Toluene:
biological waste-gas treatment, toxicity and microbial adaptation

Promotor:	dr. ir. J.A.M. de Bont hoogleraar in de Industriële Microbiologie
Co-promotor:	dr. ir. S. Hartmans universitair docent bij de sectie Industriële Microbiologie

Frans J. Weber

Toluene: biological waste-gas treatment, toxicity and microbial adaptation

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in de landbouw- en milieuwetenschappen
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Stellingen

1

Enten met een specifieke cultuur kan een grote invloed hebben op de werking van een bioreactor die onder niet steriele condities werkt.

Dit proefschrift, hoofdstuk 3.

2

Ook schimmels zijn in staat om op tolueen als enige koolstof- en energiebron te groeien.

Dit proefschrift, hoofdstuk 4.

3

De HPLC-UV methode zoals beschreven door o.a. Rehman (1991) kan niet worden gebruikt voor een kwantitatieve fosfolipide bepaling.

Rehman, S.U. (1991). Rapid isocratic method for the separation and quantification of major phospholipid classes by high-performance liquid chromatography. J. Chrom. 567: 29-37.

4

De conclusie van Lepage *et al.* (1987) en Šajbidor *et al.* (1992) dat de gevonden veranderingen in de samenstelling van het celmembraan een adaptatie is aan de giftige werking van organische oplosmiddelen, is onjuist gezien de waarneming van deze auteurs dat de groei van de gebruikte micro-organismen sterk wordt geremd in de aanwezigheid van deze oplosmiddelen.

Lepage, C., F. Fayolle, M. Hermann and J.P. Vandecasteele. (1987). Changes in membrane lipid composition of Clostridium acetobutylicum during acetone-butanol fermentation: effects of solvents, growth temperature and pH. J. Gen. Microbiol. 133: 103-110.

Šajbidor, J., and J. Grego. (1992). Fatty acid alterations in Saccharomyces cerevisiae exposed to ethanol stress. FEMS Microbiol. Lett. 93: 13-16.

5

Gezien de tegenovergestelde effecten die benzeen en druk hebben op het celmembraan, lijkt het screenen van diepzeemonsters op benzeentolerante micro-organismen weinig zinvol.

Moriya, K. and K. Horikoshi. (1993). Isolation of benzene-tolerant bacterium and its hydrocarbon degradation. J. Ferm. Bioeng. 76: 168-173.

6

Het moduleren van het proces in een biofilmreactor komt neer op het schatten van 12 parameters om de 13e te kunnen voorspellen.

7

Juryleden bij verschillende sporten moeten over bovennatuurlijke gaven beschikken om het verschil tussen een 9.95 en een 10.0 te kunnen waarnemen.

8

Om de kansen voor een AIO/OIO op de arbeidsmarkt te verhogen, zou er net als bij bepaalde studies een "numerus fixus" moeten worden ingesteld voor promovendi.

9

Het optreden van de milieu-organisatie "Greenpeace" tegen het in de oceaan dumpen van het olieplatform "Brent Spar" is ongeloofwaardig nadat deze organisatie de door een explosie verwoeste "Rainbow Warrior" in de oceaan liet afzinken.

10

Het televisiekijkgenot is omgekeerd evenredig met het aantal aangeboden TV-zenders.

11

De toename in de grootte van computerprogrammatuur is geen garantie voor een evenredige verhoging van kwaliteit en snelheid.

12

De introductie van nieuwe elementen in bestaande natuurgebieden behoort niet tot de taken van een natuurbeheerder.

Stellingen behorende bij het proefschrift "Toluene: biological waste-gas treatment, toxicity and microbial adaptation", Frans Weber, Wageningen, 15 november 1995.

Voorwoord

Zonder de bijdrage van een groot aantal mensen, die mij de afgelopen jaren hebben geholpen, had ik mijn promotieonderzoek nooit kunnen afronden tot het proefschrift zoals het nu voor u ligt. Graag wil ik iedereen hiervoor bedanken, een aantal van hen wil ik met name noemen:

Allereerst Jan de Bont en Sybe Hartmans. Dankzij jullie vertrouwen en kritische blik heb ik de afgelopen jaren veel geleerd en met veel plezier aan het onderzoek gewerkt.

Hans Rijs en Ed Waalewijn van Ecosens voor prettige samenwerking en de contacten met de praktijksituatie. Met name de geboden vrijheid in het onderzoek heb ik zeer gewaardeerd. Hierdoor was het voor mij mogelijk om naast de biologische afvalgasreiniging, interessante "zij-sporen" te bestuderen.

Zoals gezegd, veel van de resultaten zijn verkregen door de inbreng van anderen. Met name noem ik Ruud Schemen, Sonja Isken, Lydia Ooijkaas, Randall Coffie, Marjon Diender, en Ko Hage. Jullie hebben als onderdeel van het docteraal onderzoek gewerkt aan vaak weer een nieuw deel van het onderzoek. Bedankt voor jullie enthousiaste inbreng en voor de fijne samenwerking. Natuurlijk bedank ik ook alle (ex)collega's en studenten van de sectie Industriële Microbiologie voor jullie belangstelling, discussies en hulp, maar met name de goede sfeer op het lab en daar buiten. Hermann Heipieper en Jan Sikkema voor verschillende discussies. Kees Teunis van de vakgroep Organische Chemie, Folkert Hoekstra, vakgroep Plantenfysiologie en Jacques Vervoort, vakgroep Biochemie voor de hulp bij respectievelijk, de GC-MS, FTIR en NMR analyses.

Tenslotte wil ik Bernadette bedanken voor de steun en het benodigde "zetje in de rug" dat vooral tijdens de afronding van mijn proefschrift welkom was, en mijn ouders voor de mogelijkheden ze mij hebben geboden. Helaas is in februari 1995 tijdens het schrijven van de laatste hoofdstukken van dit proefschrift, mijn vader plotseling overleden. Hij had zo graag bij mijn promotie willen zijn. Ik draag dit proefschrift aan hem op.

Contents

1

General introduction

Since the industrial revolution the production and use of chemicals has increased immensely. As a consequence all kinds of wastes are produced, which are released in to the environment. Many products of the petrochemical industry, like organic solvents, fuels, herbicides or insecticides, will eventually also be released into the environment, due to production or storage losses, solvent evaporation, or emissions from motor vehicles and aeroplanes. For example for toluene it has been estimated that more than 86% of the $0.5\text{-}1*10^7$ tonnes of toluene produced in 1981 was ultimately released into the environment (Anonymous, 1985).

Nowadays there is a growing awareness concerning the possible toxic or even carcinogenic effects of these xenobiotics. Therefore the release of these chemicals into the environment is restricted by legislation. In order to meet these requirements, the industry has to reduce her waste streams. In general this can be achieved by implementing another or a better control of the process. For instance, recovery and recirculation of organic solvents can significantly reduce the waste streams. However, this will often require expensive process modifications and therefore, quite often an end-of-pipe technique is used to remove contaminants from the waste streams such as incineration or biological treatment.

Fig. 1 clearly shows that the emission of organic hydrocarbons in The Netherlands is mainly to air. In the period between 1982 and 1992 treatment of waste waters and changes in production processes have resulted in a reduction of the emission

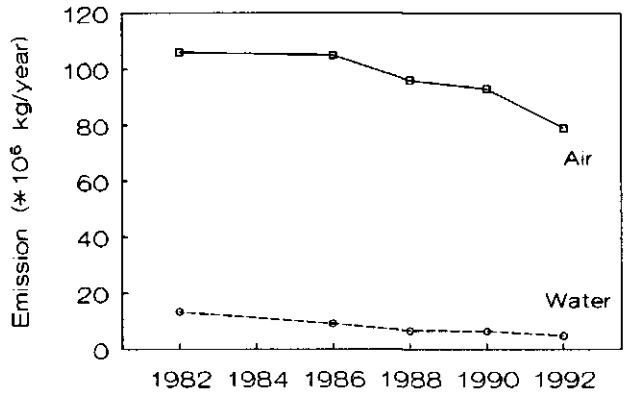

Figure 1: Industrial emission of organic hydrocarbons to air (–) and water (--) in The Netherlands between 1982 and 1992. (From van der Plas & Verhoeve, 1994)

of organic hydrocarbons to water by more than 50% (Fig. 1). Treatment of waste gases was, however, less successful, although a gradual decrease in the emission of organic hydrocarbons to air is observed since 1986. The growing environmental awareness and more stringent legislation is expected to result in a further reduction of the gaseous emissions in the future.

In The Netherlands, the release of gaseous compounds is controlled by the NER (Nederlandse Emissie Richtlijnen; Anonymous, 1992). Depending on the nature of the compound the NER describes a maximal off-gas concentration above a certain quantity of contaminant emitted. Normally, the maximal allowable off-gas concentration for an emission of more than 3.0 kg/h is 150 mg/m^3, however, for carcinogenic compounds, e.g. benzene, the limit is 5 mg/m^3 when the emission exceeds 25 g/h (Anonymous, 1992).

In order to meet these limits, various techniques for waste-gas treatment are applied and being developed. Either physico-chemical or biological techniques are used to treat waste-gas streams. Examples of physico-chemical methods are incineration, catalytic oxidation, scrubbing or adsorption on activated carbon (Heck *et al.*, 1988). Biological techniques make use of the capacity of microorganisms to degrade the toxic contaminants to less toxic products, often resulting in complete mineralization. Biological techniques are often the most cost

effective for waste gases containing less than 0.5 - 3 gram of biodegradable organic compounds per m^3 (Leson & Winer, 1991; Bohn, 1992; Kok, 1992).

BIOLOGICAL WASTE-GAS TREATMENT

Biological waste-water treatment has become an established method to reduce the release of contaminants into the environment. Biological treatment of waste gases, however, is still only applied on a minor scale, mainly for the treatment of odorous air (Ottengraf, 1987; Anonymous, 1989; Leson & Winer, 1991). These odorous waste-gas streams generally contain low concentrations of complex mixtures of compounds. Due to the increasing stringent legislation concerning the emission of volatile organic compounds, there is nowadays a growing interest to also apply biological waste-gas treatment techniques for the removal of higher concentrations of specific contaminants. For the biological removal of pollutants from waste gases several types of bioreactors have been developed (Ottengraf, 1987; Groenestijn & Hesselink, 1993) (Fig. 2).

Biofilters

Biofilters were the first bioreactors used for the biological treatment of waste gases. Already since 1920 biofilters have been used on a limited scale to remove odorous compounds from waste gases of for instance waste-water treatment plants (Leson & Winer, 1991). A biofilter is essentially a packed-bed reactor through which the waste gas is forced (Fig. 2). Microorganisms growing on the packing material, usually compost, will degrade the contaminants present in the waste-gas stream. Besides the simple and cost effective operation of biofilters, another advantage is the absence of a distinct water layer in the biofilter. Therefore, the mass transfer of poorly water soluble compounds to the biofilm is relatively good in this type of reactor.

The first biofilters were actually soil beds. Nowadays generally compost or peat, mixed with bark chips or polystyrene particles are used as packing material. The addition of wood bark or polystyrene results in a homogeneous structure of the packing with a relatively low pressure drop (Ottengraf, 1986; van Langenhove *et al.*, 1986). Since the introduction of the first biofilters another important improvement has been the development of closed biofilters, allowing a better control of the physical condition of the biofilter. In the open biofilters the packed bed is exposed to fluctuating weather conditions (temperature, rain). With the closed biofilters, the microbial activity within the biofilters can be better maintained, as temperature and humidity which strongly affect the biological activity can be better controlled (Heslinga, 1992). Usually the in-let gas stream is humidified to almost complete saturation. Generally, additional humidification of

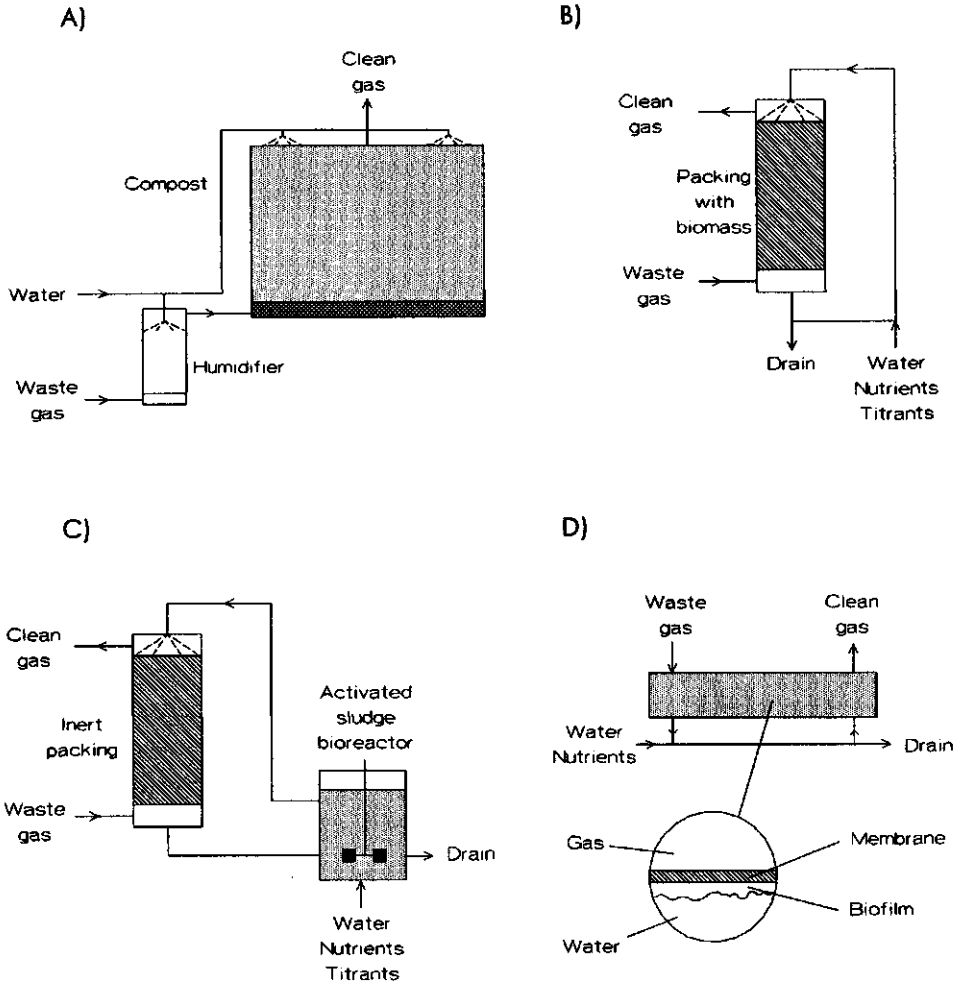

Figure 2: Various reactors for biological waste-gas treatment. (A) Biofilter, (B) Trickle-bed bioreactor, (C) Bioscrubber, (D) Membrane bioreactor.

the filter is also necessary to compensate for water loss due to an increase in temperature of the waste gas caused by the microbial activity. Besides the stringent control of the humidity other factors which are difficult to control are the pH and the supply of additional (inorganic) nutrients. Although several additives, like lime, can be mixed through the packing to buffer the pH, these additives will not be sufficient to continuously neutralise the HCl formed due to the biodegradation of chlorinated compounds. Several studies have shown that nutrient (e.g. nitrogen) limitations can strongly influence the overall performance of the biofilter (Don, 1986; Beyreitz *et al.*, 1989; Weckhuysen *et al.*, 1993). As a homogenous supply of these nutrients to the biofilter is difficult, it is expected that the reported elimination rates of more than 100 g carbon per m^3 packing material per hour (Ottengraf *et al.*, 1986; Windsperger *et al.*, 1990; Kirchner *et al.*, 1991) can not be maintained during a longer period of time. Nutrients in the compost will become exhausted, and also pH and humidity control are expected to become a problem.

Bioscrubber

In bioscrubbers the contaminants in the waste gas are transferred to a water phase in a scrubber with an inert packing. The compounds in the water are subsequently degraded in a separate reactor, which is usually a traditional activated sludge waste water treatment facility. The water phase is subsequently recirculated through the scrubber compartment (Fig. 2). In contrast to biofilters, a circulating water phase is present, which allows a better control of reactor conditions (pH, nutrients) and makes it possible to treat waste gases containing compounds like ammonia or chlorinated hydrocarbons. However, a drawback compared to the biofilter is, that this type of reactor can only be used to remove contaminants with a relatively high water solubility. In order to increase the mass transfer of less water soluble compounds, the use of organic solvents to enhance the mass transfer has been suggested (Schippert, 1994). The intermediate solvent should be non-toxic, non-volatile and not biodegradable. By applying such a solvent in a three-phase reactor the transfer of the contaminants from the gas phase to the solvent phase can be significantly enhanced. However, the biodegradation will only take place in the water phase which requires a transfer of the contaminant from the solvent to the water phase. In this situation the mass transfer from the solvent to the water phase will probably become rate limiting. An overall increase in the mass transfer is only observed when a very high specific exchange area between the solvent and water phase can be achieved (Cesário *et al.*, 1994).

Trickle-bed bioreactor

In a trickle-bed reactor some of the advantages of the biofilter and the bioscrubber are combined. The circulating water phase allows better control of the reactor conditions. Due to the combination of the scrubber and bioreactor compartments, the contaminants transferred to the water phase will be immediately degraded resulting in low contaminant concentrations in the water phase and consequently a better mass transfer of moderately water soluble compounds.

Although trickle-bed reactors are used in waste water treatment systems, they are not yet frequently used for the treatment of waste gases. Biological waste-gas treatment using trickle-bed reactors has been successfully demonstrated for the removal of dichloromethane from waste gases (Diks & Ottengraf, 1991; Hartmans & Tramper, 1991). Dichloromethane, which is moderately water soluble was shown to be eliminated with a sufficient efficiency over a longer period of time at rates of about 50 g/(m^3 h). However, this specific degradation rate amounts to only 7 grams of carbon per m^3 per h. Using other gas combinations, resulting in higher organic loads, clogging of the reactor due to excessive biomass formation was observed (Diks, 1992).

In this thesis results are presented which describe possible methods to prevent clogging of the reactor. Subsequently the application of trickle-bed reactors for the degradation of a waste-gas stream containing a high toluene load, during a longer period of time with a high degradation rate, will be demonstrated.

Membrane bioreactor

In a membrane bioreactor the gas phase and the liquid phase, containing the microorganisms, are separated by a porous hydrophobic membrane. As the membrane makes it possible to prevent the release of microorganisms into the environment, this reactor type is being evaluated for use in space-cabins to maintain a clean atmosphere during long-term manned space-flights. The pores of the hydrophobic membrane are filled with air resulting in a low mass transfer resistance (Hartmans *et al.*, 1992). As the microorganisms form a biofilm directly on the membrane, the mass transfer resistance for contaminants from the gas phase to the biofilm is expected to be low. By using a hollow-fibre reactor a large specific gas/biofilm surface can be created, resulting in a reactor with a low mass transfer resistance and a high specific surface area, and the ability to control the environment of the biofilm by the circulating water phase like in a trickle-bed reactor. It has been shown that with this type of reactor, a stable biodegradation of propene could be obtained during prolonged operation (Reij *et al.*, 1995).

MICROBIOLOGICAL ASPECTS OF WASTE-GAS TREATMENT

The first criterion for a successful biodegradation process is of course the availability of microorganisms capable of degrading the contaminant. For the biodegradation of xenobiotics which were previously thought to be non-biodegradable nowadays more and more microorganisms are being selected from nature, which are capable of degrading these compounds. The metabolic pathways for the degradation of a great number of compounds have been elucidated in many different microorganisms. Furthermore, genetic engineering has offered possibilities to combine and construct new pathways for the degradation of xenobiotics that were not degraded previously.

Although many (xenobiotic) compounds are intrinsically biodegradable, actual degradation rates depend on a number of parameters. One of the most important parameters is the contaminant (substrate) concentration. The off-gas concentrations required by legislation, especially for carcinogenic compounds, are extremely low and in some cases too low for biodegradation at an acceptable rate (Hartmans, 1994).

Application of biological waste-gas treatment techniques for the removal of higher concentrations of organic solvents seems to be promising. However, a problem could be the toxicity of the pollutants in the waste gas for the microorganisms in the bioreactor. Although many xenobiotic compounds are tolerated and degraded by microorganisms at low concentrations, they can already become toxic at slightly higher concentrations (Volsky *et al.*, 1990; Blum & Speece, 1991). Especially compounds with a good solubility in water can be expected to accumulate in the water phase of the reactor during the start-up period. This accumulation can result in the inactivation of the biomass in the reactor as the contaminant concentrations reach toxic levels. Also fluctuations in the contaminant concentrations in the waste gas could result in levels which are toxic for the microorganisms in the reactor. Interestingly, several microbial strains have been described that can adapt to these toxic levels, resulting in the capacity to tolerate and grow in the presence of these toxic solvents (Inoue & Horikoshi, 1989; Cruden *et al.*, 1992).

OUTLINE OF THIS THESIS

In this thesis several aspects of the biodegradation of xenobiotics, especially toluene, and the removal of toluene from waste gases with trickle-bed reactors are discussed. In waste gases, the concentration of the contaminants generally

fluctuate strongly. In Chapter 2 the selection and use of adsorbents to buffer these fluctuating concentrations is discussed. The application of trickle-bed reactors for the removal of xenobiotics from waste gases also depends on the ability to control the biomass content of these reactors (i.e. to prevent clogging). In Chapter 3 two possible methods are addressed with which the formation and removal of biomass from these reactors could be controlled. In one of the trickle-bed reactors used for the removal of toluene, a fungal culture developed. This organism was isolated and a tentative pathway for the biodegradation of toluene by this fungus, *Cladosporium sphaerospermum*, is presented in Chapter 4. Ultimately, most pollutants and hazardous substances are of concern because of their potential toxic effects on man. However, these xenobiotics can also be toxic for microorganisms (Chapter 5) and this might limit the application of these organisms for biodegradation processes. Generally, the toxicity of lipophilic solvents is due to interactions of these solvents with the cell membrane. Consequently the functioning of the membrane as a permeability barrier is affected. These effects of various organic solvents on the functioning of the cell membrane, and the adaptation mechanisms of microorganisms to combat these effects are reviewed and discussed in Chapter 8. *Pseudomonas putida* S12 is a strain which can grow in the presence of supersaturating solvent concentrations which are normally extremely toxic to microorganisms. In Chapter 6 the growth of *P. putida* S12 on supersaturating concentrations of styrene is presented. Aspects of the adaptation of this strain and other solvent tolerant *P. putida* strains to allow growth in the presence of supersaturating amounts of solvents are described in Chapters 7 and 8. Finally, in Chapter 9 the potential of trickle-bed reactors and solvent-tolerant bacteria for biotechnological applications are discussed.

REFERENCES

Anonymous. (1985). Toluene. Environmental Health Criteria 52, World Health Organization, Geneva.

Anonymous. (1989). Biologische Abgas-/Abluftreinigung: Biofilter. VDI-Handbuch Reinhaltung der Luft. Band 6. VDI 3477 (entwurf), Verein Deutscher Ingenieure, Düsseldorf.

Anonymous. (1992). Nederlandse emissie richtlijnen lucht. Commissie emissies lucht, Stafburo NER, Bilthoven.

Beyreitz, G., R. Hübner and M. Saake. (1989). Biotechnologische Behandelung lösemittelhaltige Abluft. *Wasser Luft und Boden* **9:** 53-57.

Blum, D.J.W. and R.E. Speece. (1991). A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. *Res. J. WPCF* **63:** 198-207.

Bohn, H. (1992). Consider biofiltration for decontaminating gases. *Chem. Eng. Prog.* (April) 34-40.

Cesário, M.T., H.H. Beeftink and J. Tramper. (1994). Removal of poorly water-soluble compounds from waste gases using water-immiscible organic solvents. *In:* L. Alberghina, L. Frontali and P. Sensi (eds.), Proceedings of the 6th European congress on biotechnology, pp. 1207-1210, Elsevier, Amsterdam.

Cruden, D.L., J.H. Wolfram, R.D. Rogers and D.T. Gibson. (1992). Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. *Appl. Environ. Microbiol.* **58:** 2723-2729.

Diks, R.M.M. and S.P.P. Ottengraf. (1991). Verification studies of a simplified model for the removal of dichloromethane from waste gases using a biological trickling filter. (Part II). *Bioproc. Eng.* **6:** 131-140.

Diks, R.M.M. (1992). The removal of dichloromethane from waste gases in a biological trickling filter. PhD-Thesis, Technical University, Eindhoven.

Don, J.A. (1986). The rapid development of biofiltration for the purification of diversified waste gas streams. *In:* VDI Berichte 561, Geruchstoffe, pp. 63-73, VDI Verlag, Düsseldorf.

Groenestijn, J.W. and P.G.M. Hesselink. (1993). Biotechniques for air pollution control. *Biodegradation* **4:** 283-301.

Hartmans, S. and J. Tramper. (1991). Dichloromethane removal from waste gases with a trickle-bed bioreactor. *Bioproc. Eng.* **6:** 83-92.

Hartmans, S., E.J.T.M. Leenen and G.T.H. Voskuilen. (1992). Membrane bioreactor with hydrophobic membranes for waste-gas treatment. *In:* A.J. Dragt, and J. van Ham (eds.), Biotechniques for air pollution abatement and odour control policies, pp. 103-106. Elsevier, Amsterdam.

Hartmans, S. (1994). Microbiological aspects of biological waste gas cleaning. *In:* VDI Berichte 1104, Biologische Abgasreinigung, pp. 1-12, VDI Verlag, Düsseldorf.

Heck, G., G. Müller and M. Ulrich. (1988). Reinigung lösungsmittelhaltiger Abluft: alternative Möglichkeiten. *Chem. Ing. Tech.* **4:** 286-297.

Heslinga, D.C. (1992). Biofiltration technology. *In:* A.J. Dragt, and J. van Ham (eds.), Biotechniques for air pollution abatement and odour control policies, pp. 13-18, Elsevier, Amsterdam.

Inoue, A. and K. Horikoshi. (1989). A *Pseudomonas* thrives in high concentrations of toluene. *Nature* **338:** 264-266.

Kirchner, K., C.A. Gossen and H.-J. Rehm. (1991). Purification of exhaust air containing organic pollutants in a trickle-bed bioreactor. *Appl. Microbiol. Biotechnol.* **35:** 396-400.

Kok, H.J.G. (1992). Bioscrubbing of air contaminated with high concentrations of hydrocarbons. *In:* A.J. Dragt, and J. van Ham (eds.), Biotechniques for air pollution abatement and odour control policies, pp. 77-82, Elsevier, Amsterdam.

van Langenhove, H., E. Wuyts and N. Schamp. (1986). Elimination of hydrogen sulphide from odorous air by a wood bark biofilter. *Wat. Res.* **20:** 1471-1476.

Leson, G. and A.M. Winer. (1991). Biofiltration: An innovative air pollution control technology for VOC emissions. *J. Air Waste Manage. Assoc.* **41:** 1045-1054.

Ottengraf, S.P.P. (1986). Exhaust gas purification. *In:* H.-J. Rehm, and G. Reed (eds.), Biotechnology, Vol. 8, pp. 426-452, VCH Verlagsgesellschaft, Weinheim.

Ottengraf, S.P.P., J.J.P. Meesters, A.H.C. van den Oever and H.R. Rozema. (1986). Biological elimination of volatile xenobiotic compounds in biofilters. *Bioproc. Eng.* **1:** 61-69.

Ottengraf, S.P.P. (1987). Biological systems for waste gas elimination. *Trends in Biotechnol.* **5:** 132-136.

van der Plas, A. and P. Verhoeve. (1994). Trends in industriële emissies van prioritaire stoffen. Periode 1981 t/m 1992. Publikatiereeks Emissieregistratie, Nr 18, juli 1994, Ministerie van VROM, 's-Gravenhage.

