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Anaerobic Biodegradation and Toxicity of Alcohol Ethoxylates

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presented by
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Zürich 2000

BTMR

for Ruth

If any one intentionally pollutes the water of another, whether the water of a spring, or collected in reservoirs, either by poisonous substances, or by digging or by theft, let the injured party bring the cause before the wardens of the city, and claim in writing the value of the loss; if the accused be found guilty of injuring the water by deleterious substances, let him not only pay damages, but purify the stream or the cistern which contains the water, in such manner as the laws of the interpreters order the purification to be made by the offender in each case.

Laws by Plato, 360 BC

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Verdankungen

Bedanken möchte ich mich herzlich

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Zusammenfassung

Alkohol Ethoxylate (AEO) sind nicht-ionische Tenside und bestehen aus einem hydrophoben Fettalkohol und einer hydrophilen Ethylenoxidkette (EO). Normalerweise kommen AEO als technische Mischungen zum Einsatz. Weltweit werden jährlich etwa 750'000 Tonnen AEO produziert und verbraucht. In der Schweiz beträgt der AEO Verbrauch etwa 6000 – 7000 Tonnen pro Jahr.

Wegen ihrem lipophilen Charakter gelangt ein grosser Anteil der AEO - sorbiert an partikuläres organisches Material - in den Faulturm der Kläranlage, wo diese Substanzen anaeroben biologischen Abbauprozessen unterworfen sind. Bis heute wurde das Abbauverhalten nur für wenige, vor allem lineare AEO untersucht. In dieser Arbeit wird das Verteilungsverhalten von linearen AEO sowie die anaerobe biologische Abbaubarkeit und Toxizität für Ethoxylate von linearen, einfach- und mehrfach verzweigten Alkoholen untersucht.

Für die biologischen Abbauexperiment wurde das Anaerobe Screening Test System (ASTS) entwickelt und für geringe Konzentrationen bis zu 10 mg C/L optimiert. Bei den Untersuchung der anaeroben biologischen Abbaubarkeit mit verschiedenen Faulschlämmen zeigte sich, dass Ethoxylate von linearen Alkoholen innert 10 – 40 Tagen vollständig abgebaut und mineralisiert wurden. Ethoxylate von einfach- und mehrfach verzweigten Alkoholen wurden hingegen nicht oder nur teilweise abgebaut. Die verschiedenen Faulschlämme zeigten ein unterschiedliches Abbauverhalten für AEO. Mit zunehmender Verzweigung des Fettalkohols nimmt die anaerobe biologische Abbaubarkeit der AEO ab.

Für die Abbauuntersuchungen mussten Konzentrationen gewählt und bestimmt werden, die keinen toxischen Effekt auf den Faulschlamm verursachen. Die Faulschlammtoxizität nimmt mit zunehmender Hydrophobizität, resp. mit abnehmender Ethoxylatkettenlänge der AEO, von 10 auf 100 mg/L zu (NOEC; Schlammkonzentration: 2 g TS/L). Der Verzweigungsgrad des Alkohols hat keinen signifikanten Einfluss auf die anaerobe Toxizität der AEO.

Die Membrantoxizität wurde mit reinen AEO untersucht. Zur Bestimmung der toxischen Membrankonzentration muss die Membran-Wasserverteilung bekannt sein. Diese wurde als Liposome-Wasser Verteilung (K_{lipw}) für 8 lineare AEO experimentell bestimmt und mit herkömmlichen K_{OW}-Abschätzungen verglichen. Die daraus berechneten Fragmente für log K_{lipw} betrugen für eine zusätzliche CH_2-Gruppe + 0.46 und für eine zusätzliche EO-Gruppe – 0.14. Die mit diesen Fragmenten berechneten K_{lipw} sind geeignete Parameter für QSARs für reine

AEO sowie für kommerziell erhältliche technischen Mischungen von AEO für die Abschätzung der Bioakkumulation, der Toxizität sowie des Sorptionsverhaltens an organisches Material.
Die Membrantoxitität von AEO wurde mit einer in-vitro Methode, basierend auf zeitlich aufgelöster Spektrophotometrie an Energie übertragenden Membranen (Chromatophoren) untersucht. Die für AEO bestimmten Effektkonzentrationen korrelierten gut mit toxischen Effekten, die an verschiedenen lebenden Organismen gemessen wurden. Toxische Effekte traten für alle AEO unterhalb der kritischen Mizellbildungskonzentration (CMC) auf. Die toxischen Membrankonzentrationen für AEO mit 5 EO, resp. ≥ 8 EO, waren 0.2 und 0.06 mol pro kg Lipid. Sie entsprechen den kritischen Körperkonzentrationen von nicht-polaren und polaren Narkotica in Fischen. Zudem konnten die gemessenen toxischen Effekte von technischen AEO mit Hilfe der Summe der Aktivität der einzelnen Komponenten modelliert werden. Dies bestätigt das Konzept der Addition der toxischen Effekte der Einzelverbindungen mit der gleichen toxischen Wirkungsweise.

Summary

Alcohol ethoxylates (AEO) are nonionic surfactants composed of a long chain fatty alcohol (hydrophobic moiety) combined with one or more ethylene oxide (EO) units (hydrophilic moiety). Worldwide approximately 750'000 tons of AEO are produced per year. In Switzerland the AEO consumption amounts to 6000 - 7000 tons per year.

Due to their hydrophobicity, a substantial part of the AEO present in the waste water reach the anaerobic reactor in the wastewater treatment plant (WTP) either sorbed to primary or activated sludge. Until now, the anaerobic biodegradation and toxicity have only been studied for the most common linear alcohol ethoxylates. Therefore, the anaerobic degradation, the toxicity towards anaerobic sludge and the partitioning behavior of AEO were investigated in this thesis.

To determine the anaerobic biodegradability of AEO and other test chemicals, the Anaerobic Screening Test System (ASTS) was developed based on the ECETOC screening test and optimized to low, non-toxic test substance concentrations (5 mg $C \cdot L^{-1} \cdot g^{-1}$).

The anaerobic biodegradation of AEO was investigated using sludge from three different wastewater treatment plants (WTP): Au-Bruggen, Wil, and Kloten/Opfikon. The linear AEO was completely degraded within 10 days by two sludges, but was not degraded by a third one within 30 days. Ethoxylates of single- and multiple branched alcohols were not or only partly degraded with the different sludges. The anaerobic biodegradability of technical AEO decreased with increasing branching of the alcohol moiety from linear to multiple-branched alcohols.

The toxicity towards anaerobic sludge, given as the no observed effect concentration (NOEC), increased with decreasing degree of ethoxylation from 10 to 100 mg/L at a sludge concentration of 2.0 ± 0.2 $g \cdot L^{-1}$. The degree of branching of the alcohol did not influence the toxicity significantly.

The mode of toxic action of various pure alcohol ethoxylates was investigated in detail with specific, membrane dependent toxicity tests. As a prerequisite the partitioning behavior between biological membranes and the water phase was experimentally determined for eight pure AEO. With these membrane-water partition coefficients it was possible to calculate the effect concentrations in the biological membrane, at which the membrane structure and functioning was non-specifically disturbed.

The membrane toxicity of AEO was investigated with an *in-vitro* method based on time-resolved spectrophotometry on energy transducing membranes. The effect concentrations obtained for this narcotic effect of AEO correlated well with the results from various toxicity tests on whole organisms. All AEO exhibited their toxic effect at concentrations well below the critical micelle concentration. The toxic membrane concentrations of AEO with 5 and ≥8 ethoxylate units were 0.2 and 0.06 mol per kg lipid, which correspond to the critical body residues of non-polar and polar narcotics in fish. In addition the toxic effects of mixtures of AEO were measured and could be modeled as the sum of activity of the single constituents, confirming the concept of concentration additivity of compounds with the same mode of toxic action.

Chapter 1
Introduction

General Remarks

Alcohol ethoxylates (AEO) are nonionic surfactants that have been used in significant amounts in industrial products since the 1930's. Usage grew after World War II in household and industrial cleaners, and from the mid-1960s in laundry products. Growth through the 1970's and 1980's has been rapid, especially in consumer products (Cahn & Lynn, 1983).

Alcohol ethoxylates are the largest volume nonionic surfactants produced, with ethoxylates based on highly linear primary alcohols the predominant type. Household laundry detergents are the largest single end-use for AEO. AEO also find much smaller volume uses in household cleaners, institutional and industrial cleaners, cosmetics, and agriculture, as well as for different purposes in industrial processes. Growth in use of linear primary AEO has been rapid over the last 20 years because of their qualities such as rapid biodegradation, low to moderate foaming ability, superior cleaning of man-made fibers, tolerance of water hardness, and ability to perform in cold water (Talmage 1994).

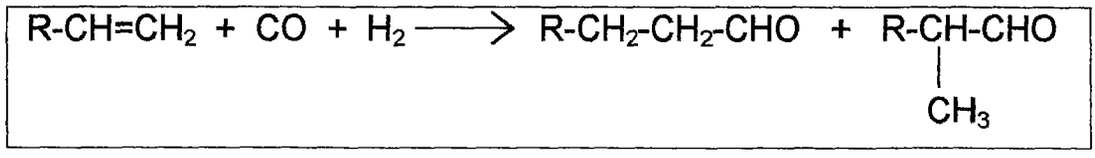
Alcohol ethoxylates with predominantly linear alkyl chains exhibit a high degree of biodegradability under aerobic and anaerobic test conditions. Both rapid primary and ultimate biodegradation have been demonstrated under a variety of laboratory and field conditions. Most of these biodegradation tests with AEO were performed under aerobic conditions, whereas under anaerobic conditions only a few studies were performed (Talmage 1994).

Although the toxicity of AEO surfactants to aquatic organisms is compound- and species-specific, several generalizations concerning chemical structure can be made. Toxicity increases with an increase in the alkyl chain length and decreases with an increase in the ethoxylate chain length (Swisher, 1987). The mode of action of AEO towards aquatic organisms is not well understood. Many nonionic surfactants interact with proteins, change the shape and activity of enzymes and solubilize structural and membrane proteins, which may result in changes in cell permeability (Helenius & Simons, 1975; Swisher, 1987; De la Maza, 1992).

Alcohol ethoxylates (AEO) are defined as follows: They are nonionic surfactants composed of a long chain fatty alcohol (hydrophobic moiety) combined, via an ether linkage, with one or more ethylene oxide (EO) units (hydrophilic moiety). The average number or range of carbon atoms in the alkyl chain (C) and the average number of ethylene oxide units (EO) are designated by subscripts (e.g.: $C_{12/14}EO_7$ means a mixture of C_{12}- and C_{14}-alcohols with an average ethoxylation degree of 7 mol EO per mol alcohol).

Production and use

Alcohol ethoxylates are produced by ethoxylation of linear or, to a small extent, of branched alcohols. The linear alcohols are based on coconut oil, palm oil, or synthetic olefins. The branched alcohols arise from the propylene trimer or tetramer with their carbon chains extended by an "OXO" process:



The ethoxylation of the alcohols proceeds with potassium hydroxide as the catalysts and a predefined molar ratio of ethylene oxide.

The reaction product is a mixture of polyethylene adducts with a broad distribution of EO-adduct chain lengths and some alcohols. The average moles of EO added per mole of alcohol define the commercial materials.

The consumption of the most important surfactants in Western Europe, USA and Japan in 1990 is shown in Figure 1. The market share of AEO was about 7 % in these countries (soap: 22 %). Approximately 200'000 tons of AEO and 120'000 of alcohol ether sulfates (AES) were produced world wide in 1988 (Talmage 1994). Kiewiet (1996) estimated the global consumption of alcohol ethoxylates to be 750'000 tons/year.

The use of surfactant in Switzerland is given in Figure 2. In a representative year (1993) the AEO consumption amounted to about 970 g per inhabitant. In Switzerland the market share of AEO has increased from 25 to 31 % in the period of 1989 to 1996. In the same period the market share of soap decreased from 20 to 13 % of total surfactant consumption.

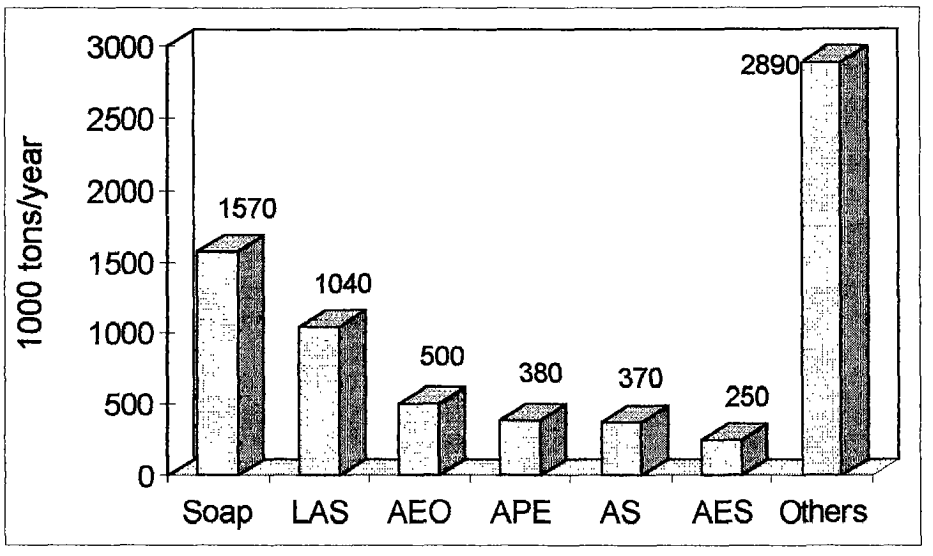


Figure 1: Consumption of surfactants in Western Europe, USA and Japan in 1990 (Richtler & Knaut 1991). Soap, LAS: linear alkyl benzene sulphonate, AEO: alcohol ethoxylates, APE: alkyl phenol ethoxylate, AS: alcohol sulfate, AES: alcohol ether sulfate.

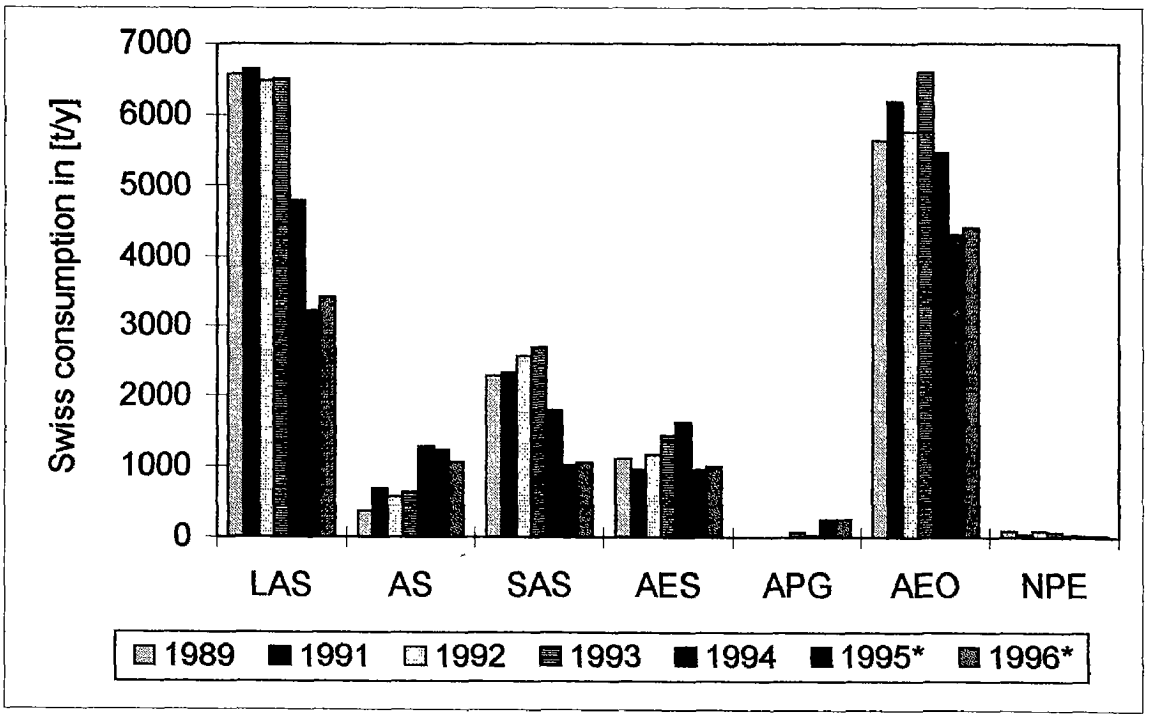


Figure 2: Swiss consumption of anionic and nonionic surfactants (1989- 1996). * in 1995-96 without Migros (share of market of about 20 - 25 %). LAS: linear alkyl benzene sulfate, AS: alcohol sulfate, SAS: secondary alkyl sulphonate, AES: alcohol ether sulfate, APG: alkyl polyglucoside, AEO: alcohol ethoxylates, NPE: nonylphenol ethoxylate (SWI: Swiss association of the soap and detergent industry).

Physical-chemical properties of surfactants

The characteristic properties of surfactants in aqueous solution, which render possible various practical applications, depend in all cases on the tendency to accumulate at interfaces between the aqueous solution and the adjacent gaseous, liquid or solid phases. Characteristic properties of surfactants are the influence on surface tension, micellation, adsorption, wetting, solubilization, emulsification, hydrophile-lipophile balance (HLB), cloud point and foaming (Schick, 1987, Swisher 1987).

The HLB scale allows rough classification of nonionic surfactants according to their solubility in water and their possible areas of application. The HLB value of a surfactant is defined as the ratio of the molecular mass of the hydrophilic fraction in the molecule M_h to the total molecular mass M of the surfactant, multiplied by 20 (Schick, 1987):

Equation 1

$$HLB = 20 \cdot \frac{M_h}{M}$$

Surfactants reduce the surface tension of water in a concentration dependent manner until the point of self-association is reached. Self-association gives rise to aggregates, either spherical, so-called micelles, or planar ones (Rosen, 1989). In these aggregates the hydrophilic headgroups remain solvated, while the hydrophobic tails are arranged in such a manner that they are shielded from water. The concentration, at which micelles are formed, is called critical micelle concentration (CMC) and is dependent on the structure of the surfactant.

The micelle forming surfactants fulfill a multitude of functions in washing powders. By reduction of the interfacial tension between the water and the fabric they improve the fabric's accessibility to water molecules. Adsorption of charged surfactants to fabric, as well as soil particles removed from the fabric, provide the surface of both with the same charge. As a result, redeposition of soil particles to the fabric is prevented electrostatically. Thirdly, the inner hydrocarbon spheres of the micelles are apolar environments, which can accommodate apolar constituents of the soil. The micelles solubilize them from the substrate during the washing process and keep them in aqueous solution (Kosswig & Stache).

Environmental fate of surfactants

After the surfactants have done their job in the cleaning process they are disposed of with the wastewater and enter the sewer. From this point on their environmental fate is considered. It is dominated by biodegradation, leading to a reduction of the amount of surfactants in the environment, and sorption to solid phases. The latter process reduces the fraction of

surfactants, which are dissolved in the aqueous phase. Volatilization from the wastewater can be neglected due to the high water solubility and the low vapor pressure of the surfactants (Tolls 1998).

By sorbing to solids surfactants are transferred from the aqueous phase to the solid phase. This occurs during sewage treatment where surfactants are removed from the wastewater stream by adsorption to the primary sludge and to the activated sludge. The primary sludge from the mechanical treatment is transferred directly into the anaerobic digester whereas the excess activated sludge is partly recycled in the wastewater treatment process and partly transferred to the anaerobic reactor. Alcohol ethoxylates are sorbed to about 65 % to 75 % to the solid phase in the raw sewage (primary sludge) and to about 40 % to 60 % to the settled sludge (Kiewiet, 1995). AEO are mainly transferred to anaerobic digesters in wastewater treatment plants where they are subjected to anaerobic biodegradation processes.

The portion of the surfactants not removed from the wastewater stream enters surface waters where, besides being biodegraded, they are subjected to sorption to suspended particles and sediments (Schwarzenbach, 1993). Surfactants can enter the terrestrial environment as a consequence of application of sewage sludge as fertilizer to soils or as a result of allowing to drain wastewater through soils. Sorption of surfactants to soils and sediments is important for the mobility of surfactants in the soil column and determines whether surfactants can enter the groundwater. In addition, sorption determines the availability of surfactants for bioaccumulation (Brownawell, 1991).

Environmental concentrations

Domestic wastewater show at the inflow of wastewater treatment plants (WTP) average nonionic surfactant concentrations of 1 - 10 mg/L (Hellemann 1979) and 50 % of which are alkyl ethoxylates (0.5 - 5 mg/L) (Swisher 1989). The 0.5 to 1.8 mg nonionic surfactants per g dry sludge were determined by Salanitro and Diaz (1995) in anaerobic sludges of 7 primary and 5 secondary digesters from 8 WTP with the CTAS- (Cobalt Thiocyanat Active Substance) and the BiAS-methods (Bismuth Active Substance), respectively. Fendinger et al. (1995) and Matthijs et al. (1995, 1996) determined alkyl ethoxylates in fresh and treated wastewater as well as in rivers before and after the effluent of the WTP with specific analytical methods such as GC-MS and HPLC-fluorescence, respectively (Table 1). The removal of alkyl ethoxylates was over 99% in different WTP and 92% at two trickling filter plants. It was not determined in these studies whether sorption or biodegradation accomplished removal. The in-sewer removal

rate of AEO was determined to range between 28 % and 58 % (average: 42 %) for C_{12}- to C_{15}-AEO (Matthijs et al. 1996).

Table 1: Concentrations of non-ionic surfactants and alcohol ethoxylates in rivers, wastewater and WTP. [1] Fendinger et al. (1995), [2] Hellmann (1979), [3] Matthijs et al. (1995, 1996), [4] Salanitro & Diaz (1995), [5] Swisher (1987), [6] Dorn et al (1993), [7] Kiewiet et al. (1995).

	Average concentration of nonionic surfactants	Average concentration of alkyl ethoxylates
Wastewater:	1 - 10 mg/L [2]	
River:	0.0042 - 0.8 mg/L [6]	0.017 - 0.037 mg/L [1]
WTP influent:	0.008 - 2.7 mg/L [6]	0.5 - 5 mg/L [1, 3, 5] 1.5 – 6.4 mg/L [7]
Effluent mechanical treatment:		2.6 - 2.8 mg/L [1] 1.2 – 3.9 mg/L [7]
WTP effluent:		0.002 - 0.07 mg/L [1, 3] 0.004 – 0.022 mg/L [7]
Anaerobic digester:	0.5 - 1.8 mg/g dry sludge [4] 2.5 - 45 mg/L sludge	

Biodegradability

The biodegradability is probably the most important factor in the environmental risk assessment of surfactants. The greater part of the surfactants used is discharged into sewage and eventually into rivers and seas. Biodegradation of the surfactants in wastewater treatment plants (WTP) is therefore of particular importance. In the determination of the biodegradability a distinction is made between the primary attack on the surfactant molecule and the ultimate degradation. The primary degradation leads to a loss of interfacial activity due to a structural change of the molecule. The ultimate degradation is the complete conversion of the surfactant into inorganic substances such as carbon dioxide and water.

The biodegradation of surfactants and alcohol ethoxylates have been reviewed by Swisher (1987) and Talmage (1994). From these reviews the following generalizations appear:

All alcohol ethoxylates derived from straight chain primary or secondary alcohol undergo rapid and ultimate biodegradation. The three features, which have a significant influence on the pathway, rate and extent of the biodegradation, are:

a) The degree of branching of the hydrophobic moiety. Ultimate biodegradation is most rapid with linear alcohols. Highly branched alcohols are slowly mineralized.

b) The chain length of the hydrophilic moiety. Up to about 11 ethoxylate units no inhibition of biodegradation is observed. However, the presence of 20 ethoxylate units reduces the rate of biodegradation.

c) Insertion of oxypropylene groups into the hydrophilic moiety. The biodegradation of alcohol ethoxy/propoxylates is inversely related to the amount of oxypropylene groups incorporated into the surfactant.

Pathway

The biodegradation of linear alcohol ethoxylates proceeds via three pathways (Swisher, 1987; Steber & Wierich, 1987):

1) A central fission between the hydrophobic and the hydrophilic moieties resulting in an alcohol or alkyl carboxylic acid and an (carboxylated) ethoxylate chain. The alkyl carboxylic acid undergoes rapid β-oxidation. The mechanisms for the ethoxylate chain degradation would seem to involve ethoxylate carboxylic acid intermediates or a diol cleavage step producing an ethoxymer with one less ethoxylate unit (Figure 3).
2) Oxidation of the terminal carbon of the alkyl chain to produce a carboxylic acid. β-Oxidation then shortens the alkyl chain by two carbon units at a time (Figure 3).
3) Oxidation of the terminal ethoxylate unit to give carboxylic acid.

Results of mechanistic and field studies show that central fission is the most important of the three mechanisms.

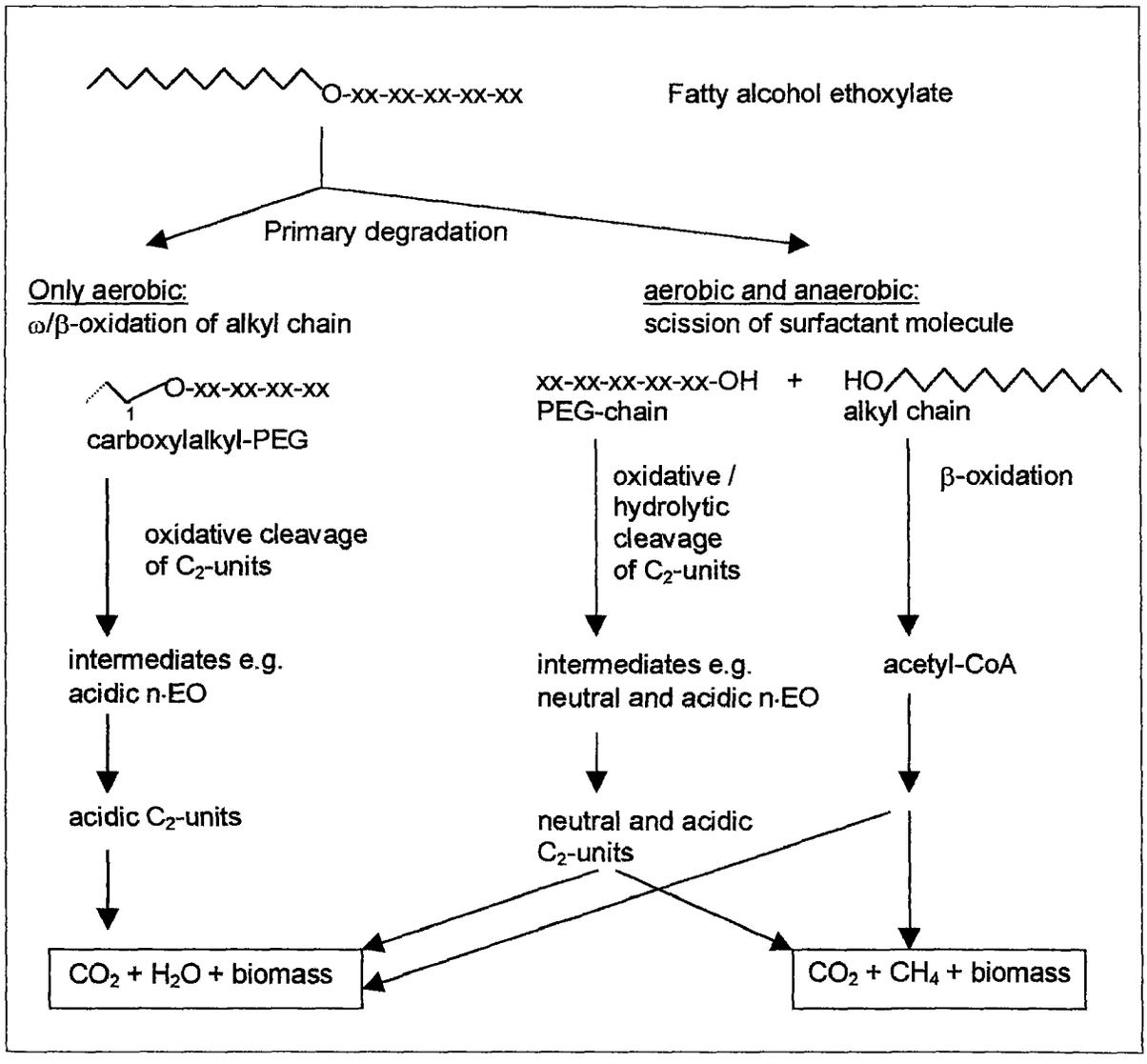


Figure 3: Biodegradation routes of fatty alcohol ethoxylates and their metabolites under aerobic and anaerobic wastewater treatment plant conditions (Steber & Wierich, 1987).

Aerobic biodegradation

As a class, AEO undergo rapid primary and ultimate aerobic biodegradation under both laboratory and field conditions. Linear AEO are degraded by hydrolysis of the ether linkage at the hydrophilic-hydrophobic interface followed by oxidation of the alkyl- and the EO-chain starting at the omega (ω) end with little or no metabolism of the alkyl chain. The aerobic biodegradability of differently branched and linear AEO was reviewed by Talmage (1994) and studied by Siegfried and Baumann (1993). It can be summarized that biodegradability is better for ethoxylates of linear (LA) and single-branched alcohols (SBA) than of multiple-branched ones (MBA). The alkyl chain length did not significantly influence biodegradation. An increasing EO-chain length up to 12 EO-units did not affect the biodegradability of the AEO.

Anaerobic biodegradation

Alcohol ethoxylates are predestined to reach anaerobic compartments due to their physical-chemical properties such as low solubility, low vapor pressure, high octanol-water partitioning coefficient and high adsorption rates towards suspended solid matter. In wastewater treatment plants these properties often lead to a good elimination. But a good elimination does not automatically mean that the substances are mineralized. The difference between primary and ultimate anaerobic degradation can amount to 20 % to 40 % for AEO (Salanitro & Diaz, 1994). For this information the ultimate biodegradation of organic compounds has to be studied in detail.

Ultimate anaerobic biodegradation was well studied for ethoxylates of linear alcohols (LA). In most experiments long adaptation periods (> 30 days) of the sludge towards AEO were observed. Ready biodegradability (> 60% within 30 days) was reported by Salanitro and Diaz (1994) for a linear alcohol ethoxylate (LA-$C_{9\text{-}11}EO_9$).

Steber and Wierich (1987) and Federle and Schwab (1992) investigated the anaerobic degradation of ^{14}C labeled alcohol ethoxylates. They concluded, that the first microbial attack on the surfactant-molecule limits the anaerobic degradation rate (Figure 3). They postulated a central fission into the alkyl and polyethylene glycol moieties. The products from the central fission, polyethylene glycol and fatty alcohols are anaerobically well degraded.

Huber (1999) found no evidence for the central fission under anaerobic conditions. He observed the ω-hydrolytic degradation of the polyethylene glycol chain, which was described before by Wagener and Schink (1988) (Figure 4).

Long adaptation periods of 30 to 90 days were observed in degradation experiments with linear AEO (Wagener & Schink 1987/1988; Steber & Wierich 1991). They can be explained with a slow first microbial attack and/or a toxicity, which results from intact surfactant molecules on some microorganisms. Inhibitory effects observed for different surfactants in anaerobic degradation experiments confirm this. Fixed bed reactor experiments with adapted sludge, where the linear AEO (LA-$C_{12}EO_{23}$) was degraded to more then 90 % at a high concentration of 1 $g \cdot L^{-1}$, are in contrast to this finding. They show that adapted microorganisms can cope with high surfactant concentrations. Nevertheless it is assumed that some anaerobic sludges are not adapted to degrade alcohol ethoxylates, that the degradation enzymes are not induced, or that the first microbial attack is inhibited by the surfactant.

No data was found in literature on the anaerobic biodegradability of ethoxylates of single- and multiple-branched alcohols (SBA, MBA).

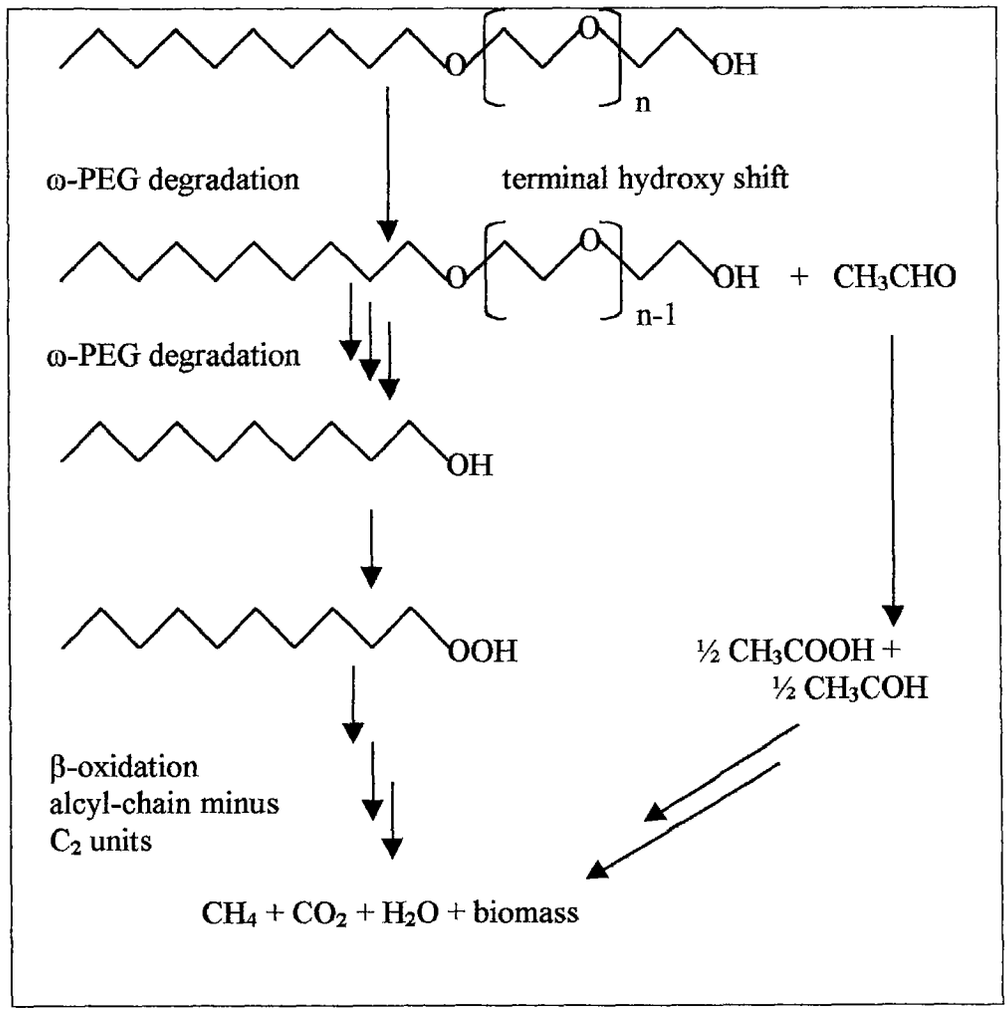


Figure 4: Biodegradation of fatty alcohol ethoxylates and their metabolites under anaerobic conditions (Wagener & Schink, 1988,).

Conditions and strategies for anaerobic degradation tests

The test conditions chosen for anaerobic degradation experiments influence the results. Origin of the sludge, pretreatment of the inoculum, and adaptation to the test substance are some of the parameters. Many groups worked with different inocula as for example sludges from various wastewater treatment plants (WTP) (Horowitz et al. 1982; Shelton & Tiedje 1984), wastewater ponds (Federle & Schwab 1992), anaerobic soils (Adriaens et al. 1995) or sediments from lakes, rivers (van Beelen et al 1994) or the sea (Urrutia et al. 1993). Others used acclimated sludge or enrichment cultures for degradation experiments with hardly-degradable substances (Wagener & Schink 1987).

Besides developing anaerobic degradation and toxicity test systems it is also important to elaborate strategies for handling negative test results. An integrated test strategy with increasing complexity was proposed by Strotmann et al. (1993). The working group Anaerobic

Biodegradation has elaborated schemes to evaluate the importance of missing anaerobic biodegradability. These schemes enable one to differentiate sensibly between substances in respect of the aquatic and the terrestrial environment (Schöberl 1994). Baumann et al. (1994) developed the BEWAG scheme to judge the potential hazard of chemicals to waters using aerobic and anaerobic biodegradation- and toxicity data.

Toxicity

Acute and chronic toxicity of linear, single branched and multiple branched alcohol ethoxylates were reviewed by Talmage (1994) for fish, daphnia, oysters, shrimps, crabs, freshwater algae, and aquatic macrophytes. The acute and chronic toxicity of AEO to Algae, daphnia and fish are in the range of 0.1 to 100 $mg \cdot L^{-1}$. It is difficult to indicate general trends in the relationship between molecular structure of nonionic surfactants and measured toxicity (Kiewiet, 1996). There are however two studies which indicate a structure toxicity relationship. Roberts (1991) shows that the toxicity of AEO to fish is primarily dependent on the hydrophobic chain, the ethoxylate chain does not exert a significant influence on the toxicity. Ribosa et al. (1993) tested the toxicity of nonionic surfactants to the *Photobacterium phosphoreum*. Again the toxicity appeared to depend on the alkyl chain length of the AEO. The toxicity was decreased, however, with increasing ethoxylate chain length.

Only a few data on bioconcentration of nonionic surfactants have been reported by Tolls et al. (1994). These data indicate, as for other surfactants, a relationship between the hydrophobicity and the bioconcentration.

Membrane toxicity

Due to their ability to absorb at interfaces, surfactants can interact with biological membranes (Sterzel 1990). This interaction depends on the concentration of the surfactant and can be described in the following sequence (Helenius & Simons 1975): In the first instance the monomeric surfactant molecule adsorbs onto the membrane. For a low surfactant-membrane ratio the permeability of the membrane and the trans-membrane solute transport are increased (De la Maza et al. 1991-1996) and leads to cell lysis at higher concentrations (Ohnishi & Sagitani 1993). At even higher concentrations, the lamellar structure of the membrane is lost and it is solubilized. A further increase in surfactant concentration results in the separation of the phospholipids from the proteins. This allows surfactant molecules to adsorb on previously hidden regions of the protein molecules. A significant solubilization of these proteins is possible only if the critical micelle concentration (CMC) is exceeded (Chang et al. 1985). As a

consequence of these interactions, surfactants are able to influence the metabolism of membrane components (De Leo 1989).

Scope of this thesis

The increasing demand for anaerobic biodegradation and toxicity data in risk assessments of chemicals requires suitable test systems and evaluation strategies. The work presented in this thesis comprises, in the first part, the anaerobic biodegradation and toxicity of technical mixtures of alcohol ethoxylates. In the second part, the focus was set on the partitioning behavior and the membrane toxicity of pure alcohol ethoxylates. The aim was, to develop Quantitative Structure Activity and Toxicity Relationships (QSAR, QSTR) for anaerobic degradation and membrane toxicity of different AEO.

Based on the actual knowledge about performing anaerobic biodegradation tests, it was the scope of the first part of this study, to develop an anaerobic screening test system for the determination of the anaerobic biodegradability and toxicity of substances, products and industrial wastewater (Chapter 2). The test systems should be small and easy to handle, useable for anaerobic degradation- and toxicity tests at small costs. The sample preparation should be effective and guarantee oxygen free conditions in the test assays. The pressure measurement should be easy and fast to perform, reproducible and accurate. The factors, which influence the performance and the reproducibility of the anaerobic degradation tests, were determined and improved for all the steps: evaluation and pre-treatment of the sludge, sample preparation, data collection, and evaluation of the test results. After these validation steps had been performed, the test system was used to assess the anaerobic biodegradability for different chemicals, e.g. surfactants and complexing agents, as well as for industrial wastewater (Chapter 2).
In Chapter 3 and Chapter 4 the focus was set on the anaerobic biodegradation and toxicity of ethoxylates of linear, single- and multiple-branched alcohols. The aim was to derive a relationship for technical AEO between their structure and their anaerobic biodegradability. In Chapter 4 the anaerobic biodegradability of technical AEO was investigated with anaerobic sludge from different wastewater treatment plants. With technical AEO it was not possible to draw a correlation between structure and toxic effects due to the broad mixtures of different alcohol ethoxylates with heterogeneous properties. Therefore, the toxicity of pure AEO towards microorganisms was investigated in the following part.

Before a chemical exerts a toxic effect in an organism, it has to build up an effective concentration at the site of toxic action. This can be reached by sorption to the membranes

followed by active or passive uptake processes. The membranes of microorganisms as possible targets for sorption and accumulation of pure alcohol ethoxylates were therefore investigated with specific test systems using membrane dependent processes. In the second part of the thesis, therefore, the partitioning behavior and the membrane toxicity of pure alcohol ethoxylates was investigated and correlated with their structure (Chapter 5 to Chapter 7).

As a prerequisite, membrane-water partition coefficients were determined for defined AEO to quantify the sorption behavior of AEO to biological membranes (Chapter 5). The AEO-concentrations at the membrane and in the water phase were calculated from nominal concentration with the membrane-water partition coefficients. The partition coefficients were compared to estimated octanol-water partition coefficients and the structural elements of the AEO. From this, a new fragment method was developed to estimate membrane-water partition coefficients for AEO. They can be used to model the partitioning behavior and the bioaccumulation of AEO in wastewater treatment plants or in natural systems.

Pure AEO were assessed with two specific test systems to determine membrane toxicity. First, the phosphate uptake system of *Acinetobacter johnsonii 210 A* was used to determine inhibitory effects caused by linear AEO on active phosphate transport via the membrane (Chapter 6). Second, the membrane toxicity of different pure alcohol ethoxylates (AEO) and technical mixtures were investigated with a time-resolved spectrophotometer using photosynthetic membranes of *Rhodobacter sphaeroides*. Quantitative Structure Toxicity Relationships (QSTR) were calculated according to the structure of the investigated linear alcohol ethoxylates.

Under Conclusions, the results of this thesis are summarized and final conclusions are drawn on the measurements and calculations of the anaerobic biodegradability and toxicity of different alcohol ethoxylates.

Chapter 2

The Anaerobic Screening Test System (ASTS)

Markus T. Müller, Urs Baumann, Alexander J.B. Zehnder

Abstract

Based on the ECETOC screening test system (ECETOC 1988) the Anaerobic Screening Test System (ASTS) was developed to determine anaerobic biodegradability and toxicity of chemicals, mixtures and waste waters. The external pressure measurement was optimized by a reduction of the dead volume and of the concentration of the test solution to 10-15 mg C/liter. The ASTS fulfills the requirements of screening large sets of chemicals, mixtures or waste waters. The performance was tested with different C_2- to C_{16}-compounds, sodium laurylsulfate, technical mixtures of alcohol ethoxylates (AEO), nitrilo triacetate (NTA), phosphonic acids and industrial waste waters from food- and textile industry. An apparatus for the anaerobic sludge transfer was also developed to optimize the sample preparation under anaerobic conditions. Different sludge pre-treatments, such as washing and pre-digestion of the sludge, were evaluated to reduce the endogenous biogas production and to improve the reproducibility of the test results. Different degradation behavior and lag-phases were observed in experiments with eight substances and sludges from three municipal wastewater treatment plants (WTP). A number of methods for the purpose of calculating the anaerobic biodegradability were compared. As a result a combination of a set of defined methods was proposed for data evaluation. The limit of significance was determined for the ASTS with degradation tests at low substance concentrations (c = 10 mg C/L).

Introduction

Anaerobic degradation tests have been used for the last 120 years to determine the possibility of producing biogas from organic waste material. Buswell (1937) presented a good review on the extensive literature of the years 1880 to 1937 available on this subject. Because of the oil

crisis in the early seventies anaerobic degradation and biogas production became suddenly of interest again. In the eighties the focus of anaerobic biodegradation tests was on environmental aspects such as the full biological degradability. In the last two decades, different anaerobic degradation and toxicity tests have been proposed and international standards were set up.

Test systems

Over time the methods changed from volumetric to automated pressure measurements of the biogas produced, all of which are based on different concepts of calculating the theoretical biogas production. These methods are described below and summarized in Table 2.

Owen et al (1979) introduced a method with a volumetric measurement of the evolved biogas and the quantification of methane by gas chromatography (GC). The amount of biogas was quantified by syringe or with gas collection tubes and the measured volume was corrected to standard pressure. Shelton and Tiedje (1984) measured the biogas in the headspace of the test bottle as pressure or by gas chromatography of methane and carbon dioxide. The amount of water soluble carbon dioxide was calculated from the formula of the compound and the stoichiometry of the reaction according to Tarvin and Buswell (1934). A similar method, based on the total organic carbon content (TOC), was presented by an ECETOC working group (ECETOC 1988, Birch et al. 1989). It was assumed that the maximum amount of C_1-gases produced during degradation is equal to the TOC of the substance tested. Therefore, the biogas pressure was measured with an external pressure transducer and the amount of dissolved carbon dioxide was quantified as inorganic carbon at the end of the test. A precondition for a correct result is a low carbonate concentration of the inoculum, which was achieved by different washing steps of the sludge material and by replacing the carbonate by a phosphate buffer. Baumann and Schefer (1990) presented a test system, based on the quantification of the methane by pressure measurement with an integrated mercury manometer and a carbon dioxide trap inside the bottle, which was used to absorb the carbon dioxide at the end of the experiment. The theoretical methane pressure was calculated directly from the chemical oxygen demand (COD) of the test material. This test system was then automated with a piezoelectric pressure transducer, integrated into the cover of the test bottle (Baumann & Rezzonico 1993). The advantages are that the automated pressure measurement and the data collection can be independently recorded. The evaluation of the data can be done at any given time.

The characteristics of the test systems mentioned above are compared in Table 2. The anaerobic screening test system presented in the following is also included into the table.

Beside all these batch test systems, different upflow anaerobic sludge bed reactors (UASB) were described (Strotmann et al. 1993, Ramirez et al. 1994, Baumann & Müller 1996) but not used for routine tests due to their higher complexity.

Table 2: Comparison of different anaerobic degradation test systems. **BMP**: biochemical methane potential; **ATA**: anaerobic toxicity assay; **GC**: gas chromatography; **COD**: chemical oxygen demand; **DOC**: dissolved organic carbon; **TGP**: theoretical gas production, **S&T**: Shelton & Tiedje (1984)

reference	**test system and substance concentration**	**measurement of biogas production**	**evaluation concept**
Owen et al (1979)	250 mL serum bottle (BMP), with 150 mg COD. 160 mL serum bottle (ATA).	Volumetrically with syringe, CH_4 of headspace with GC.	0.350 m^3 CH_4 per kg COD (Owen et al. 1964).
Shelton & Tiedje (1984): **S&T**	160 mL serum bottles, with 50 mg C/L.	Measured with a pressure transducer and GC of CH_4 and CO_2.	TGP = 0.35 CO_2 + 0.95 CH_4. TGP was calculated with Buswell equation and substance formula. Well degradable if TGP > 75 %.
Battersby & Wilson (1988)	160 mL serum bottles, with 50 mg C/L.	Precision pressure transducer, pressure was equalized to atmospheric pressure after each reading.	TGP (S&T)
ECETOC (1988) Birch et al. (1989) ISO-11734 (1991)	>160 mL bottles, substance formula (Buswell equation) or TOC of the substance.	Volumetrically, by pressure measurement or with GC.	- TGP (S&T) - CH_4 of gas phase (GC) - Theoretical amount of C_1-Biogas (see Equation 4)
Baumann & Schefer (1990)	1 L glass bottle, cover equipped with a Hg-manometer, an injection port and small vial to absorb the CO_2 of the gas phase. COD = 600 mg O/L.	Reading of biogas pressure (Torr) and CH_4 pressure after addition of NaOH to small vial at the end of the experiment.	Theoretical amount of methane: (see Equation 5)
Baumann & Rezzonico (1993)	1 L glass bottles with DEGRAMAT cover. TOC and/or COD.	Automated measurement: DEGRAMAT cover with integrated pressure transducer, injection port and small vial (absorption of CO_2).	Theoretical amount of methane: (see Equation 5)
Müller et al. (1996)	ASTS: 250 mL glass bottles, cover with injection port and ball valve for external pressure measurement. TOC: 20-100 mg C/L and COD: 50-300 mg O/L	Pressure measurement with an external pressure transducer with a low dead volume	Combination of - C_1-Biogas (Equation 4) - CH_4-Biogas (Equation 5) - DOC removal.

