Biodegradation **7**: 501–506, 1996.

Degradation of bis(2-ethylhexyl) phthalate constituents under methanogenic conditions

J. Ejlertsson & B. H. Svensson
Department of Water and Environmental Studies, Linköping University S-581 83 Linköping, Sweden

Accepted 30 December 1996

Key words: biodegradation, Bis(2-ethylhexyl) phthalate, 2-ethylhexanol, 2-ethylhexanoic acid, methane formation, mono(2-ethylhexyl) phthalate, phthalic acid esters

Abstract

The degradation of bis(2-ethylhexyl) phthalate (DEHP) and its intermediary hydrolysis products 2-ethylhexanol (2-EH) and mono(2-ethylhexyl) phthalate (MEHP) was investigated in a methanogenic phthalic acid ester-degrading enrichment culture at 37°C. 2-Ethylhexanoic acid (2-EHA), a plausible degradation product of 2-EH, was also studied. The culture readily degraded 2-EH via 2-EHA to methane which was formed in stoichiometric amounts assuming complete degradation of 2-EH to methane and carbon dioxide. MEHP was degraded to stoichiometric amounts of methane with phthalic acid as a transient intermediate. DEHP remained unaffected throughout the experimental period (330 days).

Abbreviations: 2-EH – 2-ethylhexyl alcohol, 2-EHA – 2-ethylhexanoic acid, BBP – butylbenzyl phthalate, Be-CoA – benzoyl Coenzyme A, CoA – Coenzyme A, DEHP – bis(2-ethylhexyl) phthalate, MEHP – mono(2-ethylhexyl) phthalate, MSW – municipal solid waste, PA – phthalic acid, PAE – phthalic acid ester, TMS – trimethylsilyl derivative

Introduction

Phthalic acid esters (PAEs) constitute a diverse group of organic compounds used in large quantities in most countries. They are mainly used as plasticizers in PVC-plastics and can account for up to 67% of the total weight of such materials (Giam et al. 1984). They are also important additives in cosmetics, inks, ammunition, etc. During, 1994, 4.2 million tons of PAEs were synthesised and used worldwide (A.-L. Rykfors, Neste Oxo AB, pers. comm.). Bis(2-ethylhexyl) phthalate (DEHP), the most commonly used PAE, accounts for 40-50% of the global annual production of PAEs (KEMI 1990). As a result of their extensive use and transport in the atmosphere, PAEs are ubiquitous and can be detected at almost all trophic levels in even the most pristine ecosystems (Giam et al. 1984).

During microbial degradation under oxic and anoxic conditions, PAEs are initially hydrolysed via the monoesters to phthalic acid (PA) and alcohols (Engelhardt et al. 1975; Shelton et al. 1984; Ejlertsson et al. 1996a). During aerobic growth on DEHP, organisms have been shown to hydrolyse DEHP to PA and 2-ethylhexanol (2-EH) within hours with lipases having a very broad substrate specificity (Kurane et al. 1984). Unspecific enzymatic lipase hydrolysis may also be of importance for the diester cleavage (Kurane et al. 1984; Ejlertsson et al. 1996b). Abiotic hydrolysis of PAEs may occur as well, but at much lower rates compared with the enzyme hydrolyses. For example, the abiotic hydrolysis of DEHP to mono(2-ethylhexyl) phthalate (MEHP) and 2-EH would occur so slowly that the half-life of DEHP would be approximately 2000 years if this was the only hydrolysis mechanism operating (Giam et al. 1984).

Degradation of PA can occur via two biochemical pathways which differ depending on whether the conditions are aerobic or anaerobic. Aerobically, PA is transformed to protochatecuate (Ribbons et al. 1984; Nomura et al. 1992) and then further degraded via

Table 1. $\Delta G^{o'}$ for the equations presented in this investigation and the partial pressure of hydrogen needed to achieve $\Delta G^{o'} \leq 0$

	$\Delta G^{o'}$ (kJ·mol^{-1})*	P_{H2} (bar)
Equation 1	+ 46.3	$10^{-2.7}$
Equation 2	+ 20.2	$10^{-1\,8}$
Equation 3	+ 47.5	$10^{-4.2}$
Equation 4	+ 48.2	$10^{-4.3}$

* = Free energies of formation (ΔG°_f) were taken from Thauer et al. (1977) except for 2-EH and 2-EHA. 2-EH was calculated from the ΔG°_f of heptanol and 2-EHA from the ΔG°_f of capronic acid by an increase of 8.37 kJ per CH_2-group.