Reij, M.W., K.D. de Gooijer, J.A.M. de Bont and S. Hartmans. (1995). Membrane bioreactor with a porous hydrophobic membrane as a gas-liquid contactor for waste gas treatment. *Biotechnol. Bioeng.* **45:** 107-115.

Schippert, E. (1994). Biowäschertechnologie. *In:* VDI Berichte 1104, Biologische Abgasreinigung, pp. 39-56, VDI Verlag, Düsseldorf.

Volsky Jr., V.T., C.P.L. Grady Jr. and H.H. Tabak. (1990). Effect of selected RCRA compounds on activated sludge activity. *Res. J. WPCF* **62:** 654-664.

Weckhuysen, B., L. Vriens and H. Verachtert. (1993). The effect of nutrient supplementation on the biofiltration removal of butanal in contaminated air. *Appl. Microbiol. Biotechnol.* **39:** 395-399.

Windsperger, A., R. Buchner and K. Stefan. (1990). Reinigung lösungsmittelhaltiger Abluft mit Biofiltern. Teil I: Laboruntersuchungen und Ergebnisse aus verschiedenen Industriezweigen. *Staub-Reinhaltung der Luft* **50:** 465-470.

2

Use of activated carbon as a buffer in biofiltration of waste gases with fluctuating concentrations of toluene

Frans J. Weber
and Sybe Hartmans

Summary: Fluctuations in contaminant concentrations often adversely influence the effectiveness of bioreactors for waste-gas treatment. Application of an adsorbent to minimize such fluctuations could improve the overall process. Therefore the buffer capacity of a number of activated carbons and other adsorbents was tested. The buffer capacity of the adsorbents depends on the desired concentration range of the contaminants entering the bioreactor and on the time available for desorption. When fluctuations between 0 and 1000 mg toluene per m^3 were supplied to a biofilter this resulted in significant concentrations of toluene leaving the biofilter. Using one selected type of activated carbon it was demonstrated that these fluctuations could be decreased to a value of about 300 mg/m^3, which was subsequently completely degraded in the biofilter.

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INTRODUCTION

It has been shown that biological waste-gas treatment is possible with high efficiencies and degradation velocities using biofilters or trickle-bed reactors (Ottengraf, 1986; Diks & Ottengraf, 1991; Hartmans & Tramper, 1991). However, fluctuating concentrations of the contaminants in the waste gas can have a negative influence on these performances. Temporary high concentrations can be toxic for the microorganisms in the reactor, resulting in inactivation of the system. Furthermore, for a reliable operation the design of the reactor should be based on the peak concentrations in the waste gas, which is not an economically favourable situation. Therefore, it would be desirable to buffer the fluctuations in the concentration of contaminants in waste gases by means of an adsorbent, so that a constant supply of contaminants to the bioreactor can be achieved.

MATERIALS AND METHODS

Equilibrium isotherm

The equilibrium isotherm of the amount of toluene adsorbed versus the gas-phase concentration was determined by placing 10 g of adsorbent in 250-ml flasks. These flasks were closed with a Teflon valve. Known amounts of toluene were added to these flasks which were placed in a shaking water-bath at 30°C. After 24 hours equilibrium was reached and the toluene concentration in the gas phase was determined.

Desorption profile

To determine the desorption rate the adsorbent was first loaded with toluene in a 1-l flask closed with a Teflon valve (24 h at 30°C). Enough toluene was added to achieve a gas phase equilibrium concentration of about 5000 mg/m^3. The adsorbent loaded with toluene was subsequently placed in a thermostated column (15 cm x 28 mm, 30°C). Through this column air was blown at a flow rate of 1.5 l/min (1000 h^{-1}), and the toluene concentration in the effluent gas was monitored continuously with an on-line gas-analyzer.

Experimental set-up of biofilter

The removal efficiency of fluctuating concentrations of toluene was determined in a biofilter (40 cm x 98 mm) packed with compost and polystyrene (20% v/v). Air was saturated with toluene by passing it through a bubble column with toluene at 30°C and was diluted with fresh air to the desired concentration. The total air flow was subsequently saturated with water and blown through the biofilter at a flow-rate of 0.65 m^3/h (210 h^{-1}). When activated carbon (450 ml) was mixed through

the compost, no polystyrene was added. For some experiments a separate activated-carbon column (25 cm x 50 mm) was placed in series with the biofilter. The air was humidified before entering the biofilter after it had passed through the activated-carbon column. The toluene concentrations in the air entering and leaving the activated-carbon column and/or the biofilter were analyzed with an on-line gas-analyzer.

Analytical methods

The toluene concentration in the gas phase was either determined by analyzing head-space samples on a gas-chromatograph or with an on-line gas-analyzer. The head-space samples (100 µl) were analyzed on a Packard 430 gas-chromatograph with a Porapak R column (100-120 mesh, 110 cm x 1/8 inch i.d.). For the on-line gas analysis a Chrompack CP2001 gas-analyzer with a OV-1 column (4 m) was used. Carbon dioxide was measured using the on-line gas-analyzer with a Hayesep A column (25 cm).

Materials

The activated carbons were a gift of Norit N.V., Amersfoort, The Netherlands. Silica gel was obtained from Merck, and Al_2O_3 from Aldrich.

RESULTS

Equilibrium isotherm

Different amounts of toluene were added to activated carbon in closed flasks. After equilibrium was reached between the toluene which adsorbed to the adsorbent and the amount left in the gas phase the toluene concentration in the gas phase was determined. In Fig. 1 such an equilibrium isotherm is shown for Norit R3 Extra at 30°C. Using these isotherms the amount of toluene adsorbed on the activated carbon at gas-phase concentrations of 100 mg/m^3 and 1000 mg/m^3 was determined. From these data the buffer capacity was calculated (Table 1). The equilibrium isotherm was also used to calculate the concentration of toluene in air that was passed through a column of Norit R3 Extra loaded with 400 mg toluene/g at a flow-rate of 1000 h^{-1} (Fig. 2).

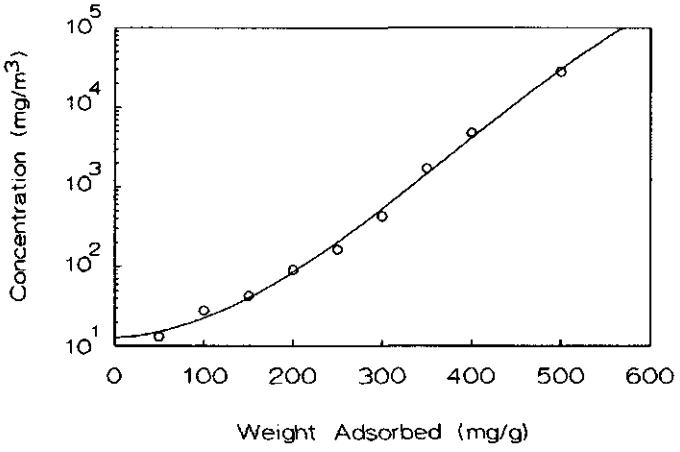

Figure 1: Adsorption isotherm of Norit R3 Extra - toluene at 30°C.

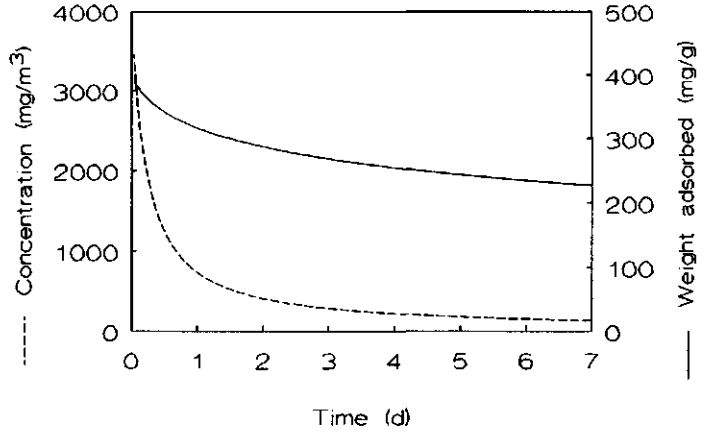

Figure 2: Theoretical desorption of toluene from Norit R3 Extra by an air-flow of 1000 h^{-1} at 30°C. The toluene concentration in the air leaving a column of activated carbon and the amount of toluene remaining adsorbed are calculated.

Table 1: Effective buffer capacities of adsorbents for toluene. The effective buffer capacity was determined from the adsorption isotherm or the desorption profiles in the concentration range 100 - 1000 mg toluene per m^3 air. The time required for a decrease in gas-phase concentration of 1000 to 100 mg/m^3 at a flow rate of 1000 h^{-1} and the amount desorbed (starting from an equilibrium gas-phase concentration of 1000 mg/m^3) in 12 h at this flow rate were determined from the desorption profiles.

Adsorbent	Effective buffering capacity (mg/g)		Time (d)	Desorbed in 12 hours (mg/g)
	Equilibrium isotherm	Desorption profile		
Norit RB3	70	18	1.0	14
Norit ROX0.8	110	62	3.5	20
Norit R3 Extra	120	74	4.3	23
Norit RO3	80	45	3.0	18
Norit ROW0.8 Supra	115	46	2.9	18
Norit Car 115 [1]	225	84	4.8	24
Silica gel	24	24	2.6	11
Al_2O_3	<25	-		-
Water	0.0034	-		-

[1] Car 115 is an experimental type of activated carbon.

Desorption profile

All the adsorbents were loaded with toluene and placed in a column. Through this column a constant air flow was blown and the toluene concentration in the off-gas was determined. Such a desorption profile is shown in Fig. 3. Using these profiles again the buffer capacities between 100 and 1000 mg/m^3 were calculated (Table 1). Also shown in this table is the time required to reduce the gas-phase concentration leaving the carbon column from 1000 to 100 mg/m^3 and the amount of toluene which is desorbed in 12 hours.

Biofilter

Norit R3 Extra was chosen to study the removal of fluctuating concentrations of toluene from waste gases with a biofilter. Three different configurations were tested. In the first configuration a biofilter was used without activated carbon. In the second set-up activated carbon was mixed with the compost in the biofilter. In the third system a separate activated-carbon column in series with the biofilter was used. Air containing 900 mg toluene per m^3 was supplied to these three reactors for 8 h a day. The biofilter without activated carbon had a removal

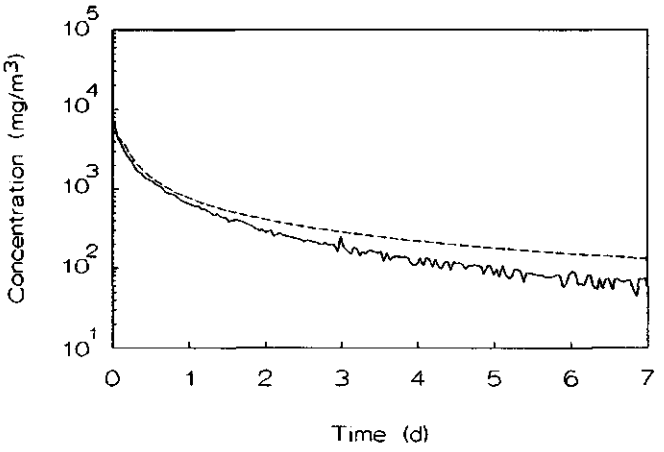

Figure 3: Theoretical desorption (---), calculated with the adsorption isotherm and assuming equilibrium, and measured (—) desorption of toluene from Norit R3 Extra. T: 30°C, Flow-rate: 1000 h^{-1}.

efficiency of about 50% (Fig. 4A). A similar removal efficiency was observed for the reactor in which activated carbon was mixed with the compost (Fig. 4B). When a separate column with activated carbon was placed before the biofilter the fluctuating toluene concentrations were buffered to a concentration of about 300 mg/m^3, which was completely degraded in the biofilter (Fig. 4C). The degradation velocities observed for the three configurations are shown in Table 2. Also shown are the observed degradation velocities when toluene was supplied continuously to these reactors.

DISCUSSION

Biological waste-gas treatment is especially useful to remove low concentrations of contaminants from waste gases. Higher concentrations of contaminants can be treated more effectively and cheaper using other systems (as for instance incineration) (Dragt, 1992). Therefore a concentration of 1000 mg/m^3 was chosen as the maximum concentration which should be allowed to enter a reactor for biological waste-gas treatment. In practise it is expected that contaminant concentrations in waste gases will fluctuate strongly, depending on the source. For a reliable system it would therefore be desirable to be able to buffer temporarily high (toxic) concentrations of contaminants by using an adsorbent

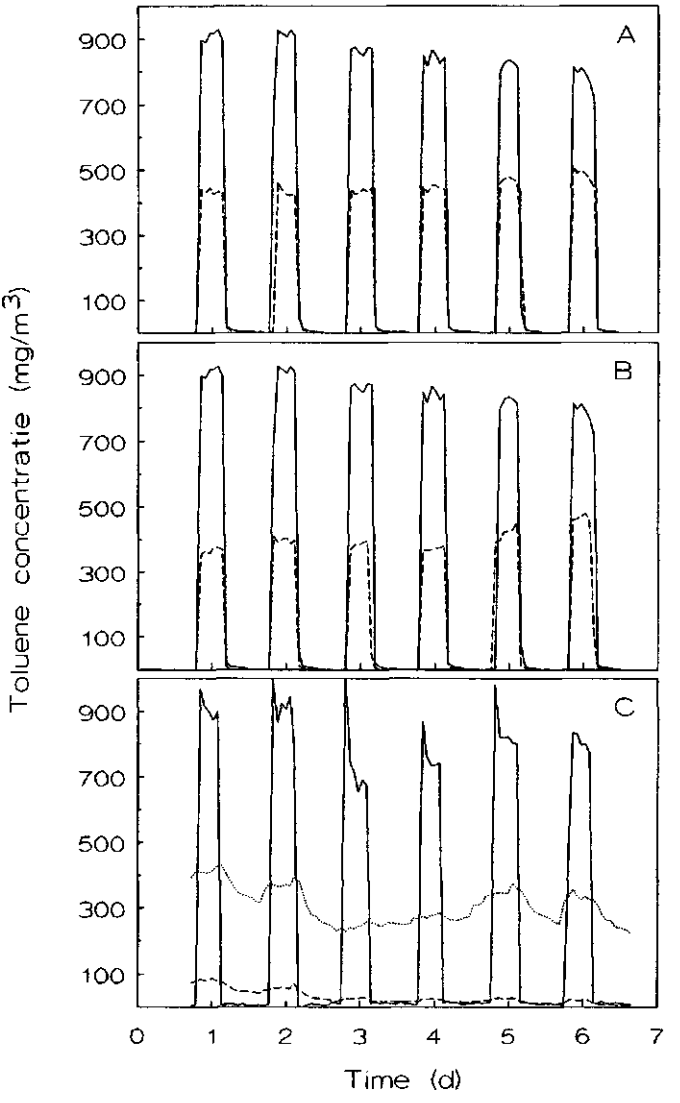

Figure 4A-C: Effect of Norit R3 Extra on the removal of fluctuating concentrations of toluene in a biofilter. (A) Biofilter without activated carbon. (B) Biofilter with activated carbon mixed with compost. (C) Biofilter with a separate activated carbon filter placed before the biofilter. (—) Toluene concentration of inlet air, (···) gas-phase concentration leaving activated-carbon column, (---) concentration in air leaving the biofilter. The results shown were obtained 20 to 30 days after start-up of the biofilters.

Table 2: Degradation rates of biofilters with and without activated carbon supplied with a fluctuating or a constant concentration of toluene.

Toluene supply:	Fluctuating				Continuous			
	Organic load [1]		Removal rate		Organic load		Removal rate	
	g-C/(m³ h)	g-C/(m³ day)	g-C/(m³ h)	g-C/(m³ day)	g-C/(m³ h)	g-C/(m³ day)	g-C/(m³ h)	g-C/(m³ day)
Activated-carbon column + biofilter	154	1240	52	1240	-	-	-	-
Biofilter mixed with activated carbon	189	1510	90	720	176	4220	39	936
Biofilter without activated carbon	189	1510	85	680	176	4220	44	1056

[1] for 8 h per day.

During periods with low contaminant concentrations in the waste gas, desorption should take place. In this manner there will be less fluctuations in the contaminant concentration supplied to the bioreactor. A useful adsorbent should therefore have a high buffer capacity below an equilibrium gas-phase concentration of 1000 mg/m^3.

At a concentration of 1000 mg toluene/m^3 in the gas phase Norit R3 Extra can adsorb about 300 mg/g (Fig. 1). However, the effective buffer capacity will be much lower than this as it will take months for the toluene to desorb totally again. This is shown more clearly when a desorption profile for Norit R3 Extra loaded with 400 mg toluene/g is calculated (Fig. 2). For this calculation it was assumed that there was equilibrium between the gas phase and the activated carbon. At gas-phase concentrations below 100 mg/m^3 a significant amount of toluene is still adsorbed on the carbon, which is only very slowly removed. Thus a significant amount of toluene adsorbed to the carbon will not be removed by desorption. This capacity will therefore not be available to buffer fluctuations in the contaminant concentration. Therefore we have defined the effective buffer capacity as the amount which can be adsorbed at equilibrium gas-phase concentrations between 100 and 1000 mg/m^3. Desorption resulting in equilibrium gas-phase concentrations below 100 mg/m^3 are not expected in practise as it can take more than a week to reach this equilibrium concentration (Fig. 2). These effective buffer capacities between 100 and 1000 mg/m^3 vary from 24 to 225 mg/g depending on the type of adsorbent (Table 1).
The actual amount of toluene desorbed from the adsorbent is less than predicted by the adsorption isotherm (Fig. 3). This difference between the calculated and measured desorption was probably due to the short residence time of the air in the column. An empty-bed contact time of 4 s was apparently too short for equilibrium between air and activated carbon to be reached. This results in lower toluene concentrations in the gas phase than calculated with the equilibrium isotherm. Owing to this effect the effective buffer capacity (between 100 and 1000 mg/m^3) is lower than calculated from the equilibrium isotherm.

If a flow rate of 1000 h^{-1} is used it still takes more than 4 days before enough toluene is desorbed to reduce the outlet gas-phase concentration from 1000 to 100 mg/m^3 (Table 1). These long desorption times are not very realistic in view of the fluctuations in contaminant concentrations observed under practical conditions, which are often the result of the 8-h working day. The effective buffer capacity therefore strongly depends on the time available for desorption. In 12 h only 23 mg toluene/g is desorbed from Norit R3 Extra loaded with 300 mg/g (equilibrium gas-phase concentration: 1000 mg/m^3) at a flow rate of 1000 h^{-1}.

Although the buffer capacity of the tested adsorbents for toluene is particularly strongly influenced by the desorption time, they can probably still be used as a buffering agent in waste-gas treatment. Of all tested adsorbents Norit Car 115 and Norit R3 Extra had the highest buffer capacity for toluene in the concentration range between 100 and 1000 mg/m^3. Norit R3 Extra was chosen for further study as a buffer in biological waste-gas treatment.

Mixing the activated carbon with the compost in the biofilter resulted in removal efficiencies comparable to those in a biofilter without activated carbon. During the 16 hours when no toluene was supplied to the reactor, almost no carbon dioxide production (signifying degradation) was observed, indicating that toluene was not buffered by the activated carbon in the biofilter. This is probably caused by the water present in the biofilter. The contaminant first has to diffuse through a water layer to reach the carbon surface. As liquid-phase diffusion is much slower than gas-phase diffusion almost no toluene will be adsorbed at these short contact times. For this reason much longer residence times (several hours) are generally used in waste water treatment to buffer shock loads of contaminants (Najm *et al.*, 1991; Chatzopoulus *et al.*, 1993). Ottengraf (1986) also concluded that water present in a biofilter diminished the buffer capacity of activated carbon.

By using a separate activated-carbon column placed before the biofilter, the presence of water in the carbon filter can be prevented. Under these conditions fluctuating toluene concentration could indeed be effectively buffered (Fig. 4C). Toluene (900 mg/m^3) supplied to this system during 8 h a day was buffered to a stable concentration of about 300 mg/m^3, which was completely degraded in the biofilter resulting in a specific removal rate of 1240 g-C/(m^3 day).

The degradation rate in the biofilter without activated carbon during the 8 h that toluene was supplied to the reactor was about 85 g-C/(m^3 h). When toluene was supplied continuously, the observed degradation rates were halved. This effect has also been observed for the degradation of ethyl acetate in a biofilter (Nolte, 1992). These differences in maximal specific removal rates are remarkable but can be explained when the degradation rate is assumed to be limited by mineralization processes in the biofilter. Release of inorganic nutrients from inactive biomass (mineralization) will take place continuously, and this can explain the higher degradation rates observed when toluene is supplied discontinuously to the biofilter. The mineralization rate is apparently sufficient to allow a continuous removal rate of about 40 g-C/(m^3 h) or a two- to three-times higher activity when contaminant removal only takes place during 8 h per 24 h.

Several experiments have indeed shown that the degradation rate in biofilters is limited by the available amount of nutrients (Don, 1986; Beyreitz *et al.*, 1989; Weckhuysen *et al.*, 1993).

In conclusion, we have demonstrated that application of an activated-carbon filter before treatment of waste gases with a fluctuating contaminant content can result in a better overall performance of a biofilter. As a consequence smaller biofilters can sometimes be applied for the treatment of a specific waste gas. It should, however, be emphasized that the waste gas entering the carbon filter has to be relatively dry. For the treatment of waste gases with a high water content the process seems less suitable.

ACKNOWLEDGEMENTS

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REFERENCES

Beyreitz, G., R. Hübner and M. Saake. (1989). Biotechnologische Behandelung lösemittelhaltige Abluft. *Wasser Luft und Boden* **9:** 53-57.

Chatzopoulus, D., A. Varma and R.L. Irvine. (1993). Activated carbon adsorption and desorption of toluene in the aqueous phase. *Am. Inst. Chem. Eng. J.* **39:** 2027-2041.

Diks, R.M.M. and S.P.P. Ottengraf. (1991). Verification studies of a simplified model for the removal of dichloromethane from waste gases using a biological trickling filter. (Part II). *Bioproc. Eng.* **6:** 131-140.

Don, J.A. (1986). The rapid development of biofiltration for the purification of diversified waste gas streams. *In:* VDI Berichte 561: Geruchstoffe, pp. 63-73, VDI Verlag, Düsseldorf.

Dragt, A.J. (1992). Opening address. *In:* A.J. Dragt, and J. van Ham (eds.), Biotechniques for air pollution abatement and odour control policies, pp. 3-9, Elsevier, Amsterdam.

Hartmans, S. and J. Tramper. (1991). Dichloromethane removal from waste gases with a trickle-bed bioreactor. *Bioproc. Eng.* **6:** 83-92.

Najm, I.M., V.L. Snoeyink, B.W. Lykins Jr. and J.Q. Adams. (1991). Using powdered activated carbon: a critical review. *J. Am. Wat. Works Ass.* **83:** 65-76.

Nolte, F.J. (1992). Ermittlung der Eliminationsleistung eines Biofilters bei der Behundlung von Ethylacetatablüften - Halbtechnische Pilotanlage. *In:* A.J. Dragt, and J. van Ham (eds.), Biotechniques for air pollution abatement and odour control policies, pp. 297-301, Elsevier, Amsterdam.

Ottengraf, S.P.P. (1986). Exhaust gas purification. *In:* H.-J. Rehm, and G. Reed (eds.), Biotechnology, vol. 8, pp. 426-452, VCH Verlagsgesellschaft, Weinheim.

Weckhuysen, B., L. Vriens and H. Verachtert. (1993). The effect of nutrient supplementation on the biofiltration removal of butanal in contaminated air. *Appl. Microbiol. Biotechnol.* **39:** 395-399.

3

Removal of toluene from contaminated air with a biological trickle-bed reactor

Frans J. Weber
and Sybe Hartmans

Summary: Generally removal of relatively high concentrations of organic compounds like toluene from waste gases in a trickle-bed reactor results in clogging of the reactor due to the formation of an excessive amount of biomass. We therefore limited the amount of nutrients available for growth, to prevent clogging of the reactor. As a consequence of this nutrient limitation a low removal rate was observed. However, when a fungal culture was used to inoculate the reactor, the toluene removal rate under nutrient-limited conditions was higher. Over a period of 375 days an average removal rate of 27 g-C/(m^3 h) was obtained with this fungal culture. We also studied the application of a NaOH-wash to remove excess biomass, as a method to prevent clogging. Under these conditions an average toluene removal rate of 35 g-C/(m^3 h) was obtained. After about 50 days there was no net increase in the biomass content of the reactor. The amount of biomass which was formed in the reactor equalled the amount removed by the NaOH-wash.

Submitted for publication

INTRODUCTION

Biological treatment can be an effective and relatively cheap technique for the removal of relatively low concentrations (<1 g/m^3) of contaminants from waste gases (Dragt, 1992; Ottengraf & Diks, 1992; Groenestijn & Hesselink, 1993). In Germany and The Netherlands biofilters have become an accepted technique, especially for the treatment of odorous waste-gas streams (Anonymous, 1989; Mildenberger, 1992). In biofilters the waste gas is blown through a packed bed of compost, peat, bark or other organic material, in which the natural microbial flora will degrade the contaminants present in the waste gas. A limitation of biofilters is that nutrient levels (e.g. nitrogen) and the pH are difficult to control. Humidification of the waste gas and maintaining a constant moisture content of the filter can also be problematic. Treatment of waste-gas streams containing chlorinated hydrocarbons is not possible in biofilters, due to the formation of hydrochloric acid. In a trickle-bed reactor these parameters can easily be controlled due to the presence of a circulating water phase. Previously, efficient removal of dichloromethane and dimethylacetamide using a trickle-bed reactor has been demonstrated (Diks & Ottengraf, 1991; Hartmans & Tramper, 1991; Waalewijn *et al.*, 1994).

Although trickle-bed reactors have certain advantages compared to biofilters, a major disadvantage can be a reduction of reactor performance due to the formation of excessive amounts of biomass. A stable removal of dichloromethane from waste gases over a period of more than 600 days has been reported (Diks & Ottengraf, 1991). However, the simultaneous removal of dichloromethane and acetone resulted in serious clogging of the reactor within 50 days (Diks, 1992). A reduce reactor performance due to clogging was also observed with several other compounds (Kirchner *et al.*, 1991; Diks, 1992; van Lith *et al.*, 1994; Weber & Hartmans, 1994).

Previously, we have shown that these clogging problems not only occurred due to the amount of biomass formed but also because of the poor biofilm formation on the packing material. Most of the biomass was present in lumps between the packing material (Weber & Hartmans, 1994). As most bacteria and packing materials are charged negatively an electrostatic repulsion exists between bacteria and between bacteria and the packing which prevents the formation of a stable biofilm. A higher ionic strength of the medium results in decreased repulsion and thus better biofilm formation (Oliveira, 1992; Rijnaarts *et al.*, 1993; Zita & Hermansson, 1994). Although an increase of the ionic strength of the water phase resulted in a better biofilm formation (Weber & Hartmans, 1994), excessive

biomass formation still can result in clogging and reduced reactor performance and should thus be prevented.

Especially, the removal efficiency of toluene in a trickle-bed reactor was shown to be sensitive to changes in the conditions in the reactor. The toluene removal rate sharply decreased when some clogging of the reactor occurred (Weber & Hartmans, 1994). The degradation of other compounds like butanone and butylacetate was not significantly affected under these conditions. The moderate water solubility of toluene probably causes this high sensitivity to changes in the reactor conditions. Therefore, toluene was chosen as a model contaminant to further study and optimize the performance of trickle-bed reactors. In this report we describe two approaches to prevent clogging of the bioreactor.