The test systems summarized in Table 2 can be used for the determination of toxicity of different compounds towards anaerobic sludge (Owen et al. 1979, Woods et al. 1990) and sediments (Remde & Traunspurger 1994).

The International Organization for Standardization (ISO) decided 1991 to standardize the ECETOC method (1988) the screening test for the evaluation of the anaerobic biodegradability of organic compounds (ISO-11734). One decade after the ECETOC screening test was

proposed, it has gained large acceptance and extensive studies on chemical structure and their biodegradability in the anaerobic environment have been made (Battersby & Wilson 1989; Henkel & Unilever 1995; Pagga 1997). Inspite of the fact, that the results of the different ring tests (Strujis & Stoltenkamp-Wouterse 1992, Pagga & Beimborn 1993) confirmed the reliability of this method, ECETOC and OECD have not yet ratified it.

Development of the Anaerobic Screening Test System (ASTS)

The results of anaerobic degradation- and toxicity tests are needed to complete the overall concept to determine the environmental hazard of compounds not only in the aerobic but also in the anaerobic environment (Baumann et al. 1994, Schöberl 1994). In wastewater treatment plants, for example, hydrophobic compounds can sorb to primary or activated sludge and reach the anaerobic reactor. Chemicals sorbed to particulate organic matter (POM) can reach anaerobic sediments in the aquatic environment. The philosophy of completely degradable and non-toxic compounds demands the determination not only of the aerobic, but also of the anaerobic biodegradation and/or toxicity. The ASTS was developed to perform anaerobic degradation and toxicity tests for a large number of substances. The test system was optimized by focussing on known weaknesses of the already existing test systems described in Table 2. This was achieved by optimizing the design of the hardware and the different steps from sample preparation to data evaluation by focusing on:

- the design of the test system: cover, connections, seals,
- the design of the pressure transducer,
- the sample preparation and *anaerobic* sludge transfer to the test bottles,
- a better evaluation process of the degradation results,
- the assessment of the sludge quality and / or activity,
- and a better understanding of the influence of sludge pre-treatment and pre-digestion.

The ASTS was developed for high throughput screening with accurate and reproducible results. The ASTS also meets the requirements formulated by ISO and the ECETOC for a standardized anaerobic degradation test.

Quantification of the mineralization

The anaerobic degradation of a test substance can be directly determined in the solution by the disappearance of either the individual chemical, the dissolved organic carbon (DOC), or the chemical oxygen demand (COD). It can also be determined indirectly by the biogas production,

which can be measured either by volume or pressure. A combination of different methods reduces the risk of misinterpretations. The degree of mineralization is quantified by a number of equations.

Shelton and Tiedje (1984) based their anaerobic degradation test on Equation 2 (Tarvin & Buswell, 1934) to calculate the maximal amount of carbon dioxide (CO_2) and methane (CH_4) from the formula of the substance to be degraded ($C_dH_aO_b$):

Equation 2

$$C_dH_aO_b + \left[d - \frac{a}{4} - \frac{b}{2}\right] H_2O \rightarrow \left[\frac{d}{2} - \frac{a}{8} + \frac{b}{4}\right] CO_2 + \left[\frac{d}{2} + \frac{a}{8} - \frac{b}{4}\right] CH_4$$

They included correction factors for the water solubility of the two gases, methane and carbon dioxide, to calculate the theoretical biogas production (C_1-biogas), which is directly measurable as biogas pressure (Equation 3). This method of Shelton and Tiedje (1984) is in the following abbreviated as S&T.

Equation 3

$$C_1\text{-biogas} = 0.95 \cdot CH_4 + 0.35 \cdot CO_2 \qquad \text{(S\&T)}$$

Birch et al. (1989) and ECETOC (1988) proposed a method based on the total organic carbon (TOC) content of the test substances (Equation 4). The carbon balance for total biodegradation postulates that the anaerobic microbial community transforms all the organic carbon into C_1-gases (CH_4 and CO_2) and a small amount of biomass (< 10%), which can be neglected. To determine the biodegradation without knowing the formula of the test substance, information on the total biogas pressure and the inorganic carbon (IC) of the test solution must be available. The sludge must be pre-washed to remove carbonates and the buffer medium should not contain carbonate.

Equation 4:

$$m(C_{1\ total}) = \frac{TOC_{test}}{12\frac{g}{mol}}$$

$m(C_{1\ total})$: total C_1-biogas production [mol]

TOC_{test}: TOC of test substance in the assay [g C]

Based on the Buswell equation (Equation 2), Baumann and Schefer (1990) derived Equation 5 to calculate the theoretical methane production directly from the chemical oxygen demand (COD) of the test substance.

Equation 5:

$$m(CH_4) = \frac{COD_{test}}{64\frac{g}{mol}}$$

$m(CH_4)$: methane-production [mol]

COD_{test}: COD of the test substance in the assay [g]

The method of Baumann and Schefer (1990) is based on the knowledge of the COD of the test substance. The theoretical methane gas production is calculated by using Equation 5 and the ideal gas law at test conditions. The methane gas pressure is determined at the end of the experiment at least one day after the addition of concentrated sodium hydroxide solution to absorb all the carbon dioxide as sodium carbonate in the liquid phase.

The arithmetic mean of the endogenous methane production is then subtracted from the total methane production. This result is divided by the calculated theoretical methane production of the test substance to get the amount degraded by anaerobic biodegradation. Additional information, e.g. the removal of the test substance, can be obtained by determining the DOC and/or COD from the test solution at the beginning and at the end of the experiment. The removal includes both sorption to the sludge as well as biodegradation. Together with the information about biogas production (mineralization), it is possible to differentiate between removal by sorption or biodegradation.

Each method discussed here has its specific advantage and disadvantage. With a combination of two or more different evaluation methods, the reliability of the results can be improved significantly, therefore errors can be avoided.

In this study a combination of different quantification methods was used. Equation 4 and Equation 5 were used to determine the theoretical amount of CO_2 and CH_4. In this procedure only the TOC and the COD of the test substance or waste water had to be known. In order to control the present amount of biodegradation during the experiment, Equation 2 was applied. At the end of the experiment carbon dioxide was removed from the gas phase by adding sodium hydroxide to the test solution. Subsequently methane pressure in the test bottle was measured. The amount of biodegradation was calculated from the results of the measured and the theoretical methane pressure (Equation 5). Finally, to check the experimental results, DOC removal was determined.

Calculation of the theoretical CH_4-pressure

The theoretical methane pressure is here defined as the maximum pressure increase due to methane production after complete anaerobic biodegradation. It can be calculated from the COD value of the test assay and Equation 8, which was derived from Equation 5 and Equation 7.

Equation 6: $$\Delta p(CH_4)_{theoretical} = \frac{m(CH_4)}{V_{test}} \cdot R \cdot T \cdot \frac{V_{test}}{V_{gas}} \cdot f = m(CH_4) \cdot R \cdot T \cdot \frac{1}{V_{gas}} \cdot f$$

$\Delta p(CH_4)_{theoretical}$	theoretical methane pressure [mbar]	T	absolute temperature [K]
$m(CH_4)$	methane-gas-production [mol]	V_{test}	volume of the test liquid [L]
R	ideal gas constant $[\frac{J}{K \cdot mol}]$	V_{gas}	volume of the gas headspace [mL]
COD_{test}	chemical oxygen demand [g/L]	$f = 10^4$	conversion factor $[\frac{mbar * mL}{Pa * m^3}]$

Equation 7: $$\Delta p(CH_4)_{theoretical} = \frac{COD_{test}}{64 \frac{g}{mol}} 8.3144 \cdot \frac{J}{K \cdot mol} \cdot 308.15\ K \cdot \frac{V_{test}}{V_{gas}} \cdot 10^4 \frac{mbar \cdot mL}{Pa \cdot m^3}$$

Equation 8: $$\Delta p(CH_4)_{theoretical}[mbar] = COD_{test}\left[\frac{g}{L}\right] \cdot \frac{V_{test}[L]}{V_{gas}[mL]} \cdot 400'325\left[\frac{mbar \cdot mL}{g}\right]$$

Calculation of the anaerobic biodegradability

To determine the anaerobic biodegradation during the experiment according to Shelton and Tiedje (1984), the biogas pressure p_1 was used (Equation 9). 24 hours after the addition of hydrochloric acid to the test assay (option) the total pressure p_2 was measured (Equation 10). The result together with Equation 4 was used for the calculation of the biodegradation. To measure methane pressure p_3 (Equation 11) sodium hydroxide has to be added to the test assay in order to remove the gaseous carbon dioxide, which takes about 24 hours.

Equation 9: $p_1 = p(biogas) = p(CH_4) + p(CO_{2\,(gas)})$ (during experiment)

Equation 10: $p_2 = p(C_{1\,(total)}) = p(CH_4) + p(CO_{2\,(total)})$ (after acid addition)

Equation 11: $p_3 = p(CH_4)$ (after base addition)

In the following we use the sample (i) and the blank (j). The sample contains anaerobic sludge and test substance, whereas the blank is used to measure the endogenous biogas production of the sludge only.

The initial pressure at the start ($p_i(t=0)$ or $p_j(t=0)$) was subtracted from each pressure measurement of a sample i or blank j at a time t ($p_i(t)$ or $p_j(t)$) (Equation 12 and Equation 13). The average endogenous biogas production was calculated according to the following Equations:

Equation 12: $$\Delta p_i = p_i(t) - p_i(t=0)$$

Equation 13: $$\Delta p_j = p_j(t) - p_j(t=0)$$

Equation 14: $$\overline{\Delta p_j} = \frac{\sum_{j=1}^{n} \Delta p_j}{n}$$ n: number of experiments

The test methane pressure of a sample δp_i was determined with Equation 15 by subtracting $\overline{\Delta p_j}$ (Equation 14) from Δp_i (Equation 12).

Equation 15: $$\delta p_i = \Delta p_i - \overline{\Delta p_j}$$

The anaerobic biodegradability of sample i ($f_{anaerobic,\ i}$) is calculated with Equation 16, where the test methane pressure δp_i is divided by the theoretical methane pressure (Equation 8).

Equation 16: $$f_{anaerobic,\ i} = \frac{\delta p_i}{\Delta p(CH_4)_{theoretical}} \cdot 100\%$$

Materials and methods

Anaerobic Screening Test System (ASTS)

The set-up of the Anaerobic Screening Test System (ASTS) is shown in Figure 5. It consists of 250 mL Duran glass bottles with an open ISO GL 45 cover (Schott GmbH, Mainz, Germany). The hole in the cover fits a butyl rubber stopper No. 9 (Faust AG, Schaffhausen, Switzerland). Two glass tubes are inserted into the rubber stoppers, one 70 mm long, diameter 10 mm with ISO GL 14 winding, the other about the same length but with a diameter of 4 mm. The larger tube is sealed with a 5 mm thick silicon septum which is fixed with a connection cover. The outer part of the thinner glass tube is connected to a Tygon pumping tube (cross-linked PVC) with an inner diameter of 2.79 mm (Ismatec SA, Glattbrugg, Switzerland) containing a glass ball of a diameter of 3-4 mm. This device acts as a ball valve. The pressure transducer is beeing connected to the Tygon tubing when needed.

DEGRAMAT

The DEGRAMAT test system consists of different test units such as: A one liter Duran glass bottle with ISO GL 45 winding (Schott GmbH, Mainz, Germany), the DEGRAMAT cover with integrated pressure transducer and sample port, the DEGRAMAT data station and the DEGRAMAT software (RST-productions AG, Berg, Switzerland). The different test bottles work independently and can be connected to the DEGRAMAT data station for data exchange.

The DEGRAMAT covers can be individually programmed. The data is independently stored in each cover to be downloaded afterwards to the DEGRAMAT data station. From here they can be transferred to a computer for the evaluation using the DEGRAMAT software. Baumann and Rezzonico (1993) described the DEGRAMAT test system in detail.

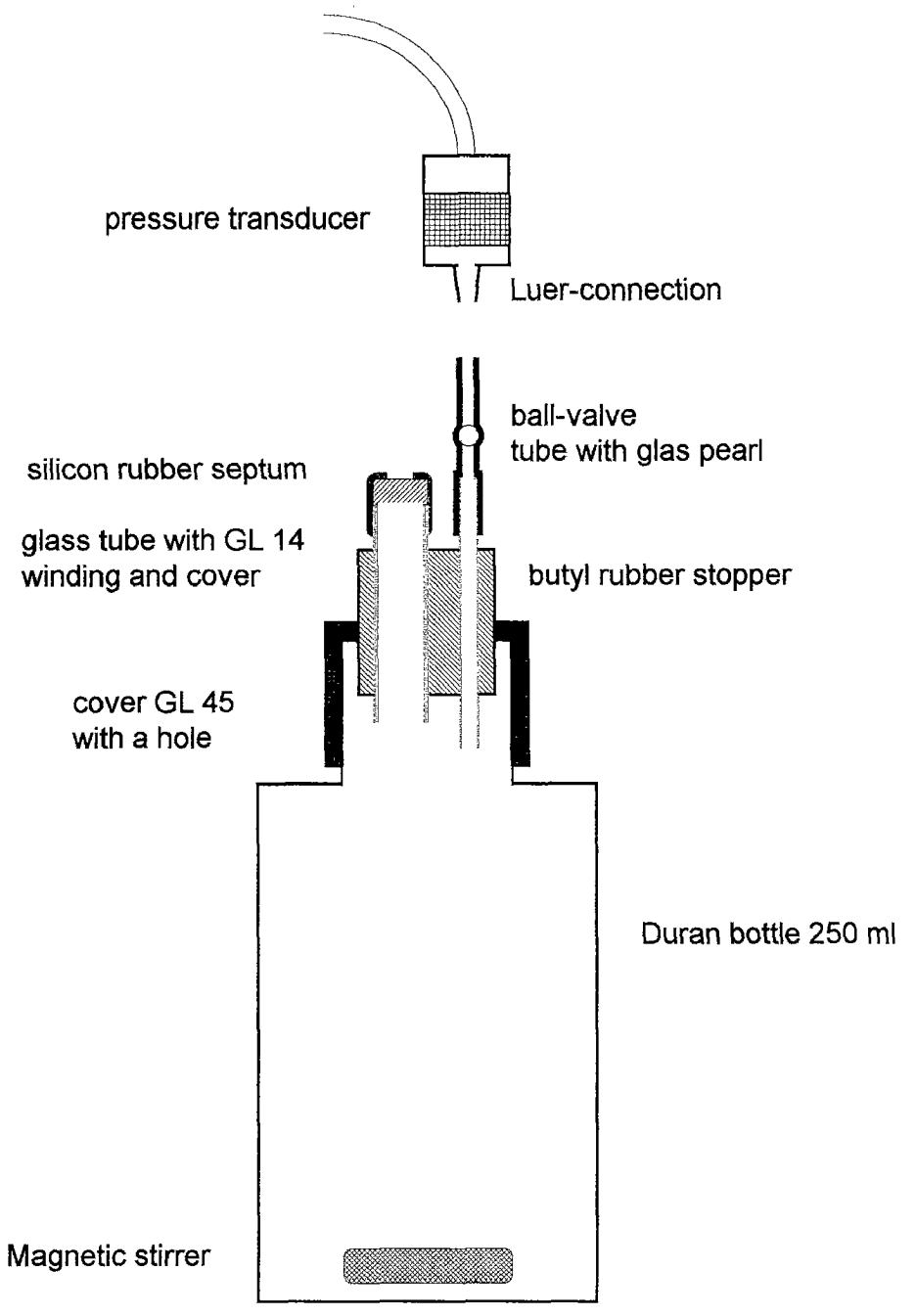


Figure 5: Test bottle and pressure transducer of the Anaerobic Screening Test System (ASTS).

Apparatus for the anaerobic sample preparation

To fill the test bottle under fully anaerobic conditions with homogeneous and precise aliquots of digested sludge, the apparatus for the anaerobic sludge transfer was developed (Figure 6).

The inoculum solution (1) must be well stirred to guarantee a homogenous sludge concentration for 30 to 50 test bottles. To fill the test bottles (3) anaerobically the following procedure applies:

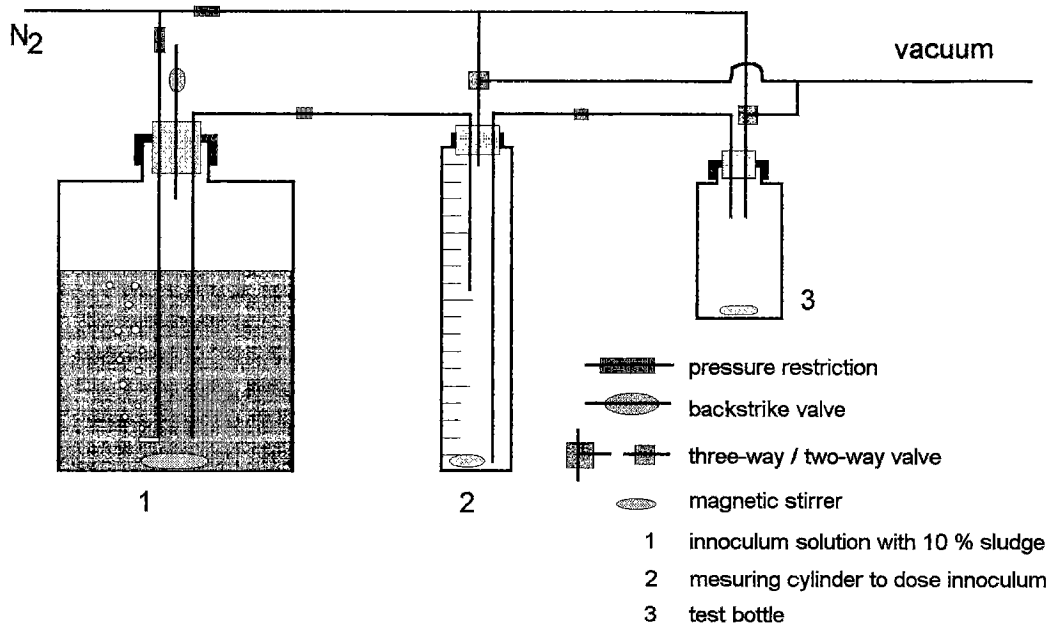


Figure 6: Apparatus for the anaerobic sludge transfer to the test bottle.

1. After removing the ball valve tube and the GL 14 cover containing the septum, the test bottle (3) is connected directly to the preparation unit directly via two glass tubes. The glass tube for sludge transfer is then sealed with a rubber ring and screwed directly onto the glass winding GL 14 (Figure 5). A rubber tube providing nitrogen and vacuum is connected to the small glass tube.
2. To remove oxygen from the liquid, the test bottle (3) is evacuated and flushed with nitrogen three times with the aid of the three-way valve on top of the test bottle (3).
3. The diluted sludge is then sucked into the measuring cylinder (2) by applying a slight underpressure using the three-way valve on top of the cylinder (2), followed by opening the two-way valve (1-2). The excess sludge will be pushed back with nitrogen to the large bottle (1) by turning the three-way valve on top of the measuring cylinder. Then the two-way valve (1-2) is closed.
4. The sludge transfer from the cylinder (2) to the test bottle occurs by means of a small vacuum.
5. To remove traces of oxygen from the test bottle (3) step two of the procedure is repeated.

6. The sludge transfer tube is disconnected from the test bottle (3) under nitrogen. The test chemicals could then be added, if not done before. Subsequently, it is closed with the GL14 cover containing the septum. Finally, the nitrogen supply tube is replaced by the ball valve.

Determination of TOC and COD

The total organic carbon (TOC), the inorganic carbon (IC) and the dissolved organic carbon (DOC) were determined using the total organic carbon analyzer Shimadzu TOC-500 (Burkard Instrumente AG, Geroldswil, Switzerland). The chemical oxygen demand (COD) was measured photospectrometrically using the COD-test-kit LCK 114 of Dr. B. Lange AG, Hegnau, Switzerland.

Chemicals

The technical alcohol ethoxylates LA-$C_{12/14}EO_{20}$ and 2-Ethyl hexanol + 10EO, were offered free of charge by Dr. W. Kolb AG, Hedingen, Switzerland. The sodium salts of the technical complexing agents Amino tri(methylene phosphonic acid) (ATMP), 1-Hydroxy ethylene-1,1-diphosphonic acid (HEDP); Ethylene diamine-N,N'-tetra(methylene phosphonic acid) (EDTMP) and Diethylene triamine penta(methylene phosphonic acid) (DTPMP) were a gift from Monsanto Chemicals SA. All the other chemicals used, were of analytical grade and purchased from Fluka AG, Buchs, Switzerland.

The following organic chemicals were used:

C_2-compounds: ethanol; 1,2-ethanediol; acetaldehyde; acetic acid; sodium acetate trihydrate; hydroxyacetic acid; oxoacetic acid.

C_3-compounds: 1-propanol; 2-propanol; 1,2-propanediol; 1,2,3-propanetriol; propionic acid.

C_4-compounds: 1-butanol; 2-butanol; 1,4-butanediol (dl); 2,3-butanediol (dl); butyric acid.

$C_{\geq 8}$-compounds: sodium caprylic acid (C_8); sodium lauric acid (C_{12}); sodium lauryl sulfate; sodium palmitic acid (C_{16}).

Others: sodium benzoic acid; D(+)-glucose; phenol; polyethylene glycol 400 (PEG 400); nitrilo triacetate sodium salt (NTA).

Preparation of the test chemical stock solutions

5.00 g of compound were brought into a 500 mL measuring flask and filled up with demineralized water (final concentration 1 %, 10 g/L). For the determination of the TOC and the COD the stock solutions were diluted with demineralized water 100 fold (100 mg/L).

Preparation of the anaerobic mineral salt medium

The anaerobic mineral salt solution described by Birch et al. (1989) was used. The minerals (Table 3) were dissolved in distilled water. The pH was adjusted with HCl (6 mol/L) or NaOH (6 mol/L) to 7.3. To minimize the concentration of dissolved oxygen, the mineral salt medium was flushed with nitrogen for at least 4 hours before use.

Table 3: Composition of the anaerobic mineral salt medium (Birch et al. 1989).

Chemical	Formula	MW [g/mol]	Final conc. [g/L]	Final conc. [10^{-3} mol/L]
Potassium dihydrogen phosphate	KH_2PO_4	135.2	0.270	2.00
Disodium hydrogen phosphate	Na_2HPO_4	142.0	0.460	3.24
Ammonium chloride	NH_4Cl	53.5	0.530	9.91
Calcium chloride	$CaCl_2 \cdot 2\ H_2O$	147.0	0.075	5.10
Magnesium chloride	$MgCl_2 \cdot 6\ H_2O$	203.2	0.100	4.92
Ferrous (II) chloride	$FeCl_2 \cdot 4\ H_2O$	198.8	0.020	0.10
Resazurin	$C_{12}H_7NO_4$	229.2	0.001	0.005

Preparation of the anaerobic test bottles

Depending on the vapor pressure and the surface activity of the test chemical, the stock solution was added either before or after the transfer of the anaerobic sludge to the test bottle. The amount of stock solution to be added was calculated according to the pre-defined COD in the test assay. The test bottles were warmed up to 35 °C in a water bath regulated by a thermostat. The water bath was covered with polyethylene balls to minimize evaporation and heat loss. After an equilibration of two hours the first pressure measurement was taken.

Characteristics of the sludges used

The anaerobic degradation experiments were carried out with sludge from different wastewater treatment plants (WTP) listed in Table 4. The dry solid matter of the sludges of Au-Bruggen, Wil and Kloten/Opfikon was in the range of 2 % to 3 %, which performed well with the ASTS.

The dry solid matter of the sludges of Herisau and Spreitenbach was between 5 % and 10 %, which led to an irregular and high endogenous biogas production.

Most experiments were performed with sludge from WTP Au-Bruggen in St. Gall, Switzerland (Table 5). The WTP Au-Bruggen works in three steps: a mechanical with phosphate precipitation, a biological and a sedimentation step. The excess activated sludge plus the primary sludge are digested in two anaerobic reactors, of which the first one is stirred whilst the second one is used for sedimentation. The hydraulic retention time of the sludge in the anaerobic digester is about 30 days. The digested sludge is dried on a band-pass filter followed by heating in a rotation oven. About 90 % of the dried sludge goes into the solid waste incineration plant next to the WTP, the rest is used for agricultural purposes and gardening.

Table 4: Sources of anaerobic sludge.

Origin of sludge	Origin of waste water
WTP Au-Bruggen, St. Gall	Municipal and industrial waste water (details in Table 5)
WTP Wil	Mainly municipal waste water.
WTP Herisau	Municipal and industrial waste water (textile industry).
WTP Kloten / Opfikon	Municipal and airport waste water.
WTP Spreitenbach	Anaerobic pre-treatment of industrial waste water (potato starch).

Preparation of the diluted sludge

Sludge was taken from the top of the first anaerobic reactor of the WTP Au-Bruggen, St. Gall. The stirring device in the reactor was run for at least 15 minutes to guarantee well-stirred conditions. Subsequently, the sludge samples were collected; filled into one liter polyethylene bottles and transported directly to the laboratories for washing and pre-digestion.

Washing procedure: The one liter bottles filled with sludge were centrifuged on a macro-centrifuge for 15 minutes (50'000 *g*) and the supernatant disposed off. Subsequently anaerobic mineral salt medium was added to the sedimented sludge. This mixture was shaken for 5 minutes and centrifuged again. This washing procedure was repeated two more times.

Pre-digestion of diluted sludge: One liter of the washed sludge was added to nine liters of anaerobic mineral salt medium. The 10 liters diluted sludge was usually pre-digested at 35 °C for 7 days and flushed with nitrogen for 2 hours before transferring to the test bottles.

Table 5: Characteristics of the WTP Au-Bruggen, St. Gall, Switzerland (average of Sept. 1996).

WTP Au Bruggen, St. Gall, CH	Sept. 1996	unit	DSS [g/L]
Inflow per day	21'300	m^3/d	
Activated sludge	134	m^3/d	
Biogas production	1'634	m^3/d	
Primary sludge	134	m^3/d	3.1 ± 0.5
Excess sludge	453	m^3/d	5 ± 2
Sludge input to 1st digester	140	m^3/d	23 ± 3
1st digester	35 ± 1	°C	**20.5 ± 1**

Waste water quality at inflow			**min.**	**max.**
COD ($KMnO_4$)	kg/d		2085	5695
BOD_5	kg/d		746	2117
TOC	kg C/d		399	1222
P_{tot}	kg P/d		25	57
N_{tot}	kg N/d		276	628
... after mechanical treatment		**average**	**min.**	**max.**
COD ($KMnO_4$)	kg/d	4597	1760	9246
BOD_5	kg/d	2011	673	3720
TOC	kg C/d	1034	294	2391
P_{tot}	kg P/d	52	26	96
N_{tot}	kg N/d	-	381	1065
... at the outflow		**average**	**min.**	**max.**
Water	m^3/d	21324	12140	55560
COD ($KMnO_4$)	kg/d	1052	481	2338
BOD_5	kg/d	145	28	389
TOC	kg C/d	168	83	319
DOC	kg C/d	130	73	266
P_{tot}	kg P/d	15	6	35
N_{tot}	kg N/d		392	842
insoluble substances	kg/d	302	96	1000
pH-value			7.7	8.4
Conductance	µS/cm		420	1120

Performance of WTP Sept 1996	**average %**	**min. %**	**max. %**
COD elimination ($KMnO_4$)	77	64	87
BOD_5 elimination	93	86	97
TOC ellmination	84	65	91
DOC elimination	87	74	94
P elimination	71	42	85
N elimination (maximum)	20	0	41

DSS: dry solid sludge; min: minimum; max: maximum; COD: chemical oxygen demand; BOD_5: biological oxygen demand within 5 days; TOC: total organic carbon; DOC: dissolved organic carbon; P_{tot}: total phosphor flux; N_{tot}: total nitrogen flux.

Pressure measurement

The external pressure transducer was specified and developed for the ASTS (RST-Productions AG, Berg TG, Switzerland). It is compatible with the data collection unit from the DEGRAMAT test systems (Baumann & Rezzonico 1993). It consists of a piezo-electronic

pressure transducer which is built into a stainless steel tube containing a Luer fitting. For pressure measurement, the Luer fitting was directly connected to the flexible ball-valve tube of the test bottle (Figure 5). To obtain the current pressure the ball-valve was pressed open with two fingers.

The linearity of the pressure transducer was controlled by adding stepwise 20 mL air into a 250 mL test bottle. Its response was linear (R^2=0.99997). The dead volume was small (<0.2 mL). Pressure loss was less than 5 % after 7 measurements.

Termination of the degradation test

At the end of the degradation test, a last pressure measurement was performed to determine the biogas pressure p_1 (Equation 9). Afterwards the test bottle was turned upside down and 2 mL of the test liquid were drawn from the injection port, using a single use syringe (Becton Dickinson, Braunschweig, Germany) and a disposable-needle (0.40 x 12 mm). For DOC determination, the test liquid was filtered through a pre-washed Durapore (PVDF) membrane filter unit with a pore size of 0.45 µm (Millex-HV, ∅ 0.25 mm, Millipore AG, Volketswil, Switzerland). Then the pH-value was determined and 1 mL of the filtrate was put aside for DOC measurement. In some cases 2 mL hydrochloric acid ($c = 2\ mol \cdot L^{-1}$) were added to the test bottles to convert carbonate into carbon dioxide. After stirring for 24 hours and removing 2 mL of the test liquid, the total C_1-gas-pressure in the test bottle p_2 (Equation 10) was measured.To measure only methane-gas pressure p_3 (Equation 11), 2 mL sodium hydroxide solution ($c = 4\ mol \cdot L^{-1}$) were injected into the medium to quantitatively absorb carbon dioxide. The liquid was stirred for 24 hours before measurement of the methane pressure.

Results

Pre-treatment of anaerobic sludge

The ASTS experiments were performed with 6 assays of non-washed and washed sludges without or with polyethylene glycol 400 (COD = 150 mg O/L). The pre-digestion time of the sludge was 1, 8 and 15 days. Test conditions were V_{liquid}: 200 mL; $V_{headspace}$: 90 mL; T=35 °C; 10 % diluted sludge from WTP Au-Bruggen.

The endogenous biogas production decreased with increasing time of pre-digestion (Figure 7). This result is in line with experiments of Birch et al. (1989), who found, that the endogenous biogas production was reduced by 50 % to 70 % after 2 and 4 weeks pre-digestion. The longer pre-digestion times increased the lag-phase of the degradation of PEG 400 by 1 to 8 days.

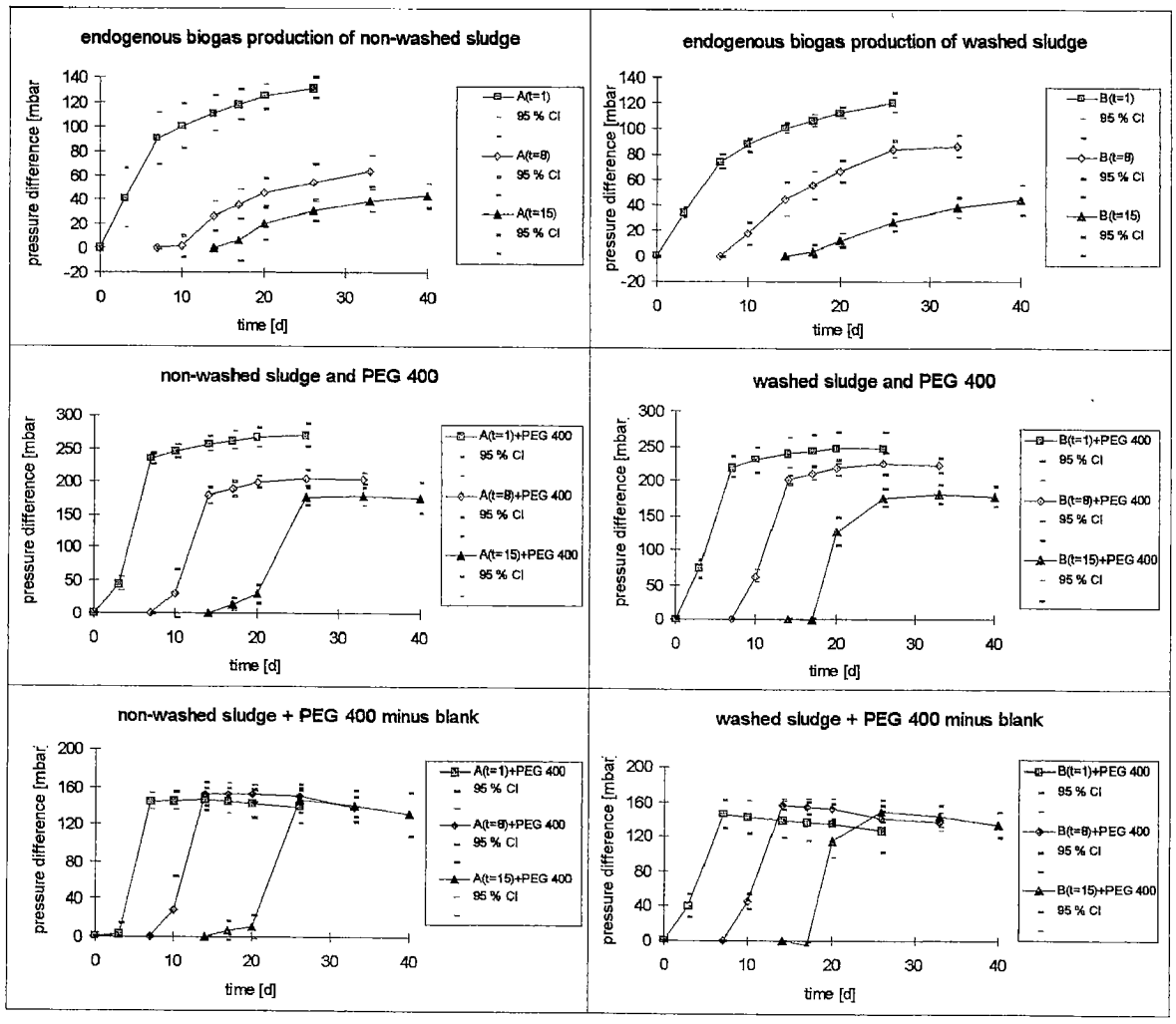


Figure 7: Pre-treatment of anaerobic sludge. Left column: non-washed sludge. Right column: washed sludge. Pre-digestion times of 1, 8 and 15 days, indicated with t=1, t=8 and t=15, respectively. Average of n=6 assays; confidence level: 95 % CI (n=6). PEG 400: polyethylene glycol (MW=400 $g \cdot mol^{-1}$).

In the PEG 400 degradation experiments with washed sludge, the lag-phases were shorter than for those with non-washed sludge (Figure 7). The degradation rates in this experiment were not influenced by the pre-treatment of the sludge. In about 8 out of 12 experiments the washing procedure appeared to improve the reproducibility, but the differences were small and statistically not significant. The degradation results of the experiments with PEG 400 were calculated with the CH_4-method of Baumann & Schefer (1990) and the C_1-biogas method of Shelton and Tiedje (1984).

The results and the confidence levels correspond well with one another (Table 6) and are independent from the sludge pre-treatment. A good reproducibility was obtained in the experiments with and without PEG 400. The absolute standard deviations were between 5 and 12 mbar. The removal of DOC was always larger than the degradation rates calculated with CH_4 and C_1-biogas (S&T). The growth of the microorganism and the incorporation of organic carbon into biomass (5 to 10 % of the substrate) can explain this difference (Gujer & Zehnder 1983).

Table 6: Results of the degradation experiments with polyethylene glycol 400 (PEG 400) using pre-digested and differently treated sludge from WTP Au-Bruggen. CH_4: calculated with the methane pressure at the end of experiment (average of 6 assays); S&T (Shelton & Tiedje 1984): calculated with the maximum biogas (C_1-gas) pressure during the experiment; DOC: removal of the DOC (start value minus end value); CI: confidence level of the average (n=6).

pre-digestion of sludge	sludge pre-treatment	CH_4 %	95 % CI	S&T %	95 % CI	DOC %	95 % CI
1 d	none	87.2	± 6.7	90.0	± 5.5	96.5	± 1.5
8 d	none	93.8	± 5.6	91.5	± 4.0	99.0	± 1.4
15 d	none	89.3	± 5.2	88.4	± 4.9	96.5	± 2.5
1 d	washing	82.9	± 9.4	82.5	± 7.5	100.9	± 1.9
8 d	washing	89.6	± 4.0	90.4	± 4.2	101.0	± 2.4
15 d	washing	86.8	± 5.1	88.8	± 4.1	103.3	± 1.5
	average	88.3	± 6.0	88.6	± 5.0	99.5	± 1.9
	standard deviation	3.6		3.2		2.7	

Washing the anaerobic sludge

The amount of dissolved organic carbon (DOC) originally present in the sludge, contributes to the endogenous biogas production. With a washing procedure it was tried to reduce the dissolved organic carbon (DOC) and the endogenous biogas production of the inoculum. Fresh sludge from the WTP Au-Bruggen was used for this study.

One liter sludge was centrifuged. In the supernatant, the DOC was determined in triplicates of 2 mL after filtration through a disposable 0.45 µm MILLEX-HV filter unit (Millipore AG, Volketswil, Switzerland). The supernatant was disposed and anaerobic mineral salt medium was added to the sedimented sludge. The bottle was then placed on an overhead shaker for an hour. After centrifugation, the DOC was determined again in the supernatant as described above. This procedure was repeated three more times.

The washing procedure significantly reduced the DOC of the anaerobic sludge (Figure 8a). Two washing cycles reduce the DOC in the inoculum to a constant level. More than two washing cycles did not improve DOC removal. DOC determined at the end of these experiments increased with longer pre-digestion times (Figure 8b). This is a result of the release of dissolved organic material during degradation of particulate organic matter. The addition of PEG 400 at the beginning of the experiment did not increase the DOC end value.

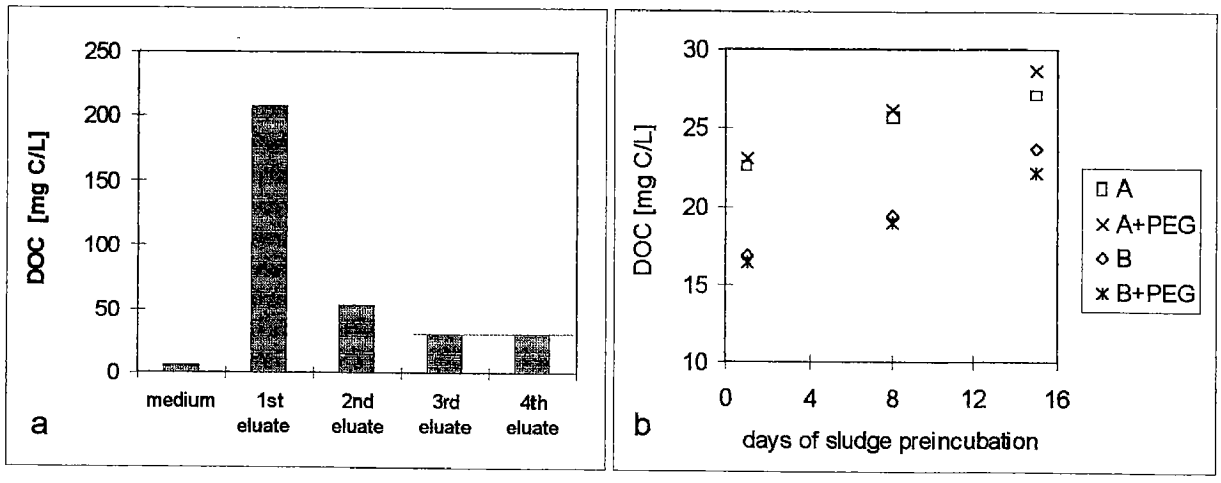


Figure 8: a) Graphic of the influence of three washing cycles on the dissolved organic carbon (DOC) in the supernatant (eluate) of the centrifuged sludge from WTP Au-Bruggen. Medium means mineral salt medium. b) DOC values determined at the end of experiments with varying sludge pre-digestion times: A: non-washed sludge, B: washed sludge, + PEG: addition of polyethylene glycol 400 at the start of the experiment.

For experiments with low test substance concentrations a small endogenous biogas production with a narrow confidence level is required. To reduce the DOC of the inoculum to a minimum, it is important to repeat the washing procedure after pre-digestion. In this particular case, the dilution of the sludge must be done after pre-digestion and washing steps.

The influence of the washing procedure on the recovery of various elements (Mo, Zn, Co, Ni, Mn, Cu, B) was studied with anaerobic sludge from WTP Au-Bruggen. For this purpose, untreated and pretreated sludge, which had been washed three times with demineralized water, was dried in a heating oven at 105 °C. The dried sludge was then pressed into 200 mg pellets. By using X-ray fluorescence spectrometry (RFS) the elements were determined. The results show that, except for boron, the washing of the sludge did not reduce the concentrations of these elements significantly (Table 7).

Table 7: The influence of the washing procedure of the anaerobic sludge on various elements, determined with X-ray fluorescence spectrometry (RFS). stdv: standard deviation (n=3).

	Mo	Zn	Co	Ni	Mn	Cu	B
	[mg·kg^{-1}]	[mg·kg^{-1}]	[mg·kg^{-1}]	[mg·kg^{-1}]	[mg·kg^{-1}]	[mg·kg^{-1}]	[mg·kg^{-1}]
1 non-washed sludge	7.9	813	12	51	813	317	26
2 non-washed sludge	5.2	887	11	54	892	334	29
3 non-washed sludge	5.4	904	11	52	878	323	29
4 washed sludge	5.0	881	11	52	857	309	16
5 washed sludge	4.8	841	10	51	862	301	17
6 washed sludge	5.1	818	10	52	834	308	17
7 demin. water [mg·L^{-1}]	0	0.113	0.047	0.067	0.106	0.10	0.09
1-3 average ± stdv	6.2 ± 1.5	868 ± 48	11.3 ± 0.6	52.3 ± 1.5	861 ± 42	325 ± 8.6	28.0 ± 1.7
4-6 average ± stdv	5.0 ± 0.2	847 ± 32	10.3 ± 0.6	51.7 ± 0.6	851 ± 15	306 ± 4.4	16.7 ± 0.6

Pre-digestion of anaerobic sludge

The endogenous biogas production of anaerobic sludge from WTP Au-Bruggen, which was pre-digested for 15 days, dropped from 130 mbar to about 45 mbar (Figure 9a). A pre-digestion of 8 days reduced the endogenous biogas production to about 50 %. Pre-digestion of the sludge did not affect the confidence interval of the biogas production (Figure 9b).

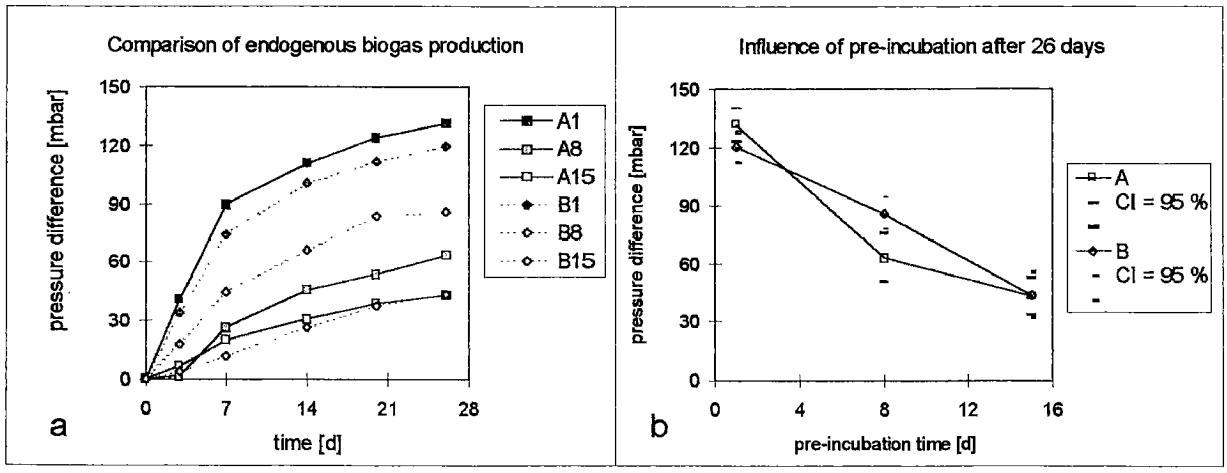


Figure 9: **a)** Average endogenous biogas production (n=6) of non-washed (A) and washed (B) sludge pre-incubated for 1, 8 and 15 days (A1, A8, A15, B1, B8, B15), respectively. **b)** Influence of the pre-incubation of non-washed (A) and washed (B) sludge on the endogenous biogas production at day 26 of the test period (n=6).

The influence of pre-digestion on the reproducibility of the biogas production, measured as confidence level 95 %, was shown in Figure 7. The lowest deviations were obtained in samples with a pre-digestion time of 8 days, followed by the samples with a pre-digestion of only one day. The absolute standard deviations were in the range of 5 ± 2 mbar for the endogenous biogas production and of 9 ± 3 mbar for the degradation of PEG 400.

Reproducibility of the anaerobic sludge transfer

The reproducibility of sludge transfer was investigated in different experiments by weighing the test bottles before and after sludge transfer (Figure 10). The actual liquid-volume was weighed in at least 10 samples, taking the average of these results as liquid-volume. Careful standardization is important, because the headspace in the test bottle strongly depends on the volume of the liquid. The error of the sludge transfer by volume was in the range of ± 0.5 to 2 % (Table 8).

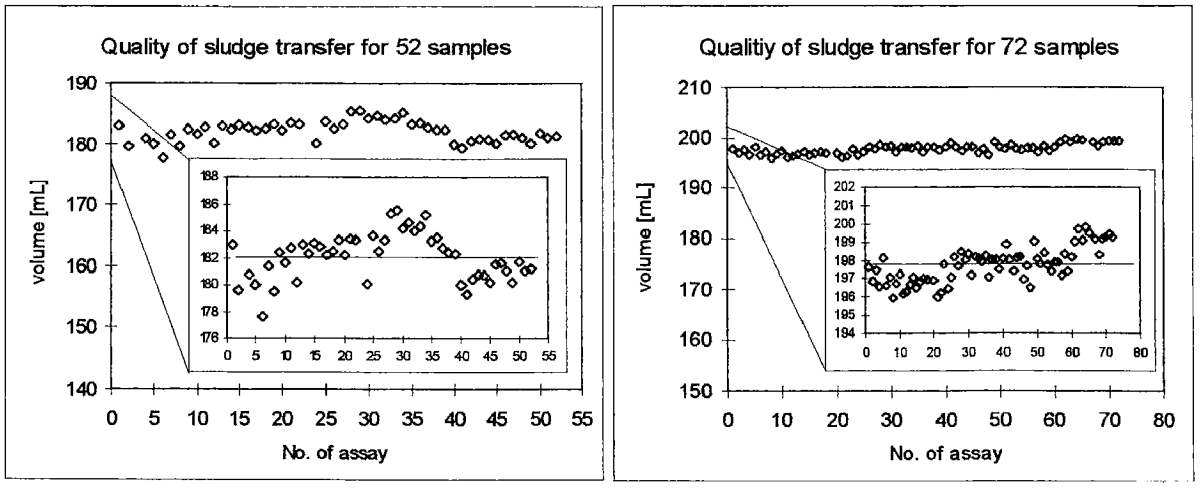


Figure 10: Reproducibility of the anaerobic sludge transfer with special regard to automatic volume measurement in two sets of experiments: Exp. 8 (left) and Exp. 14 (right).