ortho- or meta cleavage. Under anaerobic conditions, PA is most probably activated and decarboxylated to benzoyl-Coenzyme A (Be-CoA) as has been shown under denitrifying conditions (Nozawa & Maruyama 1988). The Be-CoA is then further degraded to acetate using nitrate as the terminal electron acceptor (cf. Elder & Kelly 1994). In the absence of terminal electron acceptors, such as nitrate or sulphate, hydrogen is excreted (Eq. 1), which is an endergonic reaction under standard conditions ($\Delta G^{o'}$ = + 46.3 kJ/mole).

$$\text{Benzoic acid} + 6H_2O \rightarrow 3\text{Acetate}^- + 3H^+ + CO_2 + 3H_2 \quad (1)$$

However, due to the action of hydrogen-scavenging methanogenic bacteria a low partial pressure of hydrogen is created, thereby allowing the oxidation of Be-CoA to occur (Table 1).

Several investigations have shown that DEHP can be completely degraded under aerobic conditions (Saeger & Tucker 1973; Johnson & Lulves 1975; Nakazawa & Hayashi 1977; O'Grady et al. 1985; Kurane 1986). By contrast, under anaerobic conditions, DEHP appears to be persistent (Johnson & Lulves 1975; Horowitz et al. 1982; Shelton et al. 1984; Ejlertsson et al. 1996a,b). However, in an analysis of the PAEs in leachates from landfill lysimeters simulating landfill conditions, as described by Stegmann (1981) and Lagerkvist & Chen (1993), we recently detected mono(2-ethylhexyl)phthalate (MEHP) at a concentration of 10–30 $\mu g \cdot l^{-1}$ (unpublished data), whereas the concentration of DEHP was 1–3 $\mu g \cdot l^{-1}$. The presence of MEHP indicates that DEHP must have been hydrolysed in the waste and leachate. These observations are supported by the findings of Reinhart & Pohland (1991) who reported the complete disappearance of added DEHP during four years of codisposal with municipal solid waste. According to Giam et al. (1984), the monoesters are more resistant to hydrolysis than are the diesters, which therefore may accumulate in ecosystems. However, to our knowledge, there are no reports on the occurrence of monoesters of DEHP in the environment. 2-ethylhexanol (2-EH), the product of DEHP hydrolysis, is also used in the production of DEHP, lubricant additives, wetting agents, etc. The annual (1995) worldwide production of 2-EH amounts to ca 2.3 million tons (A.-L. Rykfors, Neste Oxo AB, pers. comm.).

The aim of this study was to determine the degradability of the products of DEHP hydrolysis under anaerobic and methanogenic conditions. The compounds studied were 2-EH and MEHP. We also wanted to elucidate the degradation pathway of 2-EH under these conditions. A plausible intermediate during 2-EH degradation, 2-ethylhexanoic acid (2-EHA), was also included in the study.

Materials and methods

Chemicals

Yeast extract (Oxoid) and the chemicals used (MERCK-products) were bought from KEBO (Stockholm, Sweden). Mono(2-ethylhexyl) phthalate (MEHP) and 2-etylhexanol (2-EH) were gifts from Neste Oxo AB (Stenungsund, Sweden).

Origin of inocula

Diluted municipal solid waste (MSW) treated in a pilot plant two-stage biogas process at 37°C was previously shown to degrade selected PAEs (Ejlertsson et al. 1996b). Samples degrading butylbenzyl phthalate (BBP) were used to start enrichment cultures at a BBP concentration of 1 $g \cdot l^{-1}$ (3 mM). After one transfer, these cultures were used as inoculum for the present experiment. The dry matter content of the inoculum was 0.03 $g \cdot l^{-1}$.

Media, inoculation and sampling procedure

The media were prepared according to Ejlertsson et al. (1996a) and consisted of three stock solutions:

- a phosphate buffer with resazurin,
- a solution containing trace elements, vitamins and mineral salts (C1),
- a solution containing bicarbonate and sulphide (C2).