MATERIALS AND METHODS

Reactor

All experiments were performed in a semi-pilot scale bioreactor, consisting of a column with a diameter of 0.3 m and a height of 1.5 m. The reactor was filled to 1 m with a random-packing of Pall 50 mm rings (Vereinigte Füllkörper Fabrieken, Ramsbach-Baumbach, Germany; Specific surface area: 110 m^2/m^3). The gas stream was introduced at the bottom of the reactor, after being heated to 30°C by a heat-exchanger. The air flow was controlled at a rate of 7 m^3/h (100 $m^3/(m^2 h)$) by a swirl-meter (Fischer & Porter, Göttingen, Germany) and an automatic valve. Air saturated with toluene was added to this gas stream to give the desired inlet concentration. Toluene-saturated air was obtained by bubbling air through a thermostated bubble column (10 cm x 50 cm) with toluene, the air flow through this column was controlled by a mass-flow controller (Brooks Instrument, Veenendaal, The Netherlands). The gas-phase pressure drop over the reactor was continuously monitored by a differential pressure transducer.

A mineral salts medium was circulated counter-currently over the reactor at a flow rate of 0.5 m^3/h. The liquid was distributed on top of the packing by means of a nozzle (BETE Fog Nozzle, Spraybest, Zwanenburg, The Netherlands) and was collected at the bottom in a 70-dm^3 vessel. The temperature of the liquid in the vessel was controlled at 30°C and the pH was maintained at 7.0 by the addition of 0.5 N NaOH. Water was added to this vessel to compensate for the water lost by evaporation. Liquid and biomass were only removed from this vessel as indicated in the various experiments.

Microorganisms

A biomass suspension rich in fungi (>50% as examined by microscopy) was obtained by washing a biofilter, which has been used to remove toluene, with a 0.8% NaCl solution. This suspension is further designated as a fungal culture. An enrichment culture of especially bacteria was isolated from a water sample of the river Rhine near Wageningen, The Netherlands, using a phosphate-buffered (pH 7.0) mineral salts medium (Hartmans *et al.*, 1989) supplemented with 200 mg toluene/liter.

Medium

In the trickle-bed reactor tap water supplemented with various inorganic salts was used as the mineral salts medium. To a vessel of 70 l were added: 200 g NH_4Cl, 20 g $(NH_4)_2SO_4$, 31 g KH_2PO_4, 17 g $NaH_2PO_4 \cdot H_2O$, 14 g $MgCl_2 \cdot 7H_2O$, 2.0 g $FeSO_4$, and 2.0 g $CaCl_2$. For the experiments where the biofilm growth was controlled by a limitation of the nutrients, half the amount of nutrients were added. In all cases 820 g of NaCl was added to increase the ionic strength to 0.2 M.

Experimental conditions

The bioreactors were connected with an on-line gas-analyzer which continuously monitored the toluene and CO_2 concentrations of the inlet and off-gas streams. Routinely about 15 measurements were made from each gas flow every day. From these measurements average concentrations per day were calculated, which are used in the figures shown here.

For the nutrient limited trickle-bed reactor experiments (Fig. 1, 2 & 3), half the amount of inorganic nutrients was added on day 1. One trickle-bed reactor was inoculated with a bacterial culture, and a second reactor with a fungal culture. Both reactors were operated simultaneous under identical conditions. On day 23 the toluene removal rate at various organic loads was measured in both reactors. Various toluene concentrations were applied to the reactors and during one hour the inlet and outlet concentrations were measured every 10 minutes, after which the toluene load was changed.

In another experiment the circulation medium of the trickle-bed reactor was discharged and replaced with fresh medium every 14 days (Fig. 4). In a second reactor a NaOH wash was applied before the medium was replaced. For the NaOH-wash 280 g of NaOH was added to the reactor medium which circulated through the reactor, and after 3 hours the medium was discharged and replaced with fresh medium. As the pH of the circulation medium was still high after the medium replacement, the pH was restored to pH 7.0 by the addition of 2N HCl.

Analytical methods

Biomass concentrations were determined by weighing dried (24 h, 105°C) 10-ml samples of the liquid phase. The dry-weights were corrected for the salts present in the medium.

Toluene, 1,2-dichloroethane and CO_2 were analyzed with a CP 2001 gas-analyzer (Chrompack, Middelburg, The Netherlands) equipped with an OV-1 column (4 m) and a Hayesep A column (25 cm).

RESULTS

Nutrient limitation.

A major disadvantage, limiting the use of trickle-bed reactors for biological waste-gas treatment, can be clogging caused by the formation of excessive amounts of biomass. This excessive biomass formation should be prevented and it is expected that this can be controlled by limiting the amount of inorganic nutrients available for growth.

To test this we operated a 70-l trickle-bed reactor with a high loading rate of toluene (71 g/(m^3 h)) for 145 days with a single dose of inorganic nutrients containing 100 g NH_4Cl. This amount of nitrogen is expected to be sufficient to form a biofilm of 400 μm on the packing, assuming a biomass nitrogen-content of 9% (w/w) ($CH_{1.8}O_{0.5}N_{0.16}S_{0.0045}P_{0.0055}$) (Roels, 1983) and a biofilm density of 100 kg dry-weight/m^3.

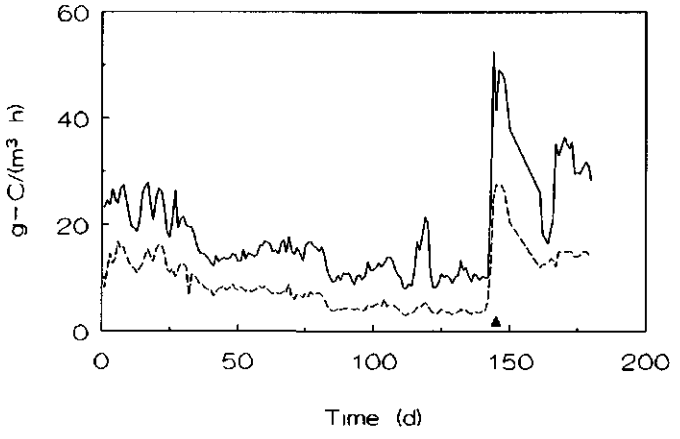

Figure 1: Toluene degradation rate (—) and CO_2 formation rate (---) in a trickle-bed reactor with an organic load of 65 g-C/(m^3 h). Additional nutrients were supplied on day 145.

In Fig. 1 the observed toluene removal rate is shown. On day 145 a second dose of inorganic nutrients was added to the reactor. Initially about 45% of the toluene present in the inlet gas-flow was degraded. After a number of weeks the degradation efficiency decreased to a stable level of about 20% (13 g-C/(m^3 h)). When additional nutrients were supplied on day 145, the degradation efficiency almost immediately increased to more than 75%, clearly illustrating that the biomass was nutrient limited.
Also shown in Fig. 1 is the CO_2 formation rate (on carbon basis). The difference in C-removal and CO_2 formation indicates that, during the whole period, a significant part of the degraded toluene is not converted into CO_2.

Effect of inoculum

The data shown in Fig. 1 are for a reactor that was inoculated with an enrichment culture from river water grown on toluene. This culture mainly contained bacteria. A second reactor was operated under the same conditions, but this time a fungal culture was used to inoculate the reactor. Interestingly, much higher toluene removal rates were observed with this reactor (Fig. 2).
During the first 120 days an average toluene removal of 48% (31 g-C/ (m^3 h)) was observed in the trickle-bed reactor with the fungal culture. During the 375 days that the reactor was operated additional nutrients were added only 4 times. The average removal rate during these 375 days was 27 g-C/(m^3 h). During this period there was no significant change in the pressure drop over the reactor (<100 Pa/m).
The difference in the removal rate between the reactors inoculated with respectively the bacterial and fungal culture are more clearly shown in Fig. 3. Here the observed degradation velocities are plotted against the toluene load for both reactors as determined on day 23.
The observed differences between the degradation rates could be caused by differences in the packing of the biofilm in both reactors resulting in differences in the specific surface area available for exchange of toluene between the gas and liquid phase. To test the mass-transfer characteristics of the two reactors we used 1,2-dichloroethane to determine the transfer rate of this compound in both reactors. Dichloroethane was chosen as it is not degraded by the biomass present in the reactors. Fresh water at 30°C was pumped through the reactors once (0.5 m^3/h), and it was determined how much dichloroethane was transferred to the water phase. At a gas-phase concentration of 950 mg/m^3 and a flow rate of 7 m^3/h, 60% of the dichloroethane was transferred to the water phase in a reactor without a biofilm. In the reactor with the bacterial and fungal cultures this was 64% and 66% respectively.

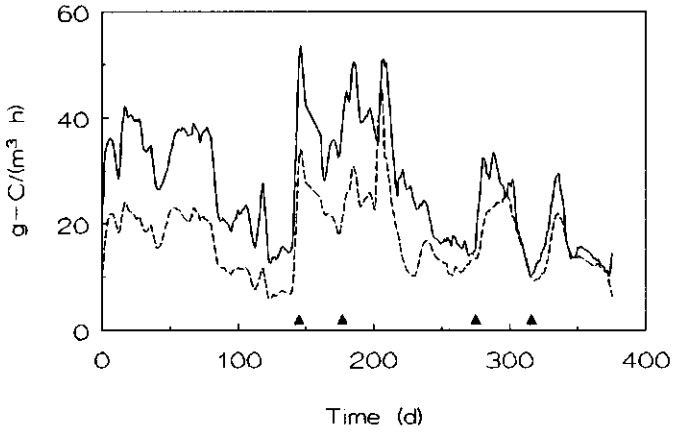

Figure 2: Toluene degradation rate (—) in a trickle-bed reactor inoculated with a fungal culture and an organic load of 64 g-C/(m³ h). (---) CO_2 formation rate. Additional nutrients were only supplied on days 145, 177, 275 and 316 (▲).

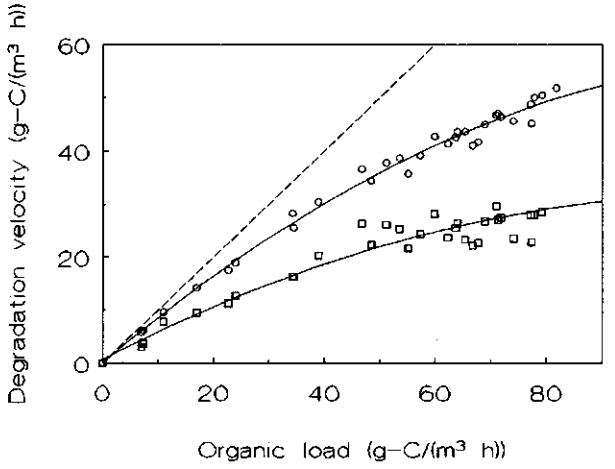

Figure 3: Observed toluene removal rates at various organic loads in a trickle-bed reactor inoculated with a bacterial culture (□) or a fungal culture (○). Dotted line corresponds to 100% removal.

Sodium hydroxide wash

By limiting the amount of nutrients available for growth, it was possible to prevent clogging of the trickle-bed reactor, but the removal rate decreased due to the nutrient limitation.

We have also operated the trickle-bed reactor with regular additions of mineral salts medium, containing 200 g NH_4Cl (Fig. 4). This clearly results in higher average toluene removal rates than under nutrient limiting conditions. The reactor was inoculated with a mixture of both the bacterial and fungal culture. The removal rate fluctuates between 15 and 45 g-C/(m^3 h) as a result of the two-weekly nutrients additions, but a gradual decrease in the removal efficiency is also

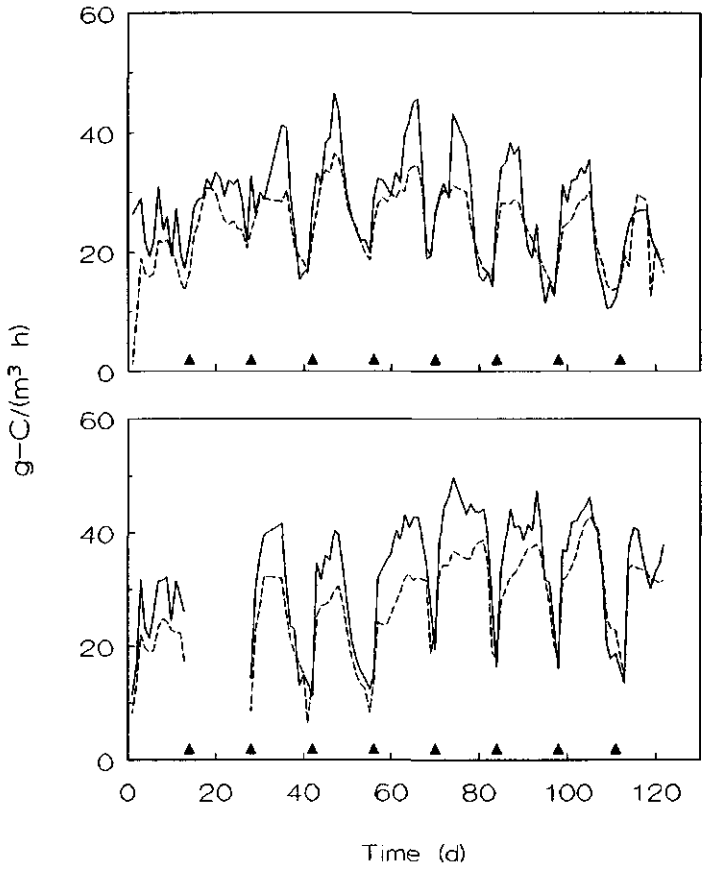

Figure 4 A-B: Toluene degradation rate (—) and CO_2 production rate (---) in two trickle-bed reactors. The circulation medium of both reactors is refreshed every 14 days (▲), in reactor B this is preceded by a NaOH-wash (0.1 M) of 3 h. The toluene load of both reactors is 65 g-C/(m^3 h).

observed. This is probably due to the increase in the biomass content of the reactor resulting in clogging and a less efficient mass-transfer. As a result of the increase in biomass in the reactor the pressure drop over the reactor fluctuated. Pressure drops as high as 1000 Pa/m were observed a number of times during a few days. Usually the pressure drop was between 100 and 200 Pa/m.

Another method to prevent clogging of the reactor can be the regular removal of biomass. Under normal conditions most of the biomass formed in the reactor remains there. Only a small percentage is removed from the packing by the liquid flowing through the packing material. We have tried to remove more biomass by introducing a sodium hydroxide wash. Every two weeks NaOH (end-concentration 0.1 M) was added to the circulation medium in the reactor. As a result excessive foaming was observed, indicating the release of protein due to the lysis of biomass. After 3 hours the medium was discharged and fresh medium was added to the reactor. The observed removal rates in the reactor operated in this manner is shown in Fig. 4B. After the NaOH-wash, the toluene removal rate was fully restored within one day (Fig. 5).
During the last 50 days, the average organic load of both trickle-bed reactors was 65 g-C/(m^3 h). In the reactor which was treated with the NaOH-wash toluene was removed at an average rate of 36 g-C/(m^3 h). In the reactor without the NaOH-wash this was 24 g-C/(m^3 h). The amount of biomass which was removed by the NaOH-wash was estimated by dry-weight determinations. An average of 3.3 g/l (1.5 - 5.3) biomass was removed, corresponding to 230 g dry-weight of biomass.

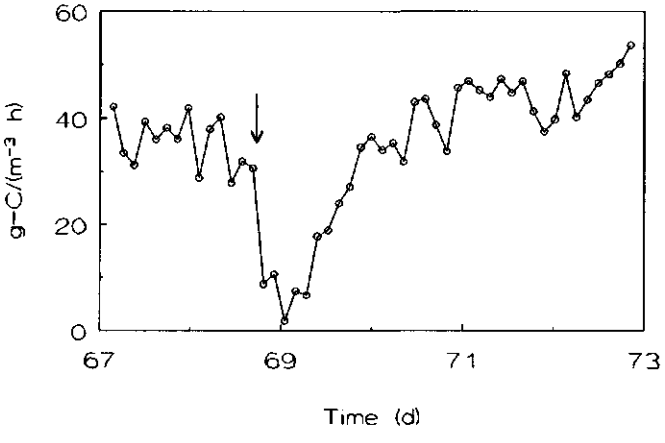

Figure 5: Toluene removal rate before and after an NaOH-wash (↓) of 3 hours.

DISCUSSION

Although, biological waste-gas treatment using trickle-bed reactors has been successfully demonstrated for the removal of dichloromethane from waste gases (Hartmans & Tramper, 1991; Diks & Ottengraf, 1991), the degradation of several other compounds resulted in the inactivation of the reactor due to clogging. To prevent this inactivation of the reactor, excessive biomass accumulation should be prevented. It is expected that by limiting the amount of nutrients available for growth the biomass formation can be controlled.

Using a bacterial culture as inoculum an initial toluene removal rate of 30 g-C/(m^3 h) was observed, under nutrient-limited conditions. However, several weeks after depletion of the nutrients present in the reactor, the toluene removal rate decreased to an average (stable) level of 13 g-C/(m^3 h). Over a period of 120 days an average toluene removal efficiency of 26% was obtained. This is obviously too low for a practical application. However, this low removal efficiency was expected because of the high toluene load applied to the reactor (65 g-C/(m^3 h)). Biofilters, which are used to treat industrial waste gases are generally operated under significantly lower loading conditions (< 15 g-C/(m^3 h)) (Anonymous, 1989; Mildenberger, 1992). At a reduced toluene load of about 10 g-C/ (m^3 h) a removal efficiency of about 80% is obtained in the trickle-bed reactor (Fig. 3). Differences in the performance of a bioreactor, however, can not be effectively studied at low loading conditions (resulting in a high removal efficiency). Interestingly, when a fungal culture was used instead of a bacterial culture to inoculate the reactor much higher removal rates were obtained. Especially during the period of nutrient depletion this difference was obvious. Over a period of 375 days an average degradation rate of 27 g-C/(m^3 h) is obtained.

In literature often much higher activities have been reported for the removal of organic compounds in bioreactors. However, it is unclear if these high activities can be maintained during longer periods of time. Compared to biofilters, which are used to treat waste gases from industry the activity of both the bacterial and fungal reactor is good. In these industrial biofilters the removal rate is often less than 15 g-C/(m^3 h) (Mildenberger, 1992). Also compared to the dichloromethane removal in a trickle-bed reactor the observed toluene removal rate is good. Over a period of 600 days an average dichloromethane elimination capacity of 7 g-C/(m^3 h) (50 g dichloromethane/(m^3 h)) has been obtained in a trickle-bed reactor. (Diks & Ottengraf, 1991).

The observed differences in degradation rates between the reactors inoculated with a bacterial or fungal culture are surprising. These results clearly show that,

Table 1: Averages of the organic load in the different experiments, and the obtained removal efficiencies and the fraction of degraded toluene which is converted into CO_2.

Reactor conditions	Average between (day)	Load (g Toluene-C/ (m^3 h))	Removal (g Toluene-C/ (m^3 h))	CO_2 formed (g CO_2-C/ (m^3 h))
Nutrient-limitation,				
"Bacteria"	1-120	65	16	8
"Fungi"	1-120	65	31	18
	1-375	63	27	18
Nutrient-additions				
normal	1-120	65	27	23
	70-120	65	24	22
NaOH-wash	1-120	65	32	27
	70-120	65	36	32

even under non-sterile conditions, inoculation of the reactor with a specific starter culture can influence the reactor performance over a prolonged period of time. As the gas-liquid transfer rate of dichloroethane (which is not degraded by the biomass in the reactor) was almost identical in both the bacterial and fungal reactor, it is not expected that differences in the biofilm structure explain the remarkable differences in degradation rate. Apparently, the fungal culture is capable to degrade more toluene under nutrient-limitated conditions than the bacterial culture.

It is expected that under nutrient-limited conditions there will be no increase in the total amount of biomass once the nutrients have been incorporated into biomass. Besides for the formation of biomass organic carbon is also required as an energy source for maintenance requirements. A maintenance coefficient of 0.02 g glucose-C/(g biomass-C h) is a average value reported in literature (Anderson & Domsch, 1985; Tijhuis *et al.*, 1993). With this maintenance coefficient and assuming that 155 g biomass-C is formed from the nutrients added to the reactor a maintenance activity of 44 g-C/(m^3 h) can be calculated. Assuming that all the biomass is still active, this activity is in the range of the activities that were observed in the reactors.

When, the reactor is running on the maintenance requirements of the biomass, a complete conversion of toluene into CO_2 is expected. However, less CO_2 is formed, indicating that even when the nutrients are depleted a substantial amount of the toluene is assimilated by the biomass resulting in the accumulation of reserve materials (e.g. glycogen). Under nutrient-limited conditions,

accumulation of these carbohydrate polymers up to 50% of the cell dry-weight has been observed (Herbert, 1961; Dawes & Senior, 1973). Extracellular polysaccharides (EPS) can also be an important carbon-sink during nutrient-limited growth. These polysaccharides are produced by the microorganisms and form a polymer which traps the microbial cells. The extracellular polymer matrix can account for 50 to 90% of the biofilm organic carbon (Bakke *et al.*, 1984).

Although the limitation of nutrients will restrict the growth of cells, a large amount of the toluene is apparently converted into biopolymers like glycogen and EPS. In the reactor with the bacterial culture, about 8 g-C/(m^3 h) is not converted into CO_2. Over the period of 120 days this corresponds to 1.6 kg-C of cells and polymers which is formed in the reactor. From the 28 g-N of nitrogen-containing compounds, which were added to the reactor, only about 310 g of biomass was expected to be formed. This would indicate that under these nutrient-limited conditions an enormous amount of EPS and other polymers are formed. Although this seems to be an unrealistic amount, we have observed that in a trickle-bed reactor to which only 17 g of nitrogen was added, more then 15 kg of wet weight biomass was formed within one month (unpublished results). When more than 1.6 kg-C biomass is present in the reactor, this will correspond to a biofilm thickness of about 3.5 mm (Assuming a biofilm density of 100 kg/m^3 with an average carbon content of 60% for the biofilm, including EPS and other polymers). Visual examination of the biofilm in the reactor did indeed show a thick biofilm.

Although the nutrient limitation did not result in a thin biofilm, a high and stable toluene removal rate was obtained with the fungal culture. As fungi are prominent decomposers of nitrogen-poor plant polymers, they may have developed special mechanisms that enable them to grow in these nitrogen-deficient environments. Several studies have suggested that hyphal outgrowth of fungi is possible without a net increase in cell cytoplasm. It is assumed that the cytoplasm is translocated to the growing hyphae (Cowling & Merill, 1966; Levi & Cowling, 1969; Watkinson *et al.*, 1981; Littke *et al.*, 1984; Paustian & Schnürer, 1987). As the cytoplasm contains most of the nitrogen and phosphate an internal recycle of the cytoplasm seems to be an effective mechanism to grow under these nutrient-limited conditions. Some other fungi may be able to further extend N resources by lysing degenerated hyphae and reassimilating the N released. (Levi *et al.*, 1968). Especially fungi are known for there capacity to grow on dead biomass (Fermor & Wood, 1981; Grant *et al.*, 1986). It is expected that this capacity of fungi to mobilize nutrients for growth is a reason for the higher degradation rate that is observed in the reactor with the fungal culture.

As also under nutrient-limited conditions the biomass content (including EPS and other polymers) of the reactor increases, this can eventually result in clogging of the reactor. The fact that no clogging was observed under the nutrient-limited conditions in our experiments is presumably due the relatively low specific surface area of the packing material we used (110 m^2/m^3). However, to be sure that clogging will not occur eventually biomass has to be removed from the reactor. With a low organic load, the amount of biomass formed in the reactor can be removed from the reactor by the liquid drain as was the case after about 200 days for a trickle-bed reactor removing dichloromethane (Diks *et al.*, 1994). The biomass yield on toluene, however, is much higher compared to dichloromethane and the amount of biomass that is removed by the liquid drain is not sufficient to counterbalance the biomass that is formed.
For a higher and stable toluene removal under non-limiting conditions, biomass removal by a NaOH-wash seems to be an effective method to prevent clogging. By washing the reactor with 0.1 M NaOH during 3 hours an average of 230 g of dry-weight biomass could be removed from the reactor every two weeks. Based on the nitrogen content of the added nutrients, 560 g of biomass ($CH_{1.8}O_{0.5}N_{0.16}$) can be formed. Based on the amount of toluene degraded and the carbon dioxide formed 235 g biomass is formed in the period proceeding the NaOH-wash. Especially the value calculated from the carbon balance over the reactor corresponds very well with the amount which is actually removed from the reactor by the NaOH-wash. Once an equilibrium is reached between the amount of biomass which is removed by the NaOH-wash and the amount of biomass which is formed in the reactor, it should be possible to maintain a high toluene removal rate without clogging of the reactor taking place.

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REFERENCES

Anderson, T.-H. and K.H. Domsch. (1985). Maintenance carbon requirements of actively-metabolizing microbial populations under in situ conditions. *Soil Biol. Biochem.* **17**:197-203.

Anonymous. (1989). Biologische Abgas-/Abluftreinigung: Biofilter. VDI-Handbuch Reinhaltung der Luft. Band 6. VDI 3477 (entwurf), Verein Deutscher Ingenieure, Düsseldorf.

Bakke, R., M.G. Trulear, J.A. Robinson and W.G. Characklis. (1984). Activity of *Pseudomonas aeruginosa* in biofilms: Steady State. *Biotechnol. Bioeng.* **26:** 1418-1424.

Cowling, E.B. and W. Merill. (1966). Nitrogen in wood and its role in wood deterioration. *Can. J. Botany* **44:** 1539-1554.

Dawes, E.A. and P.J. Senior. (1973). The role and regulation of energy reserve polymers in micro-organisms. *Adv. Microbial. Physiol.* **10:** 135-266.

Diks, R.M.M. and S.P.P. Ottengraf. (1991). Verification studies of a simplified model for the removal of dichloromethane from waste gases using a biological trickling filter. (Part II). *Bioproc. Eng.* **6:** 131-140.

Diks, R.M.M. (1992). The removal of dichloromethane from waste gases in a biological trickling filter. PhD-Thesis, Technical University, Eindhoven.

Diks, R.M.M., S.P.P. Ottengraf and S. Vrijland. (1994). The existence of a biological equilibrium in a trickling filter for waste gas purification. *Biotechnol. Bioeng.* **44:**1279-1287.

Dragt, A.J. (1992). Opening address. *In:* A.J. Dragt, and J. van Ham (eds.), Biotechniques for air pollution abatement and odour control policies, pp. 3-9, Elsevier, Amsterdam.

Fermor, T.R. and A.D. Wood. (1981). Degradation of bacteria by *Agaricus bisporus* and other fungi. *J. Gen. Microbiol.* **126:** 377-387.

Grant, W.D., L.L. Rhodes, B.A. Prosser and R.A. Asher. (1986). Production of bacteriolytic enzymes and degradation of bacteria by filamentous fungi. *J. Gen. Microbiol.* **132:** 2353-2358.

Groenestijn, J.W. and P.G.M. Hesselink. (1993). Biotechniques for air pollution control. *Biodegradation* **4:** 283-301.

Hartmans, S., J.P. Smits, M.J. van der Werf, F. Volkering and J.A.M. de Bont. (1989). Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. *Appl. Environ. Microbiol.* **55:** 2850-2855.

Hartmans, S. and J. Tramper. (1991). Dichloromethane removal from waste gases with a trickle-bed bioreactor. *Bioproc. Eng.* **6:** 83-92.

Herbert, D. (1961). The chemical composition of micro-organisms as a function of their environment. *In:* G.G. Meynell, and H. Goods (eds.), Microbial reaction to environment, pp. 391-416, Society for General Microbiology, Cambridge.

Kirchner, K., C.A. Gossen and H.-J. Rehm. (1991). Purification of exhaust air containing organic pollutants in a trickle-bed bioreactor. *Appl. Microbiol. Biotechnol.* **35:** 396-400.

Levi, M.P., W. Merill and E.B. Cowling. (1968). Role of nitrogen in wood deterioration. VI. Mycelial fractions and model nitrogen compounds as substrates for growth of *Polyporus versicolor* and other wood-destroying and wood-inhabiting fungi. *Phytopathology* **58:** 626-634.

