, W.E. (1967). Anaerobic biodegradation of DDT to DDD in soil. *Science, 156*, 116-119.

Hakulinen, R. and Salkinoja-Salonen, M. (1982). Treatment of pulp and paper industry wastewaters in an anaerobic fluidised bed reactor. *Process Biochem.* (Mar.-Apr.), 18-22.

Hill, D.W. and McCarty, P.L. (1967). Anaerobic Degradation of Selected Chlorinated Hydrocarbon Pesticides. *J. Water Pollut. Control, 39*, 1259-1277.

Holliger, C., Stams, A.J.M. and Zehnder, A.J.B. (1988). Anaerobic degradation of recalcitrant compounds. In : *Anaerobic Digestion 1988. (Adv. Water Pollut. Control* no 5) Eds. Hall, E. R. and Hobson, P. N. Proc. 5th Int. Symp., Bologna-Italy, May. Pergamon Press, Oxford, UK, pp. 221-224.

Johnson, L.D. and Young, J.C. (1983). Inhibition of anaerobic digestion by organic priority pollutants. *J. Water Pollut. Control, 55*, 1441-1449.

Jolley, R.L., Gorchen, H. and Hamilton, D.H. Jr. (1978). *Water Chlorination Environmental Impact and Health Effects*. Vol. 2, Ann Arbor Science Publishers, Inc., Ann Arbor, Mich.

Keith, L.H. and Telliard, W.A. (1979). Priority pollutants, I. A perspective view. *Environ. Sci. Technol., 13*, 416-423.

Kringstad, K.P. and Lindstrom, K. (1984). Spent liquors from pulp bleaching. *Environ. Sci. Technol., 18*, 236A-248A.

Kobayashi, H. and Rittmann, B.E. (1982). Microbial removal of hazardous organic compounds. *Environ. Sci. Technol., 16*, 170A-183A.

Lin Chou, W., Speece, R.A. and Siddiqi, R.H. (1978). Acclimation and degradation of petrochemical wastewater components by methane fermentation. *Biotech. Bioeng. Symp., 8*, 391-414.

McCarty, P.L. (1964). Anaerobic Waste Treatment Fundamentals - Part One. *Publ. Works, 95*, 107-112.

McCarty, P.L. and McKinney, R.E.(1961). Volatile acid toxicity in anaerobic digestion. *J. Water Pollut. Control Fed., 33*, 223-232.

Parkin, G.F. and Speece, R.E. (1983). Attached versus suspended growth anaerobic reactors : response to toxic substances. *Water Sci. Technol., 15*,(8/9), 261-289.

Rozzi, A. (1983). Introduction to toxicity session. In *Anaerobic Wastewater Treatment*. Proc. Eur. Symp., Noordwijkerhout, Neth., Nov. To be obtd from W.J. Van den Brink, TNA Corp. Comm. Dept., PO Box 297, NL-2501, The Hague, Neth.

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

Suflita, J.M., Gibson, S.A. and Beeman, R.E. (1988). Anaerobic biotransformations of pollutant chemicals in aquifers. *J. Ind. Microbiol., 3,* 179-194.

Suidan, M.T., Cross, W.H. and Madeline, F. (1980). Continuous bioregeneration of granular activated carbon during the anaerobic degradation of catechol. *Prog. Wat. Tech., Toronto, 12* (6), 203-214.

Tiedje, J.M., Boyd, S.A. and Fathepure, B.Z. (1987). Anaerobic degradation of chlorinated aromatic hydrocarbons. *Dev. Ind. Microbiol., 27*, 117-127.

Vogel, T.M. and McCarty P.L. (1985). Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide crudes methanogenic conditions. *Appl. Environ. Microbiol., 49*, 1080-1083.

Westrick, J.J., Mello, J.W. and Thomas, R.F. (1984). The Groundwater Supply Survey. *J. Am. Water Works Assoc., 76*, 52-59.

Zweig, G. and Sherma, J. (1972). In *Handbook of Chromatography*, Publish CRC Press, Cleveland, Ohio, USA, Vol. I, 130.