Table 8: Comparison of different sludge transfer measurements with the ASTS and the DEGRAMAT test system (Baumann & Rezzonico, 1993). Cl: confidence level; the error is calculated from the 95 % Cl; n: number of assays. * Results are shown in Figure 10.

		nominal mL	average mL	95 % Cl mL	error %	n
Experiment 8 *	ASTS	180	182.1	3.4	1.9	52
Experiment 9	DEGRAMAT	700	707.2	6.0	0.8	12
Experiment 12	ASTS	180	182.9	1.8	1.0	22
Experiment 14*	ASTS	200	197.8	2.0	1.0	72
Experiment 15	DEGRAMAT	725	722.8	2.4	0.3	12

Comparison of the degradation performance of different anaerobic sludges

Under the following conditions the degradation test was performed with the ASTS: 10 times diluted sludge (2-3 $g \cdot L^{-1}$); V_{liquid}: 185 mL; $V_{headspace}$: 105 mL; COD_{test}: 150 $mg \cdot L^{-1}$; temperature 35 °C. The experiments were performed with anaerobic sludges from different wastewater treatment plants (Table 4).

The degradation potential of these sludges was investigated with eight substances (Table 9). Their choice was based on good and bad anaerobic biodegradability, long and short lag-phases, and inhibitory effects towards the sludge. Two non-ionic surfactants, a linear and a branched alcohol ethoxylate, which were earlier studied with sludge from the WTP Au-Bruggen (Müller et al. 1996), were also included in this particular study.

The degradation results and the lag-phases of these different substances varied with the sludge used (Figure 11 and Table 10). Benzoate, phenol and polyethylene glycol (PEG 400) were

completely degraded by all studied sludges within 10 to 15 days. Palmitate and lauryl sulfate showed a lag-phase of 10 to 25 days before being entirely degraded.

Table 9: Substances used for anaerobic degradation experiments with different sludges.

Substance / Product	References of anaerobic biodegradability data
Sodium benzoate	Well degradable (Shelton & Tiedje 1984, Battersby & Wilson 1988). A short lag-phase (10 days) was observed in experiments with the ASTS and sludge A in earlier experiments (unpublished results).
Phenol	Well degradable (Shelton & Tiedje 1984).
Polyethyleneglycol (PEG 400) $HO(-CH_2-CH_2-O)_n-H$	Well degradable (Shelton & Tiedje 1984, Pagga & Beimborn 1993).
Sodium palmitate $C_{15}H_{31}-COONa$	Substance which is transferred to anaerobic digesters with the primary sludge as insoluble calcium soap. Anaerobically degraded to about 70 % within 30 days (Pagga & Beinborn 1993, Prats et al. 1996).
Sodium lauryl sulfate $C_{12}H_{25}SO_3Na$	Anionic surfactant, which inhibits anaerobic degradation as a surfactant. After sulfate reduction the C_{12}-alcohol sorbs to the sludge and is only slowly degraded. This results in a long lag-phase. Substance is degradable under anaerobic conditions.
Linear alcohol ethoxylate LA-$C_{12/14}EO_{20}$	Technical linear alcohol ethoxylate long lag-phase, anaerobically degradable (Wagener & Schink 1987, 1988; Steber & Wierich 1987, Müller et al. 1996).
2-Ethylhexanolpolyethoxylate SB-C_8EO_{10}	Partly degradable under anaerobic conditions (Müller et al. 1996).
2-Butanol $CH_3-CHOH-C_2H_5$	Anaerobic degradation of secondary alcohols in the C_2-position depends on the origin of the sludge: Shelton & Tiedje (1984) found a partial anaerobic degradation for 2-octanol; Strujis & Stoltenkamp-Wouterse (1992) determined 2-propanol in a ring test but found no positive degradation results; Henkel & Unilever (1995) determined a positive degradation result for 2-butanol in the Laboratories of Henkel. But the sludge used in the laboratories of Unilever did not degrade 2-butanol.

The nonionic surfactant 2-Ethylhexanol + 10 EO was partly degraded to about 20 - 40 % of the theoretical value. The linear alcohol ethoxylate LA-$C_{12/14}EO_{20}$ was completely degraded in the sludge from WTP Wil and Kloten/Opfikon within 10 days, whilst it was not degraded in the sludge from WTP Au-Bruggen even within 42 days. This negative result for LA-$C_{12/14}EO_{20}$ is in line with earlier experiments performed by us with sludge from WTP Au-Bruggen (Müller et al. 1996). The positive degradation results for $C_{12/14}EO_{20}$ in sludge from WTP Wil and Kloten/Opfikon are in line with data presented by Wagener & Schink (1987, 1988), Steber & Wierich (1987) and Huber (1999). Further anaerobic degradation experiments with alcohol

ethoxylates are discussed in Chapter 3 and 4. 2-Butanol was not degraded in the three sludges used. This result is in line with data presented in the study of Henkel & Unilever (1995).

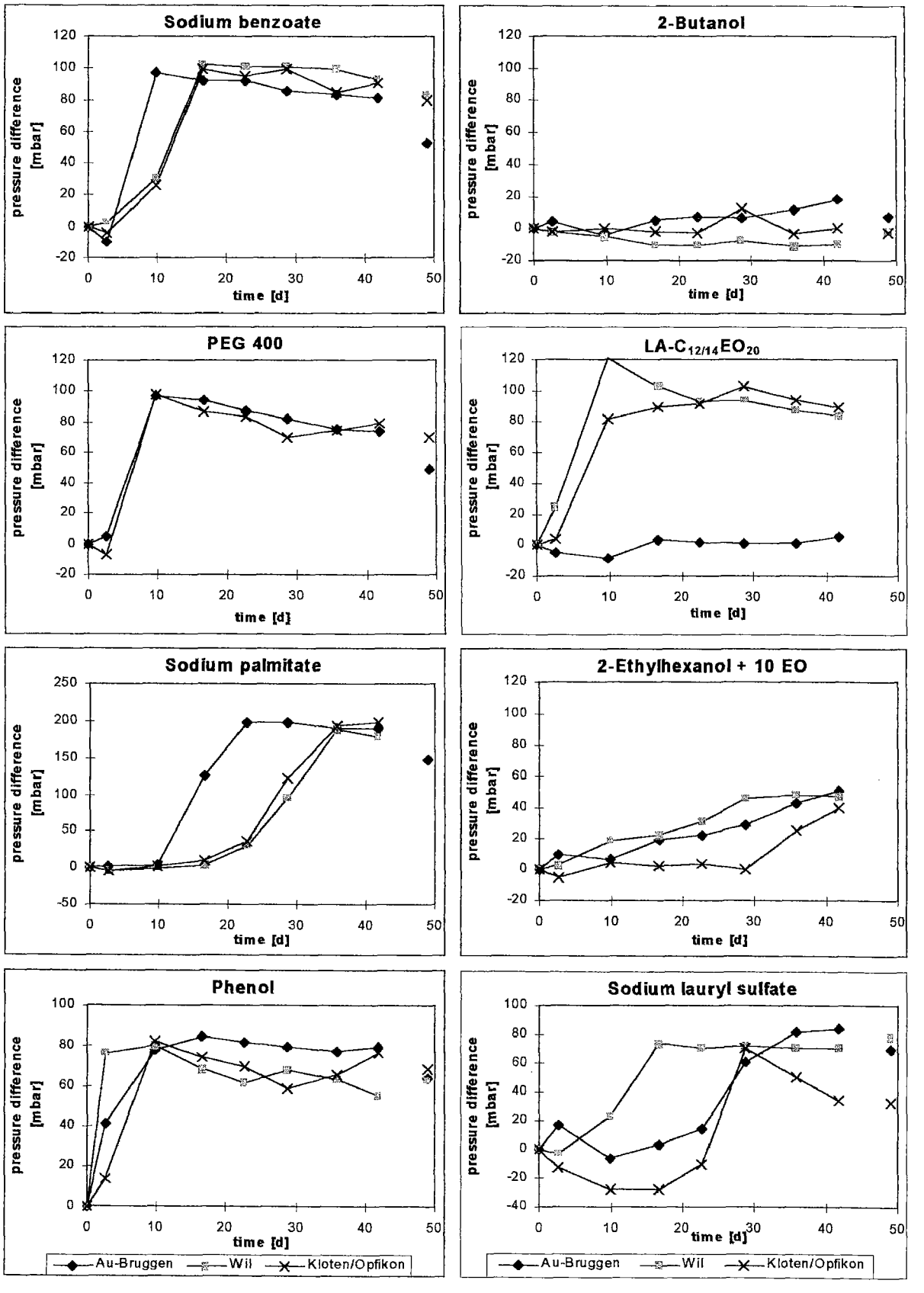


Figure 11: Experiments with different substances and sludge from three municipal WTP in Switzerland: Au-Bruggen (St. Gall), Wil and Kloten/Opfikon.

Table 10: Degradation of 8 substances with sludge from three Swiss WTP: Au-Bruggen (St.Gall), Wil (SG) and Kloten/Opfikon (ZH). Degradation rates were calculated according to S&T, CH_4 at the end of experiment and the removal of DOC. ΔDOC_{end} = DOC_{end}(test) – DOC_{end}(blank). n.d. not determined

	Sludge	ΔDOC_{end} mg C/L	S&T (C_1 max) %	CH_4 (COD) %	DOC removal %
Sodium benzoate	Au-Bruggen	-1.9	72.3	59.9	103.5
Sodium benzoate	Wil	3.7	75.6	85.6	93.2
Sodium benzoate	Kloten/Opfikon	0.9	73.9	87.5	98.4
Phenol	Au-Bruggen	-5.3	74.1	72.1	112.2
Phenol	Wil	5.3	70.0	95.1	87.8
Phenol	Kloten/Opfikon	1.1	71.9	81.5	97.6
PEG 400	Au-Bruggen	0.4	87.7	67.8	99.0
PEG 400	Wil	4.2	-	-	-
PEG 400	Kloten/Opfikon	1.1	88.7	95.6	97.4
Sodium palmitate	Au-Bruggen	-2.2	74.0	73.2	104.1
Sodium palmitate	Wil	4.1	70.4	n.d.	92.4
Sodium palmitate	Kloten/Opfikon	1.4	73.8	n.d.	97.5
Sodium lauryl sulfate	Au-Bruggen	3.5	77.1	73.6	91.2
Sodium lauryl sulfate	Wil	6.2	66.9	86.1	84.4
Sodium lauryl sulfate	Kloten/Opfikon	5.15	64.6	74.1	87.1
2-Butanol (dl)	Au-Bruggen	19.3	17.6	7.9	42.7
2-Butanol (dl)	Wil	24.2	-	-	28.2
2-Butanol (dl)	Kloten/Opfikon	17.3	12.4	n.d.	48.8
LA-$C_{12/14}EO_{20}$	Au-Bruggen	33.5	4.3	n.d.	24.1
LA-$C_{12/14}EO_{20}$	Wil	8.4	98.1	n.d.	81.0
LA-$C_{12/14}EO_{20}$	Kloten/Opfikon	4.0	83.6	n.d.	91.1
2-Ethyl hexanol + 10 EO	Au-Bruggen	23.1	41.2	n.d.	47.7
2-Ethyl hexanol + 10 EO	Wil	22.8	38.7	n.d.	48.4
2-Ethyl hexanol + 10 EO	Kloten/Opfikon	21.05	32.1	n.d.	52.4

Experimental validation of Equation 5

The quantification of the mineralization requires methods, which are independent of substance parameters such as TOC or COD. The COD-method of Baumann and Schefer (1990) was

therefore evaluated and compared to other methods, such as the total amount of C_1-gas (Birch et al. 1988) and the amount of C_1-biogas (Shelton & Tiedje, 1984).

The experimental validation of Equation 5 was performed with the results from the ASTS and DEGRAMAT test system using C_2 to C_{16}-compounds with different COD and TOC values (Table 11 and Table 12). This experimental set-up was used to vary the composition and the amount of the biogas (CH_4 + CO_2) while keeping the theoretical CH_4-production constant.

Table 11: Carbon balance at the end of the experiment for compounds with different COD. V_{liquid} of the test assay was 180 mL. Test substance concentration: 300 mg O/L. 10-times diluted sludge from WTP Au-Bruggen, incubated at 35 °C. The titles in the columns stand for: COD and TOC of test substance; IC_{end} and DOC_{end}: inorganic carbon and dissolved organic carbon at the end of experiment; Biogas: amount of C_1-biogas at the end; C_{test}: degraded amount of C measured in test assay (IC_{end} + DOC_{end} + Biogas); $C_{nominal}$: nominal amount of C in test system; C-Recovery: C_{test} versus $C_{nominal}$; Degradation = (Biogas + IC_{end}) / $C_{nominal}$: the degradation was calculated according to Birch et al. (1988).

	COD	TOC	IC_{end}	DOC_{end}	Biogas	C_{test}	$C_{nominal}$	C-Recovery		Degradation	
	g/mol	g/mol	mg C	mg C	mg C	mg C	mg C	%		%	(Birch)
Ethanol	96	24	0.6	0	5.3	5.9	6.8	86.8	± 3.9	86.8	± 3.9
Acetaldehyde	80	24	0.4	5.6	1.6	7.6	8.2	92.7	± 1.8	24.4	± 0.4
1,2-Ethanediol	80	24	0.6	0	5.3	5.9	8.2	72.0	± 5.4	72.0	± 5.4
Acetic acid	64	24	0.8	0	5.1	5.9	10.2	57.8	± 5.5	57.8	± 5.5
Sodium acetate trihydrate	56	24	2.8	1.3	5.5	9.6	11.7	82.1	± 0.7	70.9	± 1.2
Hydroxyacetic acid	48	24	1.2	1.0	6.0	8.2	13.7	59.9	± 7.0	52.6	± 2.3
Oxoacetic acid	32	24	0.7	5.9	4.3	10.9	20.5	53.2	± 2.0	24.4	± 11.9
1-Propanol	144	36	0.2	0.6	5.5	6.3	6.8	92.6	± 2.2	83.8	± 3.4
2-Propanol	144	36	0.3	6.2	1.2	7.7	6.8	113.2	± 0.6	22.1	± 1.2
1,2-Propanediol	128	36	1.0	0.5	5.1	6.6	7.7	85.7	± 5.8	79.2	± 4.1
1,2,3-Propanetriol	112	36	0.9	0.7	3.5	5.1	8.8	58.0	-	50.0	-
Propionic acid	112	36	1.0	0.8	5.8	7.6	8.8	86.4	± 1.7	77.3	± 2.7
1-Butanol	192	48	0.5	0.7	5.1	6.3	6.8	92.6	± 3.4	82.4	± 3.2
2-Butanol (dl)	192	48	0.1	5.6	0.1	5.8	6.8	85.3	± 13.1	2.9	± 10.9
1,4-Butanediol (dl)	176	48	0	6.1	1.0	7.1	7.4	95.9	± 5.1	13.5	± 12.3
2,3-Butanediol (dl)	176	48	0.6	0.6	5.3	6.5	7.4	87.8	± 2.5	79.7	± 2.1
Butyric acid	160	48	0.7	0.9	5.4	7.0	8.2	85.4	± 5.3	74.4	± 5.3
Sodium caprylate	344	96	0.7	1.2	4.6	6.5	7.6	85.5	± 2.1	69.7	± 1.3
Sodium laurate	536	144	0.5	1.2	4.2	5.9	7.3	80.8	± 0.3	64.4	± 1.8
Sodium lauryl sulfate	488	144	0	1.6	1.1	2.7	8.1	33.3	± 2.4	13.6	± 1.6
Sodium palmitate	728	192	0.3	1.2	3.2	4.7	7.2	65.3	± 9.7	48.6	± 1.9

The method of Birch et al. (1989) can be evaluated with a carbon balance (Table 11). The difficulties of taking a sample from the test solution under pressure, without loosing part of the dissolved carbon dioxide (part of the inorganic carbon: IC), limits the applicability of this method. Increasing the ratio of TOC to COD of the test substance (or the ratio of CO_2 to CH_4 in the biogas), leads to a decrease of the recovery of carbon.

The increasing ratio of CO_2 to CH_4 in the biogas depends on a decreasing COD of the test substance. This caused problems in the evaluation method of Shelton and Tiedje (1984) and lies in the defined factor for the dissolved CO_2 (Table 12). These problems do not arise if the calculation of the mineralization is based solely on the methane production.

The amount of degradation, determined in two independent experiments with different evaluation methods, corresponds well within ± 10 % in most cases.

Acetaldehyde, oxoacetic acid, 2-propanol, 2-butanol and 1,4-butanediol were not or only partially degraded during these experiments. At the end of the test, the DOC was still high compared to the nominal DOC (Table 11). Sodium lauryl sulfate was only degraded to about 30 %. Higher degradation rates of 75 % to 85 % were determined in other experiments (Table 10). Good degradation rates for lauryl sulfate of 90 to 100 % were found by Steber and Wierich (1991). The sulfate reduction, which transforms the hydrophilic lauryl sulfate to the hydrophobic dodecanol, reduced the inhibitory effect of the surfactant in the sludge (Figure 11). The low DOC at the end of the experiment can be explained by sorption of the non-degraded and hydrophobic dodecanol to the sludge (Table 11). These results are in line with the data obtained by Baumann and Müller (1997) who examined the sulfate reduction of lauryl sulfate and the sorption of dodecanol to the sludge in a continuous upflow anaerobic sludge-bed reactor.

Table 12: Comparison of the evaluation methods using the theoretical methane pressure (CH_4) (Baumann & Schefer 1990) with those using the theoretical biogas pressure (C_1-gas) (Shelton & Tiedje, 1984) and the amount of $C_{1\ total}$ (Biogas + $CO_{2(aq)}$) (Birch et al. 1988). Two independent experiments are compared (1 and 2)

Biodegradation in % (m=2)	Experiment 1 degradation CH_4 (COD) av	dev	degradation C_1-gas (S&T) av	dev	Experiment 2 degradation CH_4 (COD) av	dev	degradation C_1-gas (S&T) av	dev	degradation $C_{1\ total}$ (Birch) av	dev
Ethanol	75.8	± 9.0	63.4	± 5.9	90	± 1.3	79.8	± 0.5	86.8	± 3.9
Acetaldehyde	6.6	± 0.8	4.9	± 0.8	13.3	± 0.8	13.4	± 0.5	24.4	± 0.4
1,2-Ethanediol	109.3		77.7		86.0	± 3.5	73.5	± 4.5	72.0	± 5.4
Acetic acid	95.9	± 3.4	68.3	± 0.4	78.5		68.3		57.8	± 5.5
Sodium acetate trihydrate	105.2	± 4.1	61.5	± 4.2	94.5	± 0.8	65.5	± 0.1	70.9	± 1.2
Hydroxyacetic acid	115.2	± 0.2	66.5	± 0.1	75.2	± 2.9	64.3	± 2.6	52.6	± 2.3
Oxoacetic acid	30.1	± 7.1	50.0	± 4.6	91.2		59.6		24.4	± 11.9
1-Propanol	69.4	± 6.6	62.2	± 4.2	96.9	± 3.7	86.9	± 2.4	83.8	± 3.4
2-Propanol	24.3	± 1.1	20.9	± 2.4	16.8	± 4.7	14.2	± 5.6	22.1	± 1.2
1,2-Propanediol	89.9	± 4.7	82.2	± 5.3	89.8	± 6.0	75.4	± 5.2	79.2	± 4.1
1,2,3-Propanetriol	79.6	± 10.1	66.7	± 1.4	93.9		77.0		50.0	-
Propionic acid	79.1	± 8.6	73.4	± 6.5	101.0	± 2.8	82.1	± 2.9	77.3	± 2.7
1-Butanol	78.9	± 14.1	73.2	± 14.0	95.1	± 3.9	79.8	± 4.3	82.4	± 3.2
2-Butanol (dl)	44.7		41.8		9.8		8.3		2.9	± 10.9
1,4-Butanediol (dl)	34.7	± 11.7	31.4	± 9.6	12.1	± 9.4	10.3	± 9.2	13.5	± 12.3
2,3-Butanediol (dl)	59.2	± 16.4	60.9	± 5.9	98.0	± 0.9	83.6	± 0.4	79.7	± 2.1
Butyric acid	97.9	± 14.7	74.0	± 4.9	100.6	± 5.6	82.3	± 2.7	74.4	± 5.3
Sodium caprylate	94.6		71.7		85.7	± 0.1	77.4	± 2.2	69.7	± 1.3
Sodium laurate	68.4		56.4		75.7	± 5.1	63.5	± 4.1	64.4	± 1.8
Sodium lauryl sulfate	32.4	± 4.1	24.7	± 3.1	35.3	± 5.2	9.0	± 3.6	13.6	± 1.6
Sodium palmitate	58.0		58.0		54.3	± 1.7	57.4	± 0.5	48.6	± 1.9
D(+)-glucose	55.9	± 3.9	38.0	± 1.7						
PEG 400	63.6	± 16.3	50.3	± 8.4						

Anaerobic degradation tests at low concentrations

The ASTS is also suitable for the determination of the anaerobic biodegradability of surfactants and other inhibitory compounds (see Figure 11). These experiments ask for lower substance concentrations. In Chapter 3 the toxic levels of nonionic surfactants are discussed in detail. In this part, the detection limit of the ASTS was determined with low concentrations of PEG 400 (c=15 and 30 mg $C \cdot L^{-1}$). The results were compared with the average endogenous biogas

production of the sludge. For this experiment, the headspace was reduced by increasing the liquid volume. To decrease the endogenous biogas production and its standard deviation to a minimum, the sludge was pre-digested for 14 days in the one liter polyethylene bottle. In this experiment the washing procedure followed the pre-digestion of the sludge.

The average endogenous biogas pressure amounts to 50 ± 25 mbar (95 % confidence level, n=6) (Figure 12). The degradation of PEG 400 at a concentration of 15 mg $C \cdot L^{-1}$ leads to a biogas pressure of 100 mbar, which is significantly higher than the endogenous biogas pressure. The detection limit for this method is in the range of 10 mg $C \cdot L^{-1}$. In low substance concentrations, it is essential to know the range of the confidence level of the endogenous biogas production. If the toxicity of a substance requires even lower concentrations, ^{14}C-labeled substrates should be used.

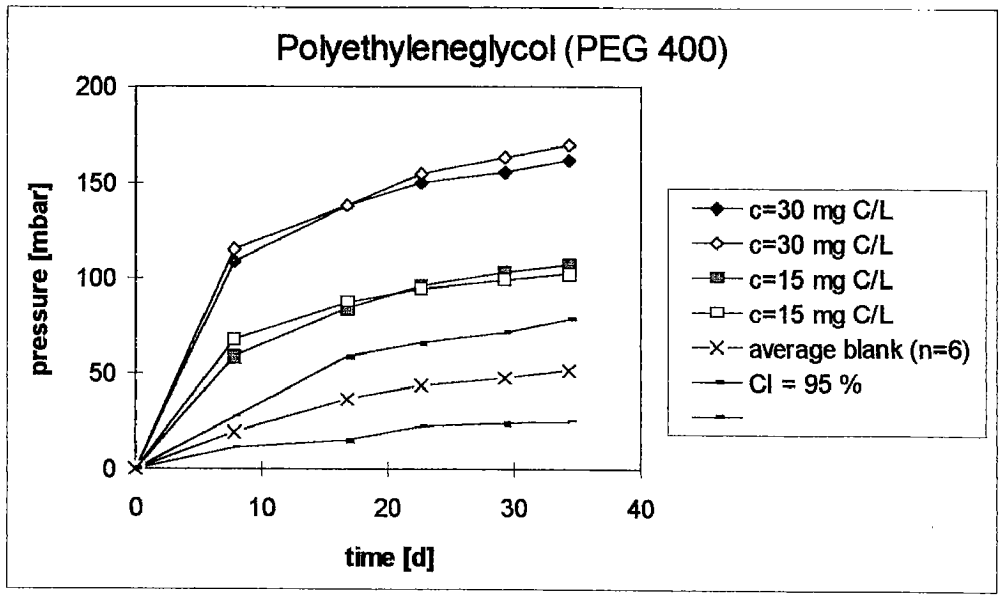


Figure 12: Validation of the test system at low concentration with polyethyleneglycol 400 (PEG 400). Blank stands for the endogenous biogas production of the sludge.

Anaerobic degradation experiments with industrial waste water

The anaerobic pre-treatment of industrial waste water can be an economic alternative to aerobic biological processes under the following conditions:

- High organic load of the incoming waste water
- Anaerobically degradable ingredients
- Missing of inhibitory compounds.

Therefore, all chemicals used in the production processes, e.g. surfactants, disinfectants or complexing agents, should at wastewater concentrations be anaerobically degradable and/or not toxic in anaerobic sludge. The ASTS was therefore tested for the investigation of the anaerobic biodegradability of industrial waste water (Table 13). The chemical oxygen demand (COD) of the wastewater samples varied between 1.5 and 3.0 g O/L, resulting in a theoretical amount of biogas of 500 to 1000 liter CH_4 per m^3 waste water. All the samples tested met the requirements of Edelmann et al. (1993), who judged waste water with a COD higher than 0.5 g O/L suitable for an anaerobic pre-treatment.

Table 13: Description and origin of the industrial wastewater samples. TOC: total organic carbon; COD: chemical oxygen demand; pH; CH_4: theoretical CH_4 per m^3 waste water.

Name	Origin	Sample place	TOC g C/L	COD g O/L	pH	CH_4 L CH_4/m^3
Reference	D(+)-glucose, Fluka, Buchs, CH		3.00	8.00	7.15	-
Food industry 1	Canned food factory Bischofszell	influent to own WTP	0.62	1.83	4.71	641
Food industry 2	Canned food factory Bischofszell	wastewater storage tank, effluent	0.46	1.54	5.55	539
Food industry 3	Canned food factory Bischofszell	effluent to municipal WTP	0.49	1.64	5.82	574
Food industry 4	Canned food factory Bischofszell	aerated storage tank	0.44	1.50	5.92	525
Dairy industry 1	Säntis AG, Gossau, CH	effluent to WTP	0.83	2.84	9.51	995
Dairy industry 2	Säntis AG, Gossau, CH	wastewater storage tank, effluent	0.72	2.58	10.18	904
Slaughterhouse	Schlachthof St. Gallen, CH	effluent to WTP	0.61	2.72	6.8	953
Textile industry 1	Cilander total, Flawil, CH	day sample of effluent to WTP	0.63	1.97	7.66	690
Textile industry 2	Cilander Entschlichtung	process water	51.6	176.0	7.04	61640

The anaerobic degradation experiments were carried out with the ASTS and the DEGRAMAT test system. These two methods and the reproducibility of the results were compared. The ASTS experiments were performed with a COD of 300 mg O/L, a liquid-volume of 180 mL and a headspace of 110 mL. The sludge taken from the WTP Au-Bruggen was diluted 10-times and used for the experiment after a pre-digestion of 7 days. The theoretical methane pressure at this COD was 180 mbar under the assumption that 10 % of the substrate was used for growth of the microorganism. The analytical uncertainties of the COD and TOC determined in the industrial waste water, were in the range of ± 5 % and should therefore be considered.

All wastewater samples from food related industries (canned food factory, dairy and slaughterhouse) were degraded within 3-5 days under anaerobic conditions; and the DOC as well as the COD were completely removed (Table 14 and Figure 13).

The two wastewater samples from textile industry were partially degraded to about 40 %, whilst the removal of DOC and COD was in the range of 80 %. This result of low mineralization and high elimination was interpreted as sorption to the sludge of slow- or non-degradable hydrophobic compounds.

Table 14: Results of ASTS experiments with industrial waste water (n=3). Mineralization determined as C_1-biogas (S&T) (Equation 3) and to methane production (CH_4) (Equation 5). Removal of DOC and COD from the liquid phase of the test assay. Digestion time: 10 – 31 days

	CH_4 degradation %	±stdev	S&T degradation %	±stdev	DOC removal %	±stdev	COD removal %
D(+)-Glucose	86.1	± 1.5	89.8	± 0.7	99.9	± 0.3	102.0
Food industry 1	91.3		85.7	± 2.1	100.0	± 1.4	102.0
Food industry 2	95.1	± 1.1	90.9	± 1.6	101.3		102.5
Food industry 3	92.0	± 3.5	87.3	± 2.6	103.5	± 3.9	103.5
Food industry 4	92.2	± 2.0	88.6	± 6.0	103.4	± 5.6	105.4
Dairy industry 1	88.2	± 0.8	85.6	± 0.8	102.7	± 4.3	99.9
Dairy industry 2	104.2	± 7.2	99.3	± 7.5	99.2		99.8
Slaughterhouse	88.3	± 3.2	79.3	± 3.8	97.6	± 0.1	98.9
Textile industry 1	40.1		32.9	± 9.2	79.8	± 0.1	84.7
Textile industry 2	35.4	± 20.9	55.0	± 11.5	82.3	± 2.0	90.9

The anaerobic biodegradability of different industrial waste waters was determined with the ASTS. All waste waters were well degradable within less than one week, except those of textile industry. The anaerobic wastewater pre-treatment is a good alternative to the aerobic treatment of waste waters from slaughterhouses, dairy- and food industries. For waste waters from textile industry, the chemicals used in the different production steps need to be anaerobically degradable and may not inhibit the activity of the anaerobic sludge. Anaerobic degradation studies with chemicals used in different processes of the textile industry are under way (Huber 1999, Haase 1997, Meier 1997)

These industrial waste waters were also tested with the DEGRAMAT test system to compare the results with those of the ASTS. These degradation results were smaller than those determined with the ASTS (Figure 14, Table 15). The low degradability of waste water from textile industry 1 was observed in both, the ASTS and the DEGRAMAT test system.

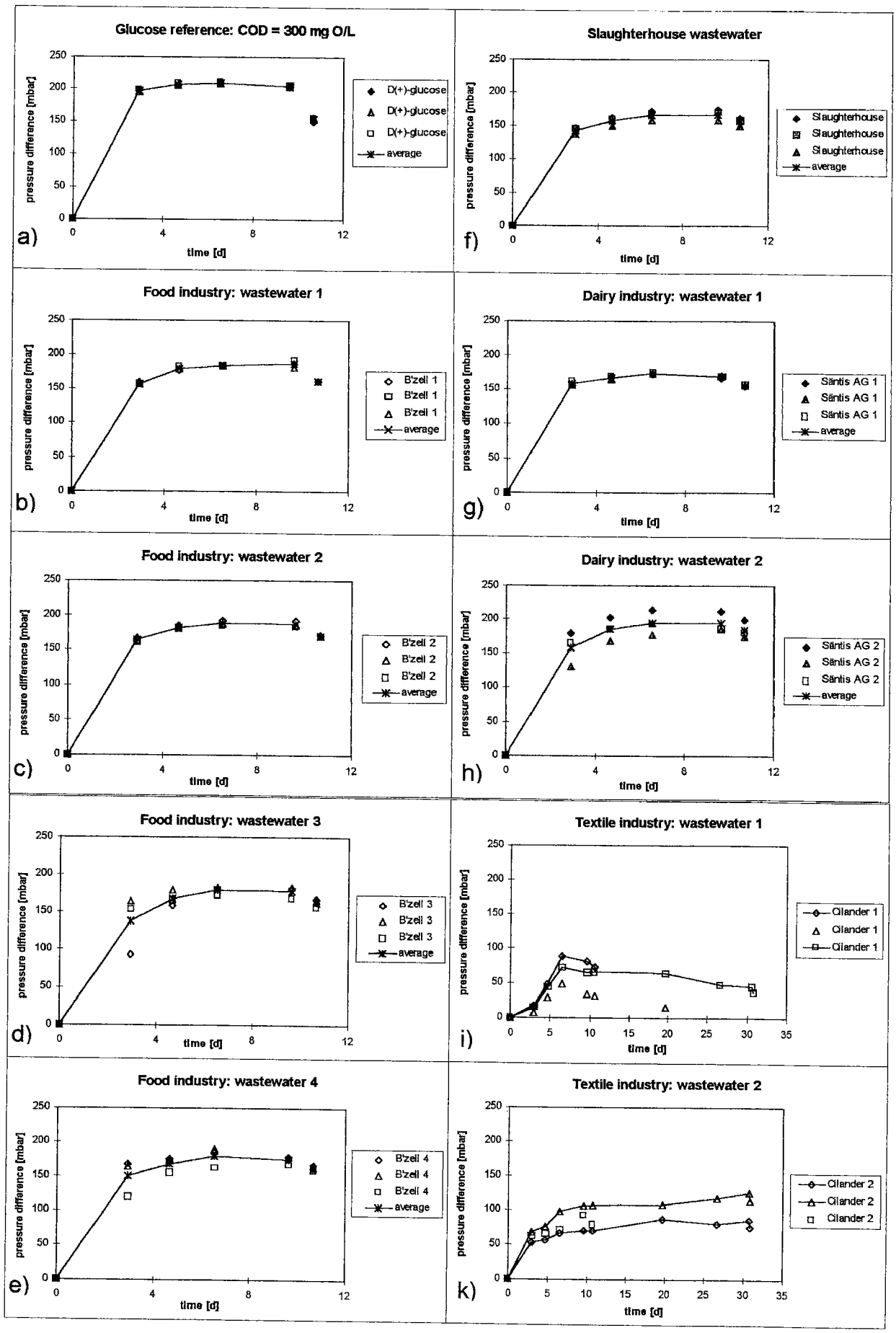


Figure 13: Results of ASTS-experiments with industrial waste waters. B'zell means Bischofszell.

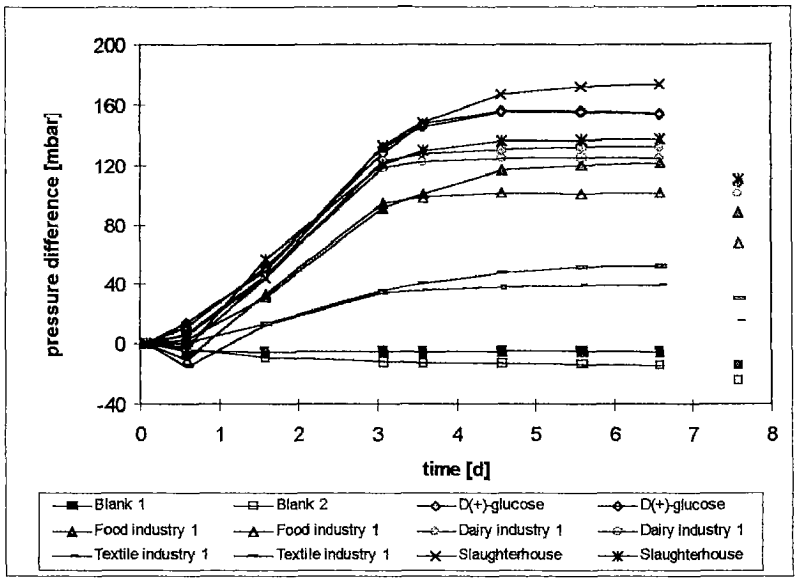


Figure 14: Results of DEGRAMAT experiments with industrial waste water. Nominal C_1-biogas-(S&T) and CH_4-pressure were 220 mbar and 167 mbar, respectively. Blank: endogenous biogas production of the sludge used. Measurement at day 7: methane pressure after the addition of 2 mL 4 M NaOH to absorb $CO_{2(gas)}$.

The degree of degradation obtained with the DEGRAMAT test system were altogether lower as compared to those obtained with the ASTS. A decrease of the biogas pressure was observed in the DEGRAMAT test system within the first 12 hours. This is most probably due to small amounts of oxygen contaminating the headspace. The low endogenous biogas production of the sludge was interpreted as a lower activity of the sludge. Neither oxygen contamination nor lower activity of the sludge did affect the degradation time, which was also 3 to 5 days like in the ASTS experiments. Comparable results were obtained when the degradation was normalized to glucose as a reference substance (data not shown).

Table 15: Results of anaerobic degradation experiments with industrial waste water (DEGRAMAT)

DEGRAMAT	% degradation calculated from CH_4 formation	% degradation according to S&T	% elimination calculated from DOC decrease
D(+)-glucose	76.9	68.4	102.1
D(+)-glucose	76.7	68.0	104.3
Food industry 1	64.5	56.9	105.0
Food industry 1	51.8	48.1	104.8
Dairy industry 1	76.1	66.5	104.7
Dairy industry 1	72.1	63.1	103.5
Textile industry 1	20.5	21.8	78.9
Textile industry 1	29.8	27.5	67.5
Slaughterhouse	-	80.8	102.4
Slaughterhouse	77.8	64.7	103.4

Anaerobic degradation experiments with NTA and phosphonates

The anaerobic biodegradation of complexing agents, such as nitrilo triacetate (NTA) and phosphonic acids (phosphonates), was investigated with the ASTS. 10 times diluted sludge from WTP Wil was used at a concentration of 2 g dry solid matter per liter. The test substances were used at a COD of 150 $mg \cdot L^{-1}$ under the following test conditions: V_{liquid} = 200 mL; $V_{headspace}$ = 90 mL; temperature: 35 °C. PEG 400 was used as a reference in triplicates, whereas the other experiments were performed in duplicates. The iron- and zinc salts of amino tri(methylene phosphonic acid) (Fe-ATMP and Zn-ATMP) were produced from the sodium salt of ATMP. Because of positive aerobic degradation results, these two salts were investigated (Nowack et al. 1997).

PEG 400 was completely degraded within 8 days. None of the other test substances were degraded to a significant amount. The degradability, determined from the C_1-biogas pressure, amounted to less than 20 % of the theoretical C_1-biogas pressure according to Shelton and Tiedje (1984).

An inhibition of the endogenous biogas pressure was observed for the zinc-salt of ATMP. The presence of zinc was assumed to be responsible for this inhibiting effect because no inhibition was observed for the sodium salt or the iron salt of the ATMP.

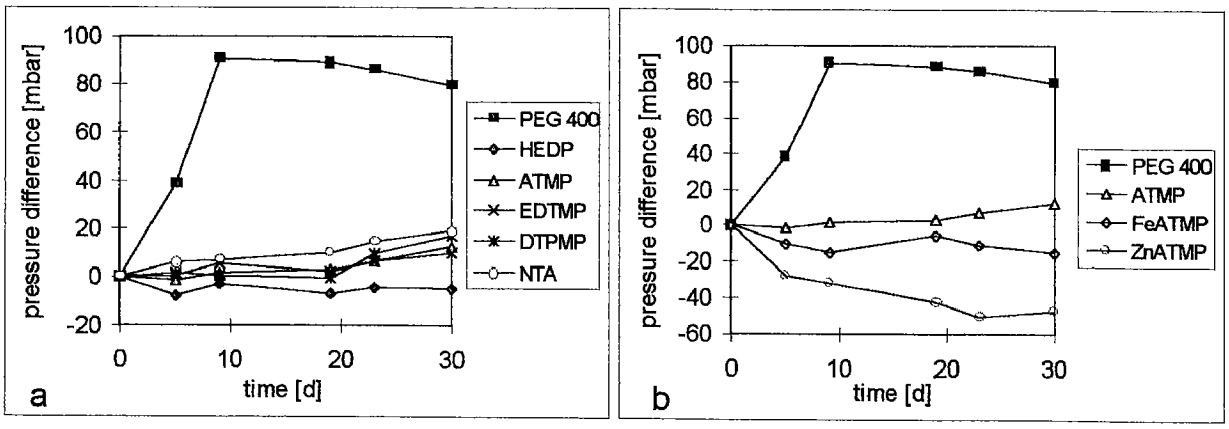


Figure 15: a) Anaerobic biodegradation of nitrilo triacetate (NTA) and phosphonic acids: HEDP: 1-hydroxy ethylene-1,1-diphosphonic acid, sodium salt; ATMP: amino tri(methylene phosphonic acid), sodium salt; EDTMP: ethylene diamine-N,N'-tetra(methylene phosphonic acid), sodium salt; DTPMP: diethylene triamine penta(methylene phosphonic acid), sodium salt. b) ATMP was tested as sodium- (ATMP), iron- (FeATMP) and zinc salt (ZnATMP).

Discussion

Pre-treatment of anaerobic sludge

In the sludge pre-treatment experiments presented here, the influence of washing and pre-digestion of the sludge was studied. The aim of the entire pre-treatment procedure was to obtain a smaller mean variation of the endogenous biogas pressure in the control assays and to increase the reproducibility of the results of degradation experiments with different substrates. By optimizing the pretreatment procedure, the following was achieved:

- The endogenous biogas production was reduced and a significantly smaller deviation was obtained.
- The influence of the washing procedure, the pre-digestion and the substrate addition was specified on the performance of the sludge, e.g. reproducibility and activity.
- The activity of the sludge was slightly increased by the washing procedure, possibly due to a removal of inhibitory substrates or intermediates.

Earlier studies also investigated pre-digestion- and washing -procedures. In the report of ECETOC (1988) pre-digestion times from 1 to 4 weeks were examined. Henkel and Unilever (1995) investigated shorter times of 1 to 3 days. Both reported, that the activity of the sludge decreased with longer pre-digestion periods. We came to the same results for pre-digestion periods longer than 8 days. The washing procedure reduced 80 % of the DOC, but did not reduce the amount of 6 trace metals or the endogenous biogas production significantly. Similar data were obtained by Birch et al. (1989) who investigated the influence of the washing procedure on the decrease of DOC and IC in the sludge.

Comparison of different anaerobic sludges

Eight different substances were used to characterize and compare the anaerobic degradation potential of three sludges. The substances used can be characterized with the following degradation behavior:

- Well degradable substances (Sodium benzoate, PEG 400, Phenol, Sodium palmitate, Sodium lauryl sulfate, linear alcohol ethoxylate LA-$C_{12/14}EO_{20}$).
- Partly and non-degradable substances (2-Ethylhexanol + 10 EO, linear alcohol ethoxylate LA-$C_{12/14}EO_{20}$, 2-Butanol).
- Substances with a longer lag-phase (Sodium benzoate, Sodium palmitate, Sodium lauryl sulfate, 2-Ethylhexanol + 10 EO).

- Substances with an inhibitory effect (Sodium lauryl sulfate).

Each sludge has its own specific potential and limitation regarding the degradation of the set of the investigated substances. The experiment with linear alcohol ethoxylate LA-$C_{12/14}EO_{20}$ shows, that working with different anaerobic sludges is one way to cope with negative anaerobic degradation results. Our findings confirm the statements of Shelton and Tiedje (1984). They investigated sludges from nine municipal WTP on the ability to degrade nine different chemicals (ethanol, PEG 20'000, *p*-cresol, phthalic acid, *m*-cresol, di-*n*-butyl-phthalate, 2-octanol, *m*-chlorobenzoic acid and propionanilide). The degradation capacities of the sludges varied from one substance to another. These variations could not be correlated with the properties of the incoming waste water of the different WTP.

Strotmann et al. (1993) proposed for non- or partly degradable substances an adaptation of the sludge, using a co-substrate. The adaptation of sludge to a test substance provides information about its theoretical biological degradability. Little has been done to predict the adaptation mechanisms at low concentrations in the waste water or the aquatic environment.

The degradation behavior of a chemical studied with fresh and non-adapted sludge provides information on the biodegradability in anaerobic digesters. If chemicals have been found to be non-degradable in a given sludge, they should be additionally tested with different sludges.

Advantages using Equation 5

To calculate the theoretical methane production of a chemical, mixture or waste water in an anaerobic degradation experiment, the chemical oxygen demand (COD) must be known (Equation 5). The advantages of this method (Baumann & Schefer, 1990) are the following:

- The method is independent of the inorganic carbon content (IC) of the liquid used by Birch et al. (1989) and of the pH-dependent partitioning of CO_2 between the gas and water phase estimated by Shelton and Tiedje (1984).
- In contrast to the evaluation method of Shelton and Tiedje (1984) (Equation 2+2), the formula of the substance is not needed here to estimate the theoretical biogas pressure.
- This method works without a cost-intensive TOC-analyzer. The COD can easily be determined photometrically with easy to use COD-test kits.

Comparison between different methods to quantify mineralization

The results obtained with the method of Baumann and Schefer (1990) were consistent in experiments with different COD of the test chemicals (Figure 16). The results, which were obtained with the methods by Shelton and Tiedje (1984) and Birch et al. (1989) for test

chemicals with varying CO_2 to CH_4 ratios, were inconsistent. The method of Birch et al. (1989) mainly failed because of analytical problems with the determination of the dissolved inorganic carbon (IC). With acidification of the test assay at the end of the experiment, followed by the pressure measurement, the total C_1-biogas ($C_{1\,total}$) could also be quantified.

The anaerobic degradability of polyethylene glycol (PEG 400) calculated with the CH_4-method (88.3 % ± 6.0 %) corresponded well with the result calculated according to Shelton and Tiedje (88.6 % ± 5.0 %) (Table 6).

Experimental degradation results can be converted into three general statements:

- anaerobically well degradable (60 % - 80 %),
- anaerobically partly degradable,
- or anaerobically non-degradable (approx. < 25 %).

The proposed levels for the criterion of the *anaerobically well degradable* varied from 60 % (Pagga & Beimborn 1993) to 80 % (Battersby & Wilson 1989). In a ring test Pagga and Beimborn (1993) proposed to use well- and non-degradable reference substances, firstly, to check the operating conditions of the test system and secondly to set the standard of *anaerobically well degradable*. Therefore, a line was drawn in Figure 16, indicating the 70 % degradability.

Based on the specific limitations of the different methods used, a combination of at least two different evaluation methods is recommended for a better interpretation of the experimental results. For example, the additional information of the removal of DOC or COD can easily be determined at the end of a test and used to control the experimental conditions. The TOC and the COD of a product or waste water must be known, to calculate the theoretical amount of CH_4 and CO_2 without knowing the substance formulas. The theoretical biogas pressure can be estimated according to Shelton and Tiedje (1984) and the theoretical methane pressure calculated according to Baumann and Schefer (1990). In addition to the products of mineralization, the removal of the test substance from the test solution can be quantified with the DOC (or COD). The combination of these two results allows to distinguish between sorption and mineralization of the test substance.

The difference between high DOC-removal and small mineralization is shown in the degradation results of sodium palmitate and sodium lauryl sulfate in Figure 16. These differences can be explained by a partial degradation combined with a sorption of the metabolites to the sludge.