Levi, M.P. and E.B. Cowling. (1969). Role of nitrogen in wood-deterioration. VII. Physiological adaptation of wood-destroying and other fungi to substrates deficient in nitrogen. *Phytopathology* **59:** 460-468.

van Lith, C.P.M., S.P.P. Ottengraf and R.M.M. Diks. (1994). The control of a biotrickling filter. *In:* VDI Berichte 1104, Biologische Abgasreinigung, pp. 169-180, VDI Verlag, Düsseldorf.

Littke, W.R., C.S. Bledsoe and R.L. Edmonds. (1984). Nitrogen uptake and growth in vitro by *Hebeloma crutuliniforme* and other Pacific Northwest mycorrhizal fungi. *Can. J. Botany* **62:** 647-652.

Mildenberger, H.-J. (1992). Biofiltersysteme zur Geruchsbeseitigung und zur Reduzierung von Organika-Emissionen auf Kläranlagen und in der chemischen Industrie. *In:* A.J. Dragt, and J. van Ham (eds.), Biotechniques for air pollution abatement and odour control policies, pp. 187-196, Elsevier, Amsterdam.

Oliveira, D.R. (1992). Physico-chemical aspects of adhesion. *In:* L.F. Melo, T.R. Both, M. Fletcher, and B. Capdeville (eds.), Biofilms - Science and technology, NATO ASI Series E, Vol. 223, pp. 45-58, Kluwer Academic Publishers, Dordrecht.

Ottengraf, S.P.P. and R.M.M. Diks. (1992). Process technology of biotechniques. *In:* A.J. Dragt, and J. van Dam (eds.), Biotechniques for air pollution abatement and odour control policies, pp. 17-31, Elsevier, Amsterdam.

Paustian, K. and J. Schnürer. (1987). Fungal growth response to carbon and nitrogen limitation: Application of a model to laboratory and field data. *Soil Biol. Biochem.* **19:** 621-629.

Rijnaarts, H.H.M., W. Norde, E.J. Bouwer, J. Lyklema and A.J.B. Zehnder. (1993). Bacterial adhesion under static and dynamic conditions. *Appl. Environ. Microbiol.* **59:** 3255-3265.

Roels, J.A. (1983). Energetics and kinetics in biotechnology. Elsevier Biomedical Press, Amsterdam.

Tijhuis, L., M.C.M. van Loosdrecht and J.J. Heijnen. (1993). A thermodynamically based correlation for maintenance Gibbs energy requirements in aerobic and anaerobic growth. *Biotechnol. Bioeng.* **42:** 509-519.

Waalewijn, E., C.D. Meijer, F.J. Weber, U.C. Duursma and P. van Rijs. (1994). Practice runs of a bio-trickling filter in the exhaust gases from a fibre factory. *In:* VDI Berichte 1104, Biologische Abgasreinigung, pp. 545-550, VDI Verlag, Düsseldorf.

Watkinson, S.C., E.M. Davidson and J. Bramah. (1981). The effect of nitrogen availability on growth and cellulolysis by *Serpula lacrimans*. *New Phytologist* **89:** 295-305.

Weber, F.J. and S. Hartmans. (1994). Toluene degradation in a trickle bed reactor. - Prevention of clogging. *In:* VDI Berichte 1104, Biologische Abgasreinigung, pp. 161-168, VDI Verlag, Düsseldorf.

Zita, A. and M. Hermansson. (1994). Effects of ionic strength on bacterial adhesion and stability of flocs in a wastewater activated sludge system. *Appl. Environ. Microbiol.* **60:** 3041-3048.

4

Growth of the fungus *Cladosporium sphaerospermum* with toluene as the sole carbon and energy source

Frans J. Weber
Ko C. Hage
and Jan A.M. de Bont

Summary: The fungus *Cladosporium sphaerospermum* was isolated from a biofilter used for the removal of toluene from waste gases. This is the first report describing growth of a eukaryotic organism with toluene as the sole source of carbon and energy. The oxygen consumption rates, as well as the measured enzyme activities, of toluene-grown *C. sphaerospermum* indicate that toluene is degraded by an initial attack on the methyl group.

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INTRODUCTION

The complete aerobic biodegradation of toluene to carbon dioxide, water, and biomass by bacteria has been studied extensively. Different initial oxidative reactions have been identified for a variety of bacteria, and the biodegradation of the resulting oxygenated compounds is well documented (Kitagawa, 1956; Claus & Walker, 1964; Gibson *et al.*, 1970; Worsey & Williams, 1975; Shields *et al.*, 1989; Kaphammer *et al.*, 1990; Shields *et al.*, 1991; Whited & Gibson, 1991). The presently known five different pathways for the utilization of toluene in bacteria are summarized in Fig. 1.

In humans and animals, the initial step in the metabolic transformation of toluene appears to be hydroxylation of toluene to benzyl alcohol (Fig. 1) by a monooxygenase. Benzyl alcohol is further converted to benzoic acid and excreted in urine, either unchanged or as its glycine conjugate, hippuric acid (Anonymous, 1985).

In contrast, utilisation of toluene by fungi as the sole carbon and energy source has not been reported. Partial degradation of toluene by fungi was observed by Holland *et al.* (1988). Cultures of *Mortierella isabellina* and *Helminthosporium* species were pregrown on a rich medium with glucose and when the mycelium was subsequently incubated in water for 72 hours, it converted toluene into benzyl alcohol. Degradation of toluene by the white rot fungus *Phanerochaete chrysosporium* has been demonstrated by using ring-labeled [^{14}C]toluene and by measuring the production of $^{14}CO_2$ (Yadav & Reddy, 1993). During these experiments, the fungus was grown for 3 weeks in media containing glucose (1%) and toluene was added at 5 mg/l. The quantity of toluene degraded was very small on the order of 1 mg of toluene degraded per g of mycelium per day. Interestingly, the degradation took place under nonligninolytic culture conditions, indicating that lignin peroxidases or other peroxidases were not involved. It is known from many other studies that these enzymes play a key role in the partial oxidation of anthracene to anthraquinone (Field *et al.*, 1992) and in the partial oxidation of many other xenobiotic aromatic compounds (Field *et al.*, 1993). A number of nonligninolytic fungi have intracellular mechanisms for the partial degradation of complex aromatic structures, as documented by Cerniglia (1984), and yeasts are also able to partially oxidize polycyclic aromatic hydrocarbons (Hofmann, 1986; MacGillivray & Shiaris, 1993).

Fungal growth with aromatic hydrocarbons other than toluene has been tested for *n*-alkylbenzenes and for styrene. Fungi isolated on *n*-alkanes were grown in the presence of *n*-alkylbenzenes to determine the effect of the side chain length (C_1 to C_{12}). The organisms did not grow in the presence of toluene, maybe as a result of the toxicity of this compound, but they grew on other compounds, for instance

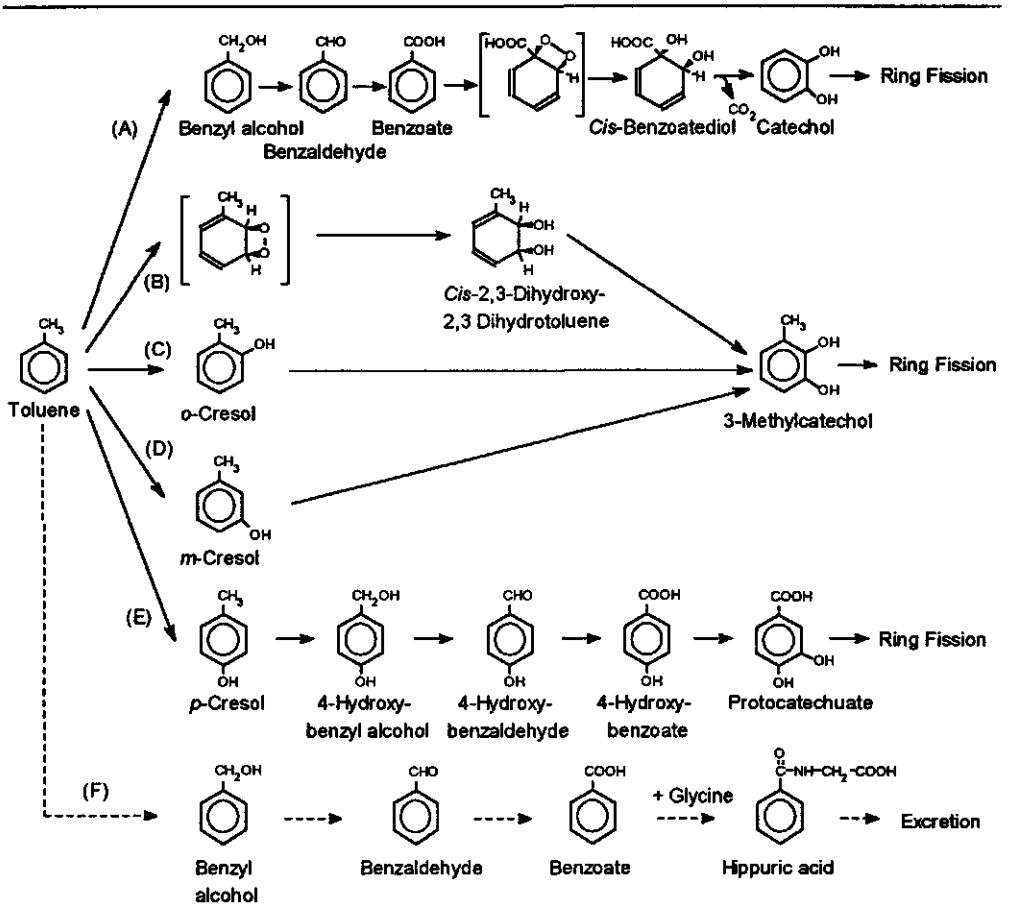

Figure 1: Toluene biodegradation routes in bacteria (–), and mammals (--). Composed after (A) Worsey & Williams (1975), (B) Gibson *et al.* (1970), (C) Shields *et al.* (1989), (D) Kaphammer *et al.* (1990) (E) Whited & Gibson (1991), and (F) Epstein & Braunstein (1931)

on dodecylbenzene, from which the organism accumulated benzoic and phenylacetic acids (Fedorak & Westlake, 1986). Two styrene-degrading fungi have been isolated (Hartmans *et al.*, 1990), and one strain identified as *Exophiala jeanselmei* was subsequently studied in more detail (Cox *et al.*, 1993a). The results obtained with *E. jeanselmei* (Cox *et al.*, 1993a) indicate that the styrene degradation pathway in the fungus was similar to a pathway observed for a styrene-degrading bacterium (Hartmans *et al.*, 1990).
The metabolism by fungi of oxygenated aromatic compounds has been amply studied, and reviews of this field are available (Cain *et al.*, 1968; Middelhoven *et al.*, 1992; Wright, 1993).

During our investigations of the removal of toluene from contaminated air by use of a compost biofilter, we macroscopically observed abundant fungal growth. Microscopic examination confirmed that fungi were predominant in this system, removing toluene from waste gases. On the basis of current knowledge of toluene biodegradation, it might be expected that the fungi only partly metabolize toluene. Alternatively, they might be involved in the degradation of partly oxidized products derived by bacteria from toluene, since it is known that fungi are able to metabolize oxygenated aromatic compounds, as, for instance, benzoate. However, in view of the abundance of the fungi in the biofiltration system, it seemed more reasonable to expect that the fungi were involved in the complete biodegradation of toluene. To test if a eukaryotic toluene-degrading organism was indeed present, we decided to isolate fungi from the biofilter and to investigate one pure culture in more detail.

MATERIALS AND METHODS

Isolation of fungi

Fungi were isolated from a compost biofilter (70 liters), which had been used to remove toluene from contaminated air (≈150 mg/m^3, 7 m^3/h) for 3 months, by washing the biofilter with a 0.8% NaCl solution. From this suspension dilutions were made, and the resulting suspensions were seeded on agar plates with a mineral salts medium (Hartmans *et al.*, 1990) and 10 µg of streptomycin per ml to minimize bacterial growth. These plates were incubated at 30°C in a desiccator containing toluene vapor, resulting in a concentration of about 200 mg/l in the solidified 'liquid' phase as calculated by using a water/air partition coefficient of 3.8 (Amoore & Hautala, 1983). Pure fungal cultures were obtained by subsequent transfers to new agar plates.

Growth with volatile aromatic compounds

For growth experiments, 250-ml flasks containing 10 ml of a phosphate buffered (pH 7) mineral salts medium (Hartmans *et al.*, 1990) were used to which the various aromatic hydrocarbons were added. The bottles were closed with Teflon valves (Mininert, Phase Separations, Waddinxveen, The Netherlands) to prevent evaporation of the various added aromatic hydrocarbons and incubated under stationary conditions at 30°C. Growth was assessed by monitoring the production of CO_2.

Preparation of washed mycelium suspensions

In order to obtain larger amounts of mycelium, *Cladosporium sphaerospermum* was grown in a flat-bottomed round flask (2 liters) with 500 ml of phosphate-buffered mineral salts medium at 30°C. Air containing approximately 3000 mg of toluene per m^3 was bubbled through the medium at a flow rate of 125 ml/min. Mycelium was harvested from the growth medium by filtration over cheesecloth, washed with 50 mM potassium phosphate buffer (pH 7.0), and resuspended in the same buffer.

Oxygen consumption experiments

The oxygen consumption of washed mycelium suspensions was determined with a Clark type oxygen electrode. The oxygen consumption of a 4-ml mycelium suspension was monitored for at least 5 min after the addition of 50 µl of a stock solution of 20 mM substrate in *N,N*-dimethylformamide. Addition of *N,N*-dimethylformamide resulted in a minimal increase in the endogenous oxygen uptake rate. After measuring the oxygen consumption rate of the substrate, we checked if the substrate concentration used was not toxic by measuring the oxygen consumption rate after the addition of 50 µl of the toluene stock solution.

Determination of enzyme activities in cell-free extracts

Washed mycelium suspensions of toluene-grown *C. sphaerospermum* were frozen at -30°C and disrupted by two passages through a prechilled (-30°C) 5-ml X-press (AB Biox, Göteborg, Sweden). After being slowly thawed the paste was diluted in 50 mM potassium phosphate buffer (pH 7.0) and centrifuged at 20,000 x *g* for 10 min at 4°C. The supernatant obtained was used for the enzyme activity determinations. The activities of the monooxygenases and dehydrogenases were determined spectrophotometrically. The reaction mixture (total volume, 1 ml) contained cell-free extract diluted in the phosphate buffer and 0.2 µmol of cofactor (NAD^+, $NADP^+$, NADH, or NADPH). The activities of both 4-hydroxybenzyl alcohol dehydrogenase and 4-hydroxybenzaldehyde dehydrogenase were corrected for the level of high adsorbance of 4-hydroxy-benzaldehyde at

340 nm. p-Cresol methylmonooxygenase was assayed by monitoring the O_2 uptake as described previously (Hopper & Taylor, 1977), except that a 50 mM potassium phosphate buffer was used. Catechol-1,2-dioxygenase was monitored by measuring the formation of *cis-cis*-muconic acid at 260 nm (Gibson, 1971b). The activity of protocatechuate-3,4-dioxygenase was measured spectrophotometrically at 290 nm (Gibson, 1971b). 3-Methylcatechol-1,2-dioxygenase was determined by measuring the formation of the ring fission product at 390 nm.

Analytical methods

Carbon dioxide concentrations were determined by injecting 100 µl head space samples into a Packard 427 gas chromatograph (Packard/Becker, Delft, The Netherlands) equipped with a Hayesep Q column (Chrompack B.V. Middelburg, The Netherlands). Protein was quantified by the Bradford method (Bradford, 1976) with bovine serum albumin as the standard. Cell dry weight was determined by weighing dried (24 h 105°C) cell suspensions.

RESULTS

Isolation of fungi from the biofilter

From a compost biofilter, which had been used to remove toluene from contaminated air, a biomass suspension rich in fungi (>50% as examined by microscopy) was obtained by washing the biofilter with an NaCl solution. From this suspension several pure fungal cultures were isolated, and they grew on mineral agar plates incubation in the presence of toluene vapor.

Growth of fungi on toluene

As these isolated fungi could possibly grow on impurities from the agar plates, the capacity of these fungi to grow on toluene was checked by using liquid medium. One of the isolated fungi grew in liquid medium with toluene as the sole carbon and energy source. This fungus was identified by the Centraalbureau voor Schimmelcultures (CBS) (Baarn, The Netherlands) as *Cladosporium sphaerospermum* Penzig.

In Fig. 2, a typical growth curve for *C. sphaerospermum* grown with an initial amount of 6 µl (56 µmol) of toluene in the incubation system (10 ml of liquid medium in a 250-ml culture bottle) is shown. This initial amount of toluene in the incubation system, on the basis of its partition coefficient, results in a concentration of 0.8 mM in the water phase. In about 10 days all the added toluene was degraded and about 210 µmol of CO_2 and about 6 mg (dry weight)

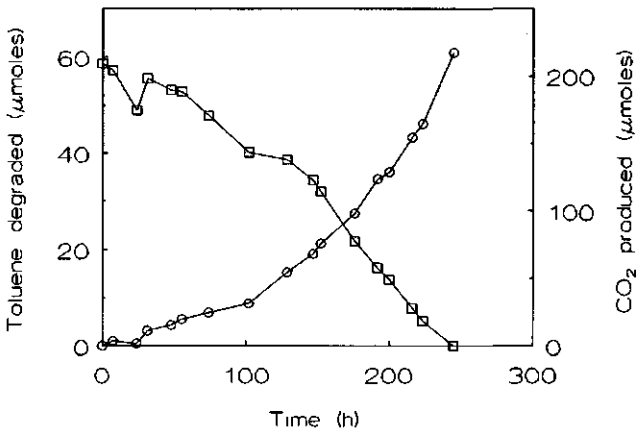

Figure 2: Growth of *C. sphaerospermum* with toluene in 10 ml of mineral salts medium. □, amount of toluene degraded; O, total amount of CO_2 produced.

of cells was formed. The growth rate was maximal at pH 7.0 under stationary conditions.

Growth of *C. sphaerospermum* with other aromatic solvents

Growth of *C. sphaerospermum* with several other aromatic hydrocarbons was also tested. As the aromatic hydrocarbons used can be toxic even at low concentrations, various amounts of the hydrocarbons were tested (10, 20, and 50 µmol). After 10 days of incubation, growth was assessed by CO_2 determination. Besides toluene, *C. sphaerospermum* could also use styrene, ethylbenzene, and propylbenzene as sole carbon and energy sources for growth at all concentrations tested (>40 µmol of CO_2 produced). No growth was observed with o-xylene, benzene or phenol at any of the concentrations tested.

Whether *C. sphaerospermum* could grow with several intermediates of the known bacterial degradation pathways of toluene was also tested (Fig. 1). Growth was observed with benzyl alcohol, benzaldehyde, benzoate, and catechol. Growth with benzoate and catechol was observed only at 10 and 20 µmol; no growth was observed at 50 µmol. No growth with 3-methylcatechol was observed at any of the concentrations tested.

Table 1: Rates of oxygen consumption by washed cell suspensions of *C. sphaerospermum* grown on toluene.

Assay substrate	Oxygen consumption (nmol O_2 / min · mg cell dry-weight)
Toluene	164
Benzyl alcohol	154
Benzaldehyde	155
Benzoate	13
Catechol	77
3-Methylcatechol	<5
o-Cresol	<5
m-Cresol	<5
p-Cresol[1]	11
4-Hydroxybenzyl alcohol	<5
4-Hydroxybenzaldehyde	128
3-Hydroxybenzoate	6
4-Hydroxybenzoate	7
Protocatechuate	<5

[1] Inhibited the toluene induced oxygen consumption rate.

Table 2: Activities of enzymes possible involved in the catabolism of toluene in cell-free extracts of *C. sphaerospermum*.

Enzyme	Cofactor	Enzyme activity (nmol / min · mg protein)
Dehydrogenases		
Benzyl alcohol	NAD^+	169
	$NADP^+$	<10
Benzaldehyde	NADH	7180
	NADPH	107
4-Hydroxybenzyl alcohol	NAD^+	15
	$NADP^+$	<1
4-Hydroxybenzaldehyde	NADH	157
	NADPH	9
Monooxygenase		
4-Hydroxybenzoate	NADH + FAD	528
	NADPH + FAD	97
Dioxygenases (intra diol)		
Catechol	-	118
Protocatechuate	-	145
3-Methylcatechol	-	<25

Oxygen consumption experiments

Oxygen consumption rates of washed suspensions of *C. sphaerospermum* grown with toluene were monitored in the presence of various possible intermediates of the toluene degradation pathway (Table 1). Addition of toluene after measurement of the *p*-cresol-induced oxygen consumption rate resulted in a reduced toluene-induced oxygen consumption rate. This inhibition of the toluene oxygen consumption rate was observed only for *p*-cresol, even when a ten-fold lower concentration of *p*-cresol was used. The results presented in Table 1 have been corrected for the endogenous oxygen uptake (96 nmol O_2 / min · mg [dry weight] of cells).

Enzyme activities in cell-free extracts

Cell-free extracts of toluene-grown *C. sphaerospermum* were used to measure the activities of various enzymes possibly involved in the degradation of toluene (Fig. 1). 4-Hydroxybenzoate monooxygenase activity could be measured only after the addition of FAD. Addition of FAD and ferrous ammonium sulfate to the reaction mixture did not enable *in vitro* monooxygenase activity to be obtained with any of the other substrates tested (toluene, benzoate, 3-hydroxybenzoate, *o*-cresol, or *p*-cresol). As many of these monooxygenases are unstable enzymes and have proven difficult to isolate (Gibson, 1971a), the inability to measure these enzyme activities is not very surprising. The activities of the measured dioxygenases and dehydrogenases of toluene-grown *C. sphaerospermum* cells are shown in Table 2.

DISCUSSION

From a biofilter used to remove toluene from contaminated air, we have isolated a fungus, *C. sphaerospermum*, which is able to grow with toluene as the sole source of carbon and energy. To our knowledge this is the first report of toluene catabolism by a eukaryotic microorganism.

The spectrum of substrates used for growth by *C. sphaerospermum* cells indicates that toluene is degraded by an initial attack on the methyl group, leading to benzoate. *C. sphaerospermum* could use benzyl alcohol, benzaldehyde and benzoate as sole carbon and energy sources.

The route for toluene degradation was further investigated by measuring the oxygen consumption rates of various possible intermediates in toluene-grown mycelium of *C. sphaerospermum*. A prerequisite for measuring a substrate-induced increase in the oxygen consumption rate by whole cells is the ability of the compound to pass through the cytoplasmic membrane. It is expected that

substrates like toluene, *o*-cresol, *m*-cresol, and *p*-cresol will enter the cell by diffusion through the cytoplasmic membrane. The fact that no significant activity was observed with *o*-cresol, *m*-cresol, and *p*-cresol indicates that these compounds are not intermediates of the toluene degradation pathway. Charged compounds like benzoate, however, may require an active transport system to enter the cell. The absence of an increase in oxygen consumption after the addition of these substrates could thus very well be caused by the lack of a suitable membrane transport system. Since an appreciable increase in oxygen consumption was observed after the addition of benzyl alcohol, benzaldehyde, and catechol and no activity was obtained with *o*-cresol, *m*-cresol, *p*-cresol, *p*-hydroxybenzyl alcohol, and 3-methylcatechol, it appears that toluene is degraded by a hydroxylation of the methyl group (Fig. 1).
The results from both the growth and oxygen consumption experiments indicated that toluene is degraded by an initial attack on the methyl group. The high activities measured for benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase confirm these results. The activities for the degradation of 4-hydroxybenzaldehyde both in cell suspensions (Table 1) and in extracts (Table 2) are not in keeping with the proposed pathway, but these activities are likely due to an aspecific reaction of the benzaldehyde dehydrogenase. It has indeed been shown that in *Pseudomonas convexa*, 4-hydroxybenzaldehyde and benzaldehyde are oxidized by the same dehydrogenase (Bhat & Vaidyanathan, 1976).

In mammals, aromatic hydrocarbons usually are oxidized to an epoxide and subsequently a *trans*-dihydrodiol is formed (Gibson, 1971a, 1993). Such a pathway has been established, for instance, for benzene (Gibson, 1971a), but toluene was degraded in a different fashion with an initial oxidation at the methyl group (Gibson, 1971a; Anonymous, 1985). Our results indicate that the mammalian and fungal degradations of toluene are similar in that the initial reaction in both cases is with the methyl group and results in benzoate. Benzoate in mammals either is an end product and is excreted or is conjugated to hippuric acid. In bacteria, benzoate is further metabolized via *cis*-dihydrodiol to catechol as the ring fission substrate. In all fungi studied, the sole pathway for benzoate metabolism is by hydroxylation of benzoate to 4-hydroxybenzoate, leading to protocatechuate as the ring fission substrate (Wright, 1993). The measured activities for 4-hydroxybenzoate monooxygenase and protocatechuate-3,4-dioxygenase indicate that benzoate in *C. sphaerospermum* is also degraded via 4-hydroxybenzoate.
In cell-free extracts of *C. sphaerospermum* a catechol dioxygenase activity, besides a protocatechuate dioxygenase activity, was also detected, indicating

that catechol is a ring fission substrate. As the protocatechuate dioxygenase activity is measured by disappearance of protocatechuate, this activity could also be caused by the conversion of protocatechuate into catechol. It has been shown in several fungi that 4-hydroxybenzoate is degraded via protocatechuate, leading to catechol as the ring fission substrate (Cain *et al.*, 1968). Whether protocatechuate is the actual ring fission substrate or is converted into catechol is uncertain and will require further investigation.

The discovery of toluene-degrading fungi is of importance for biofiltration. A disadvantage of biofilters used for waste-gas treatment is that the pH cannot be controlled, and humidification of the filter can be problematic (Ottengraf, 1986). Both these parameters should be carefully controlled to allow an extended operation of the filter. The application of aromatic hydrocarbon-degrading fungi in these biofilters might have two advantages, as fungi generally require less stringent control of both the pH and the water activity. A reduction of the water activity in the biofilter might also enhance the mass transfer of substrates poorly soluble in water (Cox *et al.*, 1993b).

REFERENCES

Amoore, J.E. and E. Hautala. (1983). Odor as an aid to chemical safety: odor thresholds compared with threshold limit values and volatilities for 214 industrial chemicals in air and water dilution. *J. Appl. Toxicol.* **3:** 272-290.

Anonymous. (1985). Toluene. Environmental Health Criteria 52, World Health Organization, Geneva.

Bhat, S.G. and C.S. Vaidyanathan. (1976). Involvement of 4-hydroxymandelic acid in the degradation of mandelic acid by *Pseudomonas convexa*. *J. Bacteriol.* **127:** 1108-1118.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.

Cain, R.B., R.F. Bilton and J.A. Darrah. (1968). The metabolism of aromatic acids by micro-organisms. *Biochem. J.* **108:** 797-828.

Cerniglia, C.E. (1984). Microbial metabolism of polycyclic aromatic hydrocarbons. *Adv. Appl. Microbiol.* **30:** 31-71.

Claus, D. and N. Walker. (1964). The decomposition of toluene by soil bacteria. *J. Gen. Microbiol.* **36:** 107-122.

Cox, H.H.J., J.H.M. Houtman, H.J. Doddema and W. Harder. (1993a). Growth of the black yeast *Exophiala jeanselmei* on styrene and styrene-related compounds. *Appl. Microbiol. Biotechnol.* **39:** 372-376.

Cox, H.H.J., J.H.M. Houtman, H.J. Doddema and W. Harder. (1993b). Enrichment of fungi and degradation of styrene in biofilters. *Biotechnol. Lett.* **15:** 737-742.

Epstein, S. and A.E. Braunstein. (1931). Über den Einfluß der sogen. sauren und basischen Nahrung auf das Schicksal aromatischer Substanzen im Organismus. II. Oxydation von Toluol. *Biochem. Z.* **235:** 328-333.