Cystein normally present in C2 was omitted during this study. DEHP and MEHP dissolved in acetone were added to empty 118-ml bottles to give a final concentration of 1 mM. After evaporation of the acetone, 40 ml phosphate buffer was added while flushing with N_2, and the bottles were closed with ethene-propene-diemmer (EPDM)-stoppers and aluminium crimps. The headspace $N_2$was then replaced by N_2/CO_2 (80:20, O_2<2 ppm). 2-EH and 2-EHA were added with a Hamilton 10-μl syringe after addition of phosphate buffer (40 ml) and replacing the headspace gas with N_2/CO_2. All experiment bottles containing phosphate buffer and substrate were autoclaved at 121°C for 30 min, whereas C1 and C2 were filter sterilized. Before inoculation, C1 (2.5 ml) and C2 (2.5 ml) were added. The bottles were then quickly opened (< 3 seconds), to allow 5 ml of inoculum to be added with an automatic pipette, whereupon they were closed and resealed with aluminium crimps. Incubation of the experiment bottles was done in the dark at 37°C.

Three sets of experimental bottles with DEHP, MEHP, 2-EH and 2-EHA, respectively, were prepared and incubated. The first set consisted of triplicate bottles for methane analysis. The second set of triplicate bottles was inoculated and autoclaved at 121°C for 30 min in order to account for the nonbiological degradation of added compounds. From the third set of bottles, also in triplicate, 0.5-ml liquid samples were withdrawn on a weekly basis. These samples were immediately frozen in Eppendorf tubes and kept frozen at -20°C for analysis of 2-EH and 2-EHA or of PA formed later on. Due to the low solubility of DEHP and MEHP, a fourth set of bottles with these two compounds was prepared. In this case whole bottles were frozen after various periods of incubation and were analysed later.

Analytical procedure

Samples (0.3 ml) for methane analysis were subjected to gas chromatography (GC) according to Örlygsson et al. (1993) and diluted when needed in the following way: After withdrawal of the 0.3-ml gas sample, the needle was pulled out until its tip was situated inside the stopper, thus preventing any gas from leaking from the syringe. The overpressure in the syringe was relieved by pulling out the plunger to the 1.0-ml mark, resulting in an underpressure. Thus when the syringe was then pulled out of the stopper, laboratory air entered the needle until the pressure was equilibrated. The contents of the syringe were then injected into a 13-ml bottle. A 0.3-ml amount of the gas from this bottle was then injected into the GC.

DEHP, MEHP, 2-EH and 2-EHA were analysed by GC (Chrompack 9000). Helium was used as carrier gas at a flow rate of 5 ml·min^{-1}, and the split flow was set at 25 ml·min^{-1}. Nitrogen was used as make-up gas at 25 ml·min^{-1}. Injector and flame ionisation detector temperatures were set at 250°C. Frozen bottles with DEHP and MEHP were thawed and kept at +2°C while adding 1 ml of concentrated H_2SO_4 and 40 ml of diethylether. The bottles were then shaken for 4 x 30 s and stored at -20°C for at least 3 h to minimize the content of water in the ether extract. The extract was then transferred to a clean bottle, and a teaspoon of $MgSO_4$ was added to eliminate remaining traces of water. Thereafter the extract was transferred to a 50-ml measuring cylinder and concentrated to 10 ml under a gentle stream of N_2. A 1-μl mixture of 50 μl concentrated extract and 50 μl TMS (2.4 ml N,O-bis(trimethylsilyl)-trifluoro acetamid, 0.24 ml chlorotrimethyl silane and 2.4 ml pyridin) was injected manually on the GC and separated on a 10 m × 0.53 mm CP SIL 5 CB (df 5μ) isothermically at 200°C. Using TMS-reagent we were able to quantify PA and MEHP in the ether extract. Frozen samples with 2-EH and 2-EHA were thawed and centrifuged at 12000 rpm for 15 min. They were then injected on the GC with an auto sampler and separated on a 25 m × 0.53 mm CP SIL 5 CB (df 1μ) column programmed to remain at an initial temperature of 80°C for 5 min, increase by 10°C·min^{-1} to 120°C, remain isothermal for 2 min, rise by 20°C·min^{-1} to 200°C and, finally, remain isothermal for 2 min. In this way volatile fatty acids (VFA) present in the 2-EH and 2-EHA samples were detected simultaneously (detection limit was 50 μM). Phthalic acid in frozen liquid samples was quantified by HPLC according to Ejlertsson et al. (1996a).

Results and discussion

Transformation of 2-EH to methane took place after a lag phase of six to eight days (Figure 1). As the 2-EH disappeared, 2-EHA accumulated. Between days six and eleven, methane formation was lower in the incubations with 2-EH than in inoculated bottles without any 2-EH, 2-EHA, MEHP or DEHP added (controls).