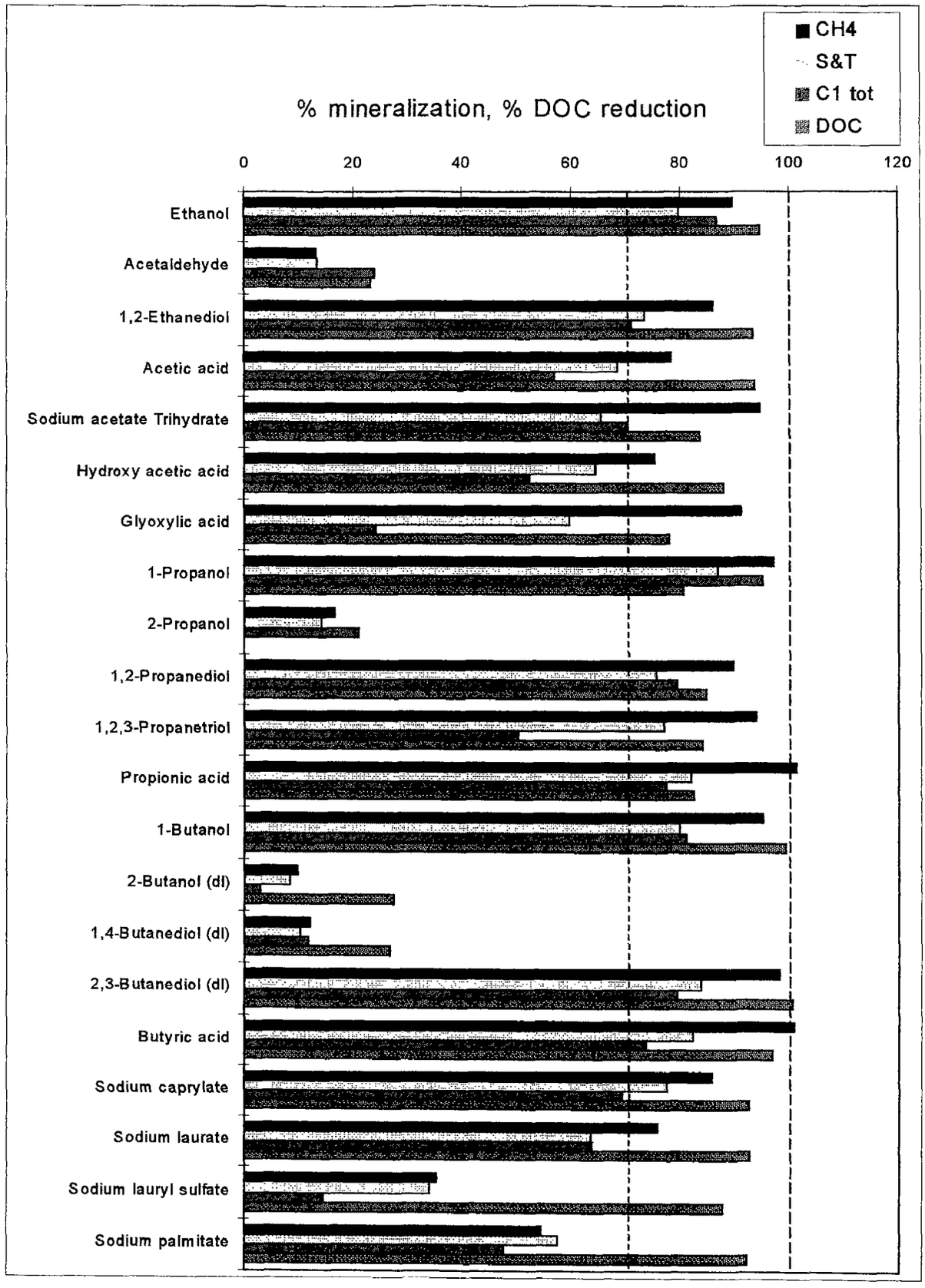


Figure 16: Comparison of the degradation results calculated with different methods to quantify mineralization: CH_4: Baumann and Schefer (1990); S&T (C_1-Biogas): Shelton and Tiedje (1984); $C_{1\,tot}$: Birch et al. (1989); DOC: DOC reduction during the experiment.

If the methods of CH_4-production, C_1-biogas production and DOC removal are combined, it is possible to determine the differences between elimination (sorption plus degradation) and mineralization. It also enables to detect experimental errors, e.g. none or double substance addition to the test assay.

The influence of oxygen contamination at the start of the experiment

The redox indicator resazurin indicates traces of oxygen by turning the test solution from colorless to pink. Traces of oxygen decrease the effective amount of methane produced (Shelton & Tiedje 1984). Oxygen consumption has been observed in the DEGRAMAT test system, where the resazurin was not completely reduced at the beginning of the experiments. Therefore a decrease of the biogas pressure was observed during the first 2 days of the experiment (Figure 17). When the biogas pressure was quantified 3 days after incubation, usually the pressure decrease could no longer be observed.

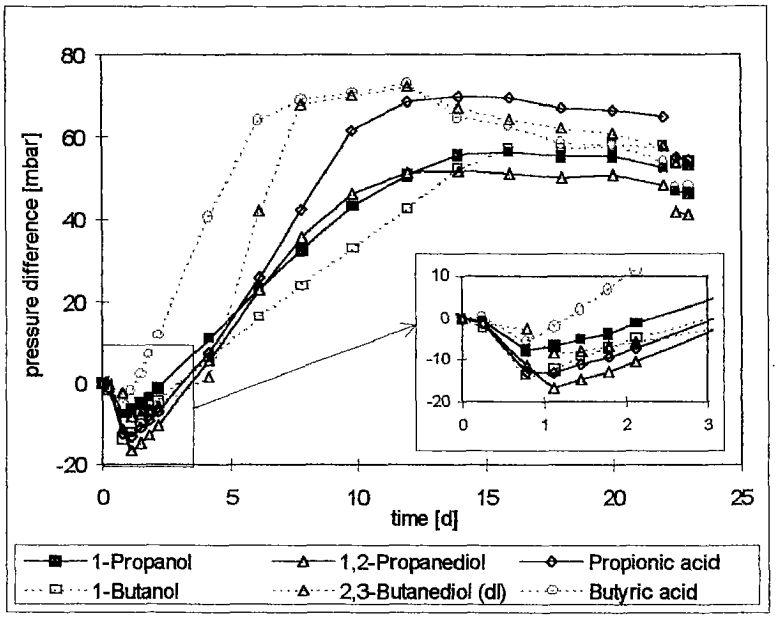


Figure 17: Influence of small amounts of oxygen on biogas production in the DEGRAMAT test systems.

The anaerobic conditions in the test systems during sample preparation could be better maintained in the smaller test bottles of the ASTS than in the one liter bottles used in the DEGRAMAT test systems. The reason is that the smaller volume of the ASTS test system allowed a faster and more effective gas exchange during the vacuum/nitrogen cycles. In addition the disconnection of the cover from the sludge transfer unit and the nitrogen supply could be easier handled with the ASTS test system.

Nevertheless, the results obtained for the different substances with the DEGRAMAT test system corresponded well with those of the ASTS (Table 16). The large liquid volume of the DEGRAMAT and thus the larger methane volume easily compensated for some methane loss by traces of oxygen.

The addition of sodium dithionite reduces the amount of dissolved oxygen. However, the oxidation product is sulfate. As terminal electron acceptor sulfate would just as oxygen increase carbon dioxide production.

Table 16: Comparison of anaerobic degradation experiments with C_3- and C_4-compounds using the DEGRAMAT test system and the ASTS. CH_4: calculated according to Equation 5; C_1: calculated according to Equation 3 (S&T).

	DEGRAMAT (n=1)		ASTS (n=2)	
	CH_4 (COD) %	C_1 (S&T) %	CH_4 (COD) %	C_1 (S&T) %
1-propanol	79.3	74.9	96.9	86.9
2-propanol	4.0	0.0	16.8	14.2
1,2-propanediol	80.8	65.6	89.8	75.4
Propionic acid	93.9	87.2	101.0	82.1
1-butanol	89.7	81.7	95.1	79.8
2-butanol (dl)	13.6	0.0	9.8	8.3
1,4-butanediol (dl)	14.5	21.0	12.1	10.3
2,3-butanediol (dl)	91.6	99.3	98.0	83.6
Butyric acid	82.7	97.5	100.6	82.3

Advantages of the Anaerobic Screening Test System ASTS

Oxygen contamination during sample preparation can be substantially minimized due to the design of the ASTS (head) in combination with the apparatus for the sludge transfer. To visualize and guarantee anaerobic conditions, it is recommended to use resazurin as a redox indicator. The reproducibility of the automated sludge transfer is excellent.

The small size of the 250 mL glass bottle asks for relatively little sludge, therefore 90 test bottles can be operated at the same time on a table of 0.80 x 2.40 m^2. Experiments can easily be performed in triplicates, to get an ascertained average result.

The pressure is determined using a pressure transducer, such, no corrections have to be made to the atmospheric pressure. The ball valve allows direct pressure measurement without perforating the septum or the butyl-rubber stopper by a needle. It also keeps the pressure loss during the measurement cycle low. The pressure decrease due to diffusion is very small or can

even be neglected. The system allows pressure measurement at a rate of 60 to 90 test bottles per hour.

The results obtained with the ASTS are as accurate as those determined with the larger EMPA test system (Baumann & Schefer 1990) or the automated DEGRAMAT test system (Baumann & Rezzonico 1993). The setup of the ASTS is in line with the proposals for a standardized anaerobic degradation test system (ISO 11734; ECETOC 1988). As in other test systems, sources of possible errors can not fully be excluded in the ASTS. However the occurrence of errors can be minimized by a proper experimental set-up, triplicate test assays, the use of reference substances and a well defined sample preparation as described here. The results of at least two methods of anaerobic biodegradability calculation should be compared, e.g. CH_4 together with S&T and/or DOC-reduction, to check and substantiate the data obtained.

Acknowledgments

A special thank you is given to Dr. Markus Brechbühl for the RFS analytic of the sludge from WTP Au-Bruggen, to Angela Hinz for the determination of the dry solid matter of the sludges, to Margot Oswald and Nina Frevel for the assistance by the preparation of the first experiments and to the helpful staff of the different WTP for their generous support.

Chapter 3

Determination of the anaerobic degradation and toxicity of different alcohol ethoxylates

Markus T. Müller, Marc Siegfried, Urs Bauman

Abstract

The anaerobic biodegradability and the toxicity on anaerobic sludge of ethoxylates of linear, single- and multiple-branched alcohols with ethoxylation degrees of approx. 5, 10 and 20 EO were investigated with test systems similar to the ECETOC Screening Test. High degradabilities were confirmed for linear $C_{12/14}$-alcohol ethoxylates at concentrations of 30 mg/L (20 mg C/L). Ethoxylates of single- and multiple branched alcohols were not or only partly degraded. Depending on the ethoxylation degree, no observed effect concentrations (NOEC) from 10 to 100 mg/L were determined for the linear and multiple-branched alcohol ethoxylates. The anaerobic toxicity increased with decreasing degree of ethoxylation.

Introduction

Numerous laboratory studies on aerobic degradation of alcohol ethoxylates (AEO) (Swisher 1987, Siegfried & Baumann 1995, Dorn et al. 1993, Talmage 1994) and large field studies (Matthijs et al. 1995/1996) have shown, that ethoxylates of linear and single-branched alcohols- which are most used today – are degraded at high rate. The elimination in wastewater treatment plants takes place almost quantitatively.

Contrary to the aerobic degradation, only little research has been done regarding the anaerobic degradation of different alcohol ethoxylates. For a full ecological assessment, the anaerobic degradability must be considered, as demanded by the committee of the European Union in its resolution of the 14th February 1995 regarding the 'Ecolabel Award Scheme' for detergents.

The anaerobic pre-treatment of highly contaminated industrial wastewater offers economical advantages. The energy balance is far more favorable and less biomass is built up compared to the aerobic treatment. If anaerobic sludge is used as fertilizer, the anaerobic degradability of chemicals is of great importance. Sludge can only be an ecological fertilizer if it is free of xenobiotics and their organic breakdown products.

The aim of this study was to compare the anaerobic biodegradation of technical AEO based on different alcohols, such as linear alcohols (**LA**), single-branched alcohols (**SBA**), and multiple-branched alcohols (**MBA**), with different degrees of ethoxylation (**5, 10, 20 EO**). Test systems, similar to the ECETOC Screening Test were used to give evidence concerning the correlation of structure and degradation behavior.

Materials and methods

Test systems

Three different anaerobic test systems were used for the experiments: The EMPA test system, the DEGRAMAT test system and the Anaerobic Screening Test System (ASTS). The EMPA test system consists of a 1 litre glass bottle and a head with an integrated mercury manometer. It was described by Baumann and Schefer (1990). The DEGRAMAT test system is the automated version of the EMPA test system (Baumann & Rezzonico, 1993). It consists of a one-liter glass bottle and a DEGRAMAT head with integrated piezoelectric pressure transducer. The Anaerobic Screening Test System (ASTS) was developed according to the screening test proposed by ECETOC (1988). It consists of a 250 mL glass bottle with a special cover adapted for the external pressure measurements with a piezoelectric pressure transducer These test systems are described in detail in Chapter 2.

Anaerobic mineral salt medium

The anaerobic mineral salt medium was prepared according to Birch et al. (1989): KH_2PO_4: 0.270 $g \cdot L^{-1}$, Na_2HPO_4: 0.460 $g \cdot L^{-1}$, NH_4Cl: 0.530 $g \cdot L^{-1}$, $CaCl_2$*2 H_2O: 0.075 $g \cdot L^{-1}$, $MgCl_2$*6 H_2O: 0.10 $g \cdot L^{-1}$, $FeCl_2 \cdot 4\ H_2O$: 0.020 $g \cdot L^{-1}$, resazurin: 0.001 $g \cdot L^{-1}$. The pH was adjusted to pH = 7.0 with HCl (6 $mol \cdot L^{-1}$) or NaOH (6 $mol \cdot L^{-1}$). To guarantee oxygen-free conditions, the solution was flushed with oxygen-free nitrogen for at least 4 hours.

Pretreatment of sludge

The sludge used in these experiments was collected in 1 L polyethylene bottles at the municipal wastewater treatment plant Au-Bruggen in St. Gall, Switzerland. The sludge was centrifuged three times for 15 minutes (50'000 *g*), then washed with anaerobic mineral salt medium and incubated for 7 days at 35 °C. After the pre-digestion, the sludge was centrifuged and washed again. One-liter of the pretreated sludge was transferred into a 10 L bottle containing 9 L of anaerobic mineral salt medium. Before use it was flushed with nitrogen for 12 hours.

Test substances and stock solutions

5.0 g of the test substance (Table 17) were transferred in a 500 mL measuring flask and filled up with water to obtain a stock solution at a substance concentration of 1 % (c=10 $g \cdot L^{-1}$). The DOC and the COD were determined.

Table 17: Characteristics of technical AEO. EO: EO-chain length; Linearity: % linear alcohol of total alcohol; TOC: total organic carbon; COD: chemical oxygen demand.

Product	EO	Linearity	C-alkyl chain distribution in %	TOC [g C/g] ± 0.02	COD [g O/g] ± 0.1
LA-$C_{12/14}EO_5$	5	>99%	C_{12}:C_{14}=70:27	0.630	2.37
LA-$C_{12/14}EO_{10}$	9.1	>99%	C_{12}:C_{14}=70:27	0.614	2.19
LA-$C_{12/14}EO_{20}$	21	>99%	C_{12}:C_{14}=70:27	0.583	2.08
LA-$C_{9\text{-}11}EO_{10}$	10	87%	C_9:C_{10}:C_{11}=18:50:30	0.532	2.24
LA-$C_{14/15}EO_{10}$	10	78%	C_{14}:C_{15}=57:42	0.596	2.37
SBA-$C_{14/15}EO_{10}$	10	39%	C_{14}:C_{15}=63:37	0.578	2.19
SBA-$C_{14/15}EO_{20}$	20	39%	C_{14}:C_{15}=63:37	0.601	2.20
SBA-C_8EO_{10} (2-ethylhexanol)	10	~0%	C_8	0.593	2.17
MBA-$C_{10}EO_{10}$ (isodecanol)	10	~0%	C_{10} > 80	0.512	2.20
MBA-$C_{13}EO_5$ (isotridecanol)	5	~0%	$C_{11:12:13:14}$ =6:30:56:8	0.638	2.37
MBA-$C_{13}EO_{10}$ (isotridecanol)	10	~0%	$C_{11:12:13:14}$ =6:30:56:8	0.620	2.38
MBA-$C_{13}EO_{20}$ (isotridecanol)	20	~0%	$C_{11:12:13:14}$ =6:30:56:8	0.590	2.11

Determination of NOEC and EC_{50}

The no observed effect concentration (NOEC) [$mg \cdot L^{-1}$] was defined as the total surfactant concentration showing no significant inhibitory effect. The inhibitory effects were defined as the negative pressure difference between the gas formed from glucose alone and the biogas formed in the presence of glucose and surfactant.

The effect concentration EC_{50}(4 d) is defined as the total surfactant concentration [mg·L^{-1}], at which the biogas production from the easily degradable glucose is reduced to 50 % after 4 days.

Results

Anaerobic degradation of LA-$C_{12/14}EO_X$ and MBA-$C_{13}EO_X$ (c = 100 mg C·L^{-1})

In a first series of degradation experiments, ethoxylates of a linear alcohol (LA-$C_{12/14}$) and a multiple branched alcohol (MBA-C_{13}: Isotridecanol) with 5, 10 and 20 EO were investigated. These experiments were performed with the EMPA Test System (Baumann & Schefer, 1990) under the following conditions: V_{liquid}: 500 mL; $V_{headspace}$ = 665 mL; 10 % digester sludge with a dry solid content of 2 % - 3 % and a test substance concentration of 100 mg C·L^{-1} corresponding to about 65 mg·g^{-1} dry sludge. The incubation time was 109 days at a temperature of 35 ± 1 °C. The biogas pressure was read periodically from the mercury manometer. At the end of the test period hydrochloric acid was injected directly into the test liquid and sodium hydroxide solution into a vial, which was placed in the headspace of the test bottle, to absorb the carbon dioxide produced. The experiments were performed in duplicates. The mean endogenous biogas pressure was subtracted from the mean pressure in the test-substance experiments. The results are shown in Figure 18.

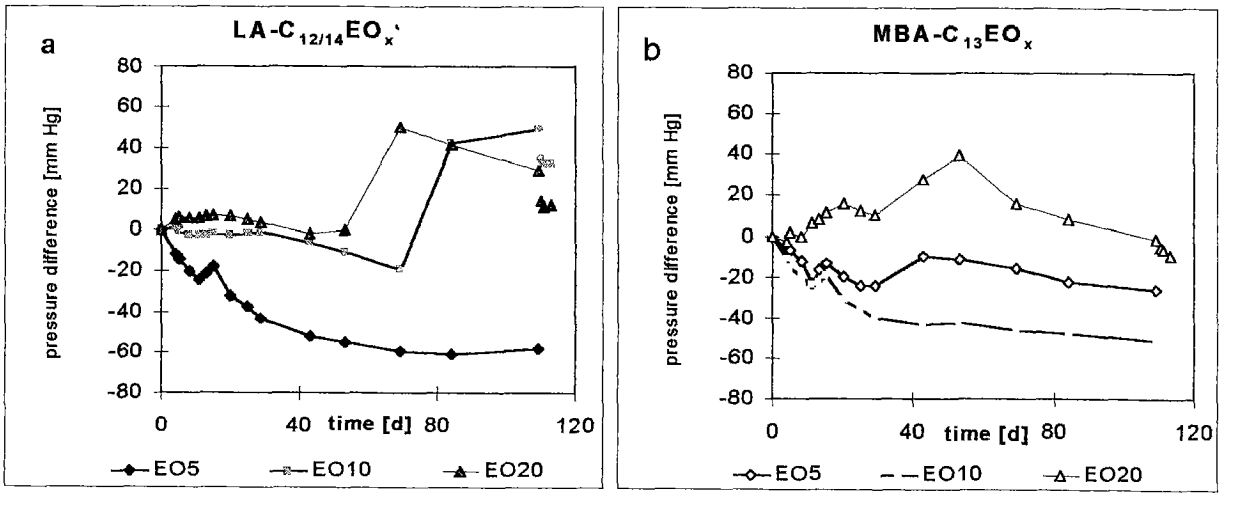


Figure 18: Anaerobic degradation of different ethoxylates of a) linear and b) multiple-branched alcohols. The pressure difference is shown in mm Hg and the average of two experiments.

LA-$C_{12/14}EO_X$: The linear AEO (EO_{10} and EO_{20}) were degraded to a significant amount within the test period of 109 days. Biogas production started after a lag-phase of 55 (LA-$C_{12/14}EO_{20}$) and 70 days (LA-$C_{12/14}EO_{10}$). Based on the theoretical methane pressure of 80 mm mercury the degradability was estimated to be about 40 %. The LA-$C_{12/14}EO_5$ inhibited the endogenous biogas production. The toxicity increased with decreasing EO chain length.

MBA-$C_{13}EO_X$: The ethoxylates of isotridecanol with an EO-chain length of 5 and 10 inhibited the biogas production. No inhibition was observed for MBA-$C_{13}EO_{20}$. Because of the inhibitory effects observed, the toxicity of these alcohol ethoxylates on anaerobic microorganisms was investigated in more detail.

Anaerobic toxicity tests with LA-$C_{12/14}EO_x$ and MBA-$C_{13}EO_x$

The anaerobic toxicity tests were performed with linear and multiple-branched AEO (LA-$C_{12/14}EO_x$ and MBA-$C_{13}EO_x$: x= 5, 10, 20) using the *Anaerobic Screening Test System* (ASTS). D(+)-Glucose (Dextrose) was added as co-substrate at a concentration of 133 $mg \cdot L^{-1}$. The sludge concentration in the assay was 10 % or 2 g dry solids$\cdot L^{-1}$. The AEO concentrations in the assays were 3, 10, 30, 100 and 300 $mg \cdot L^{-1}$ (100 $mg \cdot L^{-1} \cong 50\ mg \cdot g^{-1}$ dry sludge). V_{liquid} : $V_{headspace}$ = 150 mL : 145 mL. Nine parallel assays were made for the endogenous biogas production and six for glucose. The data represented in Figure 19 were used for reference.

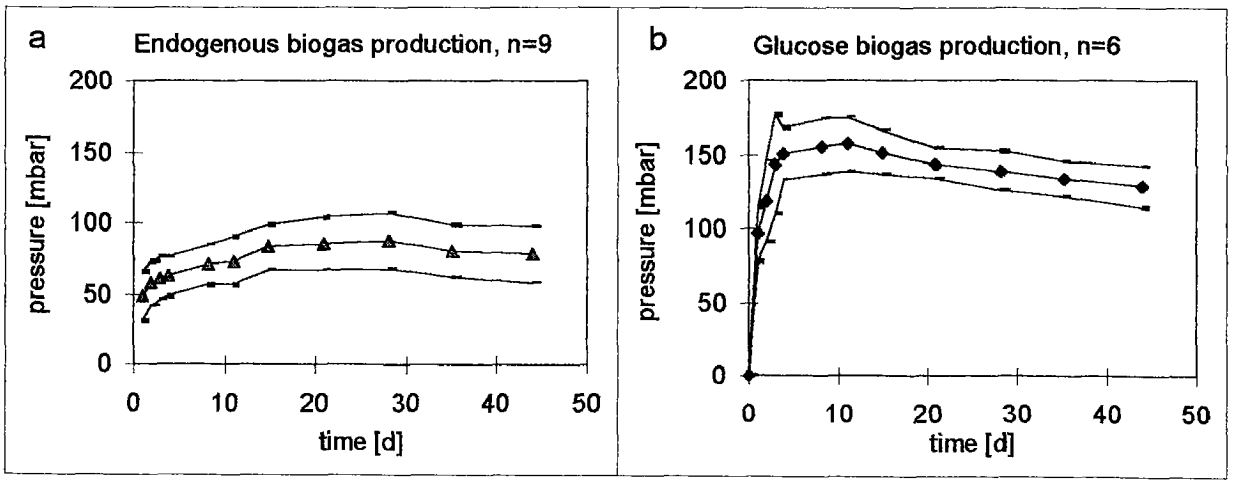


Figure 19: a) Endogenous biogas production and b) glucose biogas production in mbar. The confidence interval (CI=95%) is shown.

The progress of total biogas production for the toxicity experiments with LA-$C_{12/14}EO_{10}$ and MBA-$C_{13}EO_{10}$ is shown in Figure 20. For each concentration a single test was performed.

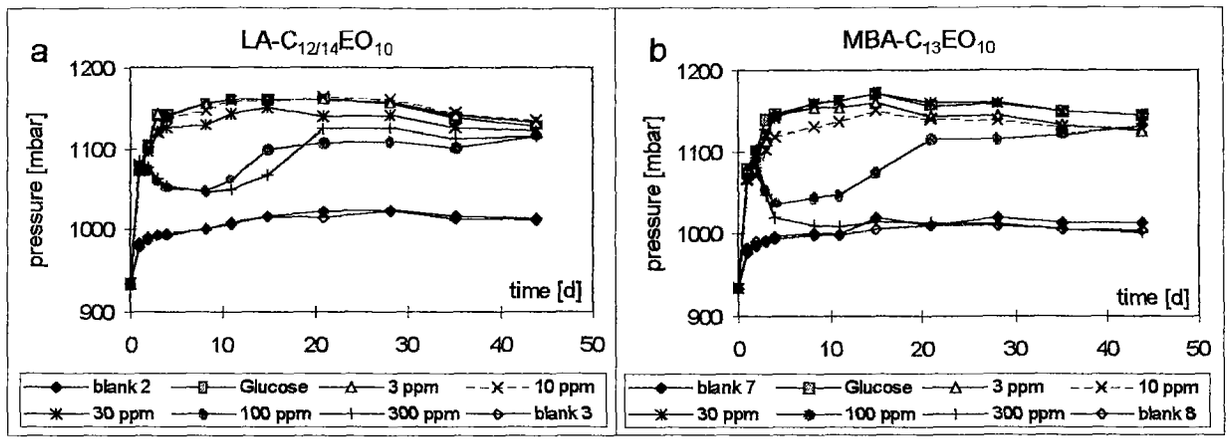


Figure 20: Anaerobic toxicity experiments with a) LA-$C_{12/14}EO_{10}$ and b) MBA-$C_{13}EO_{10}$. The blanks represent the pressure increase in the absence of glucose and AEO. ppm means $mg \cdot L^{-1}$.

The pressure differences between the mean glucose biogas production and the biogas production of the test assays of the 5, 10 and 20 EO adducts are shown in Figure 21. The biogas pressure of the reference glucose (Figure 20) was subtracted from the one of the glucose with added test substance. A negative pressure difference means that the complete degradation of glucose – observed as the biogas formation - was inhibited by the presence of the surfactant. A positive pressure difference - additional biogas production - stands for a degradation of the surfactant.

The toxicity on anaerobic bacteria decreased with the increasing of the EO chain length for ethoxylates of LA-$C_{12/14}$ and of MBA-C_{13} (Table 18). The degree of branching of the alcohol did not influence the toxicity of the ethoxylate at a given ethoxylation degree.

Table 18: Results of the toxicity experiments: NOEC = no observed effect concentration, EC_{50} (4d): 50 %-effect concentration after 4 d, duration of the inhibition at 2 concentrations. Sludge concentration: 2.0 ± 0.2 $g \cdot L^{-1}$. n.i. stands for no inhibition.

Product	NOEC [$mg \cdot L^{-1}$]	EC_{50}(4 d) [$mg \cdot L^{-1}$]	duration of inhibition 100 $mg \cdot L^{-1}$	300 $mg \cdot L^{-1}$
LA-$C_{12/14}EO_5$	8	50	30 d	> 45 d
LA-$C_{12/14}EO_{10}$	30	85	15 d	20 d
LA-$C_{12/14}EO_{20}$	100	>300	n.i.	8 d
MBA-$C_{13}EO_5$	10	60	> 45 d	> 45 d
MBA-$C_{13}EO_{10}$	30	75	20 d	> 45 d
MBA-$C_{13}EO_{20}$	100	280	n.i.	12 d

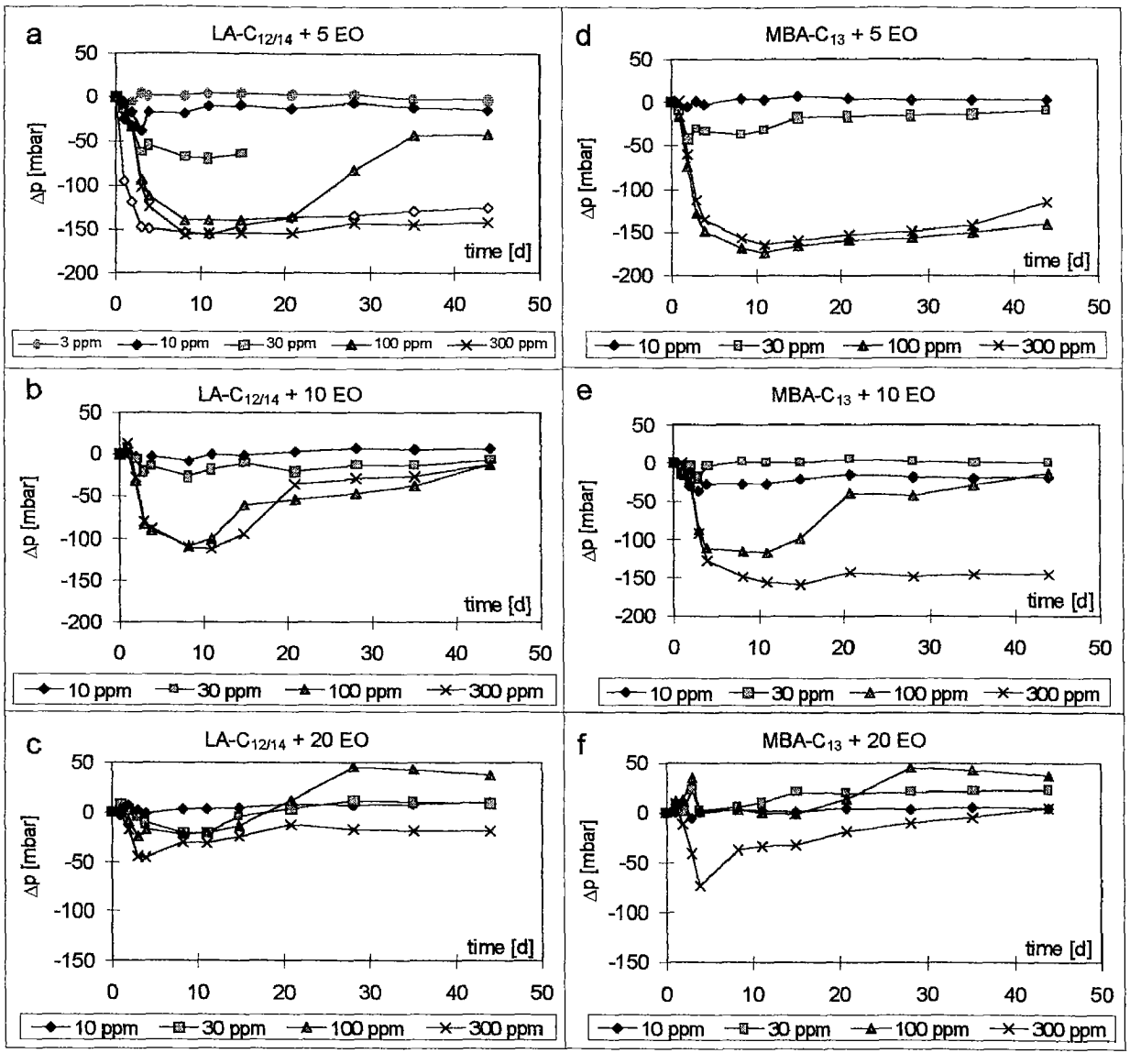


Figure 21: Progress of pressure differences of the degradation experiments with different technical AEO. The 95 % confidential interval is about ± 15 mbar (ppm = $mg \cdot L^{-1}$).

These results are in line with findings of Dorn et al (1993), which refer to biodegradation and toxicity of nonionic surfactants. They found that toxicity increased with increasing hydrophobic chain length and decreased with increasing EO chain length. Branching of the alcohol diminishes the biodegradability, but does not influence the toxicity.

The no observed effect concentrations (NOEC) for AEO with an ethoxylation degree of 5, 10, and 20 EO were about 10 $mg \cdot L^{-1}$, 30 $mg \cdot L^{-1}$, and 100 $mg \cdot L^{-1}$, respectively. The EC_{50} (4d) for the 5 and 10 EO adducts were between 50 and 85 $mg \cdot L^{-1}$; and >280 $mg \cdot L^{-1}$ for the 20 EO adducts.

$LA\text{-}C_{12/14}EO_{20}$ and $MBA\text{-}C_{13}EO_{20}$ were partly degraded at a surfactant concentration of 100 $mg \cdot L^{-1}$ with a significant biogas production. None of the other AEO was significantly degraded. However, the test conditions (V_{liquid}:$V_{headspace}$ ≈ 1:1) did not allow to obtain a

significant pressure increase at a concentration of 30 mg·L^{-1}. In order to generate a significant increase in biogas pressure the test conditions were adapted to lower test substance concentrations by decreasing the headspace (V_{liquid}:$V_{headspace}$ ≈ 2:1). The volume of the headspace was decreased by increasing the inoculum from 150 mL to about 200 mL. The total volume of the test bottle is approximately 295 mL. To avoid inhibitory effects by high surfactant concentrations, the following experiments were performed at concentrations of 30 mg·L^{-1} (c = 20 mg C·L^{-1}).

Anaerobic degradation of 12 different AEO at a concentration of 20 mg C/L

All AEO described in Table 17 were tested at a concentration of 30 mg·L^{-1} (20 mg C·L^{-1}, 12 mg/g dry sludge). The tests were performed with the ASTS at the following conditions: V_{liquid}: 205 mL; $V_{headspace}$: 90 mL; pH = 7.3, sludge concentration = 10 % (2.5 g dry solids/L). The confidence interval (95 %) of the endogenous biogas production (n =8), which was about 20 mbar, was used as significance level for anaerobic degradation. The results for the different AEO are presented in Figure 22 a-d and summarized in Table 19. The small amount of biogas lost by each measurement could be neglected. The 12 substances showed no significant inhibitory effects under the test conditions used.

Table 19: Anaerobic degradation tests with PEG 400 and different AEO (c = 20 mg C·L^{-1}). The degradability was calculated based on the theoretical methane production (CH_4) and the theoretical biogas pressure of Shelton and Tiedje (1984) (S&T). (* only one value was taken.)

Biogas pressure: all values	*time [days]*						+acid[1]	+base[2]	calculated[3]	Degradability[3]	
are given in [mbar]	*8d*	*17d*	*23d*	*43d*	*62d*	*90d*	$C_{1\,tot}$	CH_4	p(CH_4)	%CH_4	%C_1
PEG 400, c = 15 mg C/L	43.9	47.4	50.2	**50.8**			62.3	55.7	52.9	105.3%	*82.8%*
PEG 400, c = 30 mg C/L	92.4	99.7	107.3	**111.9**			129.0	110.0	105.8	104.0%	*91.1%*
LA-$C_{12/14}EO_5$	-7.1	7.1	8.5	49.7*	55.4*	**69.2***	130.7*	43.3*	76.5	56.6*%	*83.4*%*
LA-$C_{12/14}EO_{10}$	7.7	10.5	7.4	64.6	**70.6**	55.8	118	34.4	69.4	49.5%	*90.4%*
LA-$C_{12/14}EO_{20}$	32.9	32.8	29.6	**73.3**	68.0	50.2	108	20.3	69.5	29.2%	*93.9%*
LA-$C_{9\text{-}11}EO_{10}$	13.8	21.2	**21.7**	19.4	17.1	19.0	10.1	16.1	76.5	21.5*%	*25.2%*
LA-$C_{14/15}EO_{10}$	13.6	9.5	3.3	-3.8	-10.8	**19.2**	14.2	12.4	74.1	16.7%	*22.5%*
SBA-$C_{14/15}EO_{10}$	11.7	7.2	1.0	-11.3	-21.3	-20.8	-33.9	-15.0	67.6	-	-
SBA-$C_{14/15}EO_{20}$	6.5	-2.7	-11.3	-17.1	-25.7	-20.1	-35.3	-18.6	70.1	-	-
SBA-C_8EO_{10}	-4.6	-8.8	-11.5	-3.9	-7.3	-8.0	-17.9	-2.2	69.0	-	-
MBA-$C_{10}EO_{10}$	14.9	11.0	8.1	12.1	7.2	7.7	-2.8	6.5	71.2	-	-
MBA-$C_{13}EO_5$	9.6	11.6	8.3	8.5	0.7	-2.7	-3.9	-5.0	78.2	-	-
MBA-$C_{13}EO_{10}$	12.5	13.3	8.0	5.5	-2.5	-1.7	-5.4	-6.3	67.5	-	-
MBA-$C_{13}EO_{20}$	5.2	3.1	-2.4	-0.4	-4.1	4.2	-0.5	3.6	68.3	-	-

1) To measure the total C_1-Biogas pressure, 4M HCl_{aq} was added to the test assay to gas out dissolved CO_2 (Chapter 2).

2) To determine the CH_4-pressure, 4 M $NaOH_{aq}$ was added to absorb the CO_2 in the liquid (Chapter 2).

3) The determination of the theoretical methane pressure and of the degradability is described in Chapter 2.

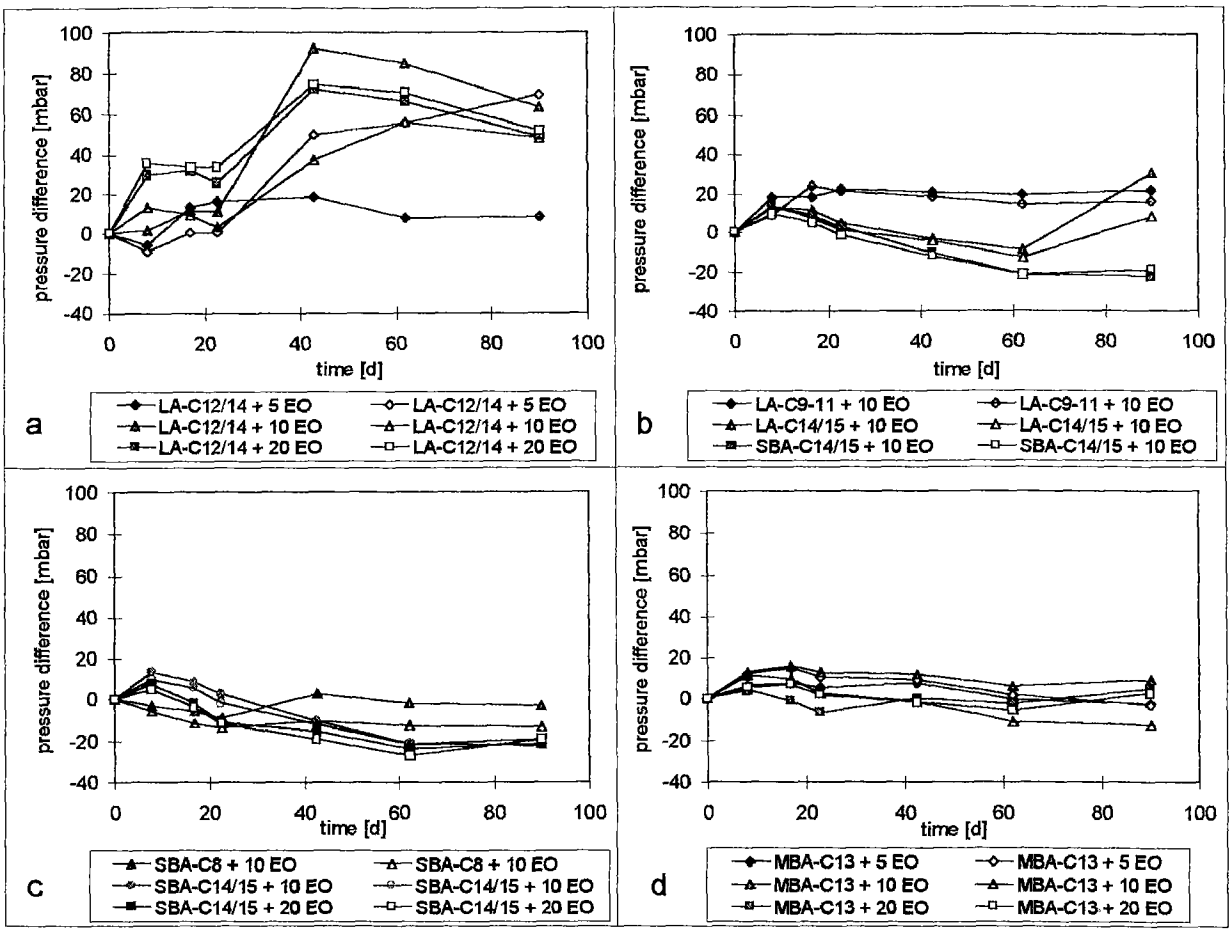


Figure 22: Anaerobic degradation of different AEO at concentrations of 30 mg·L^{-1} (20 mg C/L): LA linear alcohol, SBA: single-branched alcohol, MBA: multiple branched alcohol. The significance limit for the degradability of the test substance is ± 20 mbar (95 % confidence interval of 8 endogenous biogas production experiments). The linearity of the alcohol ethoxylates decreased from a) to d).

With the method of Shelton and Tiedje (1984) the following degradabilities were calculated from the maximum biogas production: 83 % for LA-$C_{12/14}EO_5$, 90 % for LA-$C_{12/14}EO_{10}$ and 94 % for LA-$C_{12/14}EO_{20}$ (Figure 22a). With the method of Baumann and Schäfer (1990), which is based on the theoretical methane production (CH_4), the results were: 55 % for LA-$C_{12/14}EO_5$, 50 % for LA-$C_{12/14}EO_{10}$ and 30 % for LA-$C_{12/14}EO_{20}$. The other linear AEO, LA-$C_{14/15}EO_{10}$ and LA-$C_{9\text{-}11}EO_{10}$, showed a small degradability of approx. 20 % (Figure 22b). None of the single-branched AEO reached a significant biogas pressure within 89 days (Figure 22c).

The degradation results for the LA-$C_{12/14}$ alcohol ethoxylates are in line with the data published by Steber and Wierich (1991), whereas the degradability of SBA-$C_{9\text{-}11}EO_{10}$ is significantly below the values published by Salanitro and Diaz (1995) (60 % and 84 %). MBA-$C_{13}EO_X$ showed no significant biogas production at low concentration (Figure 22d).

The difference between the degradability calculated by using either the method of Shelton and Tiedje (1984) or the COD-method of Baumann and Schäfer (1990) indicated an incomplete degradation. The high CO_2 to CH_4 ratio was also observed by Steber and Wierich (1987). A possible explanation is that the ethoxylate chain of the AEO is easier degraded than the alkyl chain.

Anaerobic degradation of LA-$C_{12/14}EO_X$ and MBA-$C_{13}EO_X$ with DEGRAMAT

The tests were performed with the DEGRAMAT Test System at the following conditions: V_{liquid}: 900 mL; $V_{headspace}$: 265 mL; pH = 7.0; sludge concentration = 10 % (≈ 1.4 g dry sludge/L); test-substance concentration = 30 mg·L^{-1} (20 mg C·L^{-1}).

The linear alcohol ethoxylates LA-$C_{12/14}EO_5$ and LA-$C_{12/14}EO_{10}$ inhibited the endogenous biogas production for about 65 and 40 days, respectively (Figure 23). LA-$C_{12/14}EO_{20}$ was degraded significantly within 15 days. The anaerobic degradabilities for the LA-$C_{12/14}EO_5$, LA-$C_{12/14}EO_{10}$ and LA-$C_{12/14}EO_{20}$ were 60 %, 50 % and 30 % (S&T), respectively.

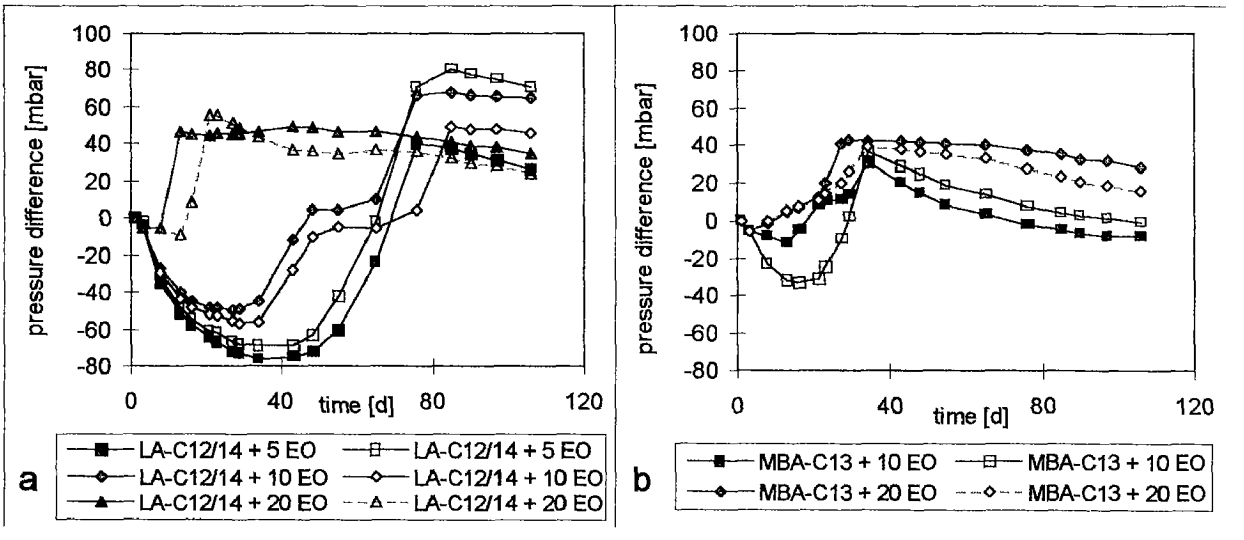


Figure 23: Anaerobic biodegradation of alkyl ethoxylates with DEGRAMAT test system (c = 20 mg C·L^{-1}): a) LA-$C_{12/14}EO_X$, (x= 5, 10, 20), b) MBA-$C_{13}EO_X$ (x=10, 20).

MBA-$C_{13}EO_{10}$ inhibited the endogenous biogas production for 25 days. Then the degradation and biogas production started and exceeded the pressure of the endogenous biogas production of the sludge without surfactant addition (blank, pressure difference = zero). After 50 days, the pressure decreased slowly to the pressure of the blank. MBA-$C_{13}EO_{20}$ was degraded to about 30 % (S&T) within 110 days. In previous experiments (see Figure 18, Figure 20 and Figure 22) an inhibition of the biogas production was observed at higher surfactant concentrations. The toxicity of surface-active substances depends on the sludge concentration, which was

smaller in the DEGRAMAT experiments. The sludge concentration was 1.4 g dry solid·L^{-1} compared to 2.0 g dry solid·L^{-1} in previous experiments and resulted in toxic effects at lower total surfactant concentrations (30 mg·L^{-1}).

Conclusions

Knowledge about the anaerobic biodegradability of alcohol ethoxylates is important, as these substances partly reach the anaerobic reactors adsorbed to the primary sludge. The AEO concentrations in anaerobic digesters range between 0.5 and 1.8 mg/g dry sludge (Salanitro & Diaz, 1984). In our experiments we used surfactant concentrations of about 15 mg/g dry sludge. At this concentration no or only small toxic effects were observed for all the alcohol ethoxylates investigated. For LA-$C_{12/14}EO_X$ and MBA-$C_{13}EO_X$, the no observed effect concentrations (NOEC) towards anaerobic sludge (2.0 g dry sludge·L^{-1}) were about 8 - 10 mg·L^{-1} (5 EO), 30 mg·L^{-1} (10 EO) and 100 mg·L^{-1} (20 EO). The EC_{50} (4d) for the 5 EO and 10 EO adducts were between 50 and 85 mg·L^{-1}, and > 280 mg·L^{-1} for the 20 EO. The degree of branching of the alcohol did not significantly influence the toxicity. The toxicity of the different technical alcohol ethoxylates towards anaerobic sludge was determined to obtain the concentration range, at which these surfactants did not inhibit the activity of the sludge in the ASTS.

The toxicity data were also compared to AEO concentrations in municipal wastewater treatment plants. Therefore the lowest NOEC (LA-$C_{12/14}EO_5$: 8 mg·L^{-1}) was divided by the sludge concentration (2.0 g dry sludge·L^{-1}) and compared with the concentration of nonionic surfactant in anaerobic digesters of 0.5 and 1.8 mg/g dry sludge (Salanitro & Diaz 1994). Thus, in the anaerobic digesters of wastewater treatment plants no inhibitory effects have to be expected for AEO concentrations below 4 mg/g dry sludge.