Fedorak, P.M. and D.W. Westlake. (1986). Fungal metabolism of *n*-alkylbenzenes. *Appl. Environ. Microbiol.* **51:** 435-437.

Field, J.A., E. de Jong, G. Feijoo-Costa and J.A.M. de Bont. (1992). Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. *Appl. Environ. Microbiol.* **58:** 2219-2226.

Field, J.A., E. de Jong, G. Feijoo-Costa and J.A.M. de Bont. (1993). Screening for ligninolytic fungi applicable for the biodegradation of xenobiotics. *Trends Biotechnol.* **11:** 44-49.

Gibson, D.T., M. Henley, H. Yoshioka and T.J. Mabry. (1970). Formation of (+)-*cis*-2,3-dihydroxy-1-methyl-cyclohexa-4,6-diene from toluene by *Pseudomonas putida*. *Biochem.* **9:** 1626-1630.

Gibson, D.T. (1971a). The microbial oxidation of aromatic hydrocarbons. *CRC Crit. Rev. Microbiol.* **1:** 199-223.

Gibson, D.T. (1971b). Assay of enzymes of aromatic metabolism. *In:* J.R. Norris, and D.W. Ribbons (eds.), Methods in microbiology, vol 6a, pp. 463-478, Academic Press, London.

Gibson, D.T. (1993). Biodegradation, biotransformation and the Belmont. *J. Ind. Microbiol.* **12:** 1-12.

Harlmans, S., M.J. van der Werf and J.A.M. de Bont. (1990). Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. *Appl. Environ. Microbiol.* **56:** 1347-1351.

Hofmann, K.H. (1986). Oxidation of naphthalene by *Saccharomyces cerevisiae* and *Candida utilis*. *J. Basic Microbiol.* **26:** 109-111.

Holland, H.L., F.M. Brown, B. Munoz and R.W. Ninniss. (1988). Side chain hydroxylation of aromatic hydrocarbons by fungi. Part 2: Isotope effects and mechanism. *J. Chem. Soc. Perkin Trans. II* **1988:** 1557-1563.

Hopper, D.J. and D.G. Taylor. (1977). The purification and properties of *p*-cresol-(acceptor) oxidoreductase (hydroxylating), a flavocytochrome from *Pseudomonas putida*. *Biochem. J.* **167:** 155-162.

Kaphammer, B., J.J. Kukor and R.H. Olsen. (1990). Cloning and characterization of a novel toluene degradative pathway from *Pseudomonas pickettii* PK01. *In:* Abstracts of the 90th Annual Meeting of the American Society for Microbiology, abstr. K-145, pp. 243, American Society for Microbiology, Washington D.C.

Kitagawa, M. (1956). Studies on the oxidation mechanisms of methyl groups. *J. Biochem (Tokyo)* **43:** 553-563.

MacGillivray, A.R. and M.P. Shiaris. (1993). Biotransformation of polycyclic aromatic hydrocarbons by yeast isolated from coastal sediments. *Appl. Environ. Microbiol.* **59:** 1613-1618.

Middelhoven, W.J., M. Koorevaar and G.W. Schuur. (1992). Degradation of benzene compounds by yeast in acidic soils. *Plant and Soil* **145:** 37-43.

Ottengraf, S.P.P. (1986). Exhaust gas purification. *In:* H.-J. Rehm, and G. Reed (eds.), Biotechnology, Vol. 8, pp. 426-452, VCH Verlagsgesellschaft, Weinheim.

Shields, M.S., S.O. Montgomery, P.J. Chapman, S.M. Cuskey and P.H. Pritchard. (1989). Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. *Appl. Environ. Microbiol.* **55:** 1624-1629.

Shields, M.S., S.O. Montgomery, M. Cuskey, S, P.J. Chapman and P.H. Pritchard. (1991). Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. *Appl. Environ. Microbiol.* **57:** 1935-1941.

Whited, G.M. and D.T. Gibson. (1991). Separation and partial characterization of the enzymes of the toluene-4-monooxygenase catabolic pathway in *Pseudomonas mendocina* KR1. *J. Bacteriol.* **173:** 3017-3020.

Worsey, M.J. and P.A. Williams. (1975). Metabolism of toluene and xylenes by *Pseudomonas putida (arvilla)* mt-2: Evidence for a new function of the TOL plasmid. *J. Bacteriol.* **124:** 7-13.

Wright, J.D. (1993). Fungal degradation of benzoic acid and related compounds. *World J. Microbiol. Biotechol.* **9:** 9-16.

Yadav, J.S. and C.A. Reddy. (1993). Degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **59:** 756-762.

5

Toxicity of contaminants in waste gases for microorganisms

Frans J. Weber
Ruud M.W. Schemen
and Sybe Hartmans

Summary: The toxicity of various volatile organic compounds frequently present as contaminants in waste gases has been determined. For both the Gram-positive *Rhodococcus* S5 and the Gram-negative *Pseudomonas* S12 the toxicity was assessed as the concentration which reduced the growth rate of the bacterium with 50%. No significant differences were observed between the IC50% values for these two bacteria. A relationship between the toxicity and hydrophobicity of various substituted benzene compounds was observed. Toxicity problems in a bioreactor for waste-gas treatment are only expected for compounds with a high affinity to dissolve in water.

Surprisingly, one of the selected bacteria, *Pseudomonas* strain S12 was able to adapt to the toxic effects caused by the contaminants. This adaptation resulted in the capacity of this strain to grow in the presence of supersaturating concentrations of toluene.

INTRODUCTION

Already for about 20 years biological waste-gas treatment has been used for the reduction of emissions from different industrial processes. Especially biofilters are used in Germany and The Netherlands for treatment of odorous waste-gas streams (Leson & Winer, 1991). Nowadays biofilters are also used to treat waste-gas streams contaminated with volatile organic solvents. Another type of reactor, the trickle-bed reactor, has especially been studied to treat waste-gas streams with higher concentrations of these organic contaminants. The application of trickle-bed reactors for the treatment of gas contaminated with dichloromethane has been demonstrated to be applicable with high efficiencies and degradation velocities (Diks & Ottengraf, 1991; Hartmans & Tramper, 1991).

The use of biological waste-gas treatment for the removal of higher concentrations of organic solvents seems to be promising. However, a serious problem could be the toxicity of the pollutants in the waste gas to the microorganisms in the reactors. Especially compounds with a good solubility in water can be expected to accumulate in the water phase of the reactor during the start-up period. This accumulation can result in the inactivation of the biomass in the reactor as the contaminant concentrations reach toxic levels. Also fluctuations in the contaminant concentrations in the waste gas could result in levels which are toxic for the microorganisms in the reactor.

In this report we will describe the toxicity of various volatile compounds to microorganisms in relation to biological waste-gas treatment.

MATERIALS AND METHODS

Microorganisms

The Gram-negative *Pseudomonas* strain S12 and the Gram-positive *Rhodococcus* strain S5 have been used for the toxicity tests. These microorganisms have previously been isolated on low concentrations of styrene as sole carbon and energy source (Hartmans *et al.*, 1990). The microorganisms were routinely maintained on yeast extract - glucose agar slants.

Toxicity test

The bacteria were grown on mineral salts medium (Hartmans *et al.*, 1989) with 40 mM sodium acetate as the carbon and energy source. From an exponentially growing preculture 1 ml of cell suspension was transferred to various 250-ml flasks with 15 ml acetate medium. To these incubation flasks various concentrations of

the contaminant to be studied were added. The flasks were closed with a Teflon Mininert valve to prevent evaporation of the contaminants and were incubated at 30°C in a water-bath with intensive shaking. The influence of the contaminants on the growth rate of the bacteria was determined by measuring carbon dioxide formation in time. The measured carbon dioxide concentrations were corrected for (1) the amount of carbon dioxide present in the bottle before inoculation, and (2) the amount of carbon dioxide that was formed by the inoculum, which is not transferred with the inoculum.

Analytical methods

Carbon dioxide was measured by analysing 100 µl-samples of the gas phase on a Packard model 427 gas chromatograph (Packard, Delft, The Netherlands) with a Hayesep Q column (Chrompack, Middelburg, The Netherlands).

RESULTS

Toxicity

The toxicity of a contaminant is measured as an inhibition of the growth rate of a bacterium growing on acetate. In Fig. 1 the carbon dioxide production of *Rhodococcus* S5 in the presence of various toluene concentrations is shown. The liquid-phase concentrations are calculated using the Henry coefficient of the

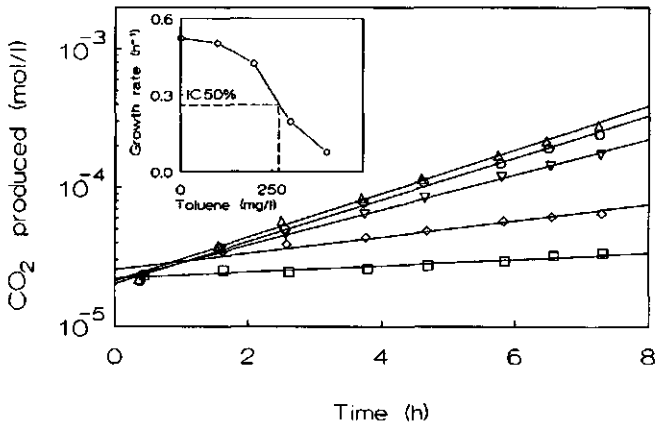

Figure 1: CO_2 production by *Rhodococcus* S5 grown on acetate medium in the presence of 0 (Δ), 100 (O), 200 (∇), 300 (◊), and 400 (□) mg/l toluene. The logarithm of the CO_2 concentration in the gas phase is shown.
Inset: Growth rate of *Rhodococcus* S5 on acetate in the presence of various concentrations of toluene.

Table 1: IC50% values of various contaminants for *Rhodococcus* S5 and *Pseudomonas* S12.

Contaminant	Log P_{OW} [a]	Water-Air distribution ratio [b]	Maximum water solubility [b]	IC 50%			
				Rhodococcus S5		*Pseudomonas* S12	
			(mg/l)	water (mg/l)	gas (g/m^3)	water (mg/l)	gas (g/m^3)
Propylbenzene	3.6	2.5[c]	55[c]	30	12	-	-
Ethylbenzene	3.1	2.9	160	145	50	135	47
Styrene	3.0	7.8	320	170	22	185	24
Toluene	2.5	3.8	540	260	68	310	82
Benzene	2.0	4.6	1800	>500	>109	410	90
Acrylonitril	-0.2	240	73000	-	-	120	0.5
Formaldehyde	-1.3	590	667000	8	0.014	20	0.03

[a] Calculated according to the method of Rekker and de Kort (1979)
[b] from Amoore & Hautala (1983)
[c] from Mackay & Shiu (1981)

contaminants. From these logarithmic plots of the carbon dioxide production in time, the growth rate was calculated. In Fig. 1b these growth rates are shown as a function of the toluene concentration in the liquid phase. Using this plot the toluene concentration that inhibited the growth rate to half of the maximum growth rate was determined (IC50%).
Similarly the IC50% values have been determined for the other contaminants. These results are summarized in Table 1. Also shown are the contaminant concentrations in the gas phase which are in equilibrium with the determined IC50% values in the liquid phase. For the substituted benzene compounds a linear relation between the toxicity and the hydrophobicity was observed (Fig. 2).

Adaptation

Surprisingly, one of the bacteria used for the toxicity tests (*Pseudomonas* strain S12) started to grow in the presence of toxic concentrations of the aromatic compounds after about 20 hours. This adaptation was studied in more detail for growth of *Pseudomonas* S12 on acetate in the presence of various concentrations of toluene. After an adaptation period growth was observed in the presence of 400 mg toluene/l and also in the presence of a supersaturating amount of toluene (1500 mg/l). When the cells were pre-cultured in the presence of 400 mg toluene/l an adaptation period was only observed when the cells were transferred to medium containing 1500 mg toluene/l. No adaptation time was observed when the cells were pre-grown in the presence of 1500 mg toluene/l.

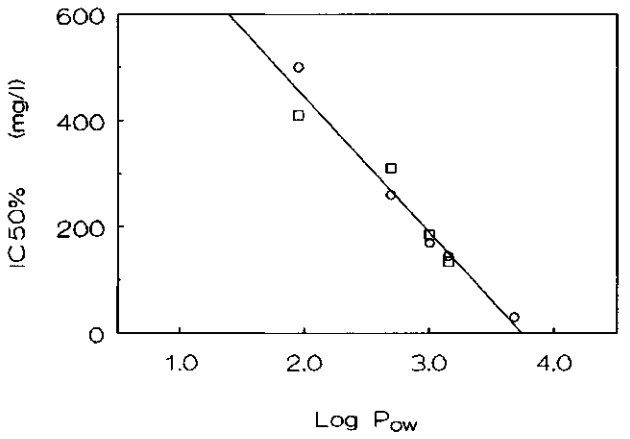

Figure 2: IC50% of the tested substituted benzene compounds versus their Log P_{OW} value. Data for (O) *Rhodococcus* S5 and (□) *Pseudomonas* S12.

DISCUSSION

Numerous studies have shown that xenobiotics present in the environment can be toxic for microorganisms (Bringmann & Kühn, 1977; Davidson & Branden, 1981; Blum & Speece, 1991; Sierra-Alvarez & Lettinga, 1991). This toxicity can seriously hamper the application of microorganisms to remove pollutants from waste streams. The contaminant concentrations in waste gases are expected to fluctuate, with temporarily high concentrations being toxic, possibly resulting in an inactivation of the system. Also during the start-up period of a bioreactor, contaminants can accumulate in the water phase of the reactor reaching toxic levels. In order to estimate the toxicity levels of various volatile compounds we have determined the IC50% value (concentrations which inhibits the growth rate by 50%) of these compounds for two types of microorganisms (the Gram-positive *Rhodococcus* S5 and the Gram-negative *Pseudomonas* S12). The observed IC50% values where similar for both types of microorganisms. This in spite of the difference in cell envelope structure of Gram-positive and Gram-negative bacteria.

For the substituted benzene compounds a relationship was observed between the toxicity and the hydrophobicity of the compound. The hydrophobicity is

expressed as the logarithm of partitioning of this compound between octanol and water (Log P_{OW}). A similar relationship has also been observed for other microorganisms and other groups of chemicals (Sierra-Alvarez & Lettinga, 1991; Vermuë *et al.*, 1993) These results indicate that the primary site of toxicity is the cell membrane. An increase in hydrophobicity correlates with in increase in toxicity, and an increase in affinity of the compound to accumulate in the cell membrane (Sikkema *et al.*, 1994).

Of the tested xenobiotics, especially the compounds with a good water solubility were already toxic at low concentrations in the gas phase. (e.g. formaldehyde, at 100 mg/m^3). Under normal operation of a bioreactor formaldehyde will be degraded and such a toxic level in the water phase will not be reached. However, during the start-up period of the bioreactor or when temporarily high concentrations are present in the waste gas these toxic levels can be reached, resulting in inactivation of the microorganisms. For a reliable operation of waste gas purification systems these situations should be prevented. Therefore, during the start-up period it is advisable to reduce the contaminant concentration or flow-rate until enough biomass is formed to degrade all of the contaminants present in the waste gas. Temporarily high concentrations, due to fluctuations, can also be buffered by using activated carbon, thereby preventing inactivation of the system (Weber & Hartmans, 1995).

Surprisingly one of the microorganisms used in the toxicity test is capable to adapt to the presence of toxic compounds. This adaptation results in the capacity of this bacterium to grow in the presence of supersaturating concentrations of toluene. Until now only a few microorganisms have been described which are able to grow in the presence of such high concentrations of toluene. Application of this type of microorganism in waste-gas treatment could result in a robust system which is not sensitive to fluctuations in contaminant concentrations.

REFERENCES

Amoore, J.E. and E. Hautala. (1983). Odor as an aid to chemical safety: odor thresholds compared with threshold limit values and volatilities for 214 industrial chemicals in air and water dilution. *J. Appl. Toxicol.* **3**: 272-290.

Blum, D.J.W. and R.E. Speece. (1991). A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. *Res. J. WPCF* **63**: 198-207.

Bringmann, G. and R. Kühn. (1977). Grenzwerte der Schadwirkung wassergefährender Stoffe gegen Bakterien (*Pseudomonas putida*) und Grünalgen (*Scenedes quadricauda*) im Zellvermehrungshemmtest. *Z. f. Wasser- und Abwasser-Forschüng* **10:** 87-98.

Davidson, P.M. and A.L. Branden. (1981). Antimicrobial activity of non-halogenated phenolic compounds. *J. Food Protection* **44:** 623-632.

Diks, R.M.M. and S.P.P. Ottengraf. (1991). Verification studies of a simplified model for the removal of dichloromethane from waste gases using a biological trickling filter. (Part II). *Bioproc. Eng.* **6:** 131-140.

Hartmans, S., J.P. Smits, M.J. van der Werf, F. Volkering and J.A.M. de Bont. (1989). Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. *Appl. Environ. Microbiol.* **55:** 2850-2855.

Hartmans, S., M.J. van der Werf and J.A.M. de Bont. (1990). Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. *Appl. Environ. Microbiol.* **56:** 1347-1351.

Hartmans, S. and J. Tramper. (1991). Dichloromethane removal from waste gases with a trickle-bed bioreactor. *Bioproc. Eng.* **6:** 83-92.

Leson, G. and A.M. Winer. (1991). Biofiltration: An innovative air pollution control technology for VOC emissions. *J. Air Waste Manage. Assoc.* **41:** 1045-1054.

Mackay, D. and Y.W. Shiu. (1981). A critical review of Henry's law constants for chemicals of environmental interest. *J. Phys. Chem. Ref. Data* **10:**1175-1199.

Rekker, R.F. and H.M. de Kort. (1979). The hydrophobic fragmental constant; an extension to a 1000 data point set. *Eur. J. Med. Chem.* **14:** 479-488.

Sierra-Alvarez, R. and G. Lettinga. (1991). The effect of the aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. *Appl. Microbiol. Biotechnol.* **34:** 544-550.

Sikkema, J., J.A.M. de Bont and B. Poolman. (1994). Interaction of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* **269:** 8022-8028.

Vermuë, M., J. Sikkema, A. Verheul, R. Bakker and J. Tramper. (1993). Toxicity of homologous series of organic solvents for the gram-positive bacteria *Arthrobacter* and *Nocardia* sp. and the gram-negative bacteria *Acinetobacter* and *Pseudomonas* sp. *Biotechnol. Bioeng.* **42:** 747-758.

Weber, F.J. and S. Hartmans. (1995). Use of activated carbon as a buffer in biofiltration of waste gases with fluctuating concentrations of toluene. *Appl. Microbiol. Biotechnol.* **43:** 365-369.

6

Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents

Frans J. Weber
Lydia P. Ooijkaas
Ruud M.W. Schemen
Sybe Hartmans
and Jan A.M. de Bont

Summary: *Pseudomonas putida* S12, previously selected for its capacity to grow on low concentrations of styrene, was shown to be able to grow on supersaturating amounts of styrene after a long lag-phase. Similarly, growth was obtained on supersaturating concentrations of octanol and heptanol.
Using acetate or propionate as a carbon and energy source, growth was obtained in the presence of solvents with a Log P_{OW} of 2.3 and higher. However, in the presence of supersaturating concentrations of toluene no growth was observed using other carbon sources. Acetate was toxic for *P. putida* S12 but cells were able to adapt to higher acetate concentrations. Only using these acetate-adapted cells adaptation to and growth in the presence of supersaturating concentrations of toluene was observed.

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INTRODUCTION

By using styrene as the sole carbon and energy source in subsaturating concentrations we have previously isolated 14 bacteria which were thought not to grow at styrene concentrations exceeding the solubility of styrene in water (Hartmans *et al.*, 1990). This substrate inhibition was not surprising since it has been established repeatedly that aromatic solvents with a log P_{OW} below 4.0, as for instance toluene and tetralin, are toxic for microorganisms even at low concentrations (de Smet *et al.*, 1978; Sikkema *et al.*, 1992). The logarithm of the partitioning coefficient of a solvent in a defined octanol-water mixture (Log P_{OW}) is commonly used as a measure for the lipophilicity of a solvent (Rekker & de Kort, 1979). Aromatic solvents with a Log P_{OW} below 4.0 can accumulate in the cytoplasmic membrane of bacteria causing impairment of membrane functions and expansion of the cell membrane, resulting in leakage of cellular metabolic products (Sikkema *et al.*, 1992). Nevertheless, two *Pseudomonas* species which grew on yeast peptone glucose medium in the presence of 50% toluene (Log P_{OW} = 2.5) have been isolated (Inoue & Horikoshi, 1989; Aono *et al.*, 1992) Recently, it has been shown that *P. putida* Idaho is not only resistant to toluene in a two-phase system, but can even use toluene at these high concentrations as the carbon and energy source (Cruden *et al.*, 1992).

Several studies demonstrate that bacteria are able to adapt to hydrophobic compounds in their environment. These adaptations may be due to the induction of degradative enzymes or alterations in the structure and dynamics of the cells (Heipieper *et al.*, 1991; Mishra & Kaur, 1991; Leying *et al.*, 1992). Possibly, such phenotypic adaptations eventually may also lead to a new genotype which would be irreversibly resistant to solvents. Thus, the constitutively toluene-resistant *Pseudomonas* species isolated by the group of Horikoshi and by Cruden *et al* (Inoue & Horikoshi, 1989; Aono *et al.*, 1992; Cruden *et al.*, 1992) may have evolved by an adaptation mechanism.

In this report we describe the adaptation of a culture of the styrene-sensitive *Pseudomonas* strain S12 to high styrene concentrations. In addition the capacity of the strain to grow in a two-phase styrene-water medium and the resistance to other toxic solvents was investigated.

MATERIALS AND METHODS

Organism and growth

Pseudomonas strain S12 has previously been isolated on styrene (Hartmans *et al.*, 1990). Cells were grown on phosphate buffered (pH 7.0) mineral salts medium (Hartmans *et al.*, 1989) with sodium acetate (60 mM) or other carbon sources (3.0 g/l). Cultures were incubated at 30°C in a horizontally shaking water bath (160 oscillations per minute, amplitude: 2.2 cm).

Growth in the presence of solvents

An inoculum of 5% (v/v) from an overnight culture of *P. putida* S12 was transferred to fresh medium. When these cells had reached the exponential growth phase ($OD_{660} \approx 0.4$) solvents were added. The culture bottles (250 ml) containing 25 ml of medium were closed with Mininert valves (Phase Separations, Waddinxveen, the Netherlands) to prevent solvent evaporation.

Bacterial survival

The number of viable cells before and after solvents were added to exponentially growing cultures ($OD_{660} \approx 0.4$) was determined by plating 0.1 ml of suitable dilutions in 0.8% (w/v) saline on agar plates with the same growth substrate (e.g. acetate or glucose) as the liquid culture. The adapted inoculum was obtained by plating a culture growing on acetate in the presence of toluene (1% v/v) on an acetate-plate.

Analytical methods

Dry weight was determined by drying washed cell suspensions at 105°C for 24 h. CO_2 concentrations were determined using a Packard model 427 gas chromatograph (Packard, Delft, The Netherlands) with a Hayesep Q column (Chrompack, Middelburg, The Netherlands). 100 µl headspace samples were analyzed. A partition coefficient of CO_2 between medium (pH 7.0) and air of 5.1 was measured and used to calculate the total amount of CO_2 produced.

RESULTS

Identification

Strain S12 was previously isolated on styrene and was tentatively identified as a *Pseudomonas* species (Hartmans *et al.*, 1990). The organism was further identified by its growth and biochemical characteristics as a *Pseudomonas putida* species (Palleroni, 1984). The results of the tests used are summarized in Table 1. The identification was confirmed by the fatty acid analysis of the strain using the

Table 1: Morphological, biochemical and growth characteristics of *P. putida* S12.

Form	Rods	Carbon sources for growth:	
Gram stain	Negative	Glucose	+
Motility	Motile	*meso*-Inositol	-
Reaction to oxygen	Aerobic	Geraniol	-
Fluorescent pigment	+	L-Valine	+
Growth at 41 °C	-	ß-Alanine	+
Arginine dehydrolase	+	DL-Arginine	+
Oxidase reaction	+		
Catalase	+		
Denitrification	-		

Microbial Identification System of MIDI (Newark, DE, USA) (Janse & Smits, 1990). Using the aerobe TSBA database of the MIDI system, strain S12 was identified as a *Pseudomonas putida* with 95% confidence.

Adaptation

Pseudomonas putida S12 was precultured on styrene and growth was assessed by determining CO_2-evolution. Exponential growth (μ = 0.6 h^{-1}) was observed without an appreciable lag-phase (Fig. 1) at an initial amount of 2 µl styrene in the incubation system (25 ml liquid medium in a total volume of 250 ml). This initial amount of styrene in the incubation system, based on its partition coefficient, results in a concentration of 0.3 mM in the water phase, which is well below the water-saturating level of 1.5 mM (Banerjee *et al.*, 1980). Growth of the organism was completely inhibited by raising the initial amount of styrene to supersaturating amounts (0.25 ml/25 ml). But surprisingly, the culture started to evolve CO_2 after about 20 h (Fig. 1). Subsequently, cells grown on this high concentration of styrene were used to inoculate fresh medium (25 ml) with either 2 µl or 0.25 ml styrene. No significant lag-time was observed for both conditions and the growth rates (0.6 h^{-1}) on basis of the CO_2 profiles were identical. Adapted cells could also be obtained following the above procedure but by starting from a single colony of *P. putida* S12 grown on acetate plates.

P. putida S12 also grew on supersaturating concentrations of octanol or heptanol as the sole carbon and energy source. Using these solvents at supersaturating amounts also resulted in lag-times of approximately 20 h.

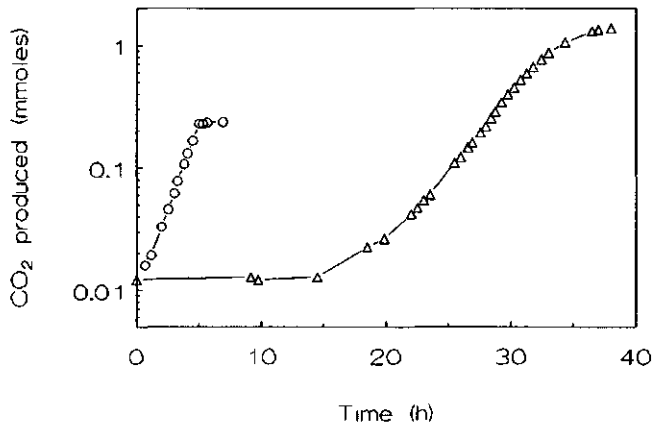

Figure 1: Growth of *P. putida* S12 on styrene at two different concentrations. Cells were precultured with 0.008% (v/v) styrene and CO_2 production was measured at low (O) and high (Δ) initial styrene concentrations (0.008% (v/v) and 1% (v/v) respectively).

Growth of cells adapted to 1% (v/v) of styrene was studied in more detail by determining dry weight and viable counts as well as CO_2-evolution. Fig. 2 shows a typical growth-curve of *P. putida* S12 growing at 1% (v/v) styrene. After a short lag-time, exponential growth was observed. In the stationary phase a rapid decline in the viable cell count was observed.

Solvent tolerance

It was also tested if a culture of the organism would develop tolerance to a solvent if it was growing on a non-toxic substrate in the presence of a non-metabolizable solvent. Unadapted cells of *P. putida* S12 were grown on several carbon sources and to the growing cultures toluene (1% v/v) was added. Only cultures growing on either acetate or propionate eventually continued to grow in the presence of toluene. No growth in the presence of toluene was observed using either glucose, fructose, glycerol, ethanol, arginine, alanine, succinate, lactate or pyruvate as carbon source. Using acetate as carbon source it was subsequently tested if unadapted cells could grow in the presence of several other solvents (Table 2). Growth was observed in the presence of solvents with a Log P_{OW} of 2.3 or higher. Lag times of more than 20 h were observed, especially for solvents with a low Log P_{OW} value.