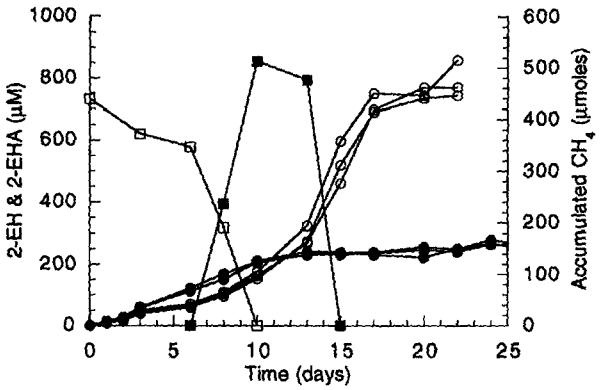

Figure 1. Degradation of 2-ethylhexanol (2-EH; □) to methane (○) via the transient intermediate 2-ethylhexanoic acid (2-EHA; ■) as compared with methane formed in inoculated bottles without 2-EH (●).

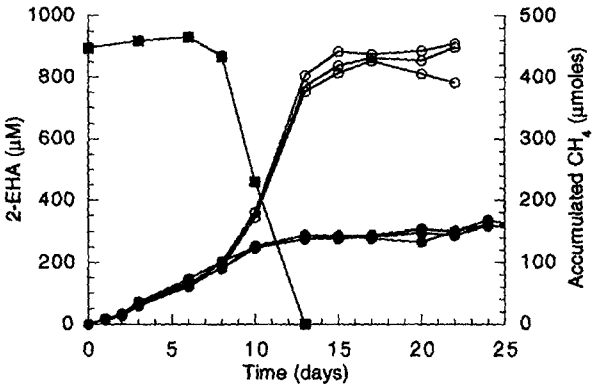

Figure 2. Degradation of 2-ethylhexanoic acid (2-EHA; ■) to methane (○) as compared with methane formed in inoculated bottles without 2-EHA (●).

The period of retarded methane formation coincided with the formation of 2-EHA (Figure 1). After 15 days, the degradation of the 2-EHA formed resulted in further methane formation. In incubations with 2-EHA (Figure 2), this compound was completely degraded to methane after a lag phase of six to eight days. The amount of methane formed corresponds to a stoichiometric degradation of 2-EHA to methane and carbon dioxide. During the disappearance of 2-EHA we were unable to detect the formation of any VFAs such as acetate and butyric acid. Neither transformation nor degradation of 2-EH or 2-EHA occurred in the autoclaved bottles.

The oxidation of 2-EH to 2-EHA (Equation 2) requires a partial presseure of hydrogen below $10^{-1.8}$ bars to proceed, since this reaction has a positive $\Delta G^{\circ'}$ at standard conditions (cf. Table 1).

$$2-\mathrm{EH} + \mathrm{H_2O} \rightarrow 2-\mathrm{EHA}^- + \mathrm{H}^+ + 2\mathrm{H_2} \quad (2)$$

The further oxidation of 2-EHA probably occurs via ß-oxidation to two molecules of butyryl-CoA, as shown for aerobic conditions by Wyatt et al. (1987) and indicated for anaerobic conditions by Yap et al. (1992). These molecules are further oxidized to acetate and hydrogen, which in turn are converted to methane and carbon dioxide. The oxidation of 2-EHA to hydrogen and acetate via butyrate has a more positive $\Delta G^{\circ'}$ than the oxidation of 2-EH to 2-EHA (equation 3 & 4; Table 1). Thus, a partial pressure below $10^{-4.2}$ bar is needed to achieve 2-EHA oxidation. Differences in the partial pressure of hydrogen needed for substrate oxidation may explain the accumulation of 2-EHA in the 2-EH incubation.

$$2-\mathrm{EHA}^- + 2\mathrm{H_2O} \rightarrow 2\mathrm{Butyrate}^- + \mathrm{H}^+ + 2\mathrm{H_2} \quad (3)$$

$$\mathrm{Butyrate}^- + 2\mathrm{H_2O} \rightarrow 2\mathrm{Acetate}^- + \mathrm{H}^+ + 2\mathrm{H_2} \quad (4)$$

Most likely, the retardation in methane formation observed in relation to the control incubations was a result of the 2-EH oxidation to 2-EHA. The oxidation reaction will occur at a partial pressure as high as $10^{-1.8}$. At this hydrogen concentration, the oxidation of probable fermentation products of the yeast extract present in the medium, such as propionate and butyrate, will not be possible. Thus, the conversion of these compounds to methane will be hampered. However, no increase in the concetration of VFAs could be seen (detection limit ca 50 µM). But evan at this level, a delay in the degradation of e.g. butyrate and propionate would give rise to a lower methane production. Therefore, the inhibition observed is believed to be an effect of the 2-EH oxidation maintaining a rather high partial pressure of hydrogen between days six and eleven.