Significant degradation results were observed for the ethoxylates (5, 10, 20 EO) of linear $C_{12/14}$-alcohols, as well as for the MBA-$C_{13}EO_{20}$ (Isotridecanol + 20 EO), although long lag-phases and inhibitory effects were observed in several experiments (Table 20). All other ethoxylates of both single- and multiple-branched alcohols did not show a significant degradation with the sludge from WTP Au-Bruggen. A clear relationship between structure and anaerobic degradability could neither be found with respect to the ethoxylation degree nor with the degree of branching of the alcohol.

Table 20: Comparison of the data obtained for anaerobic degradation of different alcohol, ethoxylates using anaerobic sludge from WTP Au-Bruggen, St. Gall, Switzerland.

substance	Exp.1 [a] (CH_4) [e]	Exp.2 [b] (*S&T*) [f]	Exp.3 [c] (CH_4) [e]; (*S&T*) [f]	Exp.4 [d] (*S&T*) [f]	Reference
LA-$C_{12/14}EO_5$	inhibition	no degrad.	56.6 %*; *83.4 %**	*60 % **	degradable [1]
LA-$C_{12/14}EO_{10}$	inhibition	no degrad.	49.5 %; *90.4 %*	*55 %*	109 ± 15 % [2]
LA-$C_{12/14}EO_{20}$	40 %	*50 %**	29.2 %; *93.9 %*	*30 %*	~90 % [3]
LA-$C_{9\text{-}11}EO_{10}$	-	-	21.5 %*; *25.2 %*	-	*60 %, 84 %* [4]
LA-$C_{14/15}EO_{10}$	-	-	16.7 %; *22.5 %*	-	
SBA-$C_{14/15}EO_{10}$	-	-	no signif. degrad.	-	
SBA-$C_{14/15}EO_{20}$	-	-	no signif. degrad.	-	
SBA-C_8EO_{10} (2-Ethylhexanol)	-	-	no signif. degrad.	-	
MBA-$C_{10}EO_{10}$ (Isodecanol)	-	-	no signif. degrad.	-	
MBA-$C_{13}EO_5$ (Isotridecanol)	inhibition	no degrad.	no signif. degrad.	-	
MBA-$C_{13}EO_{10}$ (Isotridecanol)	inhibition	no degrad.	no signif. degrad.	no degrad.	
MBA-$C_{13}EO_{20}$ (Isotridecanol)	no inhibit.	*50 % **	no signif. degrad.	*30 %*	

a) EMPA test system, C=100 mg $C \cdot L^{-1}$.

b) Toxicity test using the ASTS.

c) Degradation experiments using the ASTS with low surfactant concentrations of 20 mg $C \cdot L^{-1}$.

d) DEGRAMAT test system with C=20 mg $C \cdot L^{-1}$.

e) Biodegradability was calculated with the theoretical CH_4-pressure (CH_4): Baumann & Schäfer (1990) (see Chapter 2).

f) Biodegradability was calculated with the C_1-biogas pressure (S&T): Shelton & Tiedje (1984) (see Chapter 2).

* Result was calculated from only one experimental value.

References: [1] Wagener & Schink (1988), [2] Steber & Wierich (1991), [3] Wagener & Schink (1987), [4] Salanitro & Diaz (1995).

Chapter 4

Anaerobic degradation of technical alcohol ethoxylates with different sludges

Markus T. Müller, Urs Baumann, Alexander J.B. Zehnder

Abstract

The anaerobic biodegradation of LA-$C_{12/14}EO_{20}$ and SBA-C_8EO_{10} was studied with anaerobic sludge from three different wastewater treatment plants (WTP), Au-Bruggen, Wil and Kloten/Opfikon, using the Anaerobic Screening Test System (ASTS). LA-$C_{12/14}EO_{20}$ was completely degraded within 10 days by two sludges, but not degraded at all within 30 days by a third one. SBA-C_8EO_{10} was degraded to about 50 % by all three sludges within one month. In a second set of experiments linear, single- and multiple-branched alcohol ethoxylates (LA, SBA, MBA) with an average ethoxylation degree of 5, 10 and 20 EO were tested with one anaerobic sludge from WTP Wil. High degradation rates were found for linear $C_{12/14}$-alcohol ethoxylates at concentrations of about 30 $mg \cdot g^{-1} \cdot L^{-1}$ (COD = 150 $mg \cdot L^{-1}$; sludge concentration = 2.5 $g \cdot L^{-1}$). Ethoxylates composed of some single- and multiple-branched alcohols were degraded only partly with this digesting sludge. From the experiments presented here we can conclude that the anaerobic biodegradability of technical AEO decreased with decreasing linearity of the alcohol from linear to multiple-branched AEO.

Introduction

Alcohol ethoxylates (AEO) are surfactants that consist of molecules with a hydrophilic and a lipophilic part. Therefore they possess the properties of both, lipophilic substances and hydrophilic substances. Lipophilic substances accumulate in wastewater on particulate organic matter by sorption and reach the anaerobic reactor together with the primary sludge. Hydrophilic substances are transported with the water to the activated sludge basin where they

are completely or partially degraded by aerobic microorganisms. Partial aerobic degradation of surfactants may lead to lipophilic metabolites that sorb to the activated sludge, which is also transferred into the anaerobic reactor. Aerobic degradation tests, such as described in different OECD guidelines for testing primary and ultimate aerobic biodegradability (OECD, 1993) allow to obtain only information about the processes in the aerobic part of the wastewater treatment plant (WTP).

Sorption of a compound is controlled by its lipophilic properties, which can be estimated by the octanol-water partition coefficient (K_{OW}). Octanol-water partition coefficients are estimated in Chapter 5, and of most alcohol ethoxylates ($C_{\geq 10}$-alcohols) they are in a range where sorption to the sludge is of major importance (Chapter 5).

In Chapter 3 the anaerobic biodegradability of the major group of nonionic surfactants - alcohol ethoxylates (AEO) - was investigated with sludge from WTP Au-Bruggen. These technical AEO are mixtures of different alcohols, such as linear alcohols (LA), single-branched alcohols (SBA) and multiple-branched alcohols (MBA) with an average ethoxylation degree of 5, 10, or 20 EO. Problems with inhibition of biogas production, long lag-phases, and low activity of the sludge made the evaluation of the results in relation to the structure of the AEO difficult.

Therefore, we evaluated in this study anaerobic sludges from different WTP, Au-Bruggen (control), Wil and Kloten/Opfikon, in order to find a sludge with a higher degradation activity for AEO. The degradation experiments presented in Chapter 3 were repeated with the most active of these sludges. The aim of this study was to correlate the anaerobic biodegradation with the structure of AEO.

Materials and Methods

Anaerobic sludge

Anaerobic sludges from five different wastewater treatment plants (WTP) in Switzerland were tested for their suitability to be used in degradation tests with alcohol ethoxylates: Au-Bruggen St. Gall; Wil, Herisau, Kloten/Opfikon, and Spreitenbach. Each sludge was diluted to 10 % with anaerobic mineral salts medium and pre-incubated for 7 days at 35 °C (Chapter 2). After this time period the endogenous biogas production of the sludges from Herisau and Spreitenbach was still too high to be used in the degradation experiments, and were therefore excluded from the further study.

Test system and test conditions

The Anaerobic Screening Test System (ASTS), the anaerobic mineral salt medium (Birch et al. 1989), the determination of the total organic carbon (TOC) and the chemical oxygen demand (COD), as well as the calculation of the degradability are described in Chapter 2. The experiments were performed at a COD of 150 mg $O \cdot L^{-1}$ corresponding to a chemical concentration of about 50 mg$\cdot L^{-1}$. In all experiments the sludge concentration was 2.5 g$\cdot L^{-1}$ dry sludge and the temperature was kept at 35 °C.

For the first set of experiments with sludges originating from WTP Au-Bruggen, Wil, and Kloten/Opfikon the following test conditions were applied: V_{liquid}= 180 mL; $V_{headspace}$= 110 mL; replicates: n=1. For the second set of experiments only sludge from WTP Wil was used under the following test conditions: V_{liquid}= 200 mL; $V_{headspace}$= 90 mL; replicates: n=2.

Chemicals

All the substances for the anaerobic mineral salt medium, the D(+)-Glucose (Dextrose) and the PEG 400 were purchased from Fluka AG, Buchs, Switzerland. All technical alcohol ethoxylates were obtained from Dr. W. Kolb AG, Hedingen, Switzerland (Table 21).

Table 21: Technical alcohol ethoxylates tested. LA: linear alcohol; SBA: single-branched alcohol; MBA: multiple-branched alcohol; EO: average ethoxylate chain length in mol EO per mol alcohol; Linearity is defined as weight-% of linear alcohols to total alcohol. TOC: total organic carbon; COD: chemical oxygen demand.

Product	EO	Linearity of alcohol	C-alkyl chain distribution in %	TOC [g C/g] ± 0.02	COD [g O/g] ± 0.1
LA-$C_{12/14}EO_5$	5	>99%	C_{12}:C_{14}=70:27	0.630	2.37
LA-$C_{12/14}EO_{10}$	9.1	>99%	C_{12}:C_{14}=70:27	0.614	2.19
LA-$C_{12/14}EO_{20}$	21	>99%	C_{12}:C_{14}=70:27	0.583	2.08
LA-$C_{9-11}EO_{10}$	10	87%	C_9:C_{10}:C_{11}=18:50:30	0.532	2.24
LA-$C_{14/15}EO_{10}$	10	78%	C_{14}:C_{15}=57:42	0.596	2.37
SBA-$C_{14/15}EO_5$	5	39%	C_{14}:C_{15}=63:37	0.578	2.19
SBA-$C_{14/15}EO_{10}$	10	39%	C_{14}:C_{15}=63:37	0.578	2.19
SBA-$C_{14/15}EO_{20}$	20	39%	C_{14}:C_{15}=63:37	0.601	2.20
SBA-$C_{14/15}EO_{40}$	40	39%	C_{14}:C_{15}=63:37	0.540	2.23
SBA-C_8EO_{10} (2-ethylhexanol)	10	~0%	C_8	0.593	2.17
MBA-$C_{10}EO_{10}$ (Isodecanol)	10	~0%	C_{10} > 80	0.512	2.20
MBA-$C_{13}EO_5$ (Isotridecanol)	5	~0%	$C_{11:12:13:14}$ =6:30:56:8	0.638	2.37
MBA-$C_{13}EO_{10}$ (Isotridecanol)	10	~0%	$C_{11:12:13:14}$ =6:30:56:8	0.620	2.38
MBA-$C_{13}EO_{20}$ (Iisotridecanol)	20	~0%	$C_{11:12:13:14}$ =6:30:56:8	0.590	2.11

Results

Degradation test with different anaerobic sludges

Complete anaerobic degradation was obtained for benzoic acid within 10 to 17 days with all three sludges. Thus, benzoic acid was used as reference chemical (Figure 24). The anionic surfactant lauryl sulfate was also degraded by all three sludges within 40 days, though sludge from Au-Bruggen and Kloten/Opfikon were inhibited for about 20 days. The nonionic surfactant LA-$C_{12/14}EO_{20}$ was completely degraded by sludge from Wil and Kloten/Opfikon within 10 days. It was not degraded at all by sludge from Au-Bruggen within 42 days and the DOC was only removed by 24 %. This is in line with the results of Chapter 3 obtained with sludge from Au-Bruggen. The SBA-C_8EO_{10} was slowly degraded to about 50 % within 42 days by sludge from Au-Bruggen and Wil. A lag-phase of about 30 days was observed for sludge from Kloten/Opfikon, which was followed by a partial degradation of 50 %.

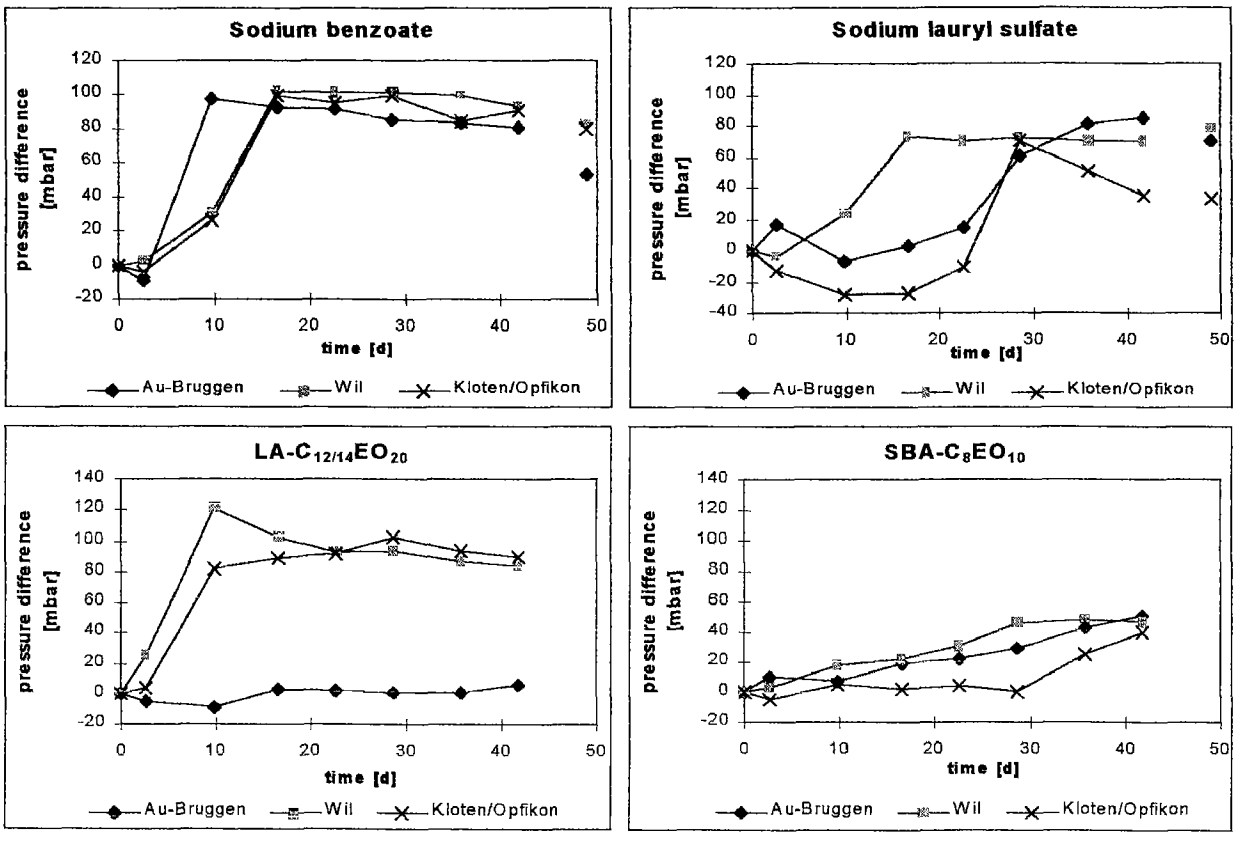


Figure 24: Anaerobic degradation of benzoic acid, lauryl-sulfate, and the nonionic surfactants SBA-C_8EO_{10} and LA-$C_{12/14}EO_{20}$ using anaerobic sludge from the wastewater treatment plants Au-Bruggen, Wil and Kloten/Opfikon (all in Switzerland).

The best degradation results were obtained with sludge from WTP Wil. Small lag-phases, no inhibitory effects and a high degradation activity characterized them for one anionic and two non-ionic surfactants. Based on its performance the anaerobic sludge from WTP Wil was chosen for further degradation experiments with different technical AEO. The results were compared with the results obtained in Chapter 3 with sludge from WTP Au-Bruggen for the same set of AEO.

Degradation experiments with sludge from WTP Wil

The endogenous biogas production of the sludge from WTP Wil was reproducible and the 95 % confidence interval was small (± 7 mbar). Glucose was used as a reference with a COD in the test assay of 150 mg $O \cdot L^{-1}$. It was completely degraded within 15 days (Figure 25). Complete degradation was supposed to be reached when more than 60 % of the theoretical methane or C_1-biogas was produced (Pagga & Beimborn, 1993). Substances, which reached between 20 % and 60 % of the theoretical methane- or C_1-biogas pressure were judged as partly degradable.

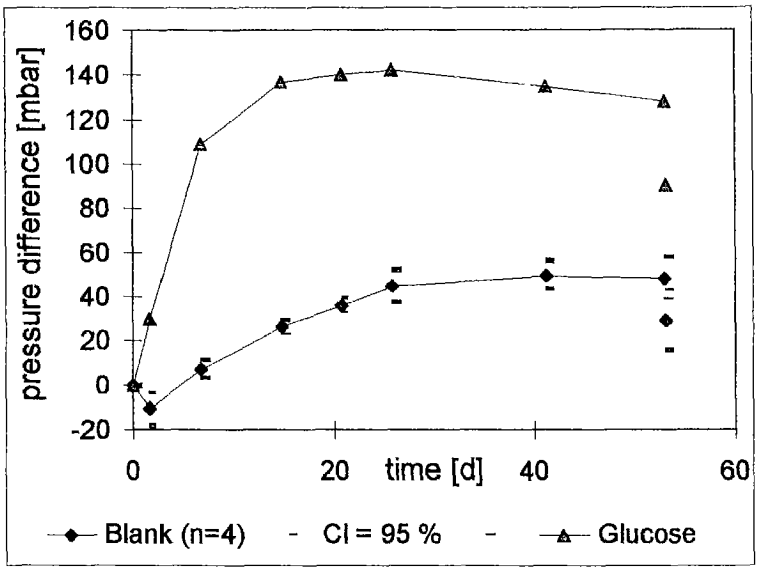


Figure 25: Biogas production from glucose and endogenous biogas production (Blank) with anaerobic sludge from WTP Wil (B). The last value off line represents the methane pressure after addition of sodium hydroxide solution.

The linear alcohol ethoxylates (LA-$C_{12/14}EO_X$) were completely degraded within 25 days (Figure 26). The lag-phases increased with decreasing EO-chain length from 7 to 16 days. A small inhibition of the endogenous biogas production was observed for LA-$C_{12/14}EO_5$. This was expected, because the test substance concentration was in the toxic range of the effect

concentrations for LA-$C_{12/14}EO_5$ (EC_{50} = 30 mg·L^{-1}) (Chapter 3). While the linear alcohol ethoxylate LA-$C_{14/15}EO_{10}$ was degraded to 70 % within 17 days, the LA-$C_{9\text{-}11}EO_{10}$ was only partly degraded to about 40 % within 25 days.

The single-branched alcohol ethoxylates (SBA-$C_{14/15}EO_{X = 5, 10, 20, 40}$; SBA-$C_8EO_{10}$) were partly degraded to 50 % - 60 % within 20 days. A small lag-phase of 7 days was observed for the EO 5 adduct.

The multiple-branched alcohol ethoxylates (MBA-$C_{10}EO_{10}$ and MBA-$C_{13}EO_X$) were partly degraded within 20 days. The degradability decreased with increasing EO chain length. A small inhibition was observed for the 5 EO adduct during the first 15 days. The small amount degraded as determined for ethoxylates of multiple-branched alcohols, were paralleled by a small DOC removal.

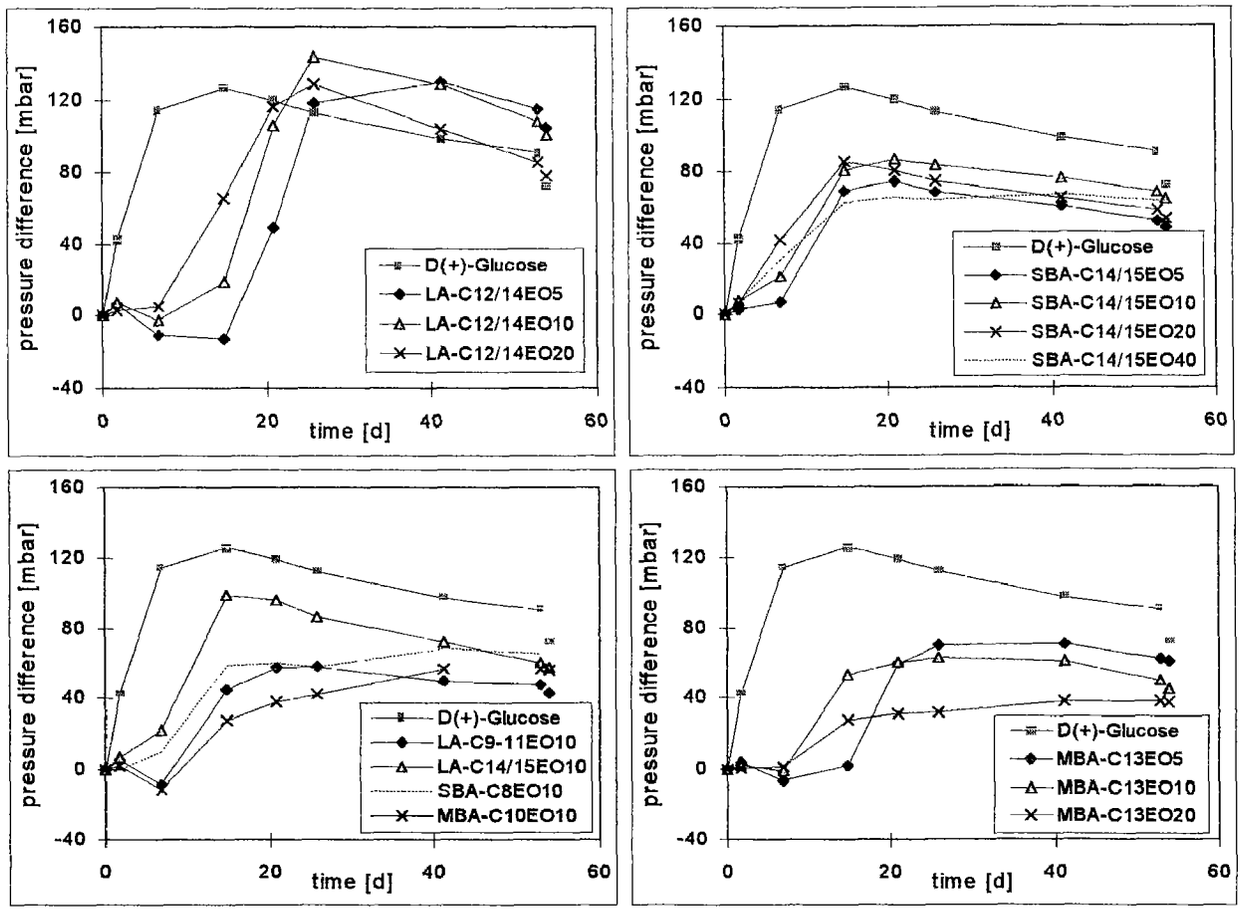


Figure 26: Degradation of technical AEO with anaerobic sludge from the WTP Wil, Switzerland.

The theoretical methane pressure (Baumann & Schefer 1990), the theoretical C_1-biogas pressure (Shelton & Tiedje 1984), and the removal of DOC were calculated and summarized in Table 22. A good anaerobic biodegradability (> 60 % of theoretical CH_4 or C_1-biogas) was found for 4 out of 5 ethoxylates of alcohols with a high linearity and for 2 out of 4 ethoxylates

of single-branched alcohols (SBA). For all of them the DOC removal was above 85 %. SBA-$C_{14/15}EO_5$ and SBA-$C_{14/15}EO_{40}$ was degraded to about 50 %. The DOC removal of 83 % and 92 % was similar to other SBA-EO.

Table 22: Anaerobic biodegradability of technical AEO with sludge from WTP Wil, Switzerland, calculated as % of the theoretical CH_4- and C_1-biogas production, and the DOC removal.

	CH_4 (COD) %	C_1-biogas (S&T) %	DOC removal %
D(+)-Glucose (reference)	75.1	72.7	100.2
LA-$C_{12/14}EO_5$	89.2	87.6	88.2
LA-$C_{12/14}EO_{10}$	100.8	92.6	86.2
LA-$C_{12/14}EO_{20}$	88.4	82.8	84.8
LA-$C_{9\text{-}11}EO_{10}$	39.3	37.3	72.2
LA-$C_{14/15}EO_{10}$	71.0	68.9	85.6
SBA-$C_{14/15}EO_5$	52.7	53.6	83.4
SBA-$C_{14/15}EO_{10}$	61.4	61.5	84.9
SBA-$C_{14/15}EO_{20}$	59.1	60.9	90.6
SBA-$C_{14/15}EO_{40}$	49.2	47.2	92.3
SBA-C_8EO_{10} (2-ethylhexanol)	49.9	49.4	61.7
MBA-$C_{10}EO_{10}$ (Isodecanol)	42.4	40.2	58.8
MBA-$C_{13}EO_5$ (Isotridecanol)	51.7	48.1	76.9
MBA-$C_{13}EO_{10}$ (Isotridecanol)	42.9	40.0	74.8
MBA-$C_{13}EO_{20}$ (Iisotridecanol)	27.9	24.3	59.0

The anaerobic biodegradability of multiple-branched alcohols (MBA) was 50 %, 40 % and 25 %, for ethoxylation degrees of 5, 10, and 20 EO, respectively. The DOC removal decreased similarly from 77 % to 59 % with increasing degree of ethoxylation (Table 2)

Discussion

The anaerobic biodegradability of ethoxylates of linear, single-branched and multiple-branched alcohols decreased with increasing branching of the alcohols. This result is in line with aerobic biodegradation studies (Dorn et al., 1993; Talmage, 1994; Siegfried & Baumann, 1995). For the ethoxylates of branched alcohols both aerobic and anaerobic biodegradation are slower and incomplete. Therefore, ethoxylates of multiple-branched alcohols should be avoided, when a good aerobic and anaerobic biodegradability is required.

The ethoxylates of linear alcohols LA-$C_{12/14}EO_X$ (x = 5, 10, 20) were anaerobically well degraded at low concentrations (COD = 150 mg·L^{-1}) within 20 days using sludge from WTP Wil. The rapid degradation with a short lag phase of only few days, suggest that the sludge was adapted to these chemicals. For linear AEO positive degradation results were also obtained with sludge from WTP Au-Bruggen (Chapter 3), whilst longer lag-phases were observed. Wagener and Schink (1987/1988) and Steber and Wierich (1987/1991) also reported positive degradation results but long lag-phases. Lag-phases of 75 days were seen by Wagener and Schink (1987) for the degradation of the linear AEO Brij 35 (LA-$C_{12}EO_{23}$) at high concentration (1 g·L^{-1}) in a fixed bed reactor. Salanitro and Diaz (1995) observed lag-phases of 19 and 26 days for the degradation of LA-$C_{9-11}EO_8$ at 50 mg C/g/L with two different anaerobic sludges (Hornsby Bend and Village Creek). In both studies the concentration of AEO seemed to influence the lag-phases of the degradation of the linear alcohol ethoxylates.

LA-$C_{9-11}EO_{10}$ was degraded to 40 %, which is below the results of Salanitro & Diaz (1995). They found a degradability of 60 % and 83 % (theoretical CH_4) for a similar LA-$C_{9-11}EO_8$ with two different sludges. Based on observed inhibitory effects in their test systems at a concentration of 50 mg C/g/L, these authors recommended test concentrations for surfactants in the range of 10 mg/g/L. The AEO concentrations in anaerobic digesters range between 0.5 and 1.8 mg/g dry solid. Our experiments dealt with concentrations of about 10 to 25 mg/g dry solid. At these concentrations no or only small toxic effects have to be expected for the alcohol ethoxylates investigated (Müller et al. 1996).

The degradation results obtained with two different sludges are compared in Table 23. While none of the ethoxylates of branched alcohols were significantly degraded by the sludge from WTP Au-Bruggen, they were at least partially degraded by anaerobic sludge from WTP Wil. We therefore confirm the statement of Steber and Birch, who say that '...a positive degradation result has a high predictive value for the real environmental situation. On the other hand, a poor degradation result must not be considered as a proof of anaerobic recalcitrance' (Henkel & Unilever, 1995). Negative degradation results, long lag-phases and inhibitory effects could be reduced here besides by lowering substance concentrations also by using sludge from a different WTP. In case substances are not degraded within the average hydraulic retention time of an anaerobic digester (about 30 days), longterm adaptation in the laboratory may still lead to degradation. However, it must be kept in mind that data from such adaptation experiments may not be relevant for a conventional WTP.

Table 23: Comparison of results of anaerobic degradation experiments with technical AEO using sludges from WTP Au-Bruggen (Chapter 3) and Wil, both in Switzerland. The anaerobic biodegradability was calculated in % of the theoretical CH_4-pressure and the theoretical C_1-biogas pressure. n.s.d: not significantly degradable.

	sludge from Au-Bruggen		sludge from Wil	
	CH_4 %	C_1-biogas %	CH_4 %	C_1-biogas %
D(+)-Glucose (reference)			75.1	72.7
PEG 400 (reference)	105.3	82.8		
LA-$C_{12/14}EO_5$	56.6	83.4	89.2	87.6
LA-$C_{12/14}EO_{10}$	49.5	90.4	100.8	92.6
LA-$C_{12/14}EO_{20}$	29.2	93.9	88.4	82.8
LA-$C_{9-11}EO_{10}$	21.5	25.2	39.3	37.3
LA-$C_{14/15}EO_{10}$	16.7	25.2	71.0	68.9
SBA-$C_{14/15}EO_5$	n.s.d	n.s.d	52.7	53.6
SBA-$C_{14/15}EO_{10}$	n.s.d	n.s.d	61.4	61.5
SBA-$C_{14/15}EO_{20}$	n.s.d	n.s.d	59.1	60.9
SBA-$C_{14/15}EO_{40}$	n.s.d	n.s.d	49.2	47.2
SBA-C_8EO_{10} (2-ethylhexanol)	n.s.d	n.s.d	49.9	49.4
MBA-$C_{10}EO_{10}$ (Isodecanol)	n.s.d	n.s.d	42.4	40.2
MBA-$C_{13}EO_5$ (Isotridecanol)	n.s.d	n.s.d	51.7	48.1
MBA-$C_{13}EO_{10}$ (Isotridecanol)	n.s.d	n.s.d	42.9	40.0
MBA-$C_{13}EO_{20}$ (Iisotridecanol)	n.s.d	30.0	27.9	24.3

Conclusions

Linear alcohol ethoxylates are anaerobically well degradable. With increasing branching of the alcohol from single- to multiple-branched alcohols the anaerobic degradability decreased. The ethoxylation degree did not influence the anaerobic degradability of AEO significantly. The anaerobic biodegradability of branched AEO was poor under laboratory conditions.

Chapter 5

Liposome-water and octanol-water partitioning of alcohol ethoxylates

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Abstract

Liposome-water partitioning coefficients, K_{lipw}, were determined for eight pure alcohol ethoxylates using equilibrium dialysis and ultracentrifugation. Both methods yielded statistically indistinguishable results. The experimentally determined log K_{lipw} were compared with log K_{ow}-values estimated with the fragment method using different literature sources for the fragment constants. Fragments of log K_{lipw} were calculated for the ethoxy-group (EO) and the CH_2-units from the experimentally determined data. An additional CH_2-unit causes an average increase of log K_{lipw} by 0.45, and an additional EO causes an average decrease of log K_{lipw} by -0.12. With these fragments, the quality of log K_{lipw} estimations can be improved significantly as compared to simple linear regression of log K_{lipw} versus log K_{ow}. The K_{lipw}-values calculated according to the new fragment method for pure compounds and for commercial mixtures are shown to be adequate descriptors for QSARs of bioaccumulation, toxicity, and sorption to natural organic material.

Introduction

The octanol water partition coefficient, K_{ow}, is the most widely used physicochemical parameter for describing hydrophobic partitioning in Quantitative Structure Activity and Toxicity Relationships (QSAR and QSTR). Hydrophobicity is of major importance for the uptake of a compound by biological organisms and for the sorption to dissolved and particular natural organic matter (NOM).

Octanol appears to be a better surrogate for NOM and biological material than other solvents, e.g. hexane, because it is an amphiphilic molecule just like NOM and membrane lipids, and capable of accommodating a greater variety of more or less hydrophobic molecules. However,

no bulk solvent can adequately describe the specific interactions of polar, charged, or amphiphilic compounds with the ordered three dimensional structure of NOM or bio-membranes.

For example, as we have shown in earlier work, the uptake of hydrophobic ions is underestimated by two or more orders of magnitude by K_{ow} as compared to the corresponding liposome-water partition coefficient K_{lipw} (Escher & Schwarzenbach 1996). Liposomes (Bangham et al. 1965) are artificial lipid bilayer vesicles that take account of the ordered structure and anisotropy of biological membranes. The liposome-water test system has been proven to be particularly well suited for describing the membrane-water partitioning behavior of hydrophobic ionizable compounds (Escher & Schwarzenbach 1996, Ottiger & Wunderli-Allenspach 1997).

The situation is more complex for surface-active compounds, not only for ionic but also for neutral surfactants such as the linear alcohol ethoxylates (AEO) investigated in this study. Surfactants strongly interact with each other and with octanol even below their critical micelle concentration (CMC) (Schwarzenbach et al. 1993). Surfactants enhance the mutual solubilities of octanol and water by formation of co-micelles. Consequently, the apparent K_{ow} of surfactants is strongly dependent on their concentration (Morall et al. 1996), and needs to be extrapolated to infinite dilution to obtain the true thermodynamic partition coefficient (Roberts & Marshall 1995). Furthermore, surfactants accumulate at interfaces. Accumulation at the octanol-water interface illustrates the inadequacy of a bulk solvent as surrogate for biomembranes or NOM, which have a high surface-to-volume ratio. In addition, emulsions are formed when one attempts to measure K_{ow} by the commonly used shake-flask method (OECD, 1995). Emulsions, which pose severe experimental problems, can be minimized with the slow stir method (Roberts & Marshall 1995; De Bruijn et al. 1989) but not principally avoided.

An alternative method is the prediction of K_{ow} from capacity factors of reversed-phase High Pressure Liquid Chromatography (HPLC) on octadecane-coated silica particles. However, for AEO, the capacity factors predict a slight increase in K_{ow} with increasing ethoxy chain length (Leeke et al. 1996). This trend is opposite to what is expected from K_{ow} predictions, where K_{ow} decreases with increasing ethoxy chain length. Hence, the HPLC method is not suitable for hydrophobicity estimations of the surface active AEO.

Most authors use K_{ow}-values estimated with the fragment method (Schwarzenbach et al. 1993, Hansch & Leo 1979) as descriptors in QSARs for AEO and alkylphenol ethoxylates (e.g. Roberts & Marschall 1995; Schüürmann 1991). Since it is impossible to validate the estimation methods by comparison to measured values, there is no consensus on the fragment values.

Values for the ethoxy fragment vary from -0.064 to -0.34 (Roberts & Marschall 1995; Hansch & Leo 1979, Lindgren et al 1996, Ahel & Giger 1993). In recent years, the fragment value of -0.1 has been applied most often. This fragment value was estimated by Schüürmann (1990) indirectly from the correlation of toxicity data to K_{ow} for longer non-terminal EO units assuming non-polar narcosis as mode of toxic action of these compounds. Shortly thereafter, this value was confirmed by Roberts (1991) by directly measured log K_{ow}-data of short ethoxylates.

AEO were chosen in this study because they represent the most important class of nonionic surfactants with the highest production volumes (Talmage 1993). Due to their good aerobic biodegradability they are usually removed in wastewater treatment plants (McAvoy et al. 1996) but they may also reach the anaerobic reactor adsorbed to primary sludge where biodegradation is delayed (Federle & Schwab 1992; Steber & Wierich 1987). AEO cover a wide range of hydrophobicity, and most of them have a potential to bioaccumulate (log $K_{ow} > 3$). AEO are known to interact with biological membranes (Florence et al. 1984). At environmentally relevant concentrations they act according to narcosis, the baseline toxicity common to all non-specifically acting compounds (Schüürmann 1991; Roberts 1991). Toxicity towards aquatic organisms increases with increasing length of the alkyl chain and decreasing degree of branching (Kaluza & Taeger 1996). Linear AEO are more toxic than the corresponding branched AEO.

In this study, liposome-water partition coefficients K_{lipw} of a series of pure linear AEO were measured with egg yolk lecithin as model lipid. Phosphatidylcholine was chosen as lipid head group because it is a zwitterion throughout all experimental conditions and because its electronic properties allow both electron donor and electron acceptor interactions with the sorbate. The fatty acids of egg yolk lecithin contain mainly long-chain fatty acids ($\approx$16 carbon units) and approximately half of the fatty acids are unsaturated. Despite this complex composition this mixture was preferred over single phospholipids that are typically used in liposome-water partitioning work, such as DMPC (dimyristoylphosphtidylcholine), because of its lower transition temperature, larger hydrophobic domain of the resulting bilayer, and because such a mixture resembles more closely to biological membranes than single phospholipids.

The measured K_{lipw}-values were compared to K_{ow} obtained with different fragment methods. A fragment method for the calculation of K_{lipw} of AEO was developed from the experimental data. The results clearly show that K_{lipw} is superior over K_{ow} as descriptor in QSAR. We therefore propose to use K_{lipw} instead of K_{ow} for QSARs, QSTRs, and risk assessment for surfactants.

Materials and Methods

Chemicals

Pure linear alcohol ethoxylates with an alkyl chain length of C_8 to C_{16} and an ethoxylation degree of 1 to 9 mol EO per mol alcohol were purchased from Fluka AG, Buchs, Switzerland. Two higher ethoxylated alcohols ($C_{14}EO_{11}$ and $C_{14}EO_{14}$) were obtained from J. Tolls (RITOX, Utrecht University, Utrecht, The Netherlands). Their physico-chemical characteristics, such as molecular weight (MW), critical micelle concentration (CMC), and hydrophilic-lipophilic balance (HLB) as well as two different estimations of the octanol-water partition coefficient (K_{ow}), are listed in Table 24.

The following chemicals were purchased from Fluka AG, Buchs, Switzerland: 3-*sn*-phosphatidylcholine (> 99%; fatty acid composition: 16:0 ≈ 33%, 18:0 ≈ 14%, 18:1 ≈ 30%, 18:2 ≈ 14%, 20:4 ≈ 4 %) from fresh egg yolk; 1-naphthoylchloride (purum ≈ 97%); 1-methylimidazole; methanol (HPLC grade); acetonitrile (HPLC grade); 3-(N-morpholino) propane sulfonic acid (MOPS), pK_a=7.2.

Table 24: Physico-chemical characteristics of alcohol ethoxylates (AEO) used in the experiments. MW: molecular weight, CMC: critical micelle concentration, HLB: hydrophilic lipophilic balance, K_{OW}: octanol-water partition coefficient

AEO	origin	MW [g/mol]	log CMC [a] [mol/L]	HLB [b] EO weight%/5	log K_{OW} [c] Roberts 1995	log K_{OW} [c] PACO
C_8EO_5	Fluka AG, Buchs, CH	350	-2.11	13.5	2.67	2.03
$C_{10}EO_5$	Fluka AG, Buchs, CH	378	-3.11	12.5	3.75	3.11
$C_{10}EO_8$	Fluka AG, Buchs, CH	510	-2.98	14.5	3.45	2.33
$C_{12}EO_5$	Fluka AG, Buchs, CH	406	-4.11	11.7	4.83	4.19
$C_{12}EO_8$	Fluka AG, Buchs, CH	538	-3.95	13.7	4.53	3.41
$C_{14}EO_5$	Fluka AG, Buchs, CH	434	-5.11	10.9	5.91	5.27
$C_{14}EO_8$	Fluka AG, Buchs, CH	566	-4.93	13.0	5.61	4.49
$C_{14}EO_{11}$	J. Tolls, RITOX, NL	698	-4.74	14.4	5.31	3.71
$C_{14}EO_{14}$	J. Tolls, RITOX, NL	830	-4.56	15.3	5.01	2.93
$C_{16}EO_8$	Fluka AG, Buchs, CH	594	-5.90	12.4	6.69	5.57

[a] The CMC was calculated according to Huibers et al. (1996).

[b] The hydrophilic lipophilic balance (HLB) is defined as follows: $HLB = \frac{\frac{\text{weight of EO - chain}}{\text{weight of the AEO - molecule}} * 100}{5}$

[c] The octanol-water partition coefficient K_{ow} was calculated with fragment values described by Roberts (1995) and by the PACO program (Lindgren et al. 1996): C_{12}-alcohol: 5.19 (Roberts 1995), $C_{12}EO_1$: 5.23 (Roberts 1995); EO fragment: -0.1 (Roberts 1995) or -0.26 (PACO); -CH_2- fragment: 0.54 (Roberts 1995).

Preparation of liposomes

For the preparation of liposomes, 3-sn-phosphatidylcholine dissolved in $CHCl_3$ was dried down to a film on a round bottom flask in a rotary evaporator. Residual traces of solvent were removed under high vacuum. The film was hydrated by shaking with aqueous buffer solution (0.01 mol·L^{-1} MOPS, pH 7.0, 1 % ($^{V}/_{V}$) of a saturated $AgCl_{aq}$ solution) to a final concentration of about 10 g·L^{-1}. The resulting suspension of multilamellar vesicles (MLV) was then exposed to ultrasonic treatment to form small unilamellar vesicles (SUV). A titanium probe (Branson Ultrasonic Co., Sonifier 450) was immersed into the lipid suspension, which was cooled in an ice bath to avoid heating. The suspension was sonicated 4 to 6 times for 1 minute at 50 W. To remove large lipid vesicles and titanium residues from the probe, the suspension was centrifuged at 20'000 *g* for 45 minutes (Escher & Schwarzenbach 1996).

Characterization of liposomes

The phospholipid content of the liposome suspension, [lip], was determined spectrophotometrically for each batch of liposomes after mineralization to inorganic phosphate and formation of a malachite green / molybdenum / phosphate complex according to Petitou (Petitou et al., 1978). Between 5 and 10 % of the liposomes were lost during preparation. The radii of the membrane vesicles were determined with a ZetaSizer (Malvern Instruments, England). The average radii of the liposomes were 120 ± 30 nm.

Determination of the liposome-water partition coefficient K_{lipw}

Sorption isotherms were determined by equilibrium dialysis (Figure 27) and by an ultracentrifugation method (Figure 28).

For the equilibrium dialysis system, the dialysis cells were made of glass and consisted of two half-cells, each of 1.2 mL volume, separated by a dialysis membrane made of regenerated cellulose with a cut-off of 10'000 to 20'000 Dalton (Thomapor, Reichelt Chemie Technik, Heidelberg, Germany). A single experiment required two dialysis cells, a reference cell and a measurement cell. One half-cell of each cell was filled with a AEO solution, the other one with buffer or a liposome suspension as reference or measurement cell, respectively. After dialyzing in a rotating tumbler shaker for 40 hours at 20° C, the AEO concentration in all half-cells without liposome suspension were analyzed with reversed phase HPLC with UV detection after derivatisation with 1-naphthoylchloride (see below). For each concentration, the experiment was performed in triplicate.

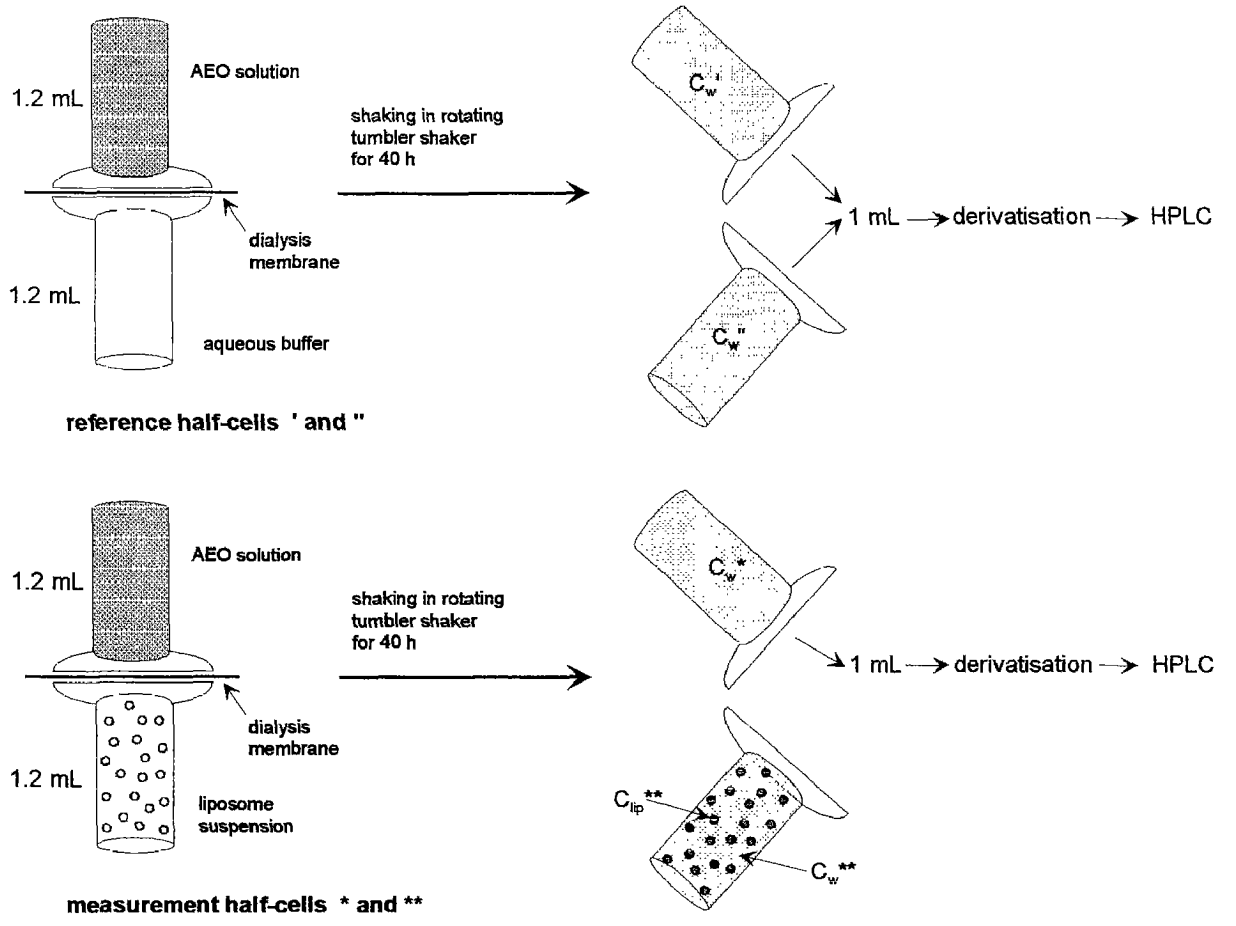


Figure 27: Determination of K_{lipw} by equilibrium dialysis.

In the linear range of the sorption isotherms, liposome-water partition ratios K_{lipw} were calculated according to Equation 17: C_w' is the molar concentration of AEO in the reference half-cell after the equilibrium was reached, C_w^* is the concentration of AEO in the liposome free compartment of the measurement half-cell, and [lip] is the concentration of the liposomes in $kg \cdot L^{-1}$, calculated per total volume of both half-cells. In most cases, [lip] accounted for less than 1% of the total volume so that changes of volume were not considered.