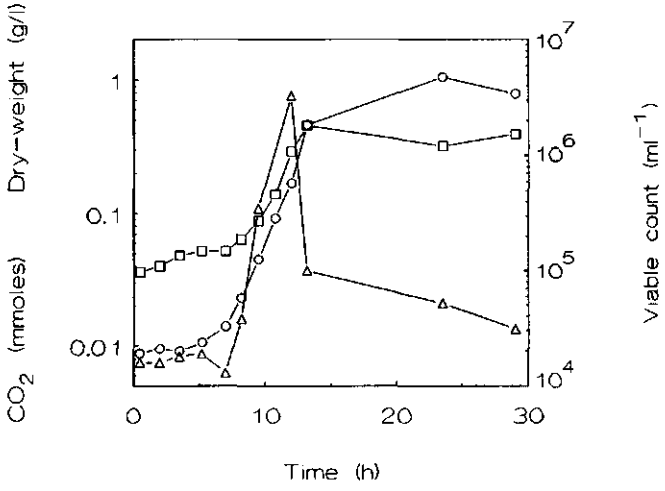

Figure 2: Growth of *P. putida* S12 on 1% (v/v) styrene in 25 ml mineral salts medium. (O) Total amount of CO_2 produced (mmoles), (□) dry weight (g/l), (Δ) viable count (cells per milliliter).

Survival after the addition of toluene

The effect of a solvent on unadapted cells was further studied by exposing either acetate-grown or glucose-grown cells to toluene. Numbers of viable cells in exponentially growing cultures were determined just before toluene additions (1% v/v) as well as 1 hour after additions (Table 3). Only 0.002% of the initial number of cells was still viable one hour after the addition of 1% (v/v) toluene to cells growing on acetate (60 mM). Growth of the culture eventually resumed as was confirmed by measuring CO_2 concentrations after 48 h. However, when glucose medium was used, no viable cells were detected one hour after the addition of toluene and no growth within 48 h was observed. It was subsequently tested if cells originating from a culture grown on acetate in the presence of toluene would retain their resistance to toluene when grown in the absence of a solvent. If such cells were grown on acetate for about 10 generations in the absence of toluene, about 10% of the cells survived the addition of toluene (Table 3). However, when cells from the solvent-adapted acetate culture were transferred to and grown in glucose medium for 10 generations, no survivors were observed one hour after the addition of toluene.

Table 2: Growth of *P. putida* S12 on solvents (1% v/v) as sole carbon source, or in presence of solvent (1% v/v) with acetate as carbon source.

Solvent	Log P_{OW}[a]	Growth[b] on:	
		Acetate in the presence of solvent (1%, v/v)	Solvent (1%, v/v)
Decane	5.6	+	-
Propylbenzene	3.6	+	-
Hexane	3.5	+	-
Cyclohexane	3.2	+	-
Ethylbenzene	3.1	+	-
p-Xylene	3.0	+	-
Styrene	3.0	+	+
Octanol	2.9	+	+
Toluene	2.5	+	-
Heptanol	2.4	+	+
Dimethylphthalate	2.3	+/-	-
Fluorobenzene	2.2	-	-
Benzene	2.0	-	-

[a] Log P_{OW} values were calculated according to the method of Rekker and de Kort (1979)

[b] Symbols: +, growth (> 0.5 mmol CO_2 produced after 48 h); +/-, growth (> 0.5 mmol CO_2 produced after 120 h); - no growth (< 0.1 mmol CO_2 produced after 120 h).

Table 3: Effect of the addition of 1% (v/v) toluene on the viable count of exponentially growing *P. putida* S12 on acetate or glucose media. Adapted cells were previously grown in the presence of 1% (v/v) toluene.

Strain	Substrate	Viable cells (ml^{-1})		Survival	Growth after 48 h
		Before	1h After		
Wild Type	Acetate (60 mM)	$3.8*10^7$	$7.8*10^2$	0.002%	yes
Wild Type	Glucose	$3.7*10^7$	<10	0%	no
Adapted	Acetate (60 mM)	$1.1*10^8$	$1.1*10^7$	10%	yes
Adapted	Glucose	$3.2*10^7$	<10	0%	no

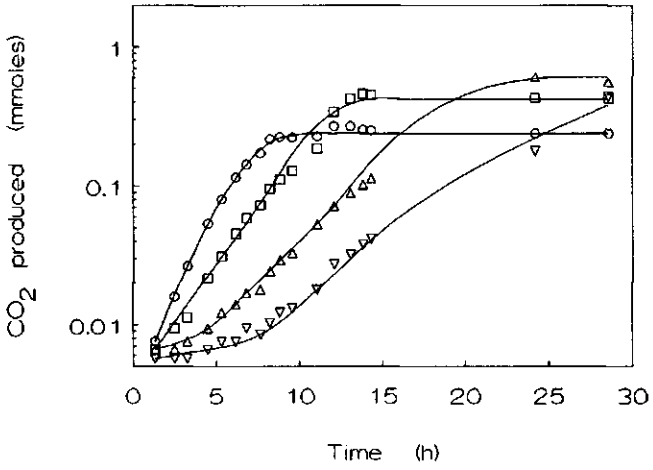

Figure 3: Growth of *P. putida* S12 at various acetate concentrations at pH 7.0. ○: 20 mM; □:40 mM; △: 60 mM; ▽: 80 mM acetate.

Toxicity of acetate

In view of the resistance of cells to toluene when grown on acetate or propionate it was tested if acetate itself is also toxic for the cells. Growth of glucose-precultured cells at different concentrations of acetate was determined and it was observed that growth of the cells was inhibited at acetate concentrations higher than 40 mM (Fig. 3). When cells had reached the stationary phase and were transferred to fresh medium with the same respective acetate concentration, no growth inhibition was observed. When toluene (1% v/v) was added to such cultures in the beginning of their exponential growth phase, growth after 48 h was only observed at initial acetate concentrations of 40 mM or higher.

DISCUSSION

Previously, we have isolated the styrene-degrader *P. putida* S12 by using subsaturating concentrations of styrene (Hartmans *et al.*, 1990). It was observed that growth of the organism was inhibited at higher concentrations of styrene. Surprisingly, we have now found that this organism is able to adapt to higher concentrations of styrene. Growth on styrene in a two-phase styrene-water system

eventually occurred and similarly growth was also observed in the presence of a second phase of heptanol or octanol. This resistance to high concentrations of a solvent, which is also used as carbon and energy source, is similar to the resistance for p-xylene observed in *P. putida* Idaho (Cruden *et al.*, 1992). The organism also grew in the presence of supersaturating concentrations of non-growth solvents with a Log P_{OW} value of 2.3 or higher using acetate as carbon source. Similar solvent-resistance has been observed in *P. putida* IH-2000 and *P. aeruginosa* ST-001 (Inoue & Horikoshi, 1989; Aono *et al.*, 1992).

Although *P. putida* S12 eventually grew on acetate in the presence of toluene, more than 99.99% of the cells were killed when toluene was added to cells growing on acetate (60 mM). The survivors eventually grew to a high cell density. However, 10% of the cells in a culture previously exposed to toluene and grown in acetate medium survived a toluene shock. Even when the cells had been transferred for 10 successive times in an acetate medium lacking solvents a similar high percentage of survivors was observed. When several other carbon sources were used instead of acetate or propionate no growth was observed after the addition of 1% toluene to a exponentially growing culture. Neither was growth in the presence of toluene observed when cells previously grown on acetate and adapted to toluene were subsequently grown on glucose as carbon source.

The response of *P. putida* S12 to the presence of a toxic solvent is quite remarkable. Cells either precultured at subsaturating concentrations of the toxic styrene or at elevated acetate concentration were able to acquire solvent resistance. The undissociated acids of acetate and propionate are toxic for microorganisms and are generally used as food preservatives (Chichester & Tanner, 1975). Sheu and Freese (1972) have reported quite similar inhibition profiles for *Bacillus subtilis*. *B. subtilis* grows at half its maximum growth velocity at 80 mM acetate (pH 6.5) whereas for *P. putida* S12 this point was reached at 60 mM acetate (pH 7.0). At 60 mM acetate (pH 7.0) and 80 mM acetate (pH 6.5) the concentrations of the undissociated acetic acids are 0.3 mM and 1.4 mM respectively. The results of Sheu and Freese (1972) suggest that microorganisms grown in the presence of short-chain fatty acids have a lower membrane fluidity. Due to this reduced membrane fluidity of acetate-grown cells a small number of *P. putida* S12 cells apparently can survive and adapt to toluene. A similar situation is expected for cells growing on subsaturating concentrations of styrene. Whereas glucose-grown cells, possessing a normal membrane fluidity, are all killed by the addition of toluene. Several studies have demonstrated that bacteria can change their lipid composition when grown in the presence of

organic solvents (Ingram, 1977; Heipieper *et al.*, 1991). Currently we are studying whether such changes are important in the adaptation of *P. putida* S12 to high concentrations of styrene as the growth substrate or to other solvents when growing on acetate.

ACKNOWLEDGEMENTS

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REFERENCES

Aono, R., M. Ito, A. Inoue, K. Horikoshi. (1992). Isolation of novel toluene-tolerant strain of *Pseudomonas aeruginosa*. *Biosci. Biotechnol. Biochem.* **1:** 145-146.

Banerjee, S., S.H. Yalkowsky, and S.C. Valvani. (1980). Water solubility and octanol/water partition coefficients of organics. Limitations of the solubility-partition coefficient correlation. *Environ. Sci. Technol.* **14:** 1227-1229.

Chichester, D.F., and F.W. Tanner Jr. (1975). Antimicrobial food additives. *In:* T.E. Furia (ed.), Handbook of food additives, pp. 115-184, CRC Press, Cleveland.

Cruden, D.L., J.H. Wolfram, R.D. Rogers, and D.T. Gibson. (1992). Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. *Appl. Environ. Microbiol.* **58:** 2723-2729.

Hartmans, S., J.P. Smits, M.J. van der Werf, F. Volkering, and J.A.M. de Bont. (1989). Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. *Appl. Environ. Microbiol.* **55:** 2850-2855.

Hartmans, S., M.J. van der Werf, and J.A.M. de Bont. (1990). Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. *Appl. Environ. Microbiol.* **56:** 1347-1351.

Heipieper, H.J., H. Keweloh, and H.J. Rehm. (1991). Influence of phenols on growth and membrane permeability of free and immobilized *Escherichia coli*. *Appl. Environ. Microbiol.* **58:** 1847-1852.

Ingram, L.O. (1977). Changes in lipid composition of *Escherichia coli* resulting from growth with organic solvents and with food additives. *Appl. Environ. Microbiol.* **33:** 1233-1236.

Inoue, A., and K. Horikoshi. (1989). A *Pseudomonas* thrives in high concentrations of toluene. *Nature* **338:** 264-266.

Janse, J.D., and P.H. Smits. (1990). Whole cell fatty acid patterns of *Xenorhabdus* species. *Lett. Appl. Microbiol.* **10:** 131-135.

Leying, H.J., K.H. Büscher, W. Cullmann, and R.L. Then. (1992). Lipopolysaccharide alterations responsible for combined quinolone and β-lactam resistance in *Pseudomonas aeruginosa*. *Chemotherapy* **38:** 82-91.

Mishra, P., and S. Kaur. (1991). Lipids as modulator of ethanol tolerance in yeast. *Appl. Microbiol. Biotechnol.* **34:** 697-702.

Palleroni, N.J. (1984). Genus I. *Pseudomonas*. *In:* N.R. Krieg, and J.G. Holt (eds.), Bergey's manual of systematic bacteriology, Vol. 1, Williams & Wilkins Co., Baltimore.

Rekker, R.F. and H.M. de Kort. (1979). The hydrophobic fragmental constant; an extension to a 1000 data point set. *Eur. J. Med. Chem. - Chimica Therapeutica* **14:** 479-488.

Sheu, C.W., and E. Freese. (1972). Effects of fatty acids on growth and envelope proteins of *Bacillus subtilis*. *J. Bacteriol.* **111:** 516-524.

Sikkema, J., B. Poolman, W.N. Konings, J.A.M. de Bont. (1992). Effects of the membrane action of tetralin on the functional and structural properties of artificial and bacterial membranes. *J. Bacteriol.* **174:** 2986-2992.

de Smet, M.J., J. Kingma, and B. Witholt. (1978). The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli*. *Biochim. Biophys. Acta* **506:** 64-80.

7

Cis/trans isomerization of fatty acids as a defence mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene

Frans J. Weber
Sonja Isken
and Jan A.M. de Bont

Summary: Defence mechanisms of three *Pseudomonas putida* strains growing in the presence of toluene up to 50% (v/v) were investigated. The three strains reacted to toxic concentrations of toluene by accumulating *trans* unsaturated fatty acids in the membrane instead of the *cis* isomers. The membranes of the toluene-adapted cells possessed a higher *trans/cis* ratio and had a higher lipid-ordering since the transition temperature was about 7°C higher compared to the non-adapted cells.

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INTRODUCTION

Aromatic solvents like toluene, styrene and tetralin are toxic for microorganisms even at subsaturating concentrations in water (de Smet *et al.*, 1978; Sikkema *et al.*, 1992). These solvents partition preferentially in the cell membrane and this excessive accumulation causes expansion of the membrane and impairment of membrane functions (Sikkema *et al.*, 1994). Notwithstanding the clear toxicity of various aromatic solvents to most microorganisms, bacteria do exist that are able to tolerate high concentrations of such compounds in their environment. Clear examples are *Pseudomonas putida* IH-2000 (Inoue & Horikoshi, 1989) and *P. putida* PpG1 (Shima *et al.*, 1991) which grew in the presence of 50% (v/v) toluene. These strains, however, were not able to metabolize toluene. Recently, two other strains have been isolated which can utilize toxic solvents as carbon and energy source. *P. putida* Idaho grew on toluene and *p*-xylene in concentrations of more than 50% (Cruden *et al.*, 1992) and *P. putida* S12 grew on supersaturating concentrations of styrene (Weber *et al.*, 1993).

The mechanism of this tolerance of these *P. putida* strains is presently unknown, but it is reasonable to expect adaptations at the level of the membrane composition. Changes in the fatty acid composition of the membrane may alter the partitioning of solvents in the membrane. An increase of the acyl chain length of the phospholipids from tetradecanoic (C14:0) to octadecanoic acid (C18:0) reduced the partitioning of lindane into liposomes 50-fold (Antunes-Madeira & Madeira, 1989). Incubations of *Escherichia coli* with apolar solvents such as benzene and octanol resulted in an increased synthesis of saturated fatty acids (Ingram, 1976, 1977). Another adaptation mechanism has been observed in a *Pseudomonas putida* species growing on phenol at high concentrations (Heipieper *et al.*, 1992). These cells adapted by converting the *cis* unsaturated fatty acids into the *trans* isomers which have a higher transition temperature and a decreased fluidity of the membrane.

In this report we describe changes in the fatty acid composition of the phospholipids of the three solvent-tolerant strains *P. putida* PpG1, *P. putida* Idaho and *P. putida* S12, in response to the presence of supersaturating concentrations of organic solvents in the growth medium.

MATERIALS AND METHODS

Organism and growth

Pseudomonas putida S12 had been isolated on styrene (Hartmans *et al.*, 1990). *P. putida* Idaho (NRRL B-18435) and *P. putida* PpG1 (ATCC 17453) were obtained from culture collections. Cells were grown in phosphate buffered (pH 7.0) mineral salts medium (Hartmans *et al.*, 1989) with sodium acetate (60 mM) or glucose (15 mM) as carbon and energy source. Cultures were incubated at 30°C with horizontal shaking in a water bath (160 oscillations per minute, amplitude: 2.2 cm).

Growth in the presence of solvents

An inoculum of 5% (v/v) from an overnight culture was transferred to fresh medium. When the culture reached the exponential growth phase ($OD_{660} \approx 0.4$) organic solvents were added. The culture bottles (250 ml) containing 25 ml of medium were closed with Mininert valves (Phase Separations, Waddinxveen, The Netherlands) to prevent evaporation. A partition coefficient of toluene between medium and air of 3.8 (Amoore & Hautala, 1983) was used to calculate the toluene concentrations in the water phase.

Chemostat

Cells were grown on mineral salts medium with 15 mM glucose in a fermentor with 700 ml working volume at 30°C, 350 rpm and at a dilution rate of 0.2 h^{-1}. Various toluene concentrations were supplied to the fermentor via the gas phase by passing an adjustable part of the air flow through a column (15 cm) of toluene which was kept at 30°C. The total air flow was kept constant at 140 ml/min.

Bacterial survival

The numbers of viable cells before and after solvents were added to exponentially growing cultures ($OD_{660} \approx 0.4$) or to cells harvested from steady state chemostat cultures were determined by plating 0.1 ml of suitable dilutions in 0.8% (w/v) saline on solidified agar-medium with the same growth substrate (e.g., acetate or glucose) as the liquid cultivation medium.

Fatty acid analysis

The total lipids of cells were extracted with chloroform/methanol (Bligh & Dyer, 1959). The fatty acids were methylated using boron trifluoride/methanol (Morrison & Smith, 1964). The fatty acid methyl esters were extracted into hexane and normally analyzed on a Chrompack CP9000 gaschromatograph with a CP-Sil 88 WCOT fused-silica column. Fatty acids identities were confirmed by GC-MS

analysis using a 5890A Hewlett/Packard chromatograph with a CP-Sil 19CB WCOT fused-silica capillary column; mass spectra were recorded on a 5970 series mass-selective detector with a 59822B Ionization Gauge Controller (Hewlett/Packard).

Phase transition temperature

The temperature-dependent vibrational frequency of the CH_2-stretch in lipids of whole bacterial cells was measured with Fourier transform infrared spectroscopy (FTIR). FTIR measurements were carried out using a Perkin-Elmer series 1750 FTIR spectrometer equipped with a 7500 data station, as described by Crowe *et al.* (1989). Exponentially growing cells were harvested and washed twice with water. The pellet was placed between two CaF_2 windows. The sample was cooled to -10°C and slowly heated and spectra were recorded after every stepwise increase (2 to 3°C) in temperature.

RESULTS

Fatty acid composition

The three solvent resistant strains, *P. putida* PpG1, *P. putida* Idaho and *P. putida* S12 were grown in mineral medium containing either glucose, acetate, or acetate in the presence of toluene (1% v/v). Exponentially growing cells from these cultures were harvested, washed and the fatty acid profiles were determined (Table 1). When glucose-grown *P. putida* S12 cells were compared to acetate-grown cells, an increase in the amount of *trans*- and a decrease in the *cis*-unsaturated fatty acids was seen. A further increase in the *trans/cis* ratio was observed when *P. putida* S12 cells were grown on acetate in the presence of toluene (1% v/v). The acetate-grown cells also had a higher percentage of cyclopropane fatty acids when compared to glucose-grown cells. However, the presence of toluene had no effect on these cyclopropane fatty acids. Similarly, in two other solvent tolerant strains *P. putida* PpG1 and *P. putida* Idaho high *trans/cis* ratios were observed when grown in the presence of high toluene concentrations.

Stability of the adaptation

Subsequently, we determined whether the observed changes in fatty acid profiles, as dependent on growth conditions, were reversible. *P. putida* S12 grown on acetate in the presence of 1% (v/v) toluene was used to inoculate (1% v/v) acetate and glucose medium without solvents. The fatty acid composition of the cells grown on acetate was almost identical to the fatty acid composition of the

Table 1: Fatty acid composition (%) of three toluene-tolerant *P. putida* strains grown on glucose, acetate, or acetate in the presence of toluene (1% v/v) respectively.

	***P. putida* S12**			***P. putida* PpG1**			***P. putida* Idaho**		
	Glucose	Acetate	Acetate + 1% Toluene	Glucose	Acetate	Acetate + 1% Toluene	Glucose	Acetate	Acetate + 1% Toluene
Fatty Acid									
16:0	35.9	31.4	31.2	30.7	34.3	34.5	39.4	38.7	39.5
16:1 *trans*	4.7	9.6	20.6	12.5	12.3	16.9	4.4	9.0	12.1
16:1 *cis*	32.8	25.9	13.5	23.2	20.4	9.4	18.9	16.7	2.9
17 cyclo	< 1.0	6.1	5.3	1.3	3.0	7.8	3.0	3.6	6.5
18:0	4.2	1.4	1.3	1.4	3.6	1.4	2.3	2.9	1.7
18:1 *trans*	1.0	3.1	10.4	1.8	1.8	7.1	4.6	8.7	17.2
18:1 *cis*	21.4	21.3	16.9	26.7	22.4	19.4	22.0	23.9	8.5
19 cyclo	< 1.0	1.2	< 1.0	1.1	2.1	3.5	5.3	4.3	11.6
Ratio									
Sat./unsat.	0.67	0.67	0.62	0.54	0.76	0.89	1.00	0.94	1.45
Trans/cis									
16:1	0.14	0.37	1.5	0.54	0.60	1.8	0.23	0.54	4.1
18:1	0.05	0.15	0.62	0.07	0.08	0.37	0.21	0.36	2.0

parent culture grown in the presence of toluene. However, a decrease in the *trans/cis* ratio was observed, when grown on glucose (Table 2).

Subsaturating concentrations of toluene

Toluene concentrations below the maximum solubility in water (640 mg/l at 30°C) (Bohon & Claussen, 1951) also slowed the growth of *P. putida* S12 on glucose or acetate medium. A toluene concentration of about 350 mg/l reduced the growth rate to one-half of the maximum. When these cells were transferred to fresh medium with the same toluene concentration, no growth inhibition was observed.

The effect of toluene on the fatty acid composition of *P. putida* S12 was also determined by growing the organism in a glucose-limited chemostat at a dilution rate of 0.2 h^{-1}. Various subsaturating toluene concentrations were applied to the fermentor via the gas phase. The fatty acid composition of the cultures were determined after a steady-state was reached in the chemostat. Toluene concentrations below 300 mg/l in the water phase did not result in changes in the fatty acid composition. But substantial changes in the *trans/cis* ratio of the unsaturated fatty acids were observed in cells grown in the presence of higher toluene concentrations (Fig. 1). The steady-state chemostat cultures were also

Table 2: Fatty acid composition (%) of toluene-adapted *P. putida* S12 grown in the absence of toluene for about 100 generations on glucose or acetate respectively.

	Glucose	Acetate
Fatty Acid		
16:0	38.4	32.5
16:1 *trans*	11.5	25.2
16:1 *cis*	19.1	12.2
17 cyclo	3.1	2.8
18:0	2.8	2.1
18:1 *trans*	2.9	6.9
18:1 *cis*	22.3	16.9
19 cyclo	< 1.0	1.0
Ratio		
Sat./Unsaturated	0.79	0.63
16:1 *trans/cis*	0.60	2.1
18:1 *trans/cis*	0.13	0.41

used to determine the effect on cell viability of a "shock" addition of toluene (1% v/v). Only a small number of survivors were recorded in cultures grown in the presence of less than 300 mg toluene per liter. However, when grown in the presence of more than 300 mg toluene per liter, a high percentage of cells survived this addition of toluene.

Phase transition temperature

Fatty acids undergoing melting from gel to liquid phase show an adsorption shift of 2 to 4 wavenumbers in the infrared spectrum (Cameron & Dluhy, 1987). The symmetric vibration bands of CH_2-groups at a frequency around 2850 cm^{-1} was measured using FTIR spectroscopy. This adsorption maximum was determined at different temperatures for *P. putida* S12 grown on glucose, acetate or acetate medium containing 400 mg toluene per liter respectively. In Fig. 2 it can be seen that cells grown in the presence of toluene possess a lipid melting temperature which is about 7°C higher than in cells grown in the absence of toluene.

DISCUSSION

Previously we have isolated 14 bacteria on subsaturating concentrations of styrene as sole carbon and energy source (Hartmans *et al.*, 1990). These bacteria were thought not to grow on supersaturating concentrations of styrene, as these high styrene concentrations were expected to be toxic (de Smet *et al.*, 1978; Sikkema *et al.*, 1992). However, one of the selected strains, *P. putida* S12,

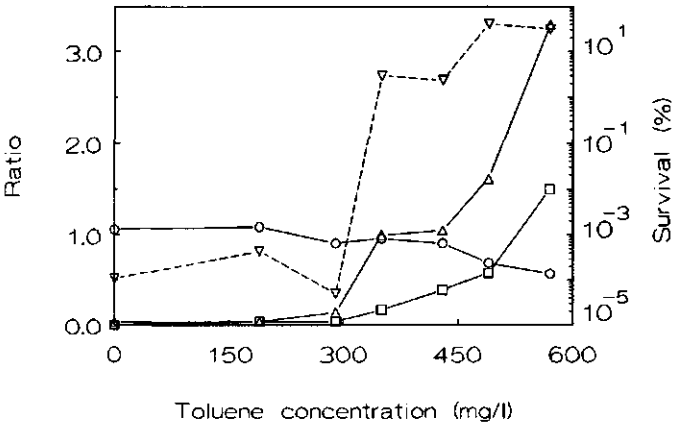

Figure 1: Effect of toluene on the fatty acid composition of *P. putida* S12 and on survival of cells given a shock addition of toluene. Cells were grown in a glucose-limited chemostat in the presence of various toluene concentrations. Ratio (—) of saturated/unsaturated fatty acids (O), *trans/cis* ratio of hexadecenoic acid (16:1) (Δ), and octadecenoic acid (18:1) (□), and the percentage survivors (---) 1h after the extra addition of toluene (1% v/v) (▽).

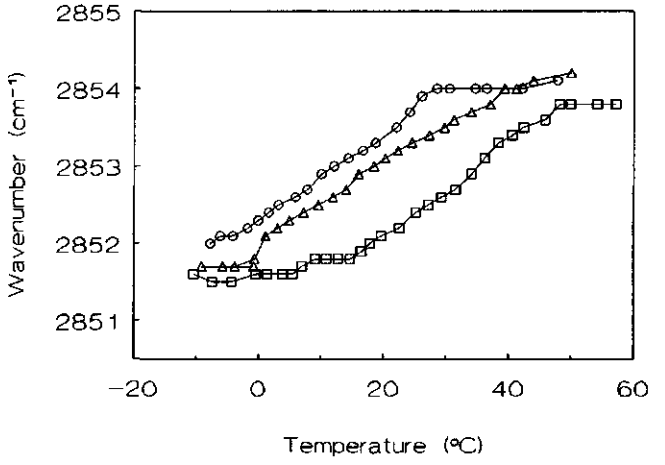

Figure 2: Phase transition melting temperature of *P. putida* S12 grown on glucose (O), acetate (Δ) and acetate medium containing 400 mg/l toluene (□) respectively. Wavenumber of the CH_2 symmetric stretch in the lipids were measured using Fourier Transforming Infrared Spectroscopy at various temperatures.

eventually grew on styrene at concentrations of more than 50% (v/v) after a long lag-phase (Weber *et al.*, 1993).

In the present investigation, significant changes in the fatty acid profiles of solvent-adapted strains have been observed. *P. putida* S12 and two other solvent-tolerant strains responded to the presence of toluene (1% v/v) by increasing the amount of *trans* fatty acids and by decreasing the corresponding *cis*-isomer. This adaptation in *P. putida* S12, occurred not only at supersaturating concentrations but also at subsaturating concentrations of toluene in water. No effects were seen below 300 mg toluene per liter, but above 300 mg/l the *trans/cis* isomer ratio of the cells increased dramatically. Cells having a high *trans/cis* isomer ratio were well equipped to survive "shock" additions of toluene (1% v/v).