MEHP was hydrolytically transformed to PA, which accumulated in nearly stoichiometric amounts (Figure 3). Traces of 2-EHA (<0.2 mM) could be detected as a transient intermediate between days 10 and 20 (data not shown). The appearance of 2-EHA was most likely due to the oxidation of 2-EH (see above). After another 3 weeks, PA was degraded to methane in an amount expected to occur when PA is completely

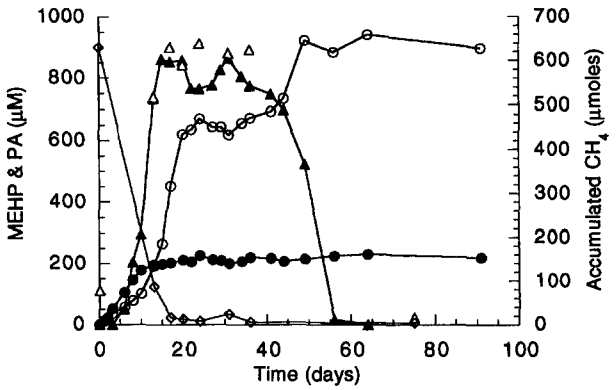

Figure 3. Degradation of mono(2-ethylhexyl) phthalate (MEHP; ◇) to methane (○) via the transient intermediate phthalic acid (PA) as compared with methane formed in inoculated bottles without MEHP (●). (▲) equals PA measured by HPLC from the samples withdrawn and (△) PA extracted from the bottles frozen along the experimental period.

Table 2. Fate of DEHP and formation of methane during 330 days of incubation as compared with incubations without addition of DEHP (control)

	DEHP (mM)	CH_4 (μmoles)
Day 0	1.00 (0.01)[1]	bd[2]
Day 330	0.94 (0.06)[1]	148 (2)[1]
Control		
Day 0	bd[2]	bd[2]
Day 330	bd[2]	153 (3)[1]

[1] = Average of triplicates and values between the brackets represents the standard deviation.
[2] = Below detection limit.

transformed to methane and carbon dioxide. As in the incubations with 2-EH, there was a small, but significant, retardation in the formation of methane, as compared with controls, in the incubations with MEHP. In the sterile incubations the MEHP concentration was unaffected. Apparently, the microorganisms have no problems in hydrolysing MEHP to PA and 2-EH. The increasing rate of methane formation as well as the increasing rate of PA formation during the disappearance of MEHP indicate that the hydrolysis of MEHP was coupled to growth.

The concentration of DEHP decreased slightly during the incubation period (330 days; Table 2). However, levels of methane formation were similar to those in the control (Table 2). MEHP was not detected in any of the frozen bottles. Even though the microorganisms are able to form 2-EH by hydrolysing MEHP, they were not able to hydrolyse DEHP. This inability could have been due to steric hindrance by the two 2-EHs or to the low solubility of DEHP in water.

Conclusions

The products resulting from the hydrolysis of DEHP, 2-EH and MEHP were shown to be completely and stoichiometrically degraded to methane. Thus, in situations where these compounds are set free, as a result of unspecific and/or specific hydrolytic activity or by abiotic means, anaerobic microorganisms should be able to transform them to methane and carbon dioxide. 2-EHA is a transient intermediate during 2-EH oxidation.

Acknowledgments

Magnus Alnervik and Marih Johnsson are greatly acknowledged for their assistance in the analysis of the substances studied. Hans Borén and Susanne Johnsson offered valuable advice concerning analytical procedures. The authors wish to thank Anna-Lena Rykfors and Jan-Olof Berntsson (Neste Oxo AB) for valuable information and for supplying the 2-EH and MEHP. We also thank Dr Anna Schnürer for valuable comments on the manuscript and Dr David Tilles for linguistic revisions. The investigation was supported by grants from the Swedish Environmental Protection Agency (contract number 802-335-93-Fr), Norsk Hydro a.s., the council of Vinyl manufacturers and the JLT faculty (SLU) research programme 'Biological waste in recirculation between urban and rural areas. Biology and flows of energy and material'.