Equation 17:
$$K_{lipw}\left(L \cdot kg^{-1}\right) = \frac{C_{lip}^{**}}{C_w^*} = \frac{C_w' - C_w^*}{C_w^* \cdot [lip]}$$

$$[K_{lipw}] = L \cdot kg^{-1}$$
$$[C_w', C_w^*] = mol \cdot L^{-1}$$
$$[C_{lip}^{**}] = mol \cdot kg^{-1}$$
$$[lip] = kg \cdot L^{-1}$$

The liposome-water partition coefficients of certain alcohol ethoxylates were determined with an ultracentrifugation method because of either problems with the determination of K_{lipw} using dialysis half-cells ($C_{12}EO_5$, $C_{14}EO_5$, $C_{14}EO_8$) or high liposome concentrations and low K_{lipw} of

the substance (C_8EO_5). The K_{lipw} of some compounds ($C_{12}EO_5$, $C_{14}EO_5$) were determined with both methods for comparison and validation of the ultracentrifugation method.

These experiments were performed in 13.5 mL ultracentrifugation tubes (Polyallomer tubes, thick wall, 16 x 76 mm, Figure 28). The ultracentrifugation was performed at about 250'000 g (45'000 rpm) for 90 minutes.

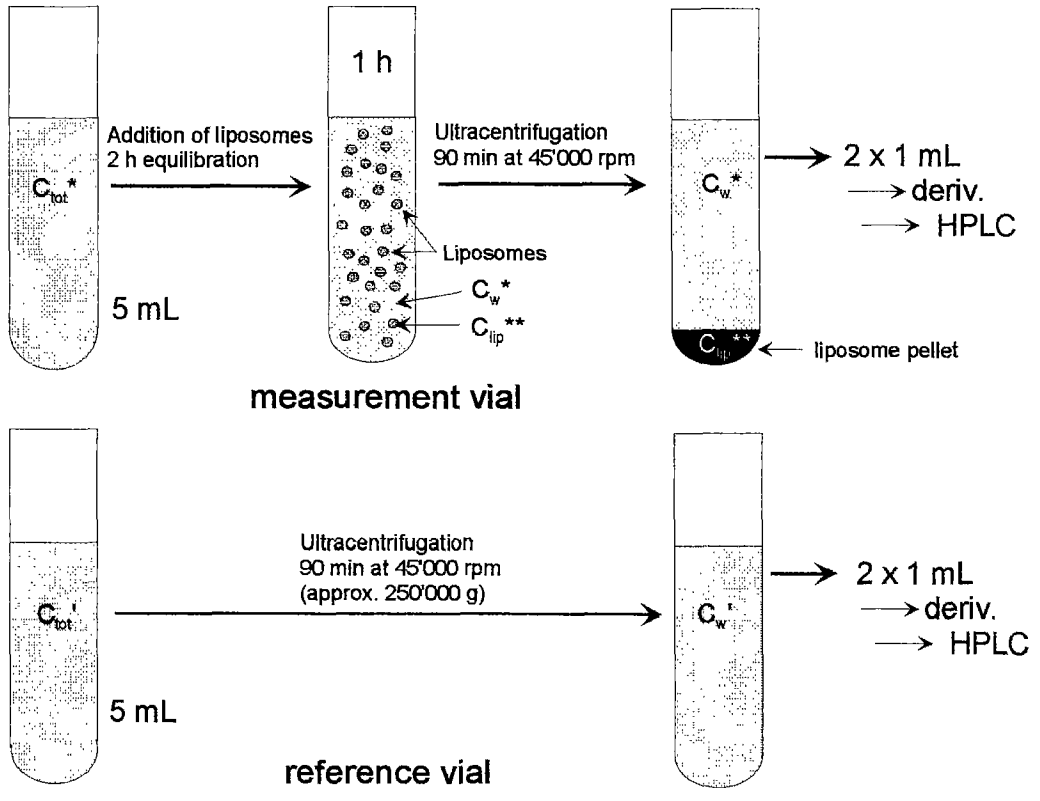


Figure 28: Experimental setup for the determination of K_{lipw} by the centrifugation method.

Model for the calculation of K_{lipw} for non-equilibrium situations

It was not possible to perform all experiments below the critical micelle concentration (CMC) due to the detection limit of the analytical method used. The sorption isotherms were determined with different AEO concentrations and different liposome concentrations.

A model was developed for the evaluation of equilibrium dialysis experiments, which were performed below and above the CMC of the surfactants investigated. The CMC is a substance property, which depends strongly on the hydrophobicity of the surfactant. For alcohol ethoxylates the CMC decreases with increasing length of the alkyl chain. The detection limit of the analytical method was in the range of the CMC for the more hydrophobic AEO. Therefore, some experiments had to be conducted at concentrations above the CMC.

At concentrations above the CMC, the sorption isotherms become non-linear due to co-micellization and solubilization of the membrane. Consequently, the experimental condition had to be chosen so that no micelles were in direct contact with the liposomes.

This condition was achieved by choosing a dialysis time that was long enough to allow equilibration of the dissolved fraction of the surfactant but short enough that micelles were not formed in the initially micelle-free compartment (Figure 29). Since only the truly dissolved surfactant can penetrate the dialysis membrane, the time to reach equilibrium between the micelle concentrations on both sides of the membrane is much longer than the time to reach equilibrium of the truly dissolved concentrations. A dialysis time of about 40 hours fulfilled the desired condition.

The general mass balance for the reference half-cells and the test half-cells are as follows:

Equation 18: $C_{tot}\,(reference) = C_{tot}' + C_{tot}'' = C_w' + C_{micelle}' + C_w''$

Equation 19: $C_{tot}\,(test) = C_{tot}^* + C_{tot}^{**} = C_w^* + C_{micelle}^* + C_w^{**} + C_{liposome}^{**} \cdot [lip]$

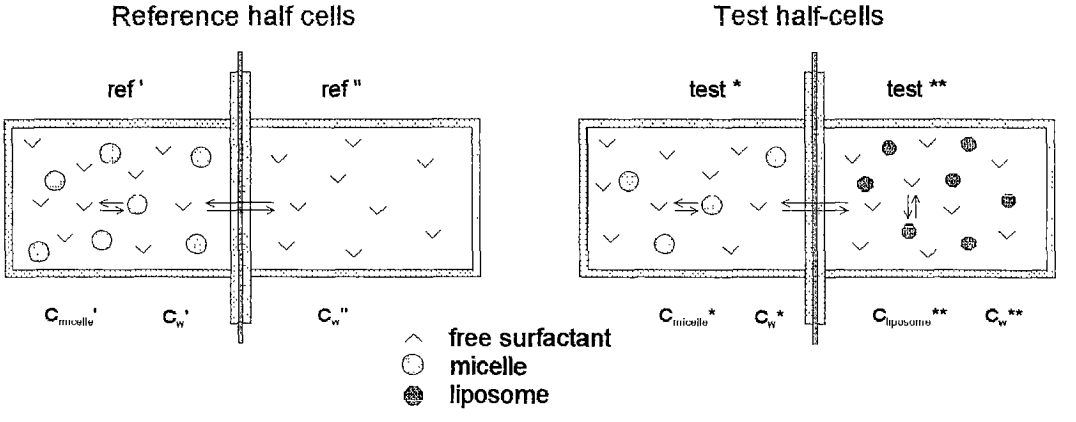


Figure 29: General situation in equilibrium dialysis experiments with surfactants, that are able to form micelles.

The following prerequisites have to be fulfilled for the experiments:

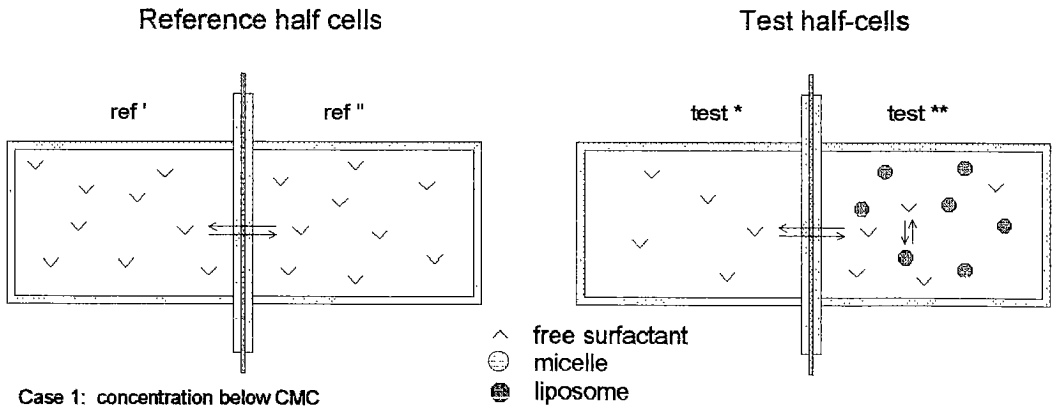
1) The equilibrium over the dialysis membrane was reached within 40 hours for the dissolved surfactants ($C_w' = C_w''$ and $C_w^* = C_w^{**}$) as long as no micelles were formed. The presence of micelles was indicated by an non-equilibrium situation in the reference cells ($C_{tot}' \neq C_{tot}''$).
2) Within 40 hours, no significant amount of micelles was formed in the cells '' and ** that contained only buffer or liposome suspension at the beginning of the experiment.
3) The following four cases have to be distinguished:

Case 1: surfactant concentrations < CMC (for AEO with alkyl chain < C_{13}) (Figure 4a).

$C_{micelle} = 0$ and $C_{tot}' = C_{tot}''$

$C_{liposome}^{**} \cdot [lip] = C_{tot}' - C_{tot}^{*}$

K_{lipw} can be calculated and determined from the slope of the sorption isotherm.



Case 1: concentration below CMC

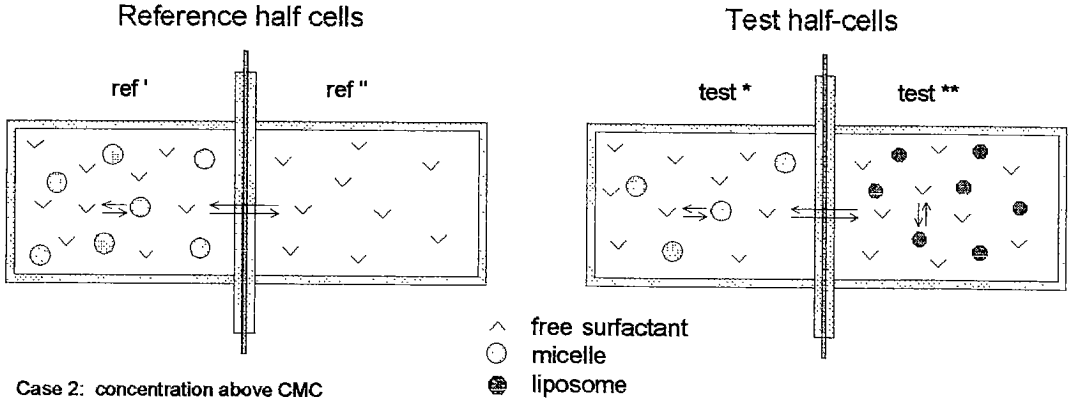
Case 2: surfactant concentrations > CMC (for AEO with alkyl chain > C_{13}) (Figure 4b).

$C_w' = C_w'' = C_w^{*} = C_w^{**} = CMC$

$C_{tot}^{*} = C_w^{*} + C_{micelle}^{*} = C_w'' + C_{micelle}^{*}$

$C_w^{*} = C_w^{**} = C_{tot}^{*} - C_{micelle}^{*}$ and $C_{liposome}^{**} \cdot [lip] = C_{tot}\,(test) - C_{tot}^{*} - C_w'$

The sorption isotherm is shifted by $C_{micelle}^{*}$ on the x-axis. K_{lipw} can be determined directly from the slope of the sorption isotherm (Figure 31a & Figure 31c). The corrected sorption isotherm is obtained after subtracting $C_{micelle}^{*}$ from C_{tot}^{*} (Figure 31b & Figure 31d).



Case 2: concentration above CMC

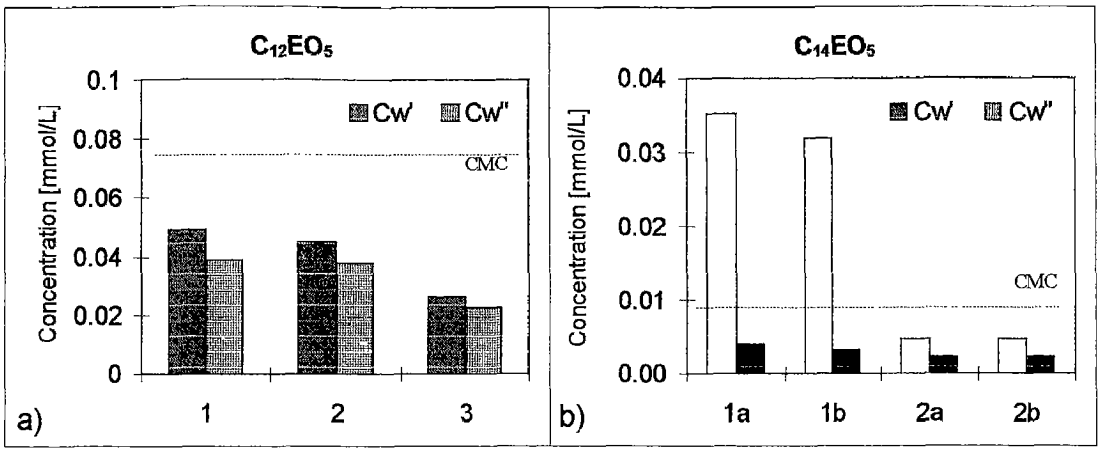


Figure 30: Comparison of experiments which were performed with concentrations below the CMC (Case 1: a) 1-3, b) 2a-2b) and above the CMC (Case 2: b) 1a-1b). The broken line corresponds to the CMC (Huibers et al. 1996). C_w' and C_w'' correspond to the concentration in the two reference half-cells.

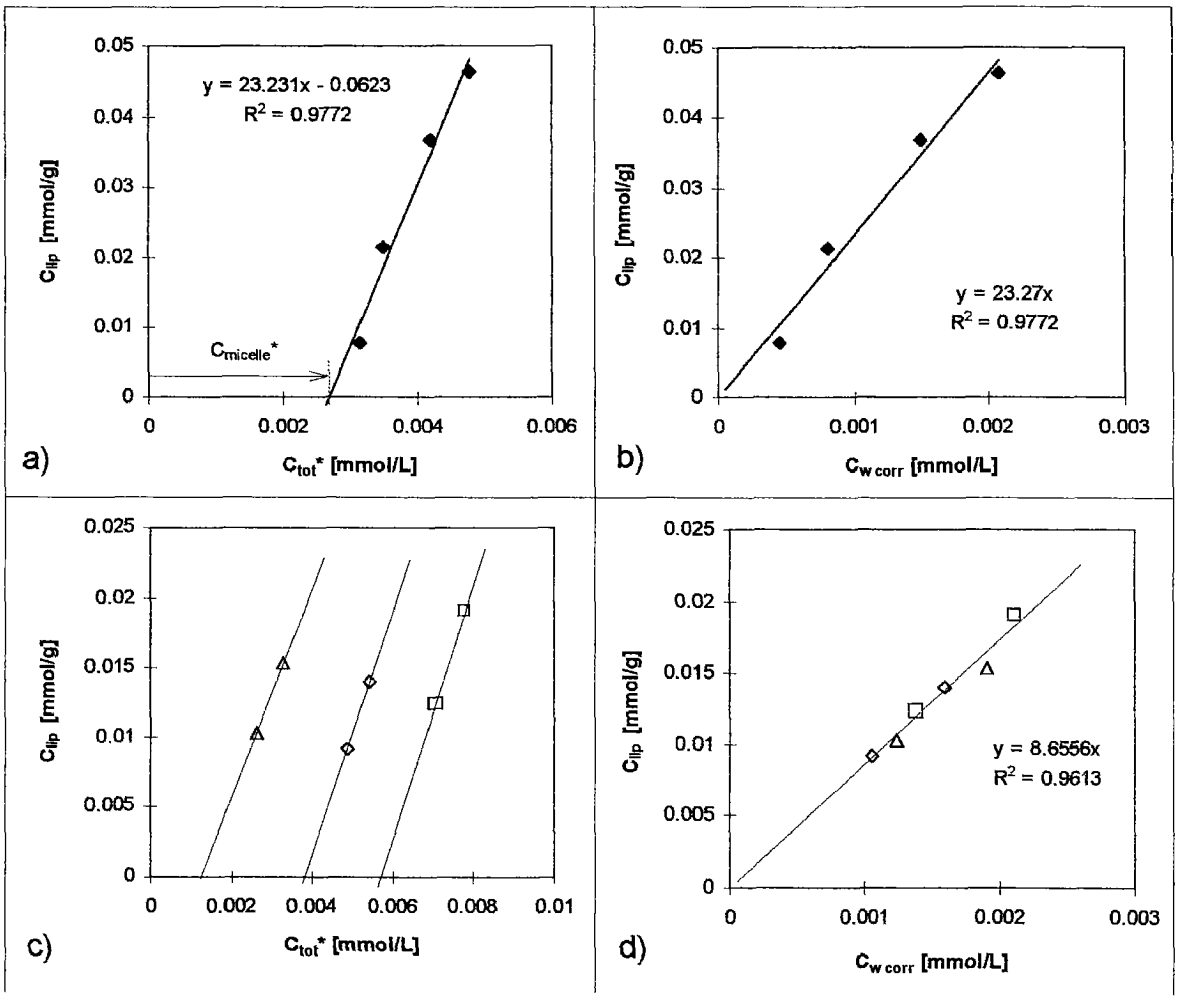
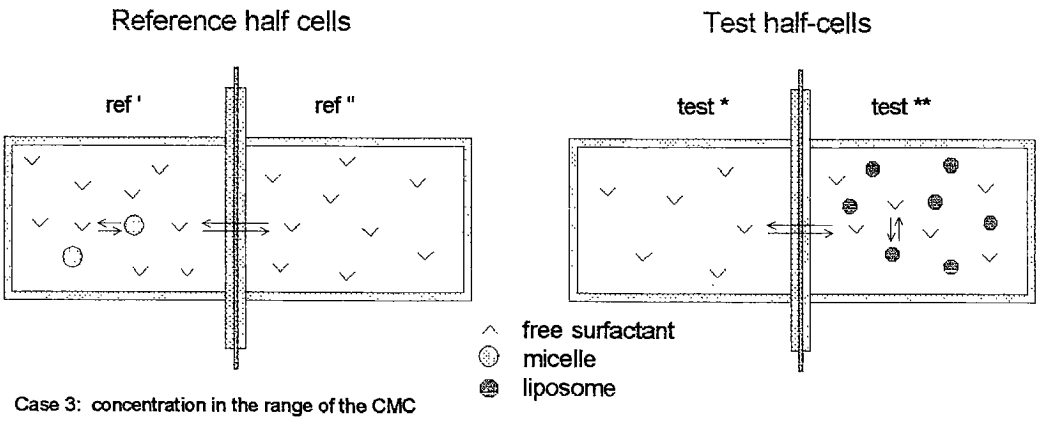


Figure 31: Correction of sorption isotherms for C_{14}-AEO according to Case 2: a) one concentration of $C_{14}EO_8$ and four different liposome concentrations; c) three concentrations of $C_{14}EO_{14}$ and two different liposome concentrations; corrected sorption isotherms for b) $C_{14}EO_8$ and d) $C_{14}EO_{14}$: $C_{w\,corr} = C_{tot}^* - C_{micelle}^*$.

Case 3: concentration ≅ CMC (micelles in reference half-cells, but not in test half-cells)

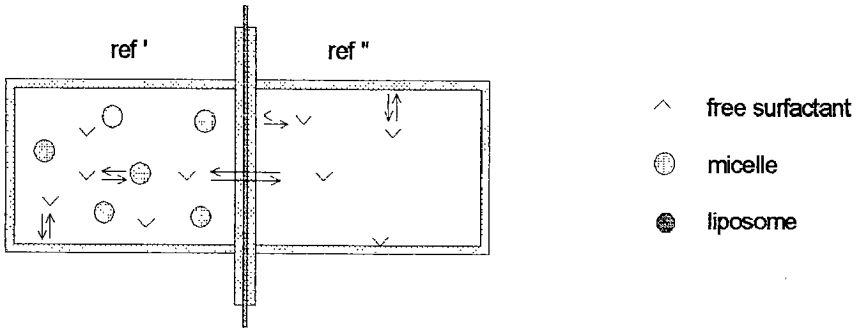
$C_w' = C_w'' = CMC > C_w^* = C_w^{**}$ and $C_{micelle}^{**} = 0$

K_{lipw} can not be determined.



Case 3: concentration in the range of the CMC

Case 4: With increasing hydrophobicity of the AEO (e.g. C_{16}-alcohols) sorption effects to the test systems and to the dialysis membrane became predominant. In this case it was not possible to determine K_{lipw}.



Case 4: low CMC and high hydrophobicity of surfactant

Quantification of alcohol ethoxylates by HPLC

The derivatisation of AEO with 1-naphthoylchloride leads to esters that can be quantified with high sensitivity using UV or fluorescence detection (Marcomini & Zanette, 1994). After derivatisation, methylimidazole was added to remove HCl. The derivatisation reaction was tested with AEO varying in alkyl chain length from C_{12}-C_{18} and a degree of ethoxylation of 0 - 20 mol EO per mol alcohol (Lux & Schmitt, 1996).

1 mL samples were transferred into mini-vials and evaporated to dryness on a heating block at a temperature of 80 °C for 90 minutes under a stream of nitrogen. After cooling to room temperature, 920 µL acetonitrile, 20 µL methylimidazole and 10 µL 1-naphthoylchloride were

added subsequently to the sample extract. The vials were thermostated in a heating block at 60 C for 15 minutes. After cooling again to room temperature, 50 µL methanol were added to the reaction mixture to quench the reaction. Aliquots of the reaction mixture were directly subjected to HPLC/UV-detection. UV absorption was determined at wavelengths of 228 and 300 nm. The stationary phase was a 150 x 4.6 mm column filled with ultracarb 5 µm ODS (Phenomex, Torrance, CA, USA), the isocratic mobile phase was a mixture of methanol and water from 9:1 (v:v) to pure methanol.

Calibrations were performed with external standards for the quantification of the different alcohol ethoxylates within the concentration range of 10^{-6} to 10^{-4} mol·L^{-1}. The correlation between peak area and alcohol ethoxylate concentration was determined by linear regression, typically with $R^2 = 0.95$. The detection limit was 10^{-6} mol·L^{-1} (signal to noise ratio of 3). The overall error of the derivatisation and the analytical step was between 5 and 10 % relative standard deviation. Experiments at one surfactant- and liposome concentration were performed in triplicates. Each sample was measured twice. The experimental and analytical error was below 5 %. For each sorption isotherm, 5 to 19 different experiments with varying surfactant concentration and/or liposome concentration were performed.

HPLC conditions

The injection volume was usually 10 µL. The stationary phase was a 150 x 4.6 mm column filled with ultracarb 5m ODS (30) (Phenomex), the mobile phase A consisted of methanol and mobile phase B of a mixture of methanol and water in a ratio of 4 to 1. Optimal separation was reached by working under different isocratic conditions (Table 25).

Table 25: HPLC conditions for the isocratic separation of different AEO.

	A: MeOH	B: MeOH / H_2O 4:1
C_8EO_5	60 %	40 %
$C_{10}EO_5$, $C_{10}EO_8$	70 %	30 %
$C_{12}EO_5$, $C_{12}EO_8$	80 %	20 %
$C_{14}EO_5$, $C_{14}EO_8$, $C_{14}EO_{11}$	90 %	10 %
$C_{14}EO_{14}$	85 %	15 %
$C_{16}EO_8$	100 %	0 %

Comparison of equilibrium dialysis and ultracentrifugation

Sorption isotherms determined with the equilibrium dialysis method and the ultracentrifugation method are compared in Figure 32 for $C_{14}EO_5$. Liposome-water partition coefficients K_{lipw} were calculated from the slopes of the isotherms and from the experimental data. The K_{lipw}-values obtained with the equilibrium dialysis method (K_{lipw} = 80'000 ± 10'000 L·kg^{-1}) and the ultracentrifugation method (K_{lipw} = 100'000 ± 20'000 L·kg^{-1}) were statistically indistinguishable which confirms that both methods are equally suited for determining K_{lipw}. Although the concentrations of C_{14}-alcohol ethoxylates were in the range of the critical micelle concentration CMC for the centrifugation experiment, the sorption isotherms were linear.

AEO with an alkyl chain of 16 C-atoms (e.g. $C_{16}EO_8$) possess a low CMC and a high K_{lipw}. Strong sorption to the test system, low substance and liposome concentrations made the determination of the K_{lipw} difficult. Sorption to the glass, to the dialysis membrane or the centrifugation vial becomes predominant for substances with a K_{lipw} in the range of 10^6 L·kg^{-1}. For substances with a K_{lipw} in the range of 10^5 L·kg^{-1} still about 20 % were sorbed to the test system.

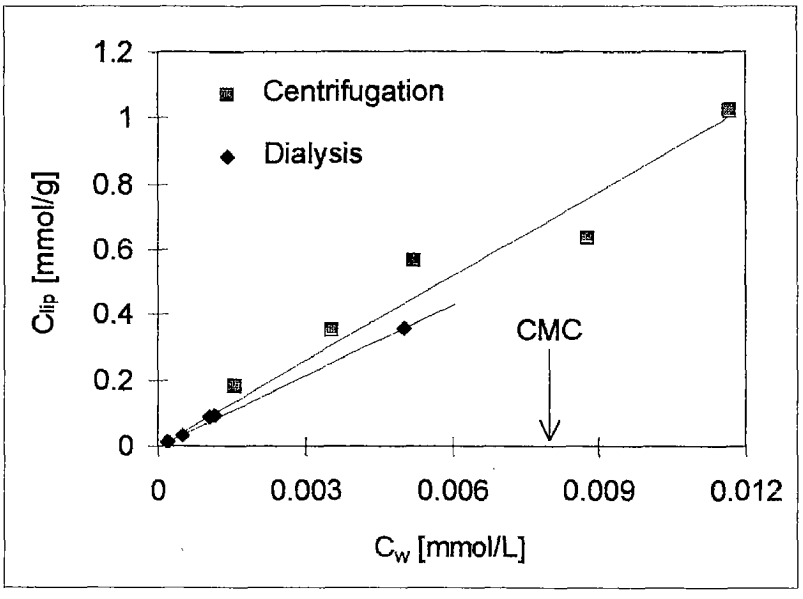


Figure 32: Comparison of the sorption isotherms of $C_{14}EO_5$ determined with the equilibrium dialysis method and the ultracentrifugation method; CMC = 7.7·10^{-6} mol·L^{-1}.

Sorption isotherms

The sorption isotherms for eight alcohol ethoxylates are shown in Figure 33. The K_{lipw}-values were derived from the slope of the sorption isotherm and are listed in Table 26. Note that some of the ultracentrifugation experiments depicted in Figure 32 were performed above the CMC. Although the isotherm still appeared linear in these experiments, the value of K_{lipw} given in Table 26 correspond to the regression without points above CMC.

The sorption isotherms of the C_{10}- and C_{12}-AEO (Figure 33a-7d) were measured below their CMC and the K_{lipw} was calculated directly from the sorption isotherms. The CMC of the C_{14}-AEO were smaller so that micelles were formed (Figure 33e-7h). The concentration of the dissolved surfactant was therefore corrected as described in Materials and Methods.

The K_{lipw} of pure linear alcohol ethoxylates depend strongly on the structure of the substance. With an increasing alkyl chain length of the AEO, for example $C_{10}EO_Y$ to $C_{12}EO_Y$, the K_{lipw} increases by a factor of approximately 10. If the EO-chain is lengthened by three ethoxylate units, for example C_XEO_5 to C_XEO_8 , the K_{lipw} decreases by a factor of approximately 3. These general trends are consistent with expected changes in hydrophobicity of the compounds.

Since the reported K_{lipw}-values are the first liposome-water partition coefficients directly determined for nonionic surfactants, comparisons with other measurements are not possible. However, the lipid- sea water partitioning of the anionic surfactant sodium dodecyl sulfate (SDS) was studied by Kalmanzon et al. (1992) with a similar dialysis assay. The partition coefficients K_{lipw} for negatively charged and neutral liposomes were 3100 ± 450 $L \cdot kg^{-1}$ and 3500 ± 600 $L \cdot kg^{-1}$, respectively. This is similar to the K_{lipw} 2600 ± 100 $L \cdot kg^{-1}$ determined for $C_{12}EO_8$. Hence, an EO-chain of about 8 EO has therefore about the same effect on the K_{lipw} of a C_{12}-alcohol as a negatively charged sulfate group. Kiewiet determined the distribution coefficient (K_d) for LA-$C_{12}EO_{10}$ between sterilised activated sludge and water (K_d = 2827 ± 117 $L \cdot kg^{-1}$) (Kiewiet et al. 1993) and the sediment-water partition coefficients (K_{SW}) for different linear alcohol ethoxylates (Table 26) (Kiewiet et al. 1996). While the K_d of LA-$C_{12}EO_{10}$ for activated sludge was in the same range as the K_{lipw} the K_{SW} differed from the K_{lipw}.

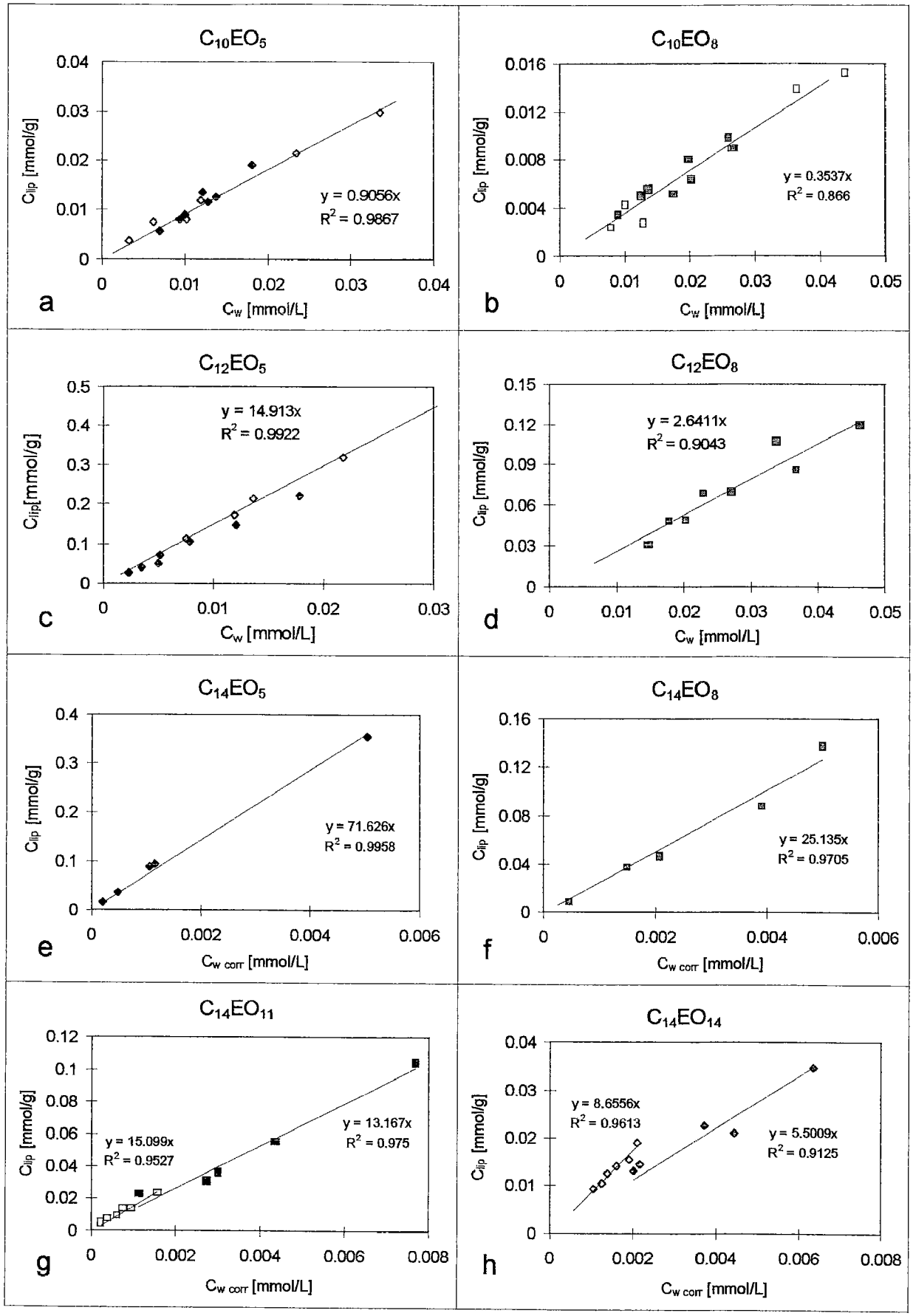


Figure 33: Sorption-isotherms for different linear alcohol ethoxylates (AEO). Open and closed symbols stand for different experiments performed with the same AEO.

Table 26: Comparison of measured and calculated K_{lipw} for different AEO.

AEO	method[a]	log CMC[b] [mol·L^{-1}]	log K_{OW}[c]	n[d]	K_{lipw}(CI[e]95%) [L·kg^{-1}]	log K_{lipw}	log K_{lipw}*[f]	log K_{lipw}**[g]
C_8EO_5 (1)	UC	-2.11	2.67	6	170 (15)	2.24 (1)	2.08	2.20
$C_{10}EO_5$	ED	-3.11	3.75	13	930 (10)	2.97	2.95	3.12
$C_{10}EO_8$	ED	-2.98	3.45	8	350 (10)	2.55	2.71	2.70
$C_{12}EO_5$	UC, ED	-4.11	4.83	19	10'300 (300)	4.01	3.83	4.04
$C_{12}EO_8$	ED	-3.95	4.53	8	2'600 (100)	3.42	3.59	3.62
$C_{14}EO_5$	UC, ED	-5.11	5.91	5	72'000 (2000)	4.86	4.71	4.96
$C_{14}EO_8$	UC, ED	-4.93	5.61	8	28'000 (1000)	4.45	4.47	4.54
$C_{14}EO_{11}$	ED	-4.74	5.31	11	13'300 (200)	4.12	4.22	4.12
$C_{14}EO_{14}$	ED	-4.56	5.01	6	8'700 (200)	3.94	3.98	3.70
$C_{16}EO_8$	UC, ED	-5.90	6.69	-	n.d.	n.d.		5.46

[a] UC: ultracentrifugation, ED: equilibrium dialysis.

[b] Critical micelle concentration calculated according to Huibers et al. (1996).

[c] Calculated with the fragment method using the values of Roberts & Marshall (1995).

[d] Number of different concentrations in the linear range of the sorption isotherm used to calculate K_{lipw}.

[e] 95 % confidence interval, calculated from standard error (se) of the slope of the linear isotherm,

$$CI\ 95\% = 2 \cdot \frac{se}{\sqrt{n}}$$

[f] log K_{lipw}* were calculated with the linear regression between log K_{OW} calculated (Roberts & Marshall 1995) and log K_{lipw} measured (Equation 22), log K_{lipw}* = 0.81·log K_{OW} - 0.09.

[g] log K_{lipw}** were estimated using the EO-fragment (-0.12 ± 0.05) and the -CH_2- fragment (+0.45 ± 0.05) from Table 27 starting with the log K_{lipw} = 4.01 ($C_{12}EO_5$).

n.d. not determined

The K_{lipw}-values were calculated for six different AEO from the raw data of triplicate measurements in equilibrium dialysis experiments (Figure 34). The rectangles provide information about the range of uncertainty of the K_{lipw} versus the surfactant concentration.

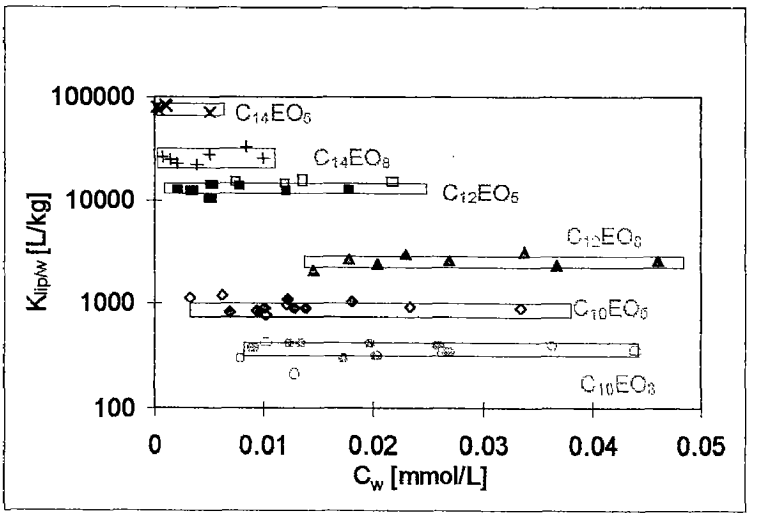


Figure 34: Liposome-water partition coefficients (K_{lipw}) of 6 linear alcohol ethoxylates.

Comparison of K_{lipw} and CMC

The critical micelle concentration CMC is the concentration of a surfactant at which micelles are formed. As such the CMC may be used to express the hydrophobicity of surfactants. The CMC decreases with increasing alkyl chain length in AEO and there is a slight decrease with decreasing number of EO units. The log CMC, which were calculated according to Huibers et al. (1996), correlate well with log K_{lipw} as is shown in Figure 35. Two separate regression lines were drawn for AEO with an ethoxylation degree of 5 EO and ≥8 EO:

Equation 20: $\log K_{lipw} (C_XEO_5) = -(0.89 \pm 0.04) \cdot \log CMC + (0.30 \pm 0.15)$ $n = 4, R^2 = 0.996$

Equation 21: $\log K_{lipw} (C_XEO_{\geq 8}) = -(0.93 \pm 0.05) \cdot \log CMC + (0.25 \pm 0.20)$ $n = 5, R^2 = 0.992$

The good correlation between log K_{lipw} and log CMC is consistent with a Linear Free-Energy Relationship (LFER) between these two phase transfer processes (Schwarzenbach et al. 1993). The free energy of the process of micelle formation and of sorption from the aqueous phase into liposomes appears to be governed by thermodynamically similar processes. The AEO dissolved in water are surrounded by oriented water molecules. Removal of the AEO from direct contact with water molecules results in a large enthalpy gain of the water molecules because water-water interactions are more attractive than water-AEO interactions. In addition there is an entropy gain although water still needs to form a cavity around the vesicles with oriented molecules at the edge of the cavity but to a much smaller extent as compared to dissolved AEO. Although the cavity formation and destruction presumably are the processes that are energetically most relevant (Schwarzenbach et al. 1993), the interactions of the alkyl chains of the AEO amongst themselves in micelles and with phospholipid molecules in liposomes are both thermodynamically favorable processes driven by a large enthalpy gain. The hydrophobic core of the lipid bilayer is composed of long-chain fatty acids and a mixture of saturated and unsaturated fatty acids to avoid unfavorable distortion of the hydrophobic domain by the long chain AEO. This may result in so-called "cut-off effect" in the adsorption of long-chain amphiphilic molecules into lipid membranes (Janoff et al. 1981; Requena & Haydon 1985). The interaction of the EO units with water molecules is energetically more favorable than interactions amongst EO units or between EO units and polar head groups of the phospholipids. However, this energetic contribution should be insignificant as compared to the other terms.

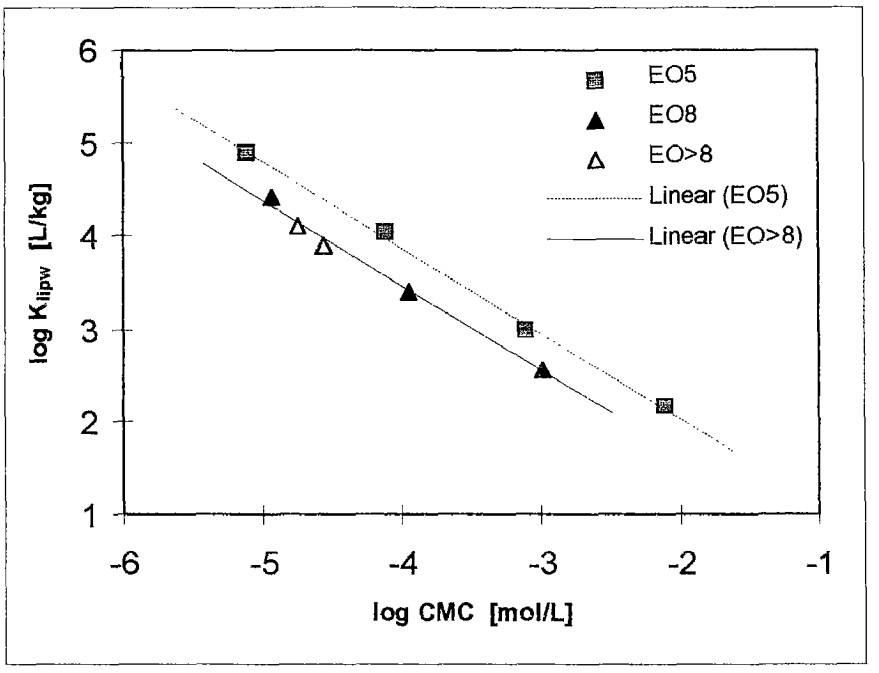


Figure 35: Comparison of log K_{lipw} and log CMC. Two linear regression lines are drawn for different ethoxylation degrees (EO_5 and EO_8)

Comparison of K_{lipw} and K_{ow}

A linear correlation was obtained between the measured K_{lipw} and the K_{OW} calculated from different fragment methods (Figure 36). The best correlation coefficients were obtained with Hansch's fragment method. The calculated K_{OW} do cover only a bit more than three orders of magnitude, whereas the K_{lipw} values cover less than three orders of magnitude. The K_{OW}-values

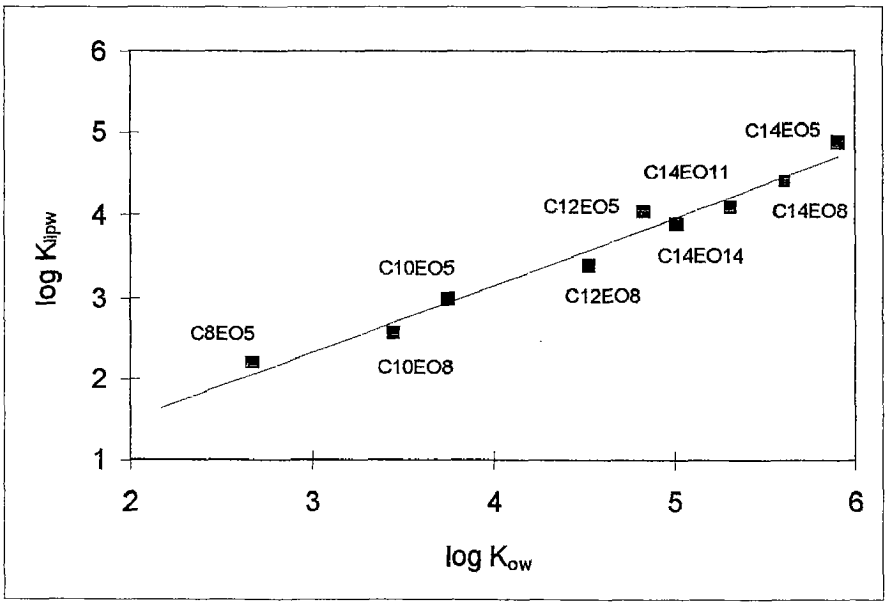


Figure 36: Comparison of log K_{ow} (Roberts 1995) and log K_{lipw} (measured) for AEO.

estimated with the method from PACO (Lindgren et al., 1996) yielded the closest to a direct one-to-one correlation but the correlation coefficient was lower than for the other regressions. The prediction with the fragment values from Roberts (1995) and Schüürmann (1991) yield the relationship given in Equation 22.

Equation 22: $\log K_{lipw}(C_XEO_Y) = (0.81 \pm 0.05) \cdot \log K_{OW} - (0.09 \pm 0.22)$ $n = 9, R^2 = 0.977$

We chose this equation for further discussions because it is of overall best quality when considering both linearity and range of values. Of course, any prediction method with a fixed increment for the CH_2-unit and a corresponding one for the EO-unit will give a parameter that correlates with log K_{lipw}. Consequently, a good correlation does not necessarily mean that the log K_{OW} estimation method is correct.

Since all AEO with 5 EO were above the regression line and all AEO with 8 EO and more were below the regression line (Figure 36), specific regressions were calculated for C_XEO_5 and $C_XEO_{\geq 8}$:

Equation 23: $\log K_{lipw}(C_XEO_5) = (0.82 \pm 0.04) \cdot \log K_{OW} - (0.02 \pm 0.17)$ $n = 4, R^2 = 0.996$

Equation 24: $\log K_{lipw}(C_XEO_{\geq 8}) = (0.88 \pm 0.03) \cdot \log K_{OW} - (0.49 \pm 0.14)$ $n = 5, R^2 = 0.997$

The AEO with 8 and more EO units have a smaller K_{lipw} and the ones with 5 EO units have a higher K_{lipw} than expected from the common regression of all values, both for the log K_{lipw} - log CMC and the log K_{lipw} - log K_{ow} regressions. This finding can be rationalized when considering that the nonpolar alkyl chain is deeply buried in the hydrophobic moiety of the membrane whereas the polar EO groups are interacting with the polar head-group region of the membrane. This latter region, however, is not very broad. It appears that the AEO with 5 EO units pose no sterically unfavorable effect whereas a higher number of EO units cannot be perfectly intercalated into the head-group region of the membrane and maybe even disturb the packing of the membrane slightly, ultimately resulting in somewhat smaller than expected K_{lipw}-values.

The results from the liposome-water partition experiments are summarized in Table 26. The K_{lipw} determined for linear AEO can be compared with estimated K_{lipw}-values and with K_{OW}-values.

Calculation of log K_{lipw} fragments for -CH_2- and EO-units

The data series for AEO with constant ethoxylate chain length were used to determine the log K_{lipw} fragment for the -CH_2- unit. The same procedure was applied for AEO with constant alkyl chain length in order to determine the log K_{lipw} fragment for an EO unit (Table 27).

The average log K_{lipw} fragment for a -CH_2- unit is 0.45 ± 0.05 (n=5, CI = 95%). This value is somewhat smaller than the -CH_2- fragment of 0.54 (Roberts 1995) from log K_{OW} -estimations. The average log K_{lipw} -fragment for an EO unit is -0.12 ± 0.05 (n=5, CI = 95%).

Table 27: Calculation of the *log K_{lipw}* fragments for additional EO or -CH_2- unit for alcohol ethoxylates. CI: confidence interval for the average.

-CH_2-	Δ log K_{lipw}	Δ log K_{lipw} (-CH_2-)	EO	Δ log K_{lipw}	Δ log K_{lipw} (EO)
C_8EO_5-$C_{10}EO_5$	0.73	0.37	$C_{10}EO_5$-$C_{10}EO_8$	-0.42	-0.14
$C_{10}EO_5$-$C_{12}EO_5$	1.04	0.52	$C_{12}EO_5$-$C_{12}EO_8$	-0.59	-0.20
$C_{10}EO_8$-$C_{12}EO_8$	0.87	0.44	$C_{14}EO_5$-$C_{14}EO_8$	-0.41	-0.14
$C_{12}EO_5$-$C_{14}EO_5$	0.84	0.42	$C_{14}EO_8$-$C_{14}EO_{11}$	-0.19	-0.06
$C_{12}EO_8$-$C_{14}EO_8$	1.02	0.51	$C_{14}EO_{11}$-$C_{14}EO_{14}$	-0.19	-0.06
average / -CH_2-		**0.45**	**average / EO**		**- 0.12**
CI[a] 95 % (n=5)		±0.05	CI[a] 95 % (n=5)		±0.05

[a] CI: 95 % confidence interval for the average.