The *cis/trans* isomerization as an adaptation mechanism in *P. putida* S12 is quite remarkable as most bacteria adapt to membrane-active compounds by changing the saturation degree of the lipids (Ingram, 1976, 1977; Ingram & Buttke, 1984; Keweloh *et al.*, 1990). Although 15 years ago *trans* monounsaturated fatty acids were regarded as non-natural fatty acids (Lehninger, 1977), over the last ten years *trans* fatty acids in bacteria have been reported in, for instance *Arcobacter cryaerophila* (Moss & Daneshvar, 1992), *Pseudomonas aeruginosa* (de Andres *et al.*, 1991), *Pseudomonas atlantica* (Guckert *et al.*, 1987), *Pseudomonas putida* (Heipieper *et al.*, 1992), *Vibrio cholerae* (Guckert *et al.*, 1986) and other *Vibrio* species (Okuyama *et al.*, 1990), a marine isolate (Gillan *et al.*, 1981) and in methane-utilizing bacteria (Makula, 1978). Recently, a *cis/trans* isomerization has been observed as an adaptation mechanism of a *P. putida* to toxic phenol concentrations (Heipieper *et al.*, 1992). From our results it now appears that this defence mechanisms is widespread in *P. putida* species.

The *trans* isomer has a steric configuration which is similar to that of saturated fatty acids. The *cis*-isomer has a kink in the acyl-chain of the fatty acids, which causes steric hindrance and results in a membrane with a high fluidity. Studies with *Acholeplasma laidlawii* membranes enriched with exogenously supplied fatty acids have shown that the phase transition temperature between membranes containing about 80% *cis* or *trans* octadecenoic acid respectively differ about 45°C (Macdonald *et al.*, 1985). In the toluene-adapted strain possessing a high *trans/cis* ratio, the transition temperature of the membrane *in vivo* was approximately 7 centigrade degrees higher compared to the non-adapted cells. This provides the cell with a mechanism to compensate for loss of membrane integrity as a result of the accumulation of lipophilic compounds. Furthermore, an

increased lipid ordering also opposes partitioning of lipophilic compounds in the membrane (Antunes-Madeira & Madeira, 1989).

Apart from an increase in the *trans/cis* ratio of the unsaturated fatty acids, an increase in the amount of cyclopropane fatty acids was observed in acetate-grown cells when compared to glucose-grown cells. The lipids of acetate-grown *P. putida* S12 consisted of about 6% cyclopropane fatty acids, whereas glucose-grown cells did not contain these fatty acids. However, adaptation to toluene either in batch or in chemostat cultures did not enhance the level of cyclopropane fatty acids.

Growth of *P. putida* S12 in batch-cultures in the presence of supersaturating toluene concentrations was only observed when using toxic concentrations of acetate or propionate as carbon source (Weber *et al.*, 1993). At 60 mM acetate (pH 7.0), about 0.3 mM of the undissociated acetic acid will be present in the medium. Results of Sheu & Freese (1972) suggest that these low concentrations of acetic acid are membrane active. Acetate-adapted cells had an increased *trans/cis* ratio of the monounsaturated fatty acids. As a result, cells from such cultures had a higher degree of lipid ordering in the membrane and consequently these cells were able to further adapt and grow in the presence of toluene. Nakajima *et al.* (1992) and Aono *et al.*, (1992) have shown that toluene-resistant bacteria can be isolated effectively by first adapting bacteria to the presence of a second phase of the less toxic solvent xylene. Pre-adaptation of cells to less toxic compounds (acetate, xylene) or to subsaturating concentrations of toxic solvents (toluene) seems to be an effective technique to isolate solvent-tolerant microorganisms.

P. putida S12 cells possessing a high *trans/cis* isomer ratio after growth on acetate medium containing toluene, did not revert back to normal fatty acid content upon several generations growth without toluene. This response seems to be quite unusual, but these cells were grown on a medium with an acetate concentration (60 mM) which is normally toxic for the cells. Apparently, the modification in the fatty acid composition induced by toluene, is of benefit for the cells to grow in the presence of toxic acetate concentrations. Upon removal of acetate, a normal fatty acid composition was observed, indicating that the *trans/cis* isomerization is an adaptation mechanism and not a mutation.

The present investigation has focused only on one possible defence mechanisms of *P. putida* strains to toxic solvents. Further investigations will be necessary to determine whether *trans/cis* isomerization of the fatty is acids is an important

factor in gaining resistance or that other, as yet undiscovered adaptations are of primary importance. In this respect it will be worthwhile to consider the known effects of alcohols on microorganisms including changed protein/lipid ratios or changes in the phospholipid classes (Ingram & Buttke, 1984).

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REFERENCES

Amoore, J.E. and E. Hautala. (1983). Odor as an aid to chemical safety: odor thresholds compared with threshold limit values and volatilities for 214 industrial chemicals in air and water dilution. *J. Appl. Toxicol.* **3:** 272-290.

de Andres, C., M.J. Espuny, M. Robert, M.E. Mercade, A. Manresa and J. Guinea. (1991). Cellular lipid accumulation by *Pseudomonas aeruginosa* 44T1. *Appl. Microbiol. Biotechnol.* **35:** 813-816.

Antunes-Madeira, M.C. and V.M.C. Madeira. (1989). Membrane fluidity as affected by the insecticide lindane. *Biochim. Biophys. Acta* **982:** 161-166.

Aono, R., M. Ito, A. Inoue and K. Horikoshi. (1992). Isolation of novel toluene-tolerant strain of *Pseudomonas aeruginose*. *Biosci. Biotechnol. Biochem.* **1:** 145-146.

Bligh, E.G. and W.J. Dyer. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911-917.

Bohon, R.L. and W.F. Claussen. (1951). The solubility of aromatic hydrocarbons in water. *J. Am. Chem. Soc.* **73:** 1571-1578.

Cameron, D.G. and R.A. Dluhy. (1987). FTIR studies of molecular conformations in biological membranes. *In:* R.M. Gendreau (ed.), Spectroscopy in the medical sciences, pp. 53-86, CRC Press, Boca Raton.

Crowe, J.H., F.A. Hoekstra, L.M. Crowe, T.J. Anchordoguy and E. Drobnis. (1989). Lipid phase transitions measured in intact cells with fourier transform infrared spectroscopy. *Cryobiology* **26:** 76-84.

Cruden, D.L., J.H. Wolfram, R.D. Rogers and D.T. Gibson. (1992). Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. *Appl. Environ. Microbiol.* **58:** 2723-2729.

Gillan, F.T., R.B. Johns, T.V. Verheyen, J.K. Volkman and H.J. Bavor. (1981). *Trans*-monounsaturated acids in a marine bacterial isolate. *Appl. Environ. Microbiol.* **41:** 849-856.

Guckert, J.B., M.A. Hood and D.C. White. (1986). Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerea*: increases in the *trans/cis* ratio and porportions of cyclopropyl fatty acids. *Appl. Environ. Microbiol.* **52:** 794-801.

Guckert, J.B., D.B. Ringelberg and D.C. White. (1987). Biosynthesis of *trans* fatty acids from acetate in the bacterium *Pseudomonas atlantica*. *Can. J. Microbiol.* **33:** 748-754.

Hartmans, S., J.P. Smits, M.J. van der Werf, F. Volkering and J.A.M. de Bont. (1989). Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. *Appl. Environ. Microbiol.* **55:** 2850-2855.

Hartmans, S., M.J. van der Werf and J.A.M. de Bont. (1990). Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. *Appl. Environ. Microbiol.* **56:** 1347-1351.

Heipieper, H.-J., R. Diefenbach and H. Keweloh. (1992). Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. *Appl. Environ. Microbiol.* **58:** 1847-1852.

Ingram, L.O. (1976). Adaption of membrane lipids to alcohols. *J. Bacteriol.* **125:** 670-678.

Ingram, L.O. (1977). Changes in lipid composition of *Escherichia coli* resulting from growth with organic solvents and with food additives. *Appl. Environ. Microbiol.* **33:** 1233-1236.

Ingram, L.O. and T.M. Buttke. (1984). Effects of alcohols on micro-organisms. *Adv. Microbiol. Physiol.* **25:**254-300.

Inoue, A. and K. Horikoshi. (1989). A *Pseudomonas* thrives in high concentrations of toluene. *Nature* **338:** 264-266.

Keweloh, H., G. Weyrauch and H.J. Rehm. (1990). Phenol-induced membrane changes in free and immobilized *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **33:** 66-71.

Lehninger, A.L. (1977). Biochemistry, Worth Publishers, New York.

Macdonald, P.M., B.D. Sykes and R.N. McElhaney. (1985). Fluorin-19 nuclear magnetic resonance studies of lipid fatty acyl chain order and dynamics in *Acholeplasma laidlawii* B membranes. A direct comparison of the effects of *cis* and *trans* cyclopropane ring and double-band substituents on orientional order. *Biochem.* **24:** 4651-4659.

Makula, R.A. (1978). Phospolipid composition of methane-utilizing bacteria. *J. Bacteriol.* **134:** 771-777.

Morrison, W.R. and L.M. Smith. (1964). Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron flouride-methanol. *J. Lipid Res.* **5:** 600-608.

Moss, C.W. and M.I. Daneshvar. (1992). Identification of some uncommon monounsaturated fatty acids of bacteria. *J. Clin. Microbiol.* **30:** 2511-2512.

Nakajima, H., H. Konayashi, R. Aono and K. Horikoshi. (1992). Effective isolation and identification of toluene-tolerant *Pseudomonas* strains. *Biosci. Biotechnol. Biochem.* **56:** 1872-1873.

Okuyama, H., S. Sasaki, S. Higaski and N. Murata. (1990). A *trans*-unsaturated fatty acid in a psychrophilic bacterium, *Vibrio* sp. strain ABE-1. *J. Bacteriol.* **172:** 3515-3518.

Sheu, C.W. and E. Freese. (1972). Effects of fatty acids on growth and envelope proteins of *Bacillus subtilis*. *J. Bacteriol.* **111:** 516-524.

Shima, H., T. Kudo and K. Horikoshi. (1991). Isolation of toluene-resistant mutants from *Pseudomonas putida* PpG1 (ATCC 17453). *Agric. Biol. Chem.* **55:** 1197-1199.

Sikkema, J., B. Poolman, W.N. Konings and J.A.M. de Bont. (1992). Effects of the membrane action of tetralin on the functional and structural properties of artificial and bacterial membranes. *J. Bacteriol.* **174:** 2986-2992.

Sikkema, J., J.A.M. de Bont and B. Poolman. (1994). Interaction of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* **269:** 8022-8028.

de Smet, M.J., J. Kingma and B. Witholt. (1978). The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli*. *Biochim. Biophys. Acta* **506:** 64-80.

Weber, F.J., L.P. Ooijkaas, R.M.W. Schemen, S. Hartmans and J.A.M. de Bont. (1993). Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. *Appl. Environ. Microbiol.* **59:** 3502-3504.

8

Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes.

Frans J. Weber
and Jan A.M. de Bont

Summary: Several organic solvents are toxic for microorganisms already at relatively low concentrations. In general, this toxicity is caused by the accumulation of these lipophilic solvents in the membrane lipid bilayer, affecting the structural and functional properties of the membrane. In this review effects of alkanols, alkanes and other hydrocarbons on membranes are summerized. The physico-chemical aspect are considered both in terms of membrane fluidity and of bilayer stability. Although organic solvents can be highly toxic for microorganisms, some microorganisms are able to adapt and grow in the presence of toxic concentrations of these solvents. The emphasis of this review will be on the ability of microorganisms to adapt their membrane structure in order to survive the deleterious effects of solvents. These adaptation mechanisms are considered in view of the effects of organic solvents on the properties and functioning of the membrane.

Submitted for publication

LIST OF ABBREVIATIONS

DGDG	Digalactosyldiglyceride
DMPC	Dimyristoyl (C14:0) phosphatidylcholine
DPG	Diphosphatidylglycerol
DPPC	Dipalmitoyl (C16:0) phosphatidylcholine
H_I	Normal hexagonal lipid configuration
H_{II}	Inverted hexagonal lipid configuration
L_α	Lamellar liquid-crystalline phase
L_β	Lamellar gel phase
$L_\beta I$	Interdigitated gel phase
MGDG	Monogalactosyldiglyceride
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
P_{MB}	Partitioning of a compound over a membrane/buffer two-phase system
P_{OW}	Partitioning of a compound over a octanol/water two-phase system
PS	Phosphatidylserine
T_{LH}	Temperature at which the transition from a lamellar configuration to an inverted hexagonal lipid phase is 50% complete
T_M	Temperature at which the transition from the lamellar gel to the lamellar liquid-crystalline is 50% complete
a	Area of the phospholipid headgroup at the water interface
l	Length of the acyl-chain in a phospholipid
v	Volume of the fatty acids in a phospholipid

CONTENTS

1. INTRODUCTION

The main function of the cell membrane of microorganisms is to form a permeability barrier, regulating the passage of solutes between the cell and the external environment. The membrane keeps essential metabolites and macromolecules inside the cell, it pumps nutrients into the cell against a concentration gradient, and it prevents the entry of certain solutes present in the environment.

In the environment, microorganisms may be confronted with lipophilic compounds which preferentially accumulate in membranes. Such compounds may be naturally occurring compounds as for instance alkanols and terpenes, or may be xenobiotics such as alkanes and aromatic hydrocarbons. This accumulation of such compounds will affect physico-chemical properties of membranes and consequently their functioning.

The physico-chemical properties of membranes as affected by solvents and the resulting biological functioning are usually described by referring to the lamellar-gel to liquid-crystalline phase transition. A great number of studies have dealt with the effects of organic solvents on the transition from the lamellar-gel to the liquid-crystalline state. Another approach is to consider the stability of membranes in lamellar structures as affected by solvents. Many phospholipids present in membranes prefer non-lamellar structures and depending on the compound this tendency will be affected positively or negatively by the solvent. Relative to studies on lamellar-gel to liquid-crystalline phase transitions, few articles have appeared dealing with this aspect.

Several aspects of the effects of a variety of solvents on the physico-chemical properties of the cell membrane, both in terms of phase transitions and lamellar stability, have been reviewed by Lohner (1991). In this review we will only summarize these physico-chemical effects of alkanols, alkanes and other hydrocarbons on membranes. The emphasis here will be on the ability of microorganisms to adapt their membrane structure in order to survive the deleterious effects of solvents. Such adaptation mechanisms include changes in the phospholipid composition and changes in the content of membrane embedded proteins, sterols, hopanoids, or carotenoids. These adaptation mechanisms will be considered in view of the effects of organic solvents on the properties and functioning of the membrane.

2. PARTITIONING OF SOLVENTS INTO MEMBRANES

Several studies have shown that a correlation exists between the lipophilicity of a compound and the partitioning of such a compound into a lipid bilayer on the one hand and the toxicity to microorganisms on the other hand (Vighi & Calamari, 1987; Osborne *et al.*, 1990; Sierra-Alvarez & Lettinga, 1991; Sikkema *et al.*, 1994a). The lipophilicity of a compound usually is expressed in terms of Log P_{OW}, which gives the partitioning of a compound over an octanol/water two-phase system. Lipophilic compounds, which possess a high affinity for the cell membrane, are more toxic than less lipophilic compounds. For instance ethanol (Log P_{OW} = -0.28) is only toxic for microorganisms at high concentrations (several %), whereas solvents like toluene (Log P_{OW} = 2.5) are already toxic in the mM range. (Heipieper *et al.*, 1994). However, this correlation does not apply for hydrophobic solvents with a Log P_{OW} > 4 - 5. These solvents are generally not toxic for microorganisms (Inoue & Horikoshi, 1991; Vermuë *et al.*, 1993) and are actually used as a non-toxic second phase that functions as a reservoir for toxic compounds in biotransformation reactions (Laane *et al.*, 1987). Although these solvents with a Log P_{OW} above 4 have a high affinity to partition into the lipid bilayer, the poor solubility of these compounds in water will prevent them from reaching high concentrations in the membrane (Osborne *et al.*, 1990). This may be illustrated by considering the accumulation of a series of homologous alkanols in membranes. As may be seen from Table 1, the most hydrophilic alcohol included (dodecanol) has a far stronger preference for partitioning in membranes than the lower alkanols. Nevertheless, the maximum concentration which this compound can reach in the membrane is lower than the less hydrophobic alkanols because of its low water solubility. The maximum membrane concentration (Table 1) was calculated from the membrane/buffer partition coefficient (P_{MB}) and the maximum water solubility. The partition

Table 1: Maximum achievable membrane alkanol concentration, calculated from the membrane partition coefficient (Osborne *et al.*, 1990) and the maximum water solubility (Riddick *et al.*, 1986)

Compound	Log P_{OW}	Log P_{MB}	Max. Aqueous solubility (mM)	Max. Membrane concentration (mM)
Ethanol (C2)	-0.26	-0.98	17040	1784
Butanol (C4)	0.80	0.05	1005	1127
Hexanol (C6)	1.86	1.09	69	849
Octanol (C8)	2.92	2.13	4.1	553
Decanol (C10)	3.98	3.17	0.25	370
Dodecanol (C12)	5.04	4.20	0.019	301

coefficient for the solutes between the membrane and aqueous buffer (P_{MB}) was calculated using Log P_{OW} values (Rekker & de Kort, 1979) and the experimentally determined relationship between these two partition coefficients (Osborne *et al.*, 1990). Such calculations are valid under the assumption that no direct contact exists between the solvent phase and the organism.

3. POSITION OF SOLVENT PARTITIONING INTO MEMBRANES

The effect which a lipophilic compound has on the integrity of a membrane depends on the position in the membrane where it accumulates. A solute near the phospholipid headgroup area will have another effect on the membrane than a solute partitioning deeply in the lipid acyl chains. Depending on the hydrophobicity of the solute, it will accumulate more or less deeply into the bilayer. Alkanols like ethanol will interact with the headgroup area and alkanes with the fatty acid acyl chains.

The position of solvents in membranes has been determined both for alkanols and alkanes using a variety of techniques, including X-ray diffraction, and magnetic resonance spectroscopy (NMR and ESR). Westerman *et al.* (1988) have used phospholipids, which were deuterium labelled at various specific places, to study the interaction of n-alkanols (butanol, octanol, dodecanol and tetradecanol) with bilayers of DMPC. Different patterns of changes in lipid order induced by the alkanols were observed by ^{2}H-NMR (Westerman *et al.*, 1988). All alkanols studied produced a large disorder in the glycerol backbone of the lipid, indicating that the terminal hydroxyl group of the alkanol is anchored near the aqueous interface of the lipid. Of the four alkanols studied, butanol produced the largest disordering in the glycerol backbone. This probably reflects the greater fraction of time spent by the hydroxyl group of butanol in the vicinity of the lipid polar headgroup compared with the other alkanols (Westerman *et al.*, 1988). A very hydrophobic compound like cholesterol had a minimal effect on the ordering in the glycerol backbone suggesting that this solute is embedded more deeply in the bilayer. These findings suggest that the amphiphatic alkanols partition into the bilayer with their hydroxyl moiety near the phospholipid polar headgroup and their aliphatic chains intercalated between the fatty acyl chains of the phospholipids. Similar results have been obtained for other amphiphatic compounds, like benzyl-alcohol (Pope *et al.*, 1986) or cannabinoids (Yang *et al.*, 1992).

Non-amphiphatic compounds like alkanes do not interact with the headgroup and accumulate more deeply in the lipid bilayer, aligning themselves parallel to

the lipid hydrocarbon chains (McIntosh *et al.*, 1980). ^{2}H-NMR studies of Pope *et al.* (1989) have shown that the phospholipid carbons 2 to 10, which are more ordered, are entropically less accessible for alkanes. They predicted that the more disorded bilayer centre is the favoured location of hydrophobic solutes (Pope *et al.*, 1989). Studies with hexane have shown that this molecule is located in the centre of the bilayer in a zone of 10 Å (White *et al.*, 1981). Short-chain alkanes are expected to fit easily into this region (King *et al.*, 1985; Pope *et al.*, 1989). As the alkane chain-length increases a point is reached when the entire alkane molecule can not be located in this disordered lipid region, but must expand to the more ordered region. Due to the interaction with this more ordered lipid region, these longer chain alkanes tend to be less soluble in the bilayer. The maximum amount of hexadecane that can be solubilized in the bilayer is about one hexadecane molecule per 6-10 lipid molecules, which is lower than for the short-chain alkanes (McIntosh *et al.*, 1980; Pope & Dubro, 1986; Pope *et al.*, 1989). Accumulation of up to 1 mol of hexane per mol of lipid has been reported (King *et al.*, 1985).

The position of molecules other than alkanols and alkanes in a lipid bilayer is not very well documented. Less hydrophobic, non-amphiphatic molecules like toluene are expected to accumulate in between the lipid acyl chains near the headgroup area (Lohner, 1991; Sikkema *et al.*, 1994b). Only for benzene several reports are available describing its position in a membrane. Unfortunately, however, there is disagreement in the literature regarding the phospholipid

Figure 1: F-NMR signal of *p*-fluorotoluene dissolved in various solvents.

regions where benzene resides. Studies with surfactant micelles and lipid bilayers indicated that benzene molecules tend to partition into the hydrophobic region (Simon *et al.*, 1982; Boden *et al.*, 1991), whereas another study showed that benzene adsorbs at the headgroup interface (Ward *et al.*, 1986).

In view of the high toxicity of these aromatic hydrocarbons for microorganisms, we are interested in the position of these compounds in the bacterial membrane. We have used 4-fluorotoluene as a model compound, because this fluorocompound gives a F-NMR signal depending on the hydrophobicity of the environment. Based on this property it is possible to determine the position of toluene in the membrane. First, we have determined the F-NMR signal of F-toluene dissolved in various solvents. In Fig. 1 these F-NMR signals of fluorotoluene are shown as a function of the hydrophobicity of the solvent used to dissolve toluene.

Subsequently, we have used lipid vesicles to determine the position of F-toluene in the membrane. From *Pseudomonas putida* S12, a Gram-negative bacterium which is able to tolerate high concentrations of toluene (Weber *et al.*, 1993), the phospholipids were extracted and further purified. These phospholipids were used to make multi-laminar vesicles to which F-toluene was added. F-toluene accumulated in these vesicles gave a F-NMR signal at 4.0, which corresponds to a Log P_{OW} of 0.8 (Fig. 1). Thus F-toluene accumulated in phospholipids vesicles in an environment with a hydrophobicity corresponding to a Log P_{OW} value of 0.8. In Fig. 2 the hydrophobicity of various parts of a phospholipid molecule is shown, as calculated using the hydrophobic fragmental set of Rekker and De Kort (1979). The environment in the lipid bilayer were toluene accumulated, has a hydrophobicity corresponding to a Log P_{OW} value of 0.8. The only place in a phospholipid were such a hydrophobicity exists is in between the lipid acyl chains,

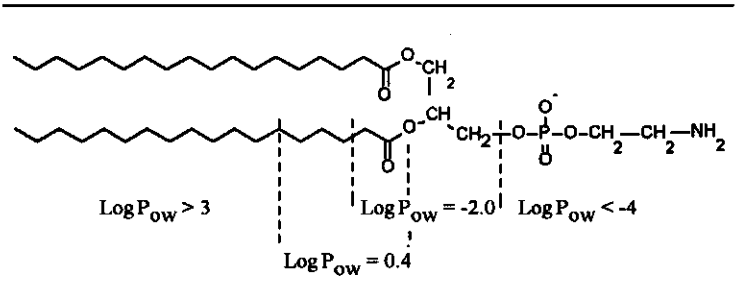

Figure 2: Hydrophobicity of various areas in a phospholipid. The Log P_{OW} values were calculated according to the method of Rekker and De Kort (1979).

near the headgroup area (Fig. 2). Using the same method it was determined that F-tetradecane accumulated in an area with a hydrophobicity with a Log P_{OW} of about 9.6, which indicates that this compound is located deeply embedded in between the lipid acyl chains. This result nicely confirms the observations of others on the position of long-chain alkanes in the membrane (Lohner, 1991; Sikkema *et al.*, 1994b).

4. EFFECT OF SOLUTE PARTITIONING ON THE BILAYER PROPERTIES

A great number of studies have shown that the accumulation of a solute in a lipid bilayer strongly influences the functioning of membranes. The Van der Waals interactions between the lipid acyl chains in a membrane will be affected by the partitioning of a solute in the membrane and the disturbance of these lipid interactions will have an effect on the fluidity of the membrane (Melchior, 1982; Shinitzky, 1984; Sikkema *et al.*, 1995). This fluidity usually is taken as a measure to describe the statues of a membrane. However, the concept of membrane fluidity is considered as an unfortunate one by some authors (Rilfors *et al.*, 1984) and in this review we will therefore also pay attention to the effects of solvents on the stability of lipid bilayers in biological membranes. Such effects of solvents on the transition of a bilayer to non-bilayer structures have, however, been studied less frequently.

4.1. Effect of alkanols on the bilayer functioning

4.1.1. Bilayer fluidity

Numerous studies have shown that interactions of alkanols with the lipid bilayer affects the fluidity of the membrane (see Shinitzky, 1984; Lohner, 1991; Sikkema *et al.*, 1995 for reviews). For a detailed description concerning the effects of alkanols on the bilayer fluidity these reviews should be consulted. As a specific example to illustrate the effects of alkanols, results obtained by Eliasz *et al.* (1976) are considered here. These authors determined the effect of several *n*-alkanols on the lamellar-gel (L_β) to the liquid-crystalline (L_α) transition temperature (T_M) of DPPC liposomes. As shown in Fig. 3, they observed that decanol had no effect on the T_M. Accumulation of *n*-alkanols with a chain length of >10 carbon atoms decreased the membrane fluidity, whereas those with a chain length <10 carbon atoms had a fluidizing effect on DPPC liposomes (Fig. 3). These *n*-alkanols will accumulate in the membrane with their hydroxyl moiety near the headgroup area and their hydrocarbon chain aligned in between the phospholipid acyl chains (Lohner, 1991). The effect of these alkanols on the T_M are therefore comparable to changes in the acyl-chain length of the phospholipids.

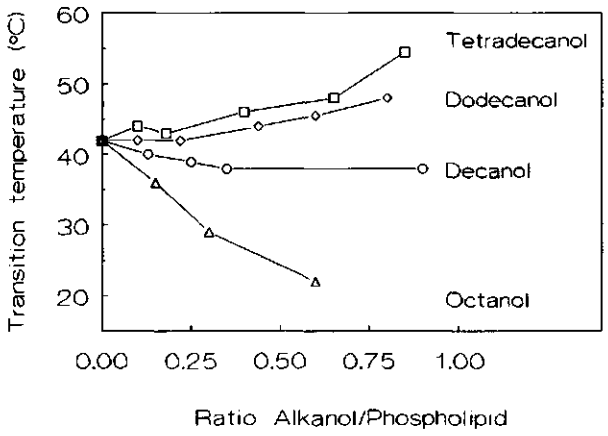

Figure 3: Effect of *n*-alkanols on the lamellar-gel to liquid-crystalline phase transition temperature of dipalmitoyl phosphatidylcholine liposomes. Alkanol concentrations are shown as the overall mole ratio of alkanol to phospholipid. (From Eliasz *et al.*, 1976).

Table 2: Membrane concentration of alkanol which causes a constant shift in transition temperature in DPPC lipid bilayers (75 mM). Membrane concentrations calculated from the experimental data of Jain and Wu (1977) using the membrane-buffer partition coefficient, calculated as described by Osborn *et al.* (1990).

	HHW'_{100} (1) (total amount of alkanol (mM) added)	Log P_{MB} (2)	Membrane concentration (mM)
Methanol	1400	-1.50	43
Ethanol	483	-0.98	46
Propanol	150	-0.47	38
Butanol	33.4	0.05	18
Pentanol	11.5	0.57	9.1
Hexanol	2	1.09	1.8
Heptanol	1.4	1.61	1.4
Octanol	0.73	2.13	0.72
Nonanol	2.1	2.65	2.1
Decanol	2.1	3.17	2.1

(1) Concentration at which the half-height width of the obtained DSC-profile is shifted 100% along the temperature axis. (Jain & Wu, 1977)

(2) Partition coefficient of a solute in a membrane/aqueous buffer system. Calculated as described by Osborn *et al.* (1990).