References

Ejlertsson J, Johansson E, Karlsson A, Meyerson U & Svensson BH (1996a) Anaerobic degradation of xenobiotics by organisms from municipal solid waste under landfilling conditions. Antonie van Leeuwenhoek 69: 67–74

Ejlertsson J, Meyerson U & Svensson BH (1996b) Degradation of phthalic acid esters in municipal solid waste under methanogenic conditions. Biodegradation 7: 345–352

Elder DJE & Kelly DJ (1994) The bacterial degradation of benzoic acid and benzenoic compounds under anaerobic conditions: Unifying trends and new perspectives. FEMS Microbiol. Rev. 13: 441–468

Engelhardt G, Wallnöfer PR & Hutzinger O (1975) The microbial metabolism of di-n-butyl phthalate and related dialkyl phthalates. Bull. Environ. Contam. Toxicol. 13: 342–347

Giam CS, Atlas A, Powers JMA & Leonard JE (1984) Phthalic acid esters. In: Hutzinger O (Ed) Handbook of Environmental Chemistry. Springer Verlag, New York, 3: 67–140

Horowitz A, Shelton DR, Cornell CP & Tiedje JM (1982) Anaerobic degradation of aromatic compounds in sediments and digested sludge. Dev. Indust. Microbiol. 23: 435–444

Johnson BT & Lulves W (1975) Biodegradation of di-n-butyl phthalate and di-2-ethylhexyl phthalate in freshwater hydrosoil. J. Fish. Res. Board Can. 32:333–339

KEMI (1990) Swedish National Chemicals Inspectorate. Appendix to Report 10/90. Description of Compounds. Print Graf, Stockholm (In Swedish)

Kurane R (1986) Microbial degradation of phthalate esters. Microbiol. Sci. 3: 92–95

Kurane R, Suzuki T & Fukuoka S (1984) Purification and some properties of a phthalate ester hydrolysing enzyme from *Nocardia erythropolis*. Appl. Microbiol. Biotechnol. 20: 378–383

Lagerkvist A & Chen H (1993) Control of two step anaerobic degradation of municipal solid waste (MSW) by enzyme addition. Wat. Sci. Tech. 27: 47–56

Nakazawa T & Hayashi E (1977) Phthalate metabolism in *Pseudomonas testosteroni*: accumulation of 4,5 dihydroxyphthalate by a mutant strain. J. Bacteriol. 131: 42–48

Nomura Y, Nakagawa M, Ogawa N, Harashima S & Oshima Y (1992) Genes in PHT plasmid encoding for the initial degradation pathway of phthalate in *Pseudomona putida*. J. Ferment. Bioeng. 74: 333–344

Nozawa T & Maruyama Y (1988) Anaerobic metabolism of phthalate and other aromatic compounds by a denitrifying bacterium. J. Bacteriol. 170: 5778–5784

O'Grady DP, Howard PH & Werner AF (1985) Activated sludge biodegradation of 12 commercial phthalate esters. Appl. Environ. Microbiol. 49: 443–445

Örlygsson J, Houwen FP & Svensson BH (1993) Anaerobic degradation of proteins and the role of methane formation in steady state thermophilic enrichment cultures. Swedish J. agric. Res. 23: 45–54

Reinhart DR & Pohland FG (1991) The assimilation of organic hazardous wastes by municipal solid waste landfills. J. Indust. Microbiol. 8: 193–200

Ribbons DW, Keyser P, Kunz DA, Tayler BF, Eaton RW & Anderson BN (1984) Microbial degradation of phthalates. In: Gibson DT (Ed) Microbial Degradation of Organic Compounds. (pp 371–394). Marcel Dekker Inc., New York

Saeger VW & Tucker ES (1973) Phthalate esters undergo ready biodegradation. Plastics Engineering 29: 46–49

Shelton DR, Boyd SA & Tiedje JM (1984) Anaerobic biodegradation of phthalic acid esters in sludge. Environ. Sci. Technol. 18: 93–97

Stegmann R (1981) Beschreibung eines Verfahrens zur Untersuchung anaerober Umsetzungsprozesse von Festen Abfallstoffen im Labormaßstab. Müll und Abfall 2

Thauer R, Jungermann KK & Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacterial. Rev. 41: 100–180

Wyatt JM, Cain RB & Higgins IJ (1987) Formation from synthetic two-stroke lubricants and degradation of 2-ethylhexanol by lakewater bacteria. Appl. Microbiol. Biotechnol. 25: 558–567

Yap MGS, Ng WJ & Chua H (1992) Performance of an anaerobic biofilter for 2-ethylhexanoic acid degradation. Biores. Technol. 41: 45–51