This increment value is in the range of different EO-fragments for log K_{OW} estimations and corresponds closest to the value of -0.1 from Schüürmann (1990) and Roberts (1991). The confidence interval is much larger as compared to the -CH_2- fragment and can be explained by a non-linear behavior of an increasing EO-chain length. The effect of an additional EO-group depends on the length of the EO chain and also the alkyl chain. If the EO chain of an AEO is lengthened from 5 to 8 or from 11 to 14, the log K_{lipw} decreases by 0.47 (average) and 0.19, respectively. The longer the EO-chain, the smaller is the effect of an additional EO-group on the change of K_{lipw}. One possible hypothesis to explain this finding is that the alkyl chain of AEO is deeply intercalated into the membrane bilayer whereas the polar ethoxylate groups are located in the outer polar region of the membrane. If the EO chain is very long, it cannot be fitted entirely into the polar region of the membrane and may stick out on the surface of the membrane.

A comparison of measured (log K_{lipw}) and calculated (log K_{lipw}* and log K_{lipw}**) liposome-water partition coefficients is given in Table 26. The log K_{lipw}* were calculated with the

estimated log K_{OW} using fragments from (Schüürmann 1990; Roberts 1991) and Equation 22. Log K_{lipw}** were estimated with the EO- (-0.12) and the -CH_2- fragment (0.45) from Table 27, starting the calculation with the log K_{lipw} determined for $C_{12}EO_5$. The K_{lipw}-values estimated with the newly developed fragment method are better estimates of the measured values than the ones calculated via the K_{ow}-fragment method and log K_{ow}- log K_{lipw}-QSAR.

In addition, K_{lipw}-values were estimated with the newly developed fragment method for commercial mixtures of AEO. The good correlation of the calculated values with toxicity and sorption data (see Equation 25 to Equation 27 given below) is an indication that the fragment method can also be applied for mixtures of AEO as they occur in commercial products.

Conclusions

We propose to use K_{lipw} instead of K_{ow} as hydrophobicity descriptor in QSARs and QSTRs of AEO for three main reasons:

1) K_{lipw} can easily be experimentally determined. Whereas the surface active property of AEO makes it principally impossible to directly measure thermodynamically correctly defined K_{ow} values, the surface activity is no problem for a surface process as is liposome-water partitioning, as long as the concentrations are below CMC.
2) The fragment method for the estimation of K_{lipw} of untested AEO or commercial mixtures is an interpolation method for most common commercial mixtures whereas estimation methods for K_{ow} extrapolate from values deduced for non-surface active compounds, e.g., $C_{12}EO_1$.
3) Membrane-water partitioning describes the true process that occurs during bioaccumulation. The target sites for the toxic effect are biological membranes where nonionic surfactants exhibit non-specific narcotic activity (Talmage, 1993). A good correlation is found between log K_{lipw} and bioconcentration, log BCF (Equation 25) (Tolls 1998), toxicity towards Fathead minnow, expressed as lethal concentration for 50 % of the organisms, -log LC_{50}, (Equation 26) (Wong et al. 1997), and toxicity towards Daphnia magna, expressed in terms of effective concentration for 50 % of the organisms, -log EC_{50} (Equation 27) (Könemann 1981).

Equation 25: $\log BCF = (1.06 \pm 0.16) \cdot \log K_{lipw} - (2.94 \pm 0.72)$ $n = 9, R^2 = 0.859$

Equation 26: $-\log LC_{50}(96\ h) = (0.78 \pm 0.10) \cdot \log K_{lipw} - (2.44 \pm 0.36)$ $n = 9, R^2 = 0.902$

Equation 27: $-\log EC_{50}(48\ h) = (0.86 \pm 0.08) \cdot \log K_{lipw} - (2.30 \pm 0.30)$ $n = 9, R^2 = 0.941$

The K_{lipw}-values for the commercial mixtures employed in the toxicity study of Wong et al. (1997) were calculated with the newly developed fragment method. The good correlation in Equation 26 and Equation 27 are hence an indication that the fragment method can also be applied for mixtures of AEO in commercial products.

There is also a qualitative correlation between log K_{lipw} and the sorption of AEO to sediment (Brownawell, 1997; Kiewiet, 1996), however, not enough experimental data is available for the development of QSARs.

More research on liposome-water partitioning of anionic, cationic, and further non-ionic surfactants is required before any final conclusion concerning the use of K_{lipw} as hydrophobicity descriptor for surfactants can be drawn. Since also in the case of hydrophobic organic ions (Escher, 1996; Ottiger, 1997) liposomes were shown to be a better surrogate for biological material than octanol, it is highly likely that the conclusions presented here can be extended in the future to anionic and cationic surfactants.

Acknowledgments

We thank Johannes Tolls for the gift of the two higher ethoxylated AEO, $C_{14}EO_{11}$ and $C_{14}EO_{14}$. We are indebted to Daniel Bürgi for the phosphate analysis of the liposomes, to Daniel Kobler for the particle size determination of the liposomes and to Joggi Rieder for his assistance in the partitioning experiments.

Chapter 6

P_i-uptake by *Acinetobacter johnsonii 210A* under the influence of different alcohol ethoxylates

Markus T. Müller, Nina Schweigert, Alexander J.B. Zehnder

Abstract

Phosphate-uptake by *Acinetobacter johnsonii 210A* was used for testing the influence of chemicals on membrane potentials in living cells. Liposome-water partition coefficient (K_{lipw}) and concentration dependent effects have been found for catechols, chlorinated catechols, and naphthalene. Up to a concentration of 10^{-4} mol/L (C_8EO_5: 35 mg/L; $C_{14}EO_{14}$: 83 mg/L) the different linear alcohol ethoxylates did not inhibit P_i-uptake of *A. johnsonii 210A* significantly. However, foam forming during aeration did not allow the testing of higher concentrations of alcohol ethoxylates, which have shown inhibition in anaerobic sludge digestion. It was therefore necessary to employ a method without aeration.

Introduction

Nonionic surfactants such as alcohol ethoxylates (AEO) interact with biological membranes and increase their permeability and the trans-membrane solute transport (Florence 1984). The mechanisms of such effects are still not fully understood. Test methods were developed for studying membrane toxic effects at the level of well known membrane processes. De la Maza et al. (1992) for example studied the effect of nonionic surfactants on the permeability of model biological membranes with liposomes from egg phosphatidylcholine. Kloecking et al. (1994) assessed the membrane toxicity of sodium dodecyl sulfate (SDS) by measurement of the release of arachidonic acid from membrane phospholipids of the promonocytic cell line U937, which is part of a signal system with which cells respond to environmental changes of

various kinds. The better the understanding of membrane dependent reactions are, the more specific type of inhibitory mechanisms can be determined.

For the membrane toxicity experiments described here, the phosphate uptake system of the bacterium *Acinetobacter johnsonii 210A* was used (Van Schie et al. 1984 & 1987, Van Groenestijn et al. 1989; Zehnder et al. 1990, Van Veen 1994). *Acinetobacter johnsonii 210A* was chosen because of the following reasons:

- It is a representative bacterium in wastewater treatment plants.
- The membrane dependent mechanisms of phosphate uptake and release are well known.
- The phosphate uptake can be enhanced by the addition of PQQ and glucose, which results in a high P_i-uptake rate within 10 to 15 minutes.
- The toxic effects of different inhibitors and uncoupler have already been determined by Van Veen (1994).

P_i-uptake of *A. johnsonii 210A* is decreased by compounds that interact with the membrane or perturbate the functioning of the membrane (Van Veen et al. 1993 A). This feature was used to examine the membrane toxicity of AEO and some reference compounds, e.g. catechol and chlorocatechols, which are uncouplers of oxidative phosphorylation, and naphthalene as an example of a narcotic agent.

Members of the genus *Acinetobacter* are aerobic, non-motile, non-fastidious gram negative bacteria, which are ubiquitously present in soil, water and sewage. The ability of *Acinetobacter* to form the acid gluconate from D-glucose and other sugars was used as taxonomic criterion within the family (Bouvet et al. 1986) and depends on the presence of a functional, cytoplasmic membrane-bound quinoprotein, aldose dehydrogenase, that catalyzes the oxidation of glucose to gluconate (Dokter et al. 1987, Gerner-Smidt et al. 1990, Van Schie et al. 1984, Van Schie et al. 1987). Most *Acinetobacter* strains as *A. johnsonii 210A* are unable to use gluconate as a carbon and energy source and release this compound into the growth medium during oxidation of glucose.

Acinetobacter johnsonii 210A is one of the most extensively studied strains of the genus (for review, see Zehnder & Van Groenestijn 1990; Kortstee et al. 1994). It grows well within a pH range of 6 to 9. In the presence of excess energy and substrates, it is able to accumulate phosphate up to 300 mg P_i per gram (dry weight) at all pH values. Concomitantly with P_i, Mg^{2+} (or Ca^{2+}) and K^+ are taken up by *A. johnsonii 210 A.* K^+ is essential for P_i-uptake. In its absence the P_i-uptake is strongly reduced.

Materials and methods

Abbreviations

catechol	1,2-dihydroxybenzene
4-MCC	4-monochlorocatechol, 4-monochloro-1,2-dihydroxybenzene
3,4-DCC	3,4-dichlorocatechol, 3,4-dichloro-1,2-dihydroxybenzene
3,4,5-TCC	3,4,5-trichlorocatechol, 3,4,5-trichloro-1,2-dihydroxybenzene
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
DNP	α-dinitrophenol
EDTA	Ethylene-diamin-tetraacetate
PQQ	4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]-quinoline-2,7,9-tricarboxylic acid

Chemicals

Radioactive potassium phosphate ($^{33}P_i$) for phopshate (P_i) uptake experiments was purchased from Amersham, Switzerland. The complete liquid scintillation count cocktail (Filter-Count™) was purchased from the Packard Instrument Company, Meriden, USA. The cellulose nitrate filters with a pore diameter of 0.45 μm (Sartorius™) were obtained from Dr. Vaudaux AG, Schönenbuch/Basel, Switzerland. Technical data and origin of the alcohol ethoxylates used are summarized in Table 1 of Chapter 5. Catechol and 4-monochloro catechol were purchased from Sigma. 3,4-dichloro catechol and 3,4,5-trichloro catechol were a gift from Jan Roelof van der Meer. All the other chemicals were purchased from Fluka AG, Buchs, Switzerland.

Determination of the protein concentration

The protein concentration was determined with the Bio-Rad protein assay (Bradford 1976) using Coomassie Brilliant Blue G-250.

Organism and culture conditions

Acinetobacter johnsonii 210A was grown aerobically at 30 °C in a buffered medium (pH 7.2) containing 2.2 $g \cdot L^{-1}$ Na-butyrate, 1.1 $g \cdot L^{-1}$ NH_4Cl, 1.25 $g \cdot L^{-1}$ $MgSO_4 \cdot 7\ H_2O$, 60 $mg \cdot L^{-1}$ $CaCl_2 \cdot 2\ H_2O$, 0.75 $g \cdot L^{-1}$ KCl, 2 mL trace element solution per liter, and 6 $g \cdot L^{-1}$ TRIS buffer + HCl (pH = 7.2). The composition of the trace element solution was 1.5 g $FeCl_3 \cdot 6\ H_2O$, 50 mg H_3BO_3, 10 mg $CuSO_4 \cdot 5\ H_2O$, 10 mg NaI, 40 mg $MnCl_2 \cdot 4\ H_2O$, 20 mg $Na_2MoO_4 \cdot 2\ H_2O$, 40 mg $ZnSO_4 \cdot 7\ H_2O$ and 50 mg $CoCl_2 \cdot 6\ H_2O$ per liter demineralised water (Groenestijn et al. 1987).

The cells were grown over night in a high-P_i-medium (0.02 mol/L) according to Van Veen (1994) using butyrate as carbon source. The cells were harvested at an OD_{600} (optical density: λ= 600 nm) of 0.5 to 0.7, centrifuged at 10'000 *g* and a temperature of 4 °C, washed three times with cooled aerated 20 mM PIPES-K_2 buffer (7.6 $g \cdot L^{-1}$, pH = 7.0) containing 2.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 7\ H_2O$ and 50 $mg \cdot L^{-1}$ chloramphenicol. After protein determination the cells were diluted to a protein concentration of 500 mg protein per liter. The cell suspension was stored on ice, aerated with water saturated air and used within 4 hours.

The EDTA treatment of the intact cells was performed according to van Veen (1993a).

P_i-uptake experiment

The $^{33}P_i$ -labeled potassium phosphate was filtered through a 0.45-mm-pore-size cellulose nitrate filter prior to use, in order to remove $^{33}P_i$ adsorbed to particles.

The P_i-uptake experiments were performed at 30 °C in 2 mL tubes. 500 µL of the cell suspension were added and aerated. The chemicals to be tested were added to the cell suspension 5 minutes before PQQ addition. Subsequently cells were pre-incubated for 3 minutes with 2 µM PQQ, followed by the addition of 3.6 $g \cdot L^{-1}$ glucose (20 mM). Two minutes later the P_i-uptake experiment was started by the addition of ^{33}P-labeled potassium phosphate (0.33 to 1.47 TBq/mol). Samples of 100 µL were taken after 0.5, 6 and 12 minutes (somtimes after 0.5, 8, 16 minutes), transferred to vials containing 1 mL of ice cooled 0.1 M LiCl and immediately filtered through 0.45 µm filters which were placed on an vacuum filtration unit for 12 filters. The filters were washed once with 4 mL ice cooled 0.1 M LiCl, transferred into scintillation vials and the filter-count-liquid added. Filters were allowed to dissolve before the vials were placed into a scintillation counter for measurement.

Estimation of K_{lipw} for chlorinated chatechols

The liposome-water partition coefficients K_{lipw} were determined with a IAM PC column for catechol (Ong et al. 1995) and estimated for chlorinated catechols with K_{lipw}/K_{OW} QSARs for substituted phenols (Escher & Schwarzenbach 1996).

Results and discussion

P_i-uptake

P_i-uptake of *A. johnsonii 210A* with PQQ/glucose were determined for each series of experiments and are shown in Figure 37a) and b). Uptake curves measured with cells from the same batch corresponded well with each other. Considerable differences are seen between various batches. The influence of an inhibitor on P_i-uptake was therefore related to the reference curve of the same batch.

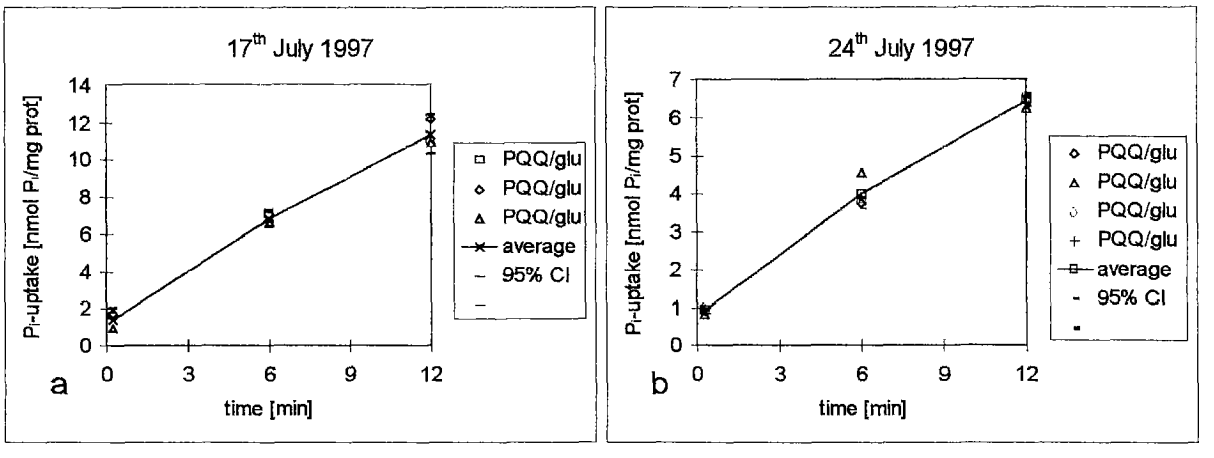


Figure 37: Reference curves of P_i-uptake experiments with PQQ/glucose at two different days: a) 17th July 1997, b) 24th July 1997. CI: confidence interval.

The P_i-uptake rates in our experiments were much lower compared to those reported by Van Veen et al. (1993a). No or only a small stimulation of P_i-uptake by PQQ/glucose was found. The PQQ used did not complement the membrane-bound glucose dehydrogenase in *A. johnsonii 210A*, even at concentrations 10 times higher than reported by van Veen et al. (1993a). New strains from Wageningen did not show higher P_i-uptake rates either. However, as the P_i-uptake rates were sufficient to measure inhibitory effects, this strain was nevertheless used to investigate different chemicals.

Test of inhibitors

Intact cells of *A. johnsonii 210A* were used for testing the effect of chemicals (Table 28) on the activity of phosphate-uptake and compared with results reported by van Veen et al. (1993a) for EDTA treated cells.

The inhibitory effects of potassium cyanide KCN, chloroform $CHCl_3$, and CCCP were in the same range as determined by Van Veen et al. (1993a). Dinitrophenol DNP showed an effect only at higher concentration. This difference can be explained by the EDTA treatment of the intact cells which permeabilizes the outer membrane and enhances the contact of the inhibitor with the inner cell membrane.

Table 28: Influence of different inhibitors and uncouplers on P_i-uptake of intact cells. The results are compared with data from van Veen et al. (1993a) which were obtained with EDTA treated cells. DNP: α-Dinitrophenol.

	26.6.97 in %	15.7.97 in %	17.7.97 in %	Van Veen in %
PQQ+Glucose	**100 ± 5**	**100**	**100**	**100**
no addition	68 ± 4	56		**30**
DNP 0.5 mM + PQQ/glu	114 ± 1			54
DNP 1.0 mM + PQQ/glu				24
DNP 5.0 mM + PQQ/glu		24		
KCN 0.5 mM + PQQ/glu	9 ± 1	7		8
$CHCl_3$ (2%) + PQQ/glu		37	38	5
20 µM CCCP + PQQ/glu				32
200 µM CCCP + PQQ/glu		4		

Validation of the membrane toxicity assay

The membrane toxicity test using the P_i-uptake system of *A. johnsonii 210 A* was validated with 3,4,5-trichlorocatechol (3,4,5-TCC), 3,4-dichlorocatechol (3,4-DCC), 4-monochloro-catechol (4-MCC) and naphthalene (Figure 38).

The 50 % effect concentrations (EC_{50}) is the concentration, at which only 50 % of the phosphate (Pi) was taken up by *A. johnsonii 210 A*. They were found for 3,4,5-TCC, 3,4-DCC, and 4-MCC to be 0.03, 0.15 and 1.2 $mmol \cdot L^{-1}$, respectively. No significant effect was observed for catechol at concentrations up to 10 $mmol \cdot L^{-1}$. Two independent experiments were performed for naphthalene and for both, the EC_{50} was 3 $mmol \cdot L^{-1}$.

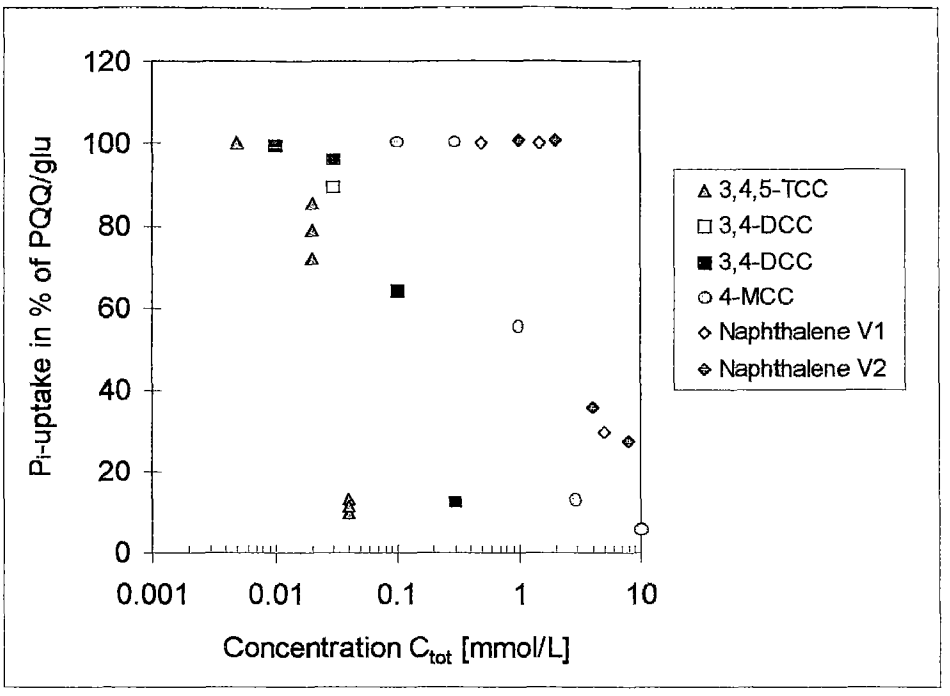


Figure 38: Relative P_i-uptake rates of *A. johnsonii 210A* under the influence of different inhibitors. 3,4-DCC and naphthalene were determined in two independent experiments.

The data presented in Table 29 suggest a certain correlation between the EC_{50} of the chlorinated catechols and their liposome-water partition coefficient (K_{lipw}). A higher K_{lipw} results in a lower EC_{50}.

Effect concentrations (EC_{50}) could be determined over a concentration range from 10^{-5} to 10^{-2} $mol \cdot L^{-1}$. The reduction of P_i-uptake can be explained by a decrease of the membrane potential either due to a permeabilization of the membrane or to uncoupling effects caused by the substances (not likely for the case of naphthalene) (Schweigert 1998).

Table 29: K_{lipw} and EC_{50} for different chlorinated catechols: K_{lipw}: liposome-water partition coefficient; EC_{50}: 50 % effect concentrations (Schweigert 1998). [1)] n.d.: not determined.

	K_{lipw} [$L \cdot kg^{-1}$]	EC_{50} [$mol \cdot L^{-1}$]
3,4,5-TCC	8920	$3 \cdot 10^{-5}$
3,4-DCC	2640	$1.5 \cdot 10^{-4}$
4-MCC	666	$1.2 \cdot 10^{-3}$
Catechol	100	$>10^{-2}$
Naphthalene	n.d.[1)]	$3 \cdot 10^{-3}$

Membrane toxicity of linear alcohol ethoxylates (AEO)

The experiments were performed with intact cells. An EDTA-treatment of the cells in order to permeabilize the outer membrane did not affect P_i-uptake under the influence of alcohol ethoxylates. Therefore, EDTA treatment was omitted here.

P_i-uptake of *A. johnsonii 210A* was not inhibited by the presence of different linear alcohol ethoxylates up to a concentration of 10^{-4} mol·L^{-1} (Table 30). Higher surfactant concentrations were not investigated with this test system because of problems with foam formation as a result of aeration. 100 µM alcohol ethoxylate did not change initial P_i-uptake rates (Figure 39).

The results presented in Table 30 and Figure 39 are comparable with toxicity data obtained for technical alcohol ethoxylates with an EC_{50}(4 d) of > 1.2 10^{-4} mol·L^{-1} (Müller et al. 1996).

Table 30 P_i-uptake of intact cells of *A. johnsonii 210A* with PQQ/glucose in the presence of different alcohol ethoxylates.

	26.6.97 %	15.7.97 %	17.7.97 %	24.7.97 %	**Average %**
PQQ/glucose	**100 ± 5**	**100**	**100**	**100**	**100**
without PQQ/glucose	68 ± 4	56	70		**64**
100 µM C_8EO_5 + PQQ/glucose			98	97	**98**
100 µM $C_{10}EO_5$ + PQQ/glucose		102	93	100	**98**
100 µM $C_{10}EO_8$ + PQQ/glucose				106	**106**
100 µM $C_{12}EO_5$ + PQQ/glucose		98	98	92	**96**
100 µM $C_{12}EO_8$ + PQQ/glucose			*51*	91	**91**
100 µM $C_{14}EO_5$ + PQQ/glucose		96	80	100	**93**
100 µM $C_{14}EO_8$ + PQQ/glucose				109	**109**
100 µM $C_{14}EO_{11}$ + PQQ/glucose				102	**102**
100 µM $C_{14}EO_{14}$ + PQQ/glucose				108	**108**
100 µM $C_{16}EO_5$ + PQQ/glucose		93	104		**98**

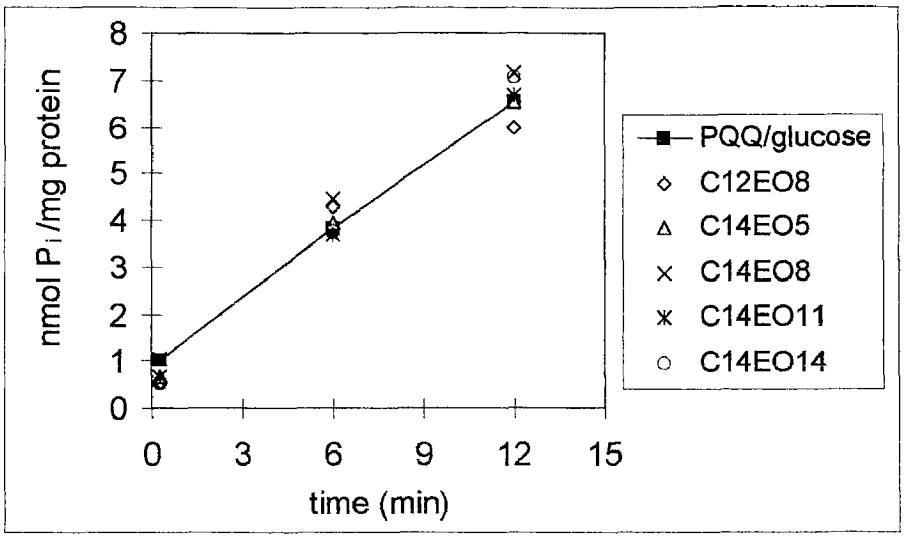


Figure 39: Effect of different alcohol ethoxylates on P_i-uptake of *A. johnsonii 210A*. All experiments were performed with AEO concentrations of 10^{-4} mol·L^{-1} and PQQ/glucose. PQQ/Glucose alone was used as a reference.

Conclusions

Phosphate-uptake by *Acinetobacter johnsonii 210A* is sensitive enough for testing chemicals for their influence on membrane potentials in living cells. K_{lipw} and concentration dependent effects have been found for catechols, chlorinated catechols, and naphthalene. Up to a concentration of 10^{-4} mol/L (C_8EO_5: 35 mg/L; $C_{14}EO_{14}$: 83 mg/L) the different linear alcohol ethoxylates did not inhibit P_i-uptake of *A. johnsonii 210A*. Foam forming during aeration did not allow the testing of higher concentrations of alcohol ethoxylates, which have shown inhibition in anaerobic sludge digestion. It was therefore necessary to employ a method without aeration. In the following Chapter such a method is presented.

Acknowledgements

A special thanks is given to Jürg Beer and Alfred Lück for their introduction and assistance in the isotope laboratory.

Chapter 7

Membrane toxicity of linear alcohol ethoxylates

Markus T. Müller, A.J.B. Zehnder, Beate Escher

Abstract

The membrane toxicity of linear alcohol ethoxylates (single reference compounds and technical mixtures) was investigated with an in-vitro method based on time-resolved spectroscopy on energy transducing membranes. The non-specific membrane perturbation of narcotic chemicals can be quantified by the degree of disturbance of build-up and relaxation of the membrane potential in membrane preparations of the purple bacterium *Rhodobacter sphaeroides*. The effect concentrations obtained for this narcotic effect correlated well with the results from various toxicity tests on whole organisms. In addition, the effect concentrations at the target site, the biological membrane, were derived from the nominal effect concentrations using membrane-water partition coefficients. The test set of linear alcohol ethoxylates comprised compounds with an alkyl chain length of 8 to 16 carbon units and 5 to 14 ethoxylate units covering more than four orders of magnitude of hydrophobicity (expressed as octanol-water partition coefficient). All alcohol ethoxylates exhibited their toxic effect at concentrations well below the critical micelle concentration. When comparing aqueous effect concentrations, toxicity increased strongly with increasing length of the alkyl chain and showed a small parabolic dependence on the number of ethoxylate units with a maximum at 8 ethoxylate units. With the toxic effect expressed in terms of membrane concentrations, all alcohol ethoxylates exhibited similar activity in the concentration range typical for narcotic chemicals. The toxic membrane concentrations of alcohol ethoxylates with 5 and $\geq$ 8 ethoxylate units were 200 and 60 mmole per kg lipid which correspond to the critical body residues of non-polar and polar narcotics in fish, respectively. In addition, the toxic effects of mixtures of alcohol ethoxylates were measured and could be modeled as the sum of activity of the single constituents, confirming the concept of concentration additivity of compounds with the same mode of toxic action.

Introduction

Alcohol ethoxylates (AEO) represent the largest volume of nonionic surfactants produced in Europe and the US (Talmage 1993). Linear primary AEO are the predominant type because they are readily biodegradable under aerobic conditions (McAvoy et al. 1998). However, in wastewater treatment plants, hydrophobic AEO may reach the anaerobic reactor adsorbed to primary sludge, where they may pose problems due to persistence and toxicity. Ethoxylates of linear alcohols have been show to be well anaerobically degradable (Huber, 1999, Steber & Wierich 1987, Müller et al. 1996). In some studies, AEO were found to be relatively recalcitrant to anaerobic biodegradation (< 60 % within 30 days), and anaerobic biodegradation required long adaptation periods (Müller et al. 1996; Wagener & Schink 1987; Steber & Wierich 1991). Because of their persistence under anaerobic conditions, it is important to assess also the toxic potential of AEO.

The inhibitory activity towards bacteria in anaerobic sludge (see Chapter 3 and 4) and the toxic effects upon higher organisms (Lewis 1991; Holt et al. 1992) have been studied extensively, mainly with mixtures of AEO from commercial products. However, there is only limited knowledge about the underlying mechanism of toxicity and about how the structure of a given AEO relates to its activity (Talmage 1993).

Surface-active compounds interact with biological membranes. At low concentrations of surfactant, the monomer adsorbs onto and absorbs into the membrane, causing the permeability of the membrane and the transmembrane solute transport to increase (Florence et al. 1984; de la Maza et al. 1991; de la Maza & Parra 1996). At higher concentrations the lamellar structure of the membrane begins to disintegrate, and the membrane-bound proteins become solubilized (Schöberl & Scholz 1993). This effect can be observed only at concentrations higher than the critical micelle concentration (CMC), which corresponds to the maximum concentration of monomers in solutions above which micelles are formed.

For environmental toxicology, toxicity of AEO at concentrations below the CMC is of major interest. Despite their good aerobic biodegradability, linear AEO with an alkyl chain length of C_{12} to C_{15} and an ethoxylation degree of 2 to 10 have to be labeled as 'dangerous for the environment' because of their high acute ecotoxicity (Kaluza / Taeger 1996). Chronic toxicity towards different aquatic organisms was found at concentrations between 0.1 and 1 $mg \cdot L^{-1}$ (Lewis 1991) with fish being slightly more sensitive than daphnia and algae (Scholz 1997). Toxicity towards aquatic organisms increased with increasing length of the alkyl chain and

decreasing degree of branching (Kaluza / Taeger 1996). In a homologous series of linear AEO with constant ethoxylate (EO) chain length, highest activity was usually found for C_{12}-alcohols with decreasing activity towards lower and higher alcohols. If the alcohol chain length was kept constant and the number of EO units was varied, a parabolic relationship between toxicity and lipophilicity was obtained with a maximum of toxic activity at 10 EO units (Florence et al. 1984).

Commercial AEO products are mostly mixtures of AEO that cover a certain range of alkyl chain length and a certain distribution of EO units. Although, so far, no study has systematically investigated the relationship between the toxicity of pure compounds and mixtures of AEO, it was commonly assumed that the overall effect of the mixture is the sum of the effects of the single constituents (Roberts 1991; Wong et al. 1997). It is well accepted that the toxicity of a mixture of compounds acting by the same mechanism can be described by the concentration addition model (Hermens et al. 1985).

Quantitative Structure-Activity Relationships (QSAR) connecting the toxicity of AEO to the hydrophobicity of the molecule, expressed in terms of the octanol-water partition coefficient (K_{ow}) (Roberts 1991; Wong et al. 1997, Roberts & Marshall 1995), were found to be very similar to the classical narcosis-QSAR developed by Könemann (1981) for a set of 50 industrial chemicals that all act according to a non-specific mechanism on biological membranes. From this comparison it was concluded that AEO act also according to non-specific narcosis. Other investigators proposed that AEO act according to another distinct mechanism, the so-called 'ethoxylate narcosis syndrome' (Schüürmann 1991).

The goal of the present study was to investigate systematically the membrane toxicity of a series of single reference AEO and compare them to the effect of two technical AEO mixtures. The membrane toxicities were investigated with time-resolved spectroscopy on energy-transducing membranes of the purple bacterium *Rhodobacter sphaeroides* (Escher et al. 1997). This method was developed initially for the simultaneous quantification of uncoupling of photophosphorylation and inhibition of the photosynthetic electron transfer chain but it may also serve to detect non-specific effects on the membrane integrity and functioning. The set-up of the test system allows one to relate the toxic effect to the concentration present at the target site, the biological membrane. Effective membrane concentrations can be calculated from nominal concentrations with liposome-water partitioning coefficients (see Chapter 5) and the membrane lipid content of the energy-tranducing membranes. Sorption to the protein fraction of this membrane is negligible as compared to sorption to the lipid fraction, which has been shown earlier for a series of neutral and charged hydrophobic compounds (Escher &

Schwarzenbach 1996). The use of target-site concentrations is advantageous over the use of nominal aqueous concentrations because it allows one to compare the effective concentration in this test system with lethal body burdens of non-polar and polar narcotics. From this comparison, conclusions regarding the mode of toxic action of AEO will be drawn.

Materials and Methods

Chemicals

Single linear alcohol ethoxylates with an alkyl chain length of C_8 to C_{16} and an ethoxylation degree of 1 to 9 mol EO per mol alcohol were purchased from Fluka (Buchs, Switzerland). Two higher ethoxylated alcohols ($C_{14}EO_{11}$ and $C_{14}EO_{14}$) were obtained from J. Tolls (RITOX, Utrecht University, The Netherlands). The technical mixtures of AEO were a gift from Dr. W. Kolb Company (Hedingen, Switzerland): LA-$C_{12/14}EO_5$ and LA-$C_{12/14}EO_{10}$ have an average number of EO of 5 and 9.1, respectively; both with linear alkyl chains with >99 % linearity, and have a C_{12}:C_{14} ratio of 7:3. The buffer used was MOPS (3-(N-morpholino)propanesulfonic acid (pK_a = 7.2)) from Fluka (Buchs, Switzerland). Chemicals used for time-resolved spectroscopy are described by Escher et al. (1997).

Determination of the membrane toxicity

Membrane vesicles (chromatophores) of *Rhodobacter sphaeroides* were prepared and characterized as described previously (Escher et al. 1997; Escher 1995). The single-beam spectrophotometer equipped with a flash excitation unit and kinetic data acquisition capabilities is described elsewhere. The measurements were performed in an anaerobic cuvette at a redox potential adjusted to 120 - 130 mV with ferricyanide/dithionite and redox mediators (2,3,5,6-tetramethyl-phenylene diamine, N-methyl phenazonium methosulfate, duroquinone, 1,2-naphtoquinone, 1,4-naphtoquinone) in 50 mM MOPS buffer containing 100 mM KCl at pH 7. One measurement cycle was conducted without AEO to obtain a control curve followed by several measurement cycles with increasing AEO concentration. After each addition of AEO, the system was equilibrated for 15 min. During one measurement cycle, 4 kinetic traces were averaged, each of which consisted of the relative absorption change at 503 nm over a 150 ms interval. The absorbance change at 503 nm is proportional to the membrane potential (Jung & Jackson 1982), which accounts for the majority of the electrochemical proton gradient in chromatophores (Melandri et al. 1984).

Compounds that disturb the membrane integrity and functioning show two types of effects on the membrane potential: The build-up of the membrane potential is decreased and the relaxation of the membrane potential after a pulse of light is disturbed. The main effect of non-specifically acting compounds is the acceleration of the decay of the membrane potential after its initial build-up. This decay was quantified as the pseudo first-order decay rate constant (k_{obs}) of the absorbance at 503 nm and hence of the membrane potential. The value of k_{obs} is normalized for the properties of a particular chromatophore preparation by dividing each value of the kinetic trace by the corresponding value of the control. More details on the measurements are given by Escher et al. (1997, 1996). The toxic endpoint chosen was the effective concentration, EC, at which the half-time of decay of the membrane potential was 500 ms. This endpoint was validated earlier by comparison with various toxicity tests using a set of substituted phenols (Escher et al. 1997).

In addition, the decrease of maximum absorbance change was used as second indicator of non-specific membrane toxicity. The corresponding endpoint was the inhibitory concentration IC_{20}, at which the maximum of the membrane potential change was 80 % of the control value. IC_{20} was chosen because the conventionally used IC_{50}-concentrations were so high that they could not be reached in many cases or were above the CMC. In addition, EC and IC_{20} lay typically in the same concentration range.

Both EC and IC_{20} were converted from nominal concentrations, EC_{tot} and $IC_{20,tot}$ to free aqueous concentrations, EC_w and $IC_{20,w}$, and to membrane-lipid concentrations, EC_{lip} and $IC_{20,lip}$ with the following equations (see Chapter 5):

Equation 28: $$C_w = \frac{C_{tot}}{[lip]K_{lipw} + 1}$$

Equation 29: $$C_{lip} = \frac{C_{tot} - C_w}{[lip]}$$

where C_{tot} is the nominal, C_w the free aqueous, and C_{lip} the membrane-lipid bound concentration. K_{lipw} is the liposome-water partition coefficient and [lip] corresponds to the concentration of lipids in the assay, which is the product of the fraction of lipids in the chromatophore membrane (~ 30 %), and the concentration of chromatophores in the assay (typically 2.7 gL^{-1}). Sorption to the proteins of the chromatophore membrane was assumed to be negligible. This assumption was based on findings of an earlier study in which liposome- and chromatophore-water partitioning of substituted phenols were compared (Escher & Schwarzenbach 1996).

Redox-controlled spectroscopic measurements

All measurements were performed under anaerobic conditions in a well-stirred cuvette under argon gas. The measuring electrode was made of thin platinum strip connected to a platinum wire. The cuvette was connected via a salt bridge to a reference electrode (Ag/AgCl/ saturated KCl; E_h = 197.6 mV). The potential was read by a potentiometer (Metrohm, model 654, Herisau, Switzerland). The platinum electrode was cleaned with 25 % ammonium solution before each redox titration. Occasionally, the cuvette was filled with nitric acid / sodium nitrite and let stand over night. The argon, containing less than 5 ppm O_2, was bubbled through water in such a way that the suspension did not loose volume during the experiment.

Prior to use, the buffer (50 mM MOPS, 100 mM K^+, pH 7.0) was made oxygen free by bubbling argon through for 10 minutes. In all experiments first 7.8 mL buffer were added to the cuvette, then 200 µL chromatophore stock solution, and finally the redox mediators (10 µM each of duroquinone, 1,2-naphtoquinone, 1,4-naphtoquinone, 1,4-benzoquinone, and 20 µM each of DAD (2,3,5,6-tetramethyl-p-phenylene-diamine, E_{mz} = 275 mV) and PMS (N-methyl-phenazonium methosulfate, E_{mz} = 85 mV)). The redox potential (E_h) was poised with sodium dithionite solution to 120 to 130 mV. Throughout the experiment the potential was held constant by adding small amounts of dithionite to decrease E_h, and ferricyanide to increase E_h. After 30 to 60 min of equilibration and redox posing in the dark, the experiment was started.

In this test system a brief, "single-turnover" flash of light creates the membrane potential. The build-up and the subsequent relaxation of the membrane potential are deduced from the change in absorbance at 503 nm. Uncouplers increase the relaxation rate. The uncoupling activity can be expressed as the pseudo first-order rate constant for the decay of absorbance, and hence membrane potential (Escher et al. 1997). The response without inhibitor served as control trace. Subsequently, increasing amounts of alcohol ethoxylates (AEO) were added. Data were collected at a sampling rate of 5 to 50 kHz. Four kinetic traces were typically averaged. One minute was allowed between each flash for complete recovery of the light-induced redox change. After increasing the AEO concentration, the suspension was allowed to equilibrate for at least 15 min before the flash experiments were continued.

The time resolved spectrophotometer (TRS)

The single beam spectrophotometer used in this study consists of a time resolved absorption measurement unit and a flash excitation unit. The particular features of the instrument are a

high time resolution of 1 μs, and the detection of small absorption changes on a high background absorption (approx. 0.1 %) in the visible region of the light. Bowyer (1979) first described this instrument which is not commercially available. In this work, the same instrumental set-up as described by Escher (1995) was used.

Single turnover of the reaction center complex (RC) of the chromatophores was activated by a xenon flash with duration of approximately 2 μs at half maximum amplitude. The flash beam was passed through a red filter (> 830 nm) placed in front of the cuvette. Minimum time between two flashes was 20 msec to assure reproducible performance of the flash.

The light path of the measuring beam is orthogonal to that of the flash. The measuring light is provided by a halogen lamp and passes through a double monochromator with a resolution of 1-2 nm. The intensity of the measuring light was so low that it did not cause excitation of photosynthetic apparatus. After passing through the measurement cuvette, the light was detected by a head-on photomultiplier tube (PMT). The photomultiplier was protected from stray actinic light by a blue glass filter. The signal was amplified in a current amplifier, and digitized in a transient recorder. The relation between high voltage and the logarithm of the signal was linear between 350 and 550 V. Routine measurements were performed with the preamplifier set at the following values: 5 to 10 μA offset, 2 μA/V sensitivity, low noise gain mode, 300 Hz to 3 kHz and 6 dB low pass filter (at a sample rate of 5 to 50 kHz).

Data were read in with a CAMAC and GPIB interface to the computer. Control of the instrument, data acquisition, and data analysis were performed using Labview, a graphical programming software (Escher 1995).

These two cases can be quantified as follows:

a) The dose-response relationships, where the total effect concentration (EC_{tot}) was determined at an endpoint of the half-decay time ($t_{1/2}$ = 0.5 sec), is related to the rate constant k_{obs} by Equation 30:

Equation 30: $$t_{1/2} = \frac{\ln 2}{k_{obs}}$$

For strong uncouplers and / or high concentrations of uncouplers, the membrane potential is reduced to zero within 10 to 500 msec. In these cases only the initial part of the trace was used to determine k_{obs} because the driving force for the uncoupling is too small when the membrane potential comes close to zero.

The total effect concentrations (EC_{tot}) of AEO were linearly interpolated at a chosen half-time of $t_{1/2}$ = 0.5 sec, at which the rate constant k_{obs} was calculated (Equation 31) for a half-decay time of $t_{1/2}$ = 0.5 sec (K_{obs} = - 1.386). These effect concentrations correlated well with those determined in other assays (Escher & Schwarzenbach 1997).

Equation 31: $$-k_{obs} = \frac{\ln 2}{t_{1/2}} = \frac{\ln 2}{0.5} = 1.386$$

The effect concentrations in the water and the membrane phase, EC_w and EC_{lip}, were calculated from the total effect concentration EC_{tot} using Equation 32 and Equation 33, respectively (see Chapter 6). Besides the total effect concentration (EC_{tot}), they use K_{lipw}, and the lipid concentration in the test assay ([lip] = 0.821 $g \cdot L^{-1}$).

Equation 32: $$EC_w = \frac{EC_{tot}}{K_{lipw}[lip]+1}$$

Equation 33: $$EC_{lip} = K_{lipw} \cdot EC_w$$

b) The end point of the reduced build-up of the membrane potential (Figure 41) was the 20 % inhibitory concentration (IC_{20}), at which the build-up of the curve was reduced by 20 % of the control value after the single-turnover flash.

Results and discussion

Effects of AEO on energy-transducing membranes

The short flash induced a biphasic rise of the membrane potential, which was first fast, then slow (Figure 40). The membrane potential is directly proportional to the relative absorbance at 503 nm (Jung & Jackson 1982). The effect of AEO was two-fold: the maximal build-up of the membrane potential was reduced, and the decay of the membrane potential was accelerated. Both effects can be interpreted as non-specific perturbation of the membrane integrity. The reduction of the maximum absorbance change was particularly pronounced for the slower phase of the build-up of the membrane potential, which corresponds to electrogenic events in the bc_1-complex of the chromatophore membrane (Jackson & Crofts 1971). This reduction is presumably not caused by a direct interaction of AEO with the membrane proteins, otherwise both phases would be affected. It is more likely that a fraction of the electrons does not reach the bc_1-complex because the quinone/quinol shuttle between reaction center complex and bc_1-complex (Snozzi & Crofts 1984) is disturbed by intercalation of the AEO in the lipid bilayer.

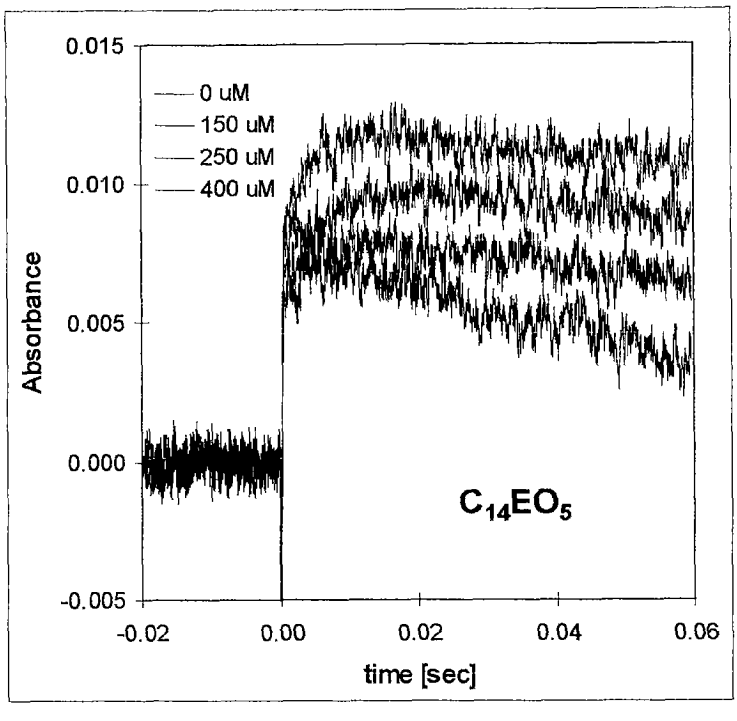


Figure 40: Original kinetic traces of the absorbance at λ = 503 nm of chromatophores of *Rb. Sphaeroides* under the influence of different concentrations of LA-$C_{14}EO_5$.

The intercalation of AEO results in an increased permeability to small charged solutes of the otherwise insulated lipid bilayer. As a consequence the decay of the membrane potential, expressed as k_{obs}, is accelerated. The reduction of the maximum absorbance was less pronounced, occurred at higher concentrations, and was much less reproducible than the acceleration of the decay of membrane potential. Hence, in the following, we use k_{obs} as indicator of the non-specific toxic effect.

The kinetic traces of the technical AEO have a different general appearance (Figure 41). The reducing effect on the maximum of absorbance was stronger than the effect on the membrane potential. Possible interpretations of this finding are discussed below in the section 'Toxicity of commercial mixtures of AEO'.

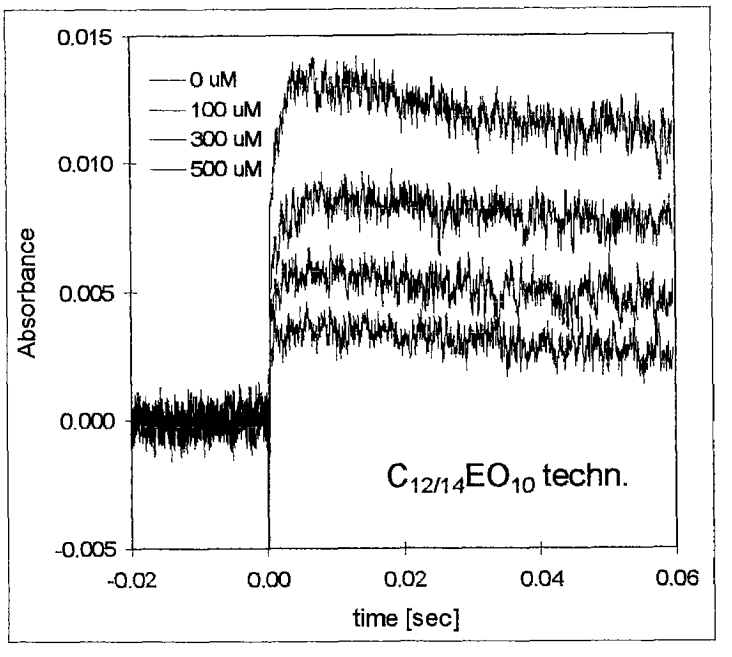


Figure 41: Original kinetic traces of the absorbance at λ = 503 nm of chromatophores of *Rb. Sphaeroides* under the influence of the technical AEO: LA-$C_{12/14}EO_{10}$.

Dose-response curves

When k_{obs} is plotted against the nominal concentrations (C_{tot}), three distinct dose-response curves were obtained (Figure 42a). The least hydrophobic C_8EO_5 was active in the millimolar concentration range. Activity of AEO with 5 EO units and $C_{10}EO_8$ occurred at tenfold smaller concentrations, and for the remaining compounds the critical concentration dropped another factor of five. The more hydrophobic compounds appeared to show effects at even lower concentrations but no quantitative correlation between C_{tot} and hydrophobicity was found.

Since the AEO investigated here cover more than three to four orders of magnitude in hydrophobicity (expressed as K_{lipw} or K_{ow}, respectively), C_{tot} does not reflect the free aqueous concentration C_w. Therefore, C_w was calculated from C_{tot} with Equation 28, and the resulting dose-response curves are plotted in (Figure 42b). The curve of the most hydrophilic compound is hardly shifted because only a small fraction of AEO is taken up into the membrane. The dose-response curves for the more hydrophobic compounds are shifted up to three orders of magnitude since these chemicals are removed to a large extent from the aqueous phase due to sorption to the membrane. Plotted on a linear scale, these curves are slightly non-linear but up to k_{obs} of 30 s^{-1} no saturation was observed (not shown). The order of the curve is between 1 and 2 as deduced from the double-logarithmic plot. No satisfactory results were obtained with a second order polynomial fit. A threshold concentration, below which no effect occurs, is a more likely explanation of the non-linearity. Some adsorption of AEO to the experimental devices (cuvette, stirrer, electrode) cannot be excluded, though they were made of glass and metal. In case of adsorption, C_w would be overestimated.

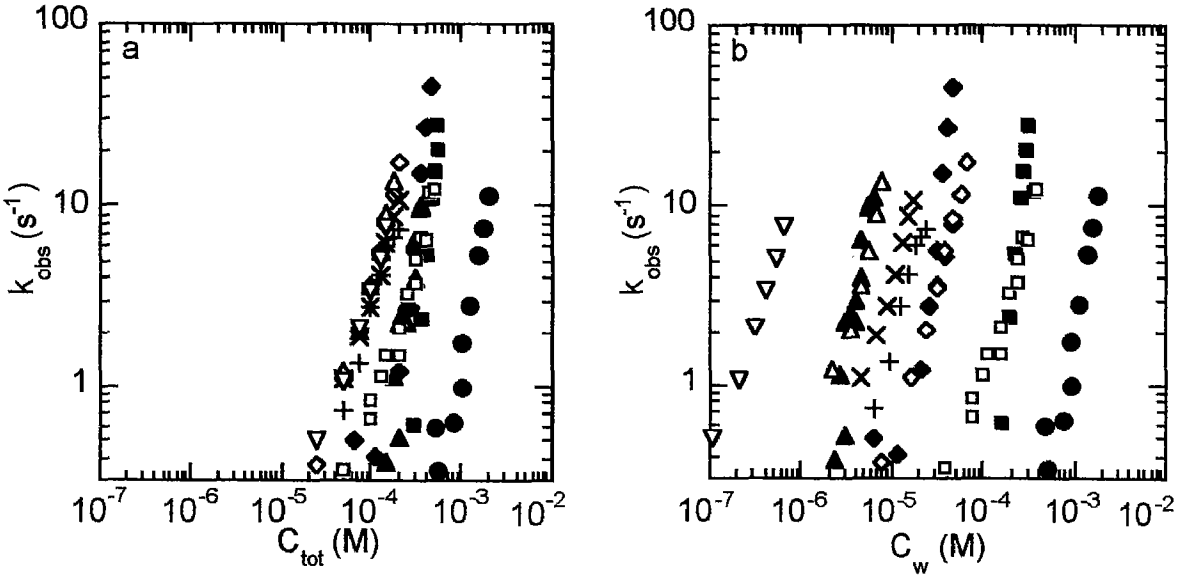


Figure 42: Dose-response curves of all single AEO investigated: k_{obs} is plotted against the (a) nominal concentration C_{tot} and (b) the free aqueous concentration C_w; calculated with Equation 28: ● C_8EO_5, ■ $C_{10}EO_5$, □ $C_{10}EO_8$, ♦ $C_{12}EO_5$, ○ $C_{12}EO_8$, ▲ $C_{14}EO_5$, △ $C_{14}EO_8$, x $C_{14}EO_{11}$, + $C_{14}EO_{14}$, ▽ $C_{16}EO_8$,

A biphasic linear response with one line for concentrations below CMC and one line above CMC did not explain the non-linearity of the dose-response curves because the intersections of the two regression lines are at concentrations that are two- to ten-fold smaller than the CMC.

The CMC-values were calculated according to the method of Huibers et al. (1996) and might lack absolute accuracy but a deviation by a factor of 10 is unlikely. Since it was not possible to find a satisfying interpretation of the shape of the dose-response curves, the effect concentrations (EC) for the endpoint decay half-time of 500 ms (k_{obs} = 1.46 s^{-1}) were not deduced from an analytical form of the dose-response curves but from a linear interpolation between the nearest neighbors on the curves.

Development of QSAR

The conventional way to express effect concentrations is in units of concentration in the surrounding aqueous phase. For development of QSARs and comparison with data from the literature, we therefore used EC_w-values although more mechanistic information can be deduced from effect concentrations in the target site, EC_{lip}.

The EC_w-values depended strongly on the hydrophobicity of the AEO. Toxicity, expressed as –log EC_w, increased linearly with increasing length of the alkyl chain as is shown in Figure 43 for AEO with 5 and 8 EO units. The influence of the alkyl chain length on the toxic effect was more pronounced than the influence of the number of EO units. The AEO with 5 EO units were only slightly less toxic than the ones with 8 EO units. The plot of the toxicity as a function of number of EO units, as shown in Figure 44 for C_{14}-AEO, is parabolic. Toxicity increased from EO_5 to EO_8, as it also did for C_{10} and C_{12}-AEO, but decreased again for higher EO numbers.

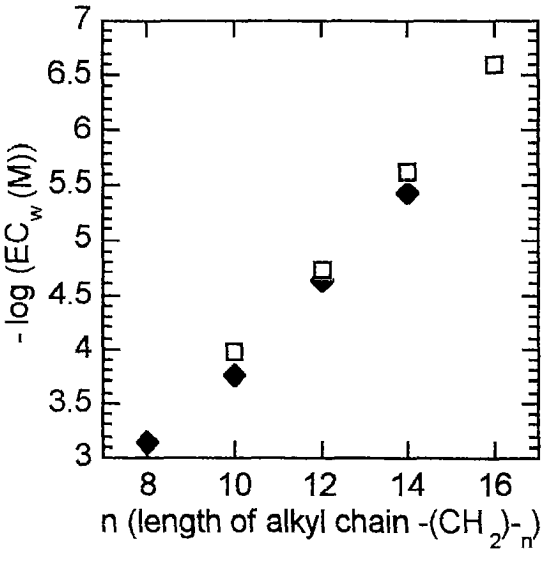


Figure 43: Effective concentration, –log EC_W, as a function of alky chain length at a constant EO-chain length (♦ EO_5 and □ EO_8).

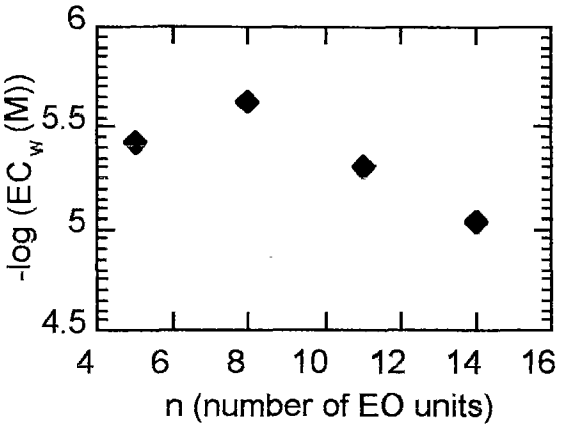


Figure 44: Effective concentration, –log EC_W, as a function of number of ethoxylate units for $C_{14}EO_Y$

When plotting the effective concentrations (-log EC_w) against the liposome-water partition coefficients (Table 31), it becomes evident that, although the toxicity increases as expected linearly with log K_{lipw} (Figure 45a), two separate linear regressions can be calculated for AEO with 5 EO units (Equation 34) and AEO with 8 and more EO units (Equation 35). These separate equations are statistically more significant than a single QSAR with all compounds (Figure 45b, Equation 36).

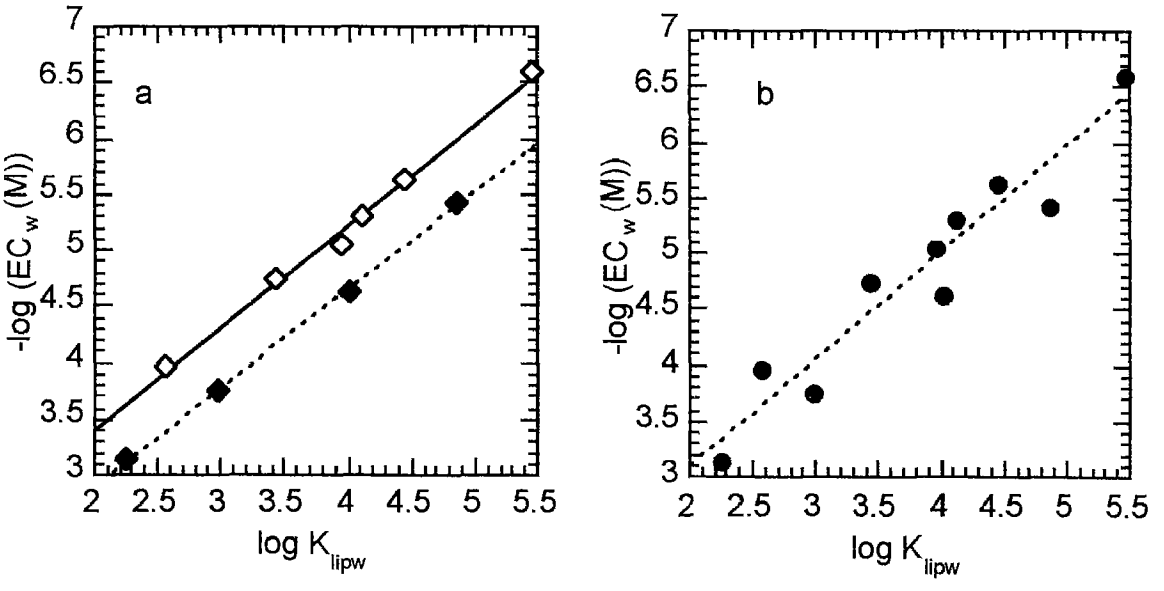


Figure 45: Linear regression of –log EC_W against log K_{lipw} a) for AEO with ♦ EO_5 and ✧ $EO_{\geq 8}$ and b) for all AEO ●.

Equation 34 $-\log EC_w(C_xEO_5)(M) = 0.87\ (\pm 0.02) \cdot \log K_{lipw} + 1.17\ (\pm 0.05)$

$n = 4,\ R^2 = 0.999,\ s = 0.029,\ F = 3564$

Equation 35 $-\log EC_w(C_xEO_{\geq 8})\ (M) = 0.91\ (\pm 0.02) \cdot \log K_{lipw} + 1.62\ (\pm 0.07)$

$n = 6,\ R^2 = 0.999,\ s = 0.034,\ F = 3336$

Equation 36 $-\log EC_w(C_xEO_Y)\ (M) = 0.96\ (\pm 0.10) \cdot \log K_{lipw} + 1.17\ (\pm 0.39)$

$n = 10,\ R^2 = 0.921,\ s = 0.303,\ F = 93$

n is the number of data points, R^2 corresponds to the coefficient of correlation, s is the standard error and F is the F-observed value.

Table 31: CMC, octanol-water, liposome-water partition coefficients, and toxicity of AEO

AEO	-log CMC[a] [mol·L^{-1}]	log K_{OW}[b]	log K_{lipw} [L·kg^{-1}]	-log EC_{tot} [mol·L^{-1}]	-log EC_w[e] [mol·L^{-1}]	-log EC_{lip}[e] [mol·kg^{-1}]	-log $IC_{20,tot}$[f] [mol·L^{-1}]
C_8EO_5	2.11	2.67	2.24[c]	3.08	3.14	0.90	3.19
$C_{10}EO_5$	3.11	3.75	2.97[c]	3.50	3.75	0.78	3.49
$C_{10}EO_8$	2.98	3.45	2.55[c]	3.84	3.96	1.41	3.52
$C_{12}EO_5$	4.11	4.83	4.01[c]	3.66	4.63	0.62	3.85
$C_{12}EO_8$	3.95	4.53	3.42[c]	4.24	4.74	1.32	3.92
$C_{14}EO_5$	5.11	5.91	4.86[c]	3.65	5.43	0.57	3.82
$C_{14}EO_8$	4.93	5.61	4.45[c]	4.25	5.63	1.18	4.03
$C_{14}EO_{11}$	4.74	5.31	4.12[c]	4.23	5.30	1.18	4.03
$C_{14}EO_{14}$	4.56	5.01	3.94[c]	4.12	5.04	1.10	3.95
$C_{16}EO_8$	5.90	6.69	5.45[d]	4.24	6.61	1.16	3.85

[a] Calculated according to Huibers et al. (1996).

[b] Calculated with the fragment method using the values of Roberts & Marshal (1995).

[c] Determined in Chapter 5.

[d] Log K_{lipw} of $C_{16}EO_8$ was estimated with the fragment method described in Chapter 5, using the EO-fragment -0.12 (± 0.05) and the $-CH_2-$ fragment +0.45 (± 0.06) starting with the log K_{lipw} = 4.01 ($C_{12}EO_5$).

[e] Calculated from EC_{tot} and K_{lipw} using Equation 32 and Equation 33.

[f] Nominal inhibitory concentration at which the build-up of the membrane potential is reduced by 20 % compared to the control value.

QSARs with log K_{ow} as descriptor (Equation 37 to Equation 39) were developed to allow comparison with literature data, although K_{lipw} is a better descriptor for the hydrophobicity of AEO in QSARs than the octanol-water partition coefficient (K_{ow}) (see Chapter 5). Log K_{ow} was calculated with the fragment method (Hansch & Leo 1995) using the increment values of Roberts & Marshall (1995).

Equation 37 $$-\log EC_w(C_xEO_5)\ (M) = 0.72\ (\pm 0.04) \cdot \log K_{ow} + 1.15\ (\pm 0.16)$$

$$n = 4,\ R^2 = 0.995,\ s = 0.085,\ F = 420$$

Equation 38 $$-\log EC_w(C_xEO_{\geq 8})\ (M) = 0.82\ (\pm 0.03) \cdot \log K_{ow} + 1.07\ (\pm 0.15)$$

$$n = 6,\ R^2 = 0.998,\ s = 0.069,\ F = 817$$

Equation 39 $$-\log EC_w(C_xEO_Y)\ (M) = 0.82\ (\pm 0.06) \cdot \log K_{ow} + 0.91\ (\pm 0.28)$$

$$n = 10,\ R^2 = 0.963,\ s = 0.206,\ F = 210$$

n is the number of data points, R^2 corresponds to the coefficient of correlation, s is the standard error and F is the F-observed value.

The two separate regression lines for EO_5 (Equation 37) and $EO_{\geq 8}$ (Equation 38) of $-\log EC_w$ versus log K_{ow} are much closer to each other than the regression with K_{lipw} and are statistically not more significant than a single QSAR. Therefore, in the following just one QSAR equation (Equation 39) was used for the correlation of $-\log EC_w$ with log K_{OW} containing the entire test set of compounds.

Equation 39 is very similar to the classical narcosis QSAR (Equation 40) that was derived by Könemann (1981) for the toxicity expressed in terms of LC_{50} (lethal concentration for 50 % of the organisms tested) of 50 non-polar industrial chemicals towards guppy fish. Könemann's QSAR has already been applied to investigate the toxicity of nonionic surfactant mixtures (Roberts & Mashall 1995).

Equation 40 $$-\log LC_{50}\ (M) = 0.87 \cdot \log K_{ow} + 1.13$$

The similarity of Equation 39 and Equation 40 demonstrates that the experimental method presented here is suitable to estimate the acute toxicity of nonionic surfactants. Direct comparisons with toxicity data from various organisms are given below in section 'Toxicity of commercial mixtures of AEO'.

Schüürmann (1991) developed a toxicity QSAR of alkyl phenol ethoxylates, which is presented in Equation 41. This equation fell in between typical QSARs for non-polar and polar narcotics,

concluding that there exists another distinct mode of action, "ethoxylate narcosis". It is interesting to note that the QSAR equation for AEO (Equation 39) is much more similar to the one for non-polar narcotics (Equation 40) than to the one for alkyl phenol ethoxylates (Equation 41).

Equation 41 $-\log LC_{50}(M) = 0.74 \cdot \log K_{ow} + 2.01$

More recently, Vaes et al. (1998) showed for non-polar and polar narcotics that the differences in toxicity-QSAR disappeared when using log K_{lipw} as hydrophobicity descriptor instead of log K_{ow}. In contrast to these findings, the confounding difference between AEO with 5 EO and ≥8 EO only appears with log K_{lipw} as descriptor. As a consequence, the difference between the two groups cannot a priori be explained by two different modes of action like non-polar and polar narcosis. Thus, it is necessary to analyze the membrane concentrations to get a clearer picture of the mode of toxic action.

Critical membrane burdens of AEO

If the effect concentration is expressed in terms of membrane concentrations, i.e. in units of mole per kg lipid, the activity of the different AEO tested varied by less than one order of magnitude as shown in Figure 46. There is again a distinct difference between AEO with 5 EO and ≥8 EO-units. The average EC_{lip} of C_xEO_5 is 200 ± 65 mmol·kg_{lip}^{-1}, and the average EC_{lip} of $C_xEO_{\geq 8}$ is 61 ± 15 mmol·kg_{lip}^{-1}. There is a slight but systematic decrease of toxicity with increasing alkyl chain length or hydrophobicity. However, the magnitude of this trend is less

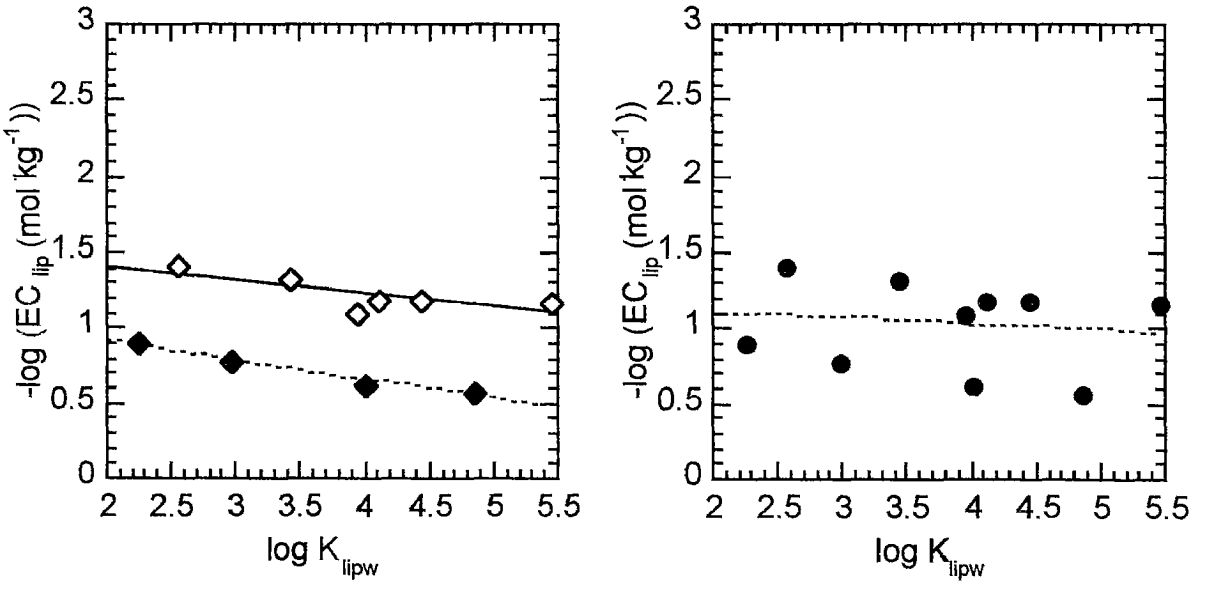


Figure 46: Linear regression of –log EC_{lip} against log K_{lipw}: a) for AEO with ♦ EO_5 and ✧ $EO_{\geq 8}$; b) for all AEO ●.

pronounced than the difference between the two classes of AEO. Statistically, the two classes are significantly different as indicated by a P-value of 0.01 (α = 5 %) obtained after submitting the data to an analysis of variance.

Constant EC_{lip}-values are in line with the concept of "critical body residues" of narcotics (van Wezel & Opperhuizen 1995). McCarty et al. (1986) pointed out that the toxicant concentration at the time of death, called lethal body burden (LBB) or critical body residue (CBR) is fairly constant for narcotic chemicals. A lower CBR points to a specific mode of action and CBRs are a means of distinguishing between non-polar and polar narcosis (McCarty et al. 1993). The EC_{lip} of C_xEO_5 are in the range of the LBB of non-polar narcotic chemicals in fish when both values are expressed in concentrations in the lipid phase, and the EC_{lip} values of $C_xEO_{\geq 8}$ correspond to LBB of polar narcotic chemicals (Figure 47).

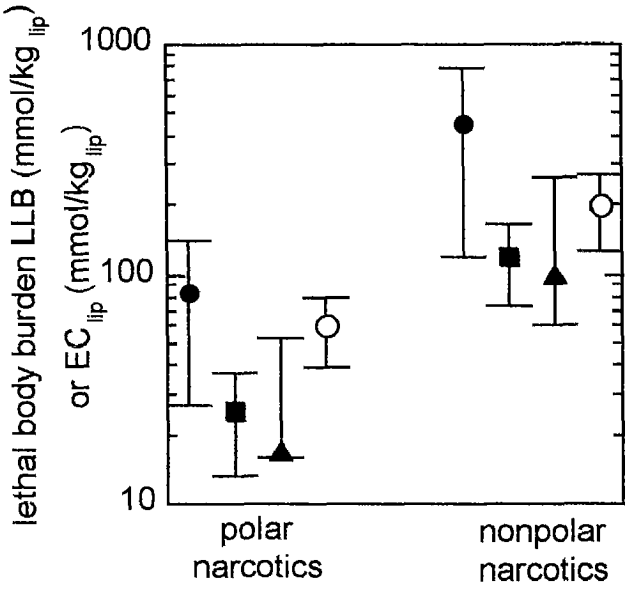


Figure 47: Comparison of lethal body burdens with EC_{lip}-values for polar and non-polar narcotics; ● (Vaes et al. 1998), ■ (McCarty et al. 1993), ▲ refs. (van Wezel & Opperhuizen 1995; van Wezel et al. 1995), o this work, left $C_xEO_{\geq 8}$, right C_xEO_5; symbols represent averages, lines represent ranges.

There is still debate over the question whether non-polar and polar narcosis represent two different mechanisms or if the difference is just an artifact of unsuitable descriptors. According to van Wezel et al. (1995), the differences between non-polar and polar narcotics can be explained by a different partitioning between target and non-target lipids. About 75 % of the total lipid content of a fish are non-polar storage lipids (non-target lipids), and the remaining fraction are polar lipids, in particular phospholipids, that make up biological membranes (target

lipids). If only partitioning into target lipids is modeled, CBR of non-polar and polar narcotics are almost equal (Vaes et al. 1998; van Wezel et al. 1995). The results presented here are not in agreement with this model because different EC_{lip}-values were observed although our test system contains only membrane lipids, i.e., target lipids.

Another possible explanation is a different distribution of polar and non-polar chemicals within the membrane, with polar chemicals accumulating at the lipid-water interface and non-polar chemicals in the hydrophobic core of the membrane (van Wezel et al. 1995). This is a reasonable explanation for amphiphilic molecules like AEO that have a very similar shape to membrane lipids with a long alkyl chain and a large polar domain, the EO-chain. It can be assumed that the AEO are deeply intercalated in the hydrophobic core with their alkyl chain and that the EO groups interact with the polar headgroups of the phospholipids. A high number of EO, however, seems to be too bulky to be fully integrated in the polar part of the membrane. Therefore, AEO with a high number of EO (≥8 EO) either disturb the polar region of the membrane more strongly and are more toxic than AEO with short EO chains, or the EO groups are located at the surface of the membrane thereby disturbing the access to the membrane.

Further work is required to elucidate fully the mode of toxic action of non-polar and polar narcotics and the specific case of amphiphilic surfactants. The test system presented here offers many advantages over previous studies that were a combination of fish toxicity studies with modeling efforts since our model system is made up of only the target site lipids.

Toxicity of commercial mixtures of AEO

It is commonly accepted that the toxicity of narcotics in mixtures is concentration-additive (Hermens et al. 1985). The concept of additivity has been applied successfully by Roberts and Marshall (1995) to describe the toxicity of commercial mixtures of AEO but was never validated experimentally for surface-active compounds. Here, the hypothesis of additivity was tested in two ways: first, EC_w-values were calculated for various commercial mixtures and were compared to toxicity data for different test organisms and endpoints from the literature. Second, the activity of two technical mixtures was determined with time-resolved spectroscopy on energy-transducing membranes.

Toxicity data for commercial mixtures and single AEO, which were not included in the test set of this study, were selected from the literature (Wong et al. 1997; Baillie et al. 1989; Morall et al. 1996). Only those data were used where the average alkyl chain length and the average number of EO units or distributions thereof were given. The corresponding K_{lipw}-values of the

single compounds were calculated with the recently developed fragment method (see Chapter 5), and K_{lipw}-values of the mixture with known distribution of ethoxamers were calculated using the method of weighted average partition coefficients (WAP) proposed by Roberts (1991). EC_w-values were calculated with the QSAR Equation 34 and Equation 35. Correlations between the thus obtained EC_w-values and toxicity towards various organisms are presented in Table 32. The high to good quality of these correlations confirms on one hand the concept of concentration additivity and proves on the other hand that the in-vitro test system presented here is a valid tool to predict non-specific narcotic effects. The sensitivity of this test is in the range of the acute toxicity tests. Our in-vitro test method is less sensitive than the test of rotifer toxicity (van Wezel et al. 1995), 96-hours fatheat minnow lethality (Wong et al. 1997), and 48-hours *Daphnia magna* acute toxicity (Wong et al. 1997) but is more sensitive than the test of 48-hours fish toxicity and daphnia toxicity (Schöberl & Scholz 1993), and the *Tetrahymena* motility test (Baillie et al 1989).

Table 32: Comparison of EC_w to toxicity data for technical mixtures from the literature

Testorganism and test (Ref.)	CH_2-range[a]	EO range[b]	Equation	n	R^2
Rotifer toxicity test (van Wezel et al. 1995)	10 - 14	4 - 8	$-\log EC_{50} = 1.33 \cdot (-\log EC_w) + 1.08$	5	0.976
Fathead minnow 96-h lethality test (Wong et al. 1997)	10 - 14.5	5 - 13	$-\log LC_{50} = 0.79 \cdot (-\log EC_w) + 2.44$	9	0.902
Daphnia magna immobilization test (Wong et al. 1997)	10 - 14.5	5 - 13	$-\log EC_{50} = 0.86 \cdot (-\log EC_w) + 2.30$	9	0.941
Inhibition test of motility of *Tetrahymena elliotti* (Baillie et al. 1989)	8 - 12	4 - 20	$-\log EC_{50} = 1.22 \cdot (-\log EC_w) - 1.30$	5	0.914

[a] Range of the carbon or $-CH_2-$ chain length of the alcohol part of the AEO.

[b] Range of the ethoxylate or –EO– chain length of the AEO.

n Number of data points, R^2 corresponds to, s is the standard error and F is the F-observed value.

R^2 corresponds to the coefficient of correlation

Two technical mixtures of linear AEO, LA-$C_{12/14}EO_5$ and LA-$C_{12/14}EO_{10}$, were tested directly with time-resolved spectroscopy. The average composition of the alkyl- and ethoxylate component is described in detail in Chapter 3. The results of the toxicity test are presented in Table 33. The nominal effect concentrations EC_{tot} agreed well with EC_{50}-values from 4-day toxicity experiments with anaerobic sludge (see Chapter 3). When toxicity was expressed in terms of EC_w, as expected, LA-$C_{12/14}EO_5$ was slightly more toxic than LA-$C_{12/14}EO_{10}$. Both

were on the regression line for C_xEO_5 (Equation 34). Note, however, the parabolic dependence of EC_w from ethoxylation degree with a maximum at approximately 8 EO units as shown in Figure 4 for $C_{14}EO_x$. The data of the technical mixtures can be qualitatively explained with such a parabolic curve. Unfortunately, there are not enough single AEO available to perform the experiment to quantitatively explain this observation.

Table 33: Toxicity of technical AEO.

AEO [a]	log K_{lipw} [b] [$L \cdot kg^{-1}$]	-log EC_{tot} [$mol \cdot L^{-1}$]	-log $EC_{50,\ anaerobic\ 4d}$ [c] [$mol \cdot L^{-1}$]	-log EC_w [d] [$mol \cdot L^{-1}$]	-log EC_{lip} [d] [$mol \cdot kg^{-1}$]	-log $IC_{20,tot}$ [e] [$mol \cdot L^{-1}$]
LA-$C_{12/14}EO_5$	4.50	3.75	3.92	5.18	0.69	4.40
LA-$C_{12/14}EO_{10}$	4.01	3.73	3.87	4.70	0.69	4.30

[a] LA-$C_{12/14}EO_5$ and LA- $C_{12/14}EO_{10}$, both with a linearity of the alcohol part of > 99 %, a C_{12} to C_{14} ratio of 7:3, and an average number of EO-units of 5 and 9.1, respectively Information provided by Kolb AG, Hedingen, Switzerland.

[b] Log K_{lipw} of two components with C_{12}- and C_{14}-alkyl chain and the respective EO average of the given technical mixture were estimated with the fragment method described in Chapter 5, using the EO-fragment (-0.12 ± 0.05) and the -CH_2- fragment (+0.45 ± 0.05) starting with the log K_{lipw} = 4.01 ($C_{12}EO_5$). The average K_{lipw} of the mixture was computed after taking antilogs, multiplying by the fraction in the mixture, summing up, and taking the logarithm.

[c] See Chapter 3.

[d] Calculated from EC_{tot} and K_{lipw} with Equation 28 and Equation 29.

[e] Nominal inhibitory concentration at which the build-up of the membrane potential is reduced by 20 % of the control value.

The membrane concentrations EC_{lip} were approximately equal (206 and 202 $mmol \cdot kg_{lip}^{-1}$) and were typical for C_xEO_5 of 200 ± 65 $mmol \cdot kg_{lip}^{-1}$ that were classified as non-polar narcotics. Taken the average composition of $C_{12/14}EO_{10}$, it should be classified as polar narcotic. However, technical AEO contain 40 to 50 ethoxy-homologues including a certain fraction of lower ethoxylated AEO and free alcohol, which are more hydrophobic and are expected to act as non-polar narcotics. Therefore one should know the distribution of ethoxamers to draw any final conclusion.

Whereas the EC-values of the technical products were a sum of the single components, the build-up of the membrane potential was affected differently by the single AEO and the mixtures. The reduction by 20 % of the build-up of the membrane potential to 80 % of the control value, as expressed by $IC_{20,tot}$, occurred at much lower concentrations for the technical

mixtures than for the single AEO. For the single AEO, there is an approximately one-to-one correlation between log $IC_{20,tot}$ and log EC_{tot} (slope = 0.966, R^2 = 0.423). The –log $IC_{20,tot}$ values for the technical mixtures are about 0.5 units higher than expected from the above correlation. This higher toxicity cannot be explained by any known concept of mixture toxicity. It is more likely that this observation is related to the broad EO distribution of the technical products with a significant fraction of non-ethoxylated alcohol, which may have different toxicological attributes than the surface-active AEO.

Conclusion

The in-vitro assay presented here offers a fast and reliable method to assess the membrane toxicity of the nonionic surfactants AEO. The results obtained with this assay do not only correlate well with acute toxicity tests using various organisms but they also contain information regarding the mode of toxic action of surface-active compounds. The series of linear AEO investigated appear to act as either non-polar or polar narcotics depending on the number of ethoxylate units. The approach described here illustrates the need to combine toxicity studies with the evaluation of bioavailability and target-site concentrations (Escher et al. 1997) in order to gain information that can be generalized for and compared to other biological systems.

Conclusions

The behavior of alcohol ethoxylates (AEO) in the anaerobic environment was investigated in this work, including anaerobic biodegradation, sorption behavior towards organic matter and toxicity towards the activity of anaerobic sludge. The motivation for these investigations was the small database for the biodegradation and toxicity of AEO under anaerobic conditions, especially for ethoxylates of branched alcohols. Both acute toxicity and the mechanisms leading to toxic effects were investigated. Different in-vitro test systems were used to determine the membrane toxicity and their results were applied to develop quantitative structure activity and toxicity relationships. The outcome of these studies explained most of the effects seen during degradation experiments under anaerobic conditions. For the prediction of the toxicity and for modeling the partitioning behavior of technical mixtures of AEO, it was necessary to understand the mechanisms on a molecular level with pure AEO.

The Anaerobic Screening Test System (ASTS)

The anaerobic screening test system (ASTS) was developed, simplified and optimized to obtain an easy access to anaerobic degradability data for AEO and other substances. This test system was designed and used for screening the anaerobic biodegradability of large amounts of chemicals under realistic conditions. Screening test systems have the disadvantage that they may produce false negative results due to either toxic effects at test concentrations or microbial variations during sludge pretreatment and during the experiment. Therefore, it is important to investigate substances that lead to negative degradation results in further detail, e.g. using lower test concentrations, different sludge pretreatment, other sludges, or a different test system. Positive degradation results, obtained with a screening test system in a reasonable time, give evidence of a good biodegradability of the substance investigated.

Anaerobic biodegradation and toxicity of alcohol ethoxylates

The anaerobic biodegradability of AEO decreased with increasing branching of the alcohols. This result was confirmed with sludge from different wastewater treatment plants. Lowering

the AEO concentration below a toxic level and using different sludges reduced toxic effects and long lag phases. During AEO degradation the toxicity can increase due to the accumulation of lower ethoxylated and more toxic AEO, which result from the stepwise degradation of the EO chain under anaerobic conditions. This increasing toxicity can hinder the anaerobic degradation of AEO compared to the aerobic degradation, where toxicity disappears after the central fission of the AEO. The toxicity of the AEO increased with the length of the alkyl chain and decreasing EO-chain length that means with increasing hydrophobicity.

The complete biodegradation of a compound under aerobic and anaerobic conditions is still the main criteria for an environmentally friendly behavior of a substance after use and can reduce the risk of other effects, such as mutagenesis or hormone-activity, which are much more difficult to detect. The no effect concentration of the AEO investigated here is above the concentration range, which is usually observed in municipal waste waters. It plays a major role for industrial waste waters, e.g. from the textile industry, where higher surfactant concentrations occur.

By combining the results from the degradation experiments with the toxicity studies of AEO performed in this work, it can be concluded that *linear* AEO are biologically better degradable than ethoxylates of branched alcohols under anaerobic and aerobic conditions. Ethoxylates of branched alcohols should therefore be replaced by linear AEO wherever possible in order to reduce the environmental risk of these substances.

Membrane-water partitioning of alcohol ethoxylates

The hydrophobic properties of alcohol ethoxylates influence the sorption and also the toxicity towards sludge. Until now there have been no descriptors for the hydrophobicity of AEO, which can directly be determined. Therefore we adapted two methods for the experimental determination of the membrane-water partition coefficient (K_{lipw}) for AEO using artificial liposomes. The membrane-water partition coefficients describe the true process that occurs during bioaccumulation. K_{lipw} of untested AEO can be estimated with the fragment method using fragment constants deduced from experimental results.

We propose to use K_{lipw} instead of K_{OW} as hydrophobicity descriptor in QSARs and QSTRs of surfactants and other substance, because liposomes model the biological membranes much better than the bulk phase experiment with octanol. K_{lipw} is a suitable descriptor for bioconcentration and toxicity towards *Fathead minnow* and *Daphnia magna*, and for the sorption of AEO to aerobic and anaerobic sludge.

Membrane toxicity of alcohol ethoxylates

The toxicity of pure alcohol ethoxylates was investigated to examine the mechanisms of toxicity and to set up a correlation between the toxic effect and the molecular composition of the AEO. Alcohol ethoxylates caused membrane toxic effects due to their hydrophobic properties by interacting with the membrane structure. The toxic effects were observed also below the critical micelle concentration (CMC), at which the membrane structure is affected. The series of linear AEO investigated appeared to act as either non-polar or polar narcotics depending on the number of ethoxylate units.

The combination of membrane-water partitioning and membrane toxicity allowed the evaluation of bioavailability and target-site concentrations. This information can be generalized for and compared to other biological systems.

Final remarks

The assessment of the environmental behavior of alcohol ethoxylates shows all the problems of substances, which are used in large amounts worldwide in many different products, such as washing powders, household detergents or industrial cleaners. Although the amount of biodegradation and toxicity data for AEO seems to be large, the understanding of the degradation behavior and the mode of toxic action are still small. In most studies technical mixtures were investigated. This can be on one hand explained by the small amount of commercially available pure reference substances, which are essential for the investigation of quantitative structure biodegradation and toxicity relationship. On the other hand, it is essential to investigate the products - the complex mixtures - with simple and easy to use test systems under experimental conditions, which are close to the field situation. In this study very specific membrane toxicity tests were used to investigate the mode of toxic action of AEO, to draw QSAR and QSTR, and to correlate these results with other toxicity data. With the results obtained on the molecular level it is possible to model and to predict the toxicity behavior. This work contributes with the anaerobic biodegradation, the partitioning and the toxicity data to complete the environmental assessment of alcohol ethoxylates.

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Abreviations

3,4,5-TCC	3,4,5-trichlorocatechol, 3,4,5-trichloro-1,2-dihydroxybenzene
3,4-DCC	3,4-dichlorocatechol, 3,4-dichloro-1,2-dihydroxybenzene
4-MCC	4-monochlorocatechol, 4-monochloro-1,2-dihydroxybenzene
AEO	alcohol ethoxylate
AES	alcohol ether sulphate
APE	alkyl phenol ethoxylate
APG	alkyl polyglucoside
AS	alcohol sulphate
catechol	1,2-dihydroxybenzene
CBR	critical body residue
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
CTAS	cobalt thiocyanat active substance
DNP	a-dinitrophenol
EDTA	ethylene-diamin-tetraacetate
EO	ethoxylate
g	gravitation constant of the earth: 9.81 $m \cdot s^{-2}$
LA	linear alcohol
LAS	linear alkyl benzene sulphonate
LBB	lethal body burden
MBA	multiple-branched alcohol
MBAS	methylene blue active substance
P_i	inorganic phosphate
PQQ	4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]-quinoline-2,7,9-tricarboxylic acid
SAS	secondary alkyl sulphonate
SBA	single-branched alcohol
TRS	time resolved spectrophotometry
WAP	weighted average partition coefficient
WTP	wastewater treatment plant

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Summary

Alcohol ethoxylates (AEO) are nonionic surfactants composed of a long chain fatty alcohol (hydrophobic moiety) combined with one or more ethylene oxide (EO) units (hydrophilic moiety). Usually, technical AEO are mixtures of different alcohols combined with a varying amount of EO-units. Approximately 750'000 tons of AEO are produced per year worldwide. In Switzerland, the AEO consumption amounts to 6000 - 7000 tons per year. In contrast to aerobic biodegradation only little is known about the fate of AEO in the anaerobic environment. Therefore, the anaerobic degradation, the toxicity towards anaerobic sludge and the partitioning behavior of AEO were investigated in this thesis.

Due to their hydrophobicity, a substantial part of the AEO present in the waste water reach the anaerobic reactor in the wastewater treatment plant (WTP) either sorbed to primary or activated sludge. Until now, the anaerobic biodegradation and toxicity have only been studied for the most common linear alcohol ethoxylates. In this work, the anaerobic biodegradability and toxicity were investigated for ethoxylates of linear, single- and multiple-branched alcohols.

To determine the anaerobic biodegradability of AEO and other test chemicals, the Anaerobic Screening Test System (ASTS) was developed based on the ECETOC screening test. The test system and the sample preparation procedure were optimized with different test substances (C_2- to C_{16}-compounds, sodium laurylsulfate, soaps, complexing agents) and industrial waste waters from food- and textile industry. The performance of the ASTS was optimized in order to decrease the test substance concentrations down to 10 mg $C \cdot L^{-1}$, because of toxic effects that were observed at higher concentrations for surfactants.

The anaerobic biodegradability and toxicity of linear, single- and multiple-branched alcohol ethoxylates were investigated with the ASTS using sludge from different wastewater treatment plants (WTP). With sludge from WTP Au-Bruggen high degradation rates were found for linear $C_{12/14}$-alcohol ethoxylates at concentrations of 30 mg/L (20 mg C/L) after 40 days. Ethoxylates of single- and multiple branched alcohols were not or only partly degraded. The

toxicity towards this sludge, given as the no observed effect concentration (NOEC), increased with decreasing degree of ethoxylation from 10 to 100 mg/L at a sludge concentration of 2.0 ± 0.2 $g \cdot L^{-1}$. The degree of branching of the alcohol did not influence the toxicity significantly.

The anaerobic biodegradation of a linear (LA-$C_{12/14}EO_{20}$) and a single-branched AEO (SBA-C_8EO_{10}) were compared with sludge from three different wastewater treatment plants (WTP): Au-Bruggen, Wil, and Kloten/Opfikon. LA-$C_{12/14}EO_{20}$ was completely degraded within 10 days by two sludges, but was not degraded by a third one within 30 days. SBA-C_8EO_{10} was partly degraded to about 50 % by all three sludges within 30 days. With sludge from WTP Wil high degradation rates were found for ethoxylates of linear $C_{12/14}$-alcohols at concentrations of about 30 $mg \cdot g^{-1} \cdot L^{-1}$ (COD: 150 $mg \cdot L^{-1}$; sludge concentration: 2.5 $g \cdot L^{-1}$). Ethoxylates of some single- and multiple branched alcohols were only partly degraded with this digesting sludge. Overall, the anaerobic biodegradability of technical AEO decreased with increasing branching of the alcohol moiety from linear to multiple-branched alcohols.

The determination of the toxicity of technical alcohol ethoxylates towards anaerobic sludge allowed to set-up the test conditions and especially to define the AEO concentrations at which toxic effects could be eliminated in the degradation studies. A trend in toxicity was observed but due to the complex composition of the technical mixtures of AEO it was not possible to draw quantitative structure toxicity relationships (QSTR) from the experiments. Therefore, the mode of toxic action of various pure alcohol ethoxylates was investigated in detail with specific, membrane dependent toxicity tests. As a prerequisite the partitioning behavior between biological membranes and the water phase was experimentally determined for eight pure AEO. With these membrane-water partition coefficients it was possible to calculate the effect concentrations in the biological membrane, at which the membrane structure and functioning was non-specifically disturbed.

The liposome-water partitioning coefficients were determined for eight pure alcohol ethoxylates with two experimental methods, equilibrium dialysis and ultracentrifugation. Both methods yielded comparable results. The experimentally determined log K_{lipw}-values were compared with log K_{ow}-values estimated with the fragment method using fragment constants from literature. Fragments of log K_{lipw} were calculated for the EO- and the CH_2-units from the experimentally determined data. An additional CH_2-unit caused an average increase of log K_{lipw} by +0.46, whilst an additional ethoxylate-group (-O-CH_2-CH_2) caused an average decrease of log K_{lipw} by -0.14. With these fragments, the quality of log K_{lipw} estimations could be improved significantly as compared to simple linear regression of log K_{lipw} versus log K_{ow}. The K_{lipw}-

values, calculated according to the new fragment method for pure compounds and for commercial mixtures, were shown to be adequate descriptors for QSARs of bioaccumulation, toxicity, and sorption to natural organic matter.

The membrane toxicity of AEO was investigated with an *in-vitro* method based on time-resolved spectrophotometry on energy transducing membranes. The effect concentrations obtained for this narcotic effect of AEO correlated well with the results from various toxicity tests on whole organisms. All AEO exhibited their toxic effect at concentrations well below the critical micelle concentration. When comparing aqueous effect concentrations, toxicity increased strongly with increasing length of the alkyl chain and showed a small parabolic dependence on the number of ethoxylate units with a maximum at 8 ethoxylate units. With the toxic effect expressed in terms of membrane concentrations, all alcohol ethoxylates exhibited similar activity in the concentration range typical for narcotic chemicals. The toxic membrane concentrations of AEO with 5 and ≥8 ethoxylate units were 0.2 and 0.06 mol per kg lipid, which correspond to the critical body residues of non-polar and polar narcotics in fish. In addition the toxic effects of mixtures of AEO were measured and could be modeled as the sum of activity of the single constituents, confirming the concept of concentration additivity of compounds with the same mode of toxic action.

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Curriculum vitae

16th of March 1967	Born in Winterthur, Switzerland.
1974-1980	Primary school in Frauenfeld.
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1986	Laboratory work in the sugar factory Frauenfeld.
1987	Military Service.
1987-1992	ETH Zurich Dep. XB: Environmental Sciences.
1989-1991	Work on the chemico-physical database for all Swiss lakes (MASAS project) at the Swiss Federal Institute of Environmental Sciences (EAWAG)
1992	Diploma of the ETH Zurich in Sciences (Dipl. Natw. ETH).
1992-1995	Chemistry and biology teacher at the gymnasium of Frauenfeld.
1994	Diploma of the higher teaching degree in chemistry (ETH Zurich).
1994-1998	Ph.D. Thesis at the Swiss Federal Institute of Materials Testing and Research (EMPA, St. Gall) and the Swiss Federal Institute of Environmental Sciences (EAWAG, Dübendorf).
1996-1997	Installation of the permanent exhibition about *Nature and Man* in the Museum of natural sciences in Frauenfeld.
1998	Chemistry teacher at the gymnasium of Frauenfeld.
1998 - 1999	Technical Service at Millipore Switzerland, Analytical Products Division.
1999 - 2000	Start-up of the European Tech Service Center of Millipore in the CSSO in Molsheim, Strasbourg, France.