Incorporation of rigid phospholipids or rigid long-chain alkanols will increase the T_M, whereas incorporation of less rigid phospholipids or short-chain alkanols will have an opposite effect.

As seen before (Table 1) the tendency of short-chain alkanols to accumulate in a biological membrane is not very high. Therefore, quite high concentrations of these alcohols have to be used to detect an effect on the membrane fluidity. Jain and Wu (1977) determined the overall alkanol concentration which was required to cause a decrease in the transition temperature by a certain magnitude. With the aid of the membrane partition coefficient (Osborne *et al.*, 1990) it is possible to calculate the actual membrane concentration of these alkanols. As can been seen in Table 2 much higher concentrations of the short-chain alkanols are required to affect the membrane fluidity than the long-chain alkanols. This can be explained by considering that alkanols partition in the bilayer with their hydroxyl moiety near the phospholipid polar headgroup. Short-chain alkanols will thus not interfere strongly with the lipid acyl chains and will therefore have a less pronounced effect on the lipid fluidity than the longer chain alkanols.

4.1.2. Bilayer stability

From theoretical calculations by Israelachvili *et al.* (1976, 1977) and others (Tanford, 1973; Mitchell *et al.*, 1983) it can be concluded that the effective geometry of lipid molecules determines the packing of the lipids into a certain configuration (e.g. lamellar or non-lamellar). From their theoretical model a simplified concept emerged, which visualized each phospholipid as a building block with a phospholipid headgroup water interfacial area '*a*', hydrocarbon chain length '*l*', and a hydrocarbon chain volume '*v*'. Lipids were simply classed as cones, cylinders or inverted cones depending on the relative packing requirements of the two regions in the phospholipid: the cross-sectional area of the hydrocarbon portion (*v/l*) compared to the optimal surface area required by the polar headgroup (*a*). Depending on these parameters three phospholipid configurations can be distinguished (Fig. 4). For instance, an unsaturated phosphatidylethanolamine has a relative small headgroup area (*a*) compared to the hydrocarbon area (*v/l*) resulting in a packing geometry >1. This lipid can be visualized as a cone and will prefer to form an inverted hexagonal (H_{II}) lipid structure (Fig. 4).

In biological membranes the situation obviously is by far more complex, because they consist of a mixture of various phospholipids and proteins. Other factors, like pH, temperature, presence of divalent cations (e.g. Ca^{2+}) or the ionic strength of the environment will also affect the packing of the phospholipids into a certain configuration (Cullis & de Kruijff, 1979; Rilfors *et al.*, 1984; Gennis, 1989; Seddon,

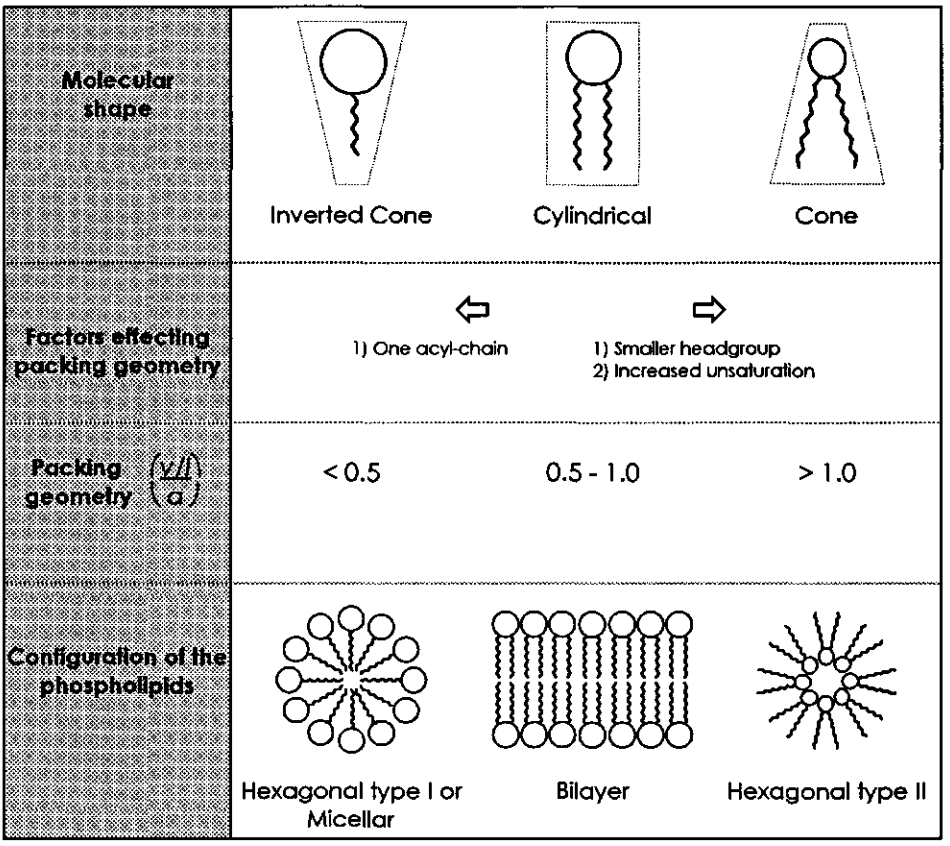

Figure 4: Molecular shape of various phospholipids and their corresponding polymorphic lipid configuration. (Adapted from Cullis & de Kruijff, 1979)

1990). Calcium ions, for instance, diminish the electrostatic repulsion between the negatively charged polar head-group of DPG and will reduce the headgroup area '*a*'. Thus calcium ions can induce the formation of a hexagonal H_{II} lipid phases of DPG model membranes (Mandersloot *et al.*, 1980).

As far as the effects of *n*-alkanols are concerned, studies have been done with artificial membranes. Egg phosphatidylethanolamine showed a transition from a lamellar to inverted hexagonal (H_{II}) lipid phase when the temperature was raised up to 30°C (Hornby & Cullis, 1981). The temperature at which this phase transition occurs is termed T_{LH}. The effect of alkanols on the T_{LH} of egg PE phospholipids has been studied by Hornby and Cullis (1981). They found that the partitioning of long-chain alkanols ($C \geq 6$) into lipid bilayers decreased the T_{LH}. In other words, the bilayer structure was made less stable by these alkanols. This observation may be explained by considering that these alkanols interact with both the headgroup area and lipid acyl chain. Although this accumulation will increase the

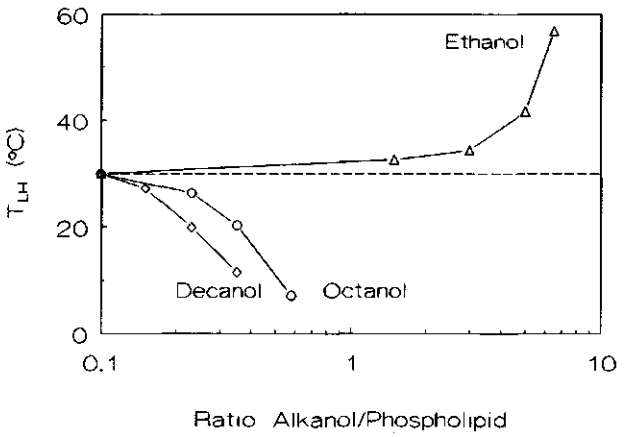

Figure 5: Influence of increasing amounts of *n*-alkanols on the lamellar to inverted hexagonal (H_{II}) phase transition temperature (T_{LH}) of egg phosphatidylethanolamine. T_{LH} is estimated as the temperature where 50% of the lipids is in the bilayer organisation and 50% is in a hexagonal structure (From Hornby & Cullis, 1981).

headgroup area '*a*', they particularly have a pronounced effect on the lipid volume '*v*', thus resulting in an increase of $(v/l)/a$ and hence promote the formation of the hexagonal type II lipid phase.

Ethanol, which partitions in membranes especially in the headgroup area of the phospholipids, will increase the headgroup area '*a*'. According to the lipid packing theory an increase in headgroup area '*a*' will oppose the formation of an inverted hexagonal (H_{II}) lipid phase. This is indeed observed, addition of ethanol to egg PE liposomes resulted in an increase of the lamellar-hexagonal transition temperature (T_{LH}) (Fig. 5). Short-chain alkanols consequently have a positive effect on the stability of bilayers, unless they would be able to transform a phospholipid bilayer into a normal hexagonal (H_I) or micellar lipid structure. However, this H_I phase is not normally observed for diacyl phospholipids (Seddon, 1990). Only lyso-phospholipids, containing only one acyl-chain, will form this structure. Also very short-chain diacyl phospholipids (six to eight carbons per fatty acid acyl chain) might form a normal hexagonal (H_I) structure (Lin *et al.*, 1987). The induction of a H_I phase by methanol or ethanol has not been observed, only tetracaine-hydrochloride has been reported to be capable of breaking up the lamellar phospholipid structure of PC into micelles (Fernández & Calderón, 1980).

This effect has been contributed to the large charged groups of tetracaine which will increase the phospholipid head-group area '*a*'.

Although phospholipids within biological membranes are organized in a bilayer, almost every biological membrane contains substantial amounts of lipids which have a strong preference for the inverted hexagonal phase (H_{II}) (Cullis & de Kruijff, 1979). In both *A. laidlawii* (Wieslander *et al.*, 1980) and *Clostridium butyricum* (Goldfine *et al.*, 1987) a proper balance between bilayer and non-bilayer forming lipids is maintained by the microorganisms in response to changes in environmental conditions. Also in *E. coli* the phase preference of the membrane lipids is regulated by adjustments in the ratio of bilayer to non-bilayer lipids (Rietveld *et al.*, 1993). Rietveld *et al.* (1993) used a mutant *E. coli* strain which lacks the ability to synthesize PE, a non-bilayer forming lipid which normally accounts for 70-80% of the *E. coli* phospholipids. In this mutant strain the absence of PE is compensated by a large increase in the levels of PG and DPG (DeChavigny *et al.*, 1991; Rietveld *et al.*, 1993). This strain also has an absolute requirement for high concentrations of divalent cations (DeChavigny *et al.*, 1991). Since divalent cations are known to induce H_{II} formation in DPG model systems, this suggests that DPG in combination with divalent cations can replace PE in membranes (DeChavigny *et al.*, 1991). The mutant strain did not grow in the absence of divalent cations. As both PG and DPG will normally form a stable bilayer these results strongly indicate that the membrane should contain phospholipids which prefer to form an inverted hexagonal (H_{II}) structure. In all experiments, a bilayer to non-bilayer transition was observed at approximately 10°C above the growth temperature (Rietveld *et al.*, 1993). By maintaining this tendency of the membrane to non-bilayer structures, it is possible that locally conditions exists which can result in the formation of non-bilayer structures. This is probably important in functional processes such as membrane fusion, cell division, and transbilayer movement of lipids and proteins (de Kruijff *et al.*, 1985; Lindblom & Rilfors, 1989; Seddon, 1990). Although short-chain alkanols like ethanol will stabilize the bilayer structure, this could possibly be unfavourable for cell-growth, as microorganisms apparently need to maintain the balance between bilayer and non-bilayer forming phospholipids in such a way that a tendency towards an inverted hexagonal (H_{II}) structure is maintained.

When comparing the effects of alkanols on both T_M and T_{LH} it is interesting to note that lower alcohols ($C \leq 3$) decrease the T_M (more fluid membrane) and increase T_{LH}, while long-chain alkanols ($C > 10$) result in more rigid (increased T_M) but less stable bilayers (decreased T_{LH}). The alkanols with a chain length between C4 and

C10 increase the membrane fluidity and destabilize the bilayer structure (decreased T_M and T_{LH}).

The interaction of short-chain alkanols with the membrane apparently stabilizes the bilayer structure since the T_{LH} increases. However, it has been demonstrated that these short-chain alkanols (C ≤ 3) can promote the formation of an unusual phospholipid configuration, the interdigitated phase ($L_\beta I$) (Simon & McIntosh, 1984; Slater & Huang, 1988; Vierl *et al.*, 1994). In the interdigitated configuration the lipid acyl-chains from the opposing monolayers are fully interpenetrated, thereby exposing the terminal methyl groups. (Fig. 6). These short-chain alkanols (C ≤ 3) will anchor with their polar moiety to the phospholipid headgroup, and with the non-polar part between the phospholipid acyl-chains. Since the non-polar moiety of these molecules is short compared to the fatty acid acyl-chains, these molecules would potentially cause voids between the lipid chains in the bilayer interior. As the energy of formation of holes between hydrocarbons is extremely large, the lipids respond by forming the interdigitated phase (Simon & McIntosh, 1984).

Although the interdigitated gel phase has been observed in various phospholipids, this lipid structure is not frequently observed in PE systems (Lohner, 1991). As PE is the major phospholipid in many microorganisms, the biological significance of the interdigitated gel phase is unclear. PE has, compared to other phospholipids, a small headgroup area. It is expected that due to this small headgroup, the interaction of the small alkanols (C ≤ 3) with the membrane will not cause the formation of an interdigitated phase. Mason and Stephenson (1990) studied the thermotropic properties of various mixed acyl PEs. They used PEs in which the chain-length of one fatty acid was constant (C18) and the other was varied between C10 and C18. Only with the C18-C10-PE a mixed

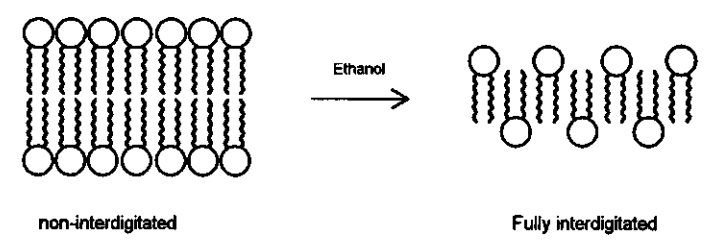

Figure 6: Schematic drawing, showing the ethanol induced conversion of DPPC bilayers from the non-interdigitated gel phase (L_β) to the fully interdigitated gel phase ($L_\beta I$).

interdigitated gel phase packing of the phospholipids was observed (Mason & Stephenson, 1990). In DMPE vesicles high concentrations of 1-propanol resulted in a phase, which had characteristics of the interdigitated gel phase, however, no direct evidence that a true interdigitated gel phase had formed is available (Centeno & O'Leary, 1990).

Besides short-chain alkanols (C ≤ 3) also several other molecules, such as benzyl alcohol, can cause the lipid hydrocarbon chains to interpenetrate and induce the formation of an interdigitated gel phase (McIntosh *et al.*, 1983). All these compounds interact with the headgroup area of the phospholipids and do not partition deeply in the lipid bilayer. Although the interdigitated lipid phase can be induced by various additives it should be noted that this lipid configuration is only observed for bilayers in the gel-phase (Simon & McIntosh, 1984; Slater & Huang, 1988; Vierl *et al.*, 1994). In microorganisms, however, most of the phospholipids are generally in the liquid-crystalline phase. It is therefore unclear if this interdigitated phase will occur or can be induced by solvents in microorganisms.

4.2. Effects of alkanes and aromatic hydrocarbons on bilayer fluidity and stability

In general *n*-alkanes have a similar effect on the membrane fluidity as the corresponding *n*-alkanols. Long-chain alkanes (C ≥ 12) increased the T_M of various phospholipids, and short-chain alkanes (C < 12) decreased the T_M (Lohner, 1991). The alignment of the rigid, long-chain alkanes parallel to the acyl-chains of the phospholipids, will have a rigidifying effect on the membrane similar to the long-chain alkanols. The lower alkanes increase the membrane fluidity, as they will disturb the Van der Waals interaction between the phospholipid acyl chains.

n-Alkanes accumulate between the acyl-chains of the phospholipids, near the centre of the bilayer. The accumulation of these alkanes can be visualized as a wedge which is placed in between the phospholipid acyl-chains and will thus destabilize the bilayer structure and promote the formation of a reversed hexagonal (H_{II}) lipid structure. Indeed incorporation of C6-C20 *n*-alkanes promoted the formation of such a non-lamellar structure (Seddon, 1990; Lohner, 1991). In general longer alkanes are more effective in reducing the L_α - H_{II} transition temperature. The C20 saturated alkane eicosane for instance lowers the T_{LH} transition by 15°C at a concentration of about 3 mole %. In contrast, the gel to liquid-crystalline transition temperature (T_M) is not significantly altered in the presence of 3% eicosane (Espand, 1985).

Compared to *n*-alkanols, the corresponding *n*-alkanes were more potent in destabilizing the bilayer structure. As the alkanols interact with the phospholipid headgroup area, they will not only increase the tail area 'v/l' but also the

headgroup area 'a' and are thus less effective in promoting an inverted hexagonal lipid phase than the corresponding alkanes (Hornby & Cullis, 1981).

The effects of aromatic hydrocarbons on the membrane fluidity and stability have received only limited attention. In an extensive study Jain and Wu (1977) showed that organic solvents like benzene, toluene and chloroform all reduced the lamellar-gel to liquid-crystalline phase transition temperature (T_M) of DPPC liposomes. As these solvents are expected to partition in between the lipid acyl-chains of the phospholipids, they will be able to induce the transformation of a lamellar lipid configuration into a hexagonal (H_{II}) configuration. The formation of an H_{II} phospholipid configuration due to benzene addition has been shown in DPPC liposomes, at a low water content (McDaniel *et al.*, 1982).

5. EFFECT OF SOLUTE PARTITIONING ON MEMBRANE FUNCTIONS

5.1. Membrane permeability

The main function of the cell membrane is a permeability barrier, regulating the passage of solutes between the cell and the external environment. The barrier properties of the cytoplasmic membrane is of special importance for the energy transduction of the cell (Nicholls, 1982). An increase in the permeability of the membrane for protons or other ions may lead to a dissipation of the proton motive force (Δp), resulting in a less effective energy transduction. Furthermore, an increased permeability of the cell might also affect the internal pH control of the cell (Sikkema *et al.*, 1995) or could result in the loss of essential metabolites.
As seen in the previous paragraphs, the accumulation of various solvents has an effect on the membrane fluidity. Studies with both liposomes and intact cell have shown repeatedly that the permeability is affected by the membrane fluidity. An increase in the fluidity due to the incorporation of more unsaturated fatty acids or lipids with a reduced chain length, resulted in an increased permeability of the membrane (de Gier *et al.*, 1968; McElhaney *et al.*, 1970, 1973; Cullen *et al.*, 1971; Romijn *et al.*, 1972; Melchior, 1982). An increase in the permeability has also been observed in various membranes, upon accumulation of membrane-fluidizing solvents like ethanol, narcotics, antibiotics, and aromatics (Bangham *et al.*, 1965; Silver & Wendt, 1967; Johnson & Bangham, 1969; Saha *et al.*, 1970; Cartwright *et al.*, 1986; Heipieper *et al.*, 1991; Szogyi *et al.*, 1993; Sikkema *et al.*, 1994a).

The effect of alkanols on the membrane permeability can be illustrated by the results obtained, already 30 years ago, by Bangham and colleagues. They determined the $^{42}K^+$-permeability of liposomes and showed that alkanols

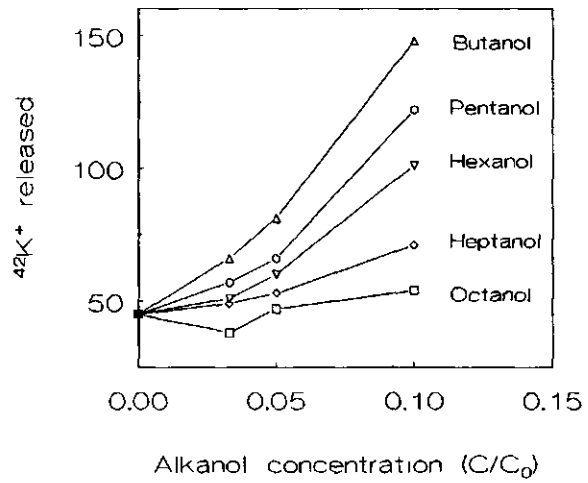

Figure 7: Leakage rate of $^{42}K^+$ from phosphatidylcholine/phosphatic acid (85:15) vesicles in the presence of various concentrations of alkanols. (Concentration is expressed as the fraction (C/C_0) of an alkanol saturated KCl solution added). Adapted from Bangham *et al.*, (1965).

significantly increased the K^+ efflux. (Bangham *et al.*, 1965) (Fig. 7). Recently, it has been shown that the toxicity of cyclic aromatic hydrocarbons for microorganisms is caused by an increase in the membrane permeability induced by these hydrocarbons. Accumulation of various cyclic hydrocarbons in the lipid bilayer enhanced the proton permeability of whole cells (Sikkema *et al.*, 1992) and of liposomes derived from *E. coli* phospholipids (Sikkema *et al.*, 1994a). As a result of this increase in proton permeability the proton-motive force of the cell was significantly decreased and in some instances completely dissipated. For all hydrocarbons tested a maximum increase in the permeability of the membrane was observed at a membrane concentration of approximately 1 solvent molecule per 2 phospholipid molecules (Sikkema *et al.*, 1994a).

As pointed out before, solvents have an effect on both the fluidity and the bilayer stability. The accumulation of a solute in the membrane can eventually result in the formation of a non-bilayer phospholipid structure or an interdigitated phase. The formation of such structures will of course strongly affect the barrier properties of the membrane. It is, however, unclear if an effect on the membrane permeability will be observed when the packing geometry of the phospholipids is only slightly changed and no non-bilayer structures are formed. Accumulation of a solute in a phospholipid monolayer might result in the spontaneous curvation

(positively or negatively) of the lipid monolayer, due to an imbalance of the forces across the layer (Seddon, 1990). In a symmetric bilayer, however, the two phospholipid monolayers want to curve the same way, and so counteract each other since they are oppositely oriented back-to-back (Gruner *et al.*, 1985; Seddon, 1990). This "state of physical frustration" in the bilayer may modify many properties of the bilayer such as thickness and permeability (Seddon, 1990). Gangliosides are normal components of the plasma membrane of vertebrate cells. These lipids have a large headgroup area (140 $Å^2$) compared to other phospholipids (e.g. egg PC: 62 $Å^2$) and will therefore form a normal hexagonal (H_I) lipid configuration (Jain, 1988). Incorporation of various gangliosides in membrane vesicles will decrease the fluidity of the membrane (Bertoli *et al.*, 1981), which is expected to result in a reduced permeability of the membrane. However, an increase in the membrane permeability is observed upon incorporation of gangliosides (Sarti *et al.*, 1990). It is likely that the "state of physical frustration" in the bilayer, caused by the large headgroup of the ganglioside will have caused this increased permeability.

The interaction of very short-chain alkanols ($C \leq 3$) with the membrane is rather complex. These alkanols can transform the normal bilayer structure in an interdigitated phospholipid configuration ($L_{\beta I}$). In this configuration the lipid acyl-chains are exposed to the phospholipid-aqueous interface (Fig. 6). As polar headgroups are important for the barrier properties of the membrane bilayer, it is expected that the formation of such lipid configurations will result in an increased permeability of the membrane. At 20°C DPPC is in the non-interdigitated gel phase at ethanol concentrations below 1.0 M (Zeng *et al.*, 1993). A dramatic increase in the proton permeability is observed at 1.2 M ethanol at 20°C and 2.0 M at 14°C. This abrupt increase in permeability occurs at almost the same ethanol concentration which causes the formation of the fully interdigitated gel phase at both temperatures (Zeng *et al.*, 1993).

5.2. Effect on membrane-embedded enzymes

The membrane is besides a barrier also a matrix for various important enzymes. These include enzymes involved in solute transport, and enzymes participating in the electron transport chains. Many studies have shown that the activity of membrane-embedded enzymes can be influenced by the physico-chemical properties of the membrane (Yeagle, 1989; In 't Veld *et al.*, 1991). The interaction of a solvent with the membrane will influence these membrane properties, and therefore will affect the activity of membrane-embedded enzymes.

In *E. coli* both the K_m and *V* of the *B*-galactoside transport system increased in the presence of *n*-alkanols (Sullivan *et al.*, 1974). A reduction of the enzyme activity by the accumulation of various cyclic hydrocarbons in the membrane was observed for cytochrome c oxidase reconstituted in liposomes (Sikkema *et al.*, 1994a). Similarly, benzyl alcohol affected the activity of various intrinsic membrane enzymes of the rat liver plasma membrane (Gordon *et al.*, 1980).
A systematic examination of how various regions of the bilayer affect the catalytic activity of sugar transport proteins has been carried out by Carruthers and Melchior (1986). Changes in the lipid composition can drastically alter the transporter's activity. However, these authors concluded that the bilayer fluidity is of minimal importance for the activity of this enzyme. Only the transition of the membrane from the gel to the liquid-crystalline phase had a limited influence on the activity in certain bilayers. Especially bilayer thickness and the type of phospholipid headgroup had an influence on the enzyme activity (Carruthers & Melchior, 1986). In 't Veld *et al* (1991) postulated that the activity of transmembrane carrier proteins is strongly affected by the degree of matching between the bilayer thickness and the hydrophobic thickness of the protein. Interaction of *n*-alkanes with membranes have been shown to increase the bilayer thickness (McIntosh *et al.*, 1980; Pope *et al.*, 1984) and will consequently affect the activity of transmembrane carrier proteins.
McElhany (1989) reviewed the influence of the membrane lipid composition on the activity of membrane-bound enzymes in *Acholeplasma laidlawii* B. NADH oxidase and *p*-nitrophylphosphatase were both unaffected by the fluidity and phase state of the membrane. For the $(Na^+ + Mg^{2+})$-ATPase the activity varied with the fatty acid composition, however, above the phospholipid phase transition temperature the ATPase activities were rather independent of the fatty acid composition. This indicates that alterations in the fatty acid composition, and thus membrane fluidity had only a small effect on the enzyme activity (McElhaney, 1989). The activity of the enzyme was, however, strongly influenced by the headgroup composition. Reconstitution of the enzyme in DMPC vesicles resulted in a fully active enzyme, but in DMPC liposomes it was fully inactive (McElhaney, 1989). The activity of the Ca^{2+}-ATPase of rabbit muscle is also strongly affected by the phospholipid headgroup composition. An increase in the activity was observed when the enzyme was reconstituted in membranes with either a high PE or MGDG content (Navarro *et al.*, 1984; Yeagle, 1989). A property that PE and MGDG have in common is the ability to form the inverted hexagonal (H_{II}) phase. Cholesterol, which can induce the H_{II} phase, also stimulated the activity of the Ca^{2+}-ATPase reconstituted in vesicles dominant in PE (Cheng *et al.*, 1986). Although the reason for these variations in enzyme activity have not yet been

discovered, it is clear that many factors, including the phase preference of the phospholipids, have an influence on the enzyme activity.
Several membrane-embedded enzymes depend for their activity on a specific boundary phospholipid (Sandermann, 1978; Carruthers & Melchior, 1986). It is expected that these phospholipids are necessary for the correct conformation and orientation of the enzyme. Displacement of these boundary lipids by the interaction of organic solvents with the membrane might result in a lipid/protein mismatch and inactivation of the enzyme (Sandermann, 1993). Furthermore, direct interactions of the solvent with hydrophobic parts of the enzyme might also occur, which are expected to affect the enzymatic activity.

6. ADAPTATION

As has been shown in the previous paragraphs the accumulation of solvents in the membrane bilayer can influence both the membrane fluidity and the bilayer stability. These changes in the physico-chemical properties of the membrane can affect the permeability of the membrane as well as the activity of membrane-embedded enzymes. Consequently, the viability of the cell will be affected. Although there are many examples of the toxicity of organic solvents for microorganisms, some microorganisms are able to adapt and grow in the presence of toxic concentrations of these solvents. Spectacular examples are *Lactobacillus heterohiochii* which is able to grow in the presence of up to 20% ethanol (Uchida, 1975a) and the adaptation of several *Pseudomonas* strains allowing them to grow in the presence of a second phase of toluene, *p*-xylene or styrene (Inoue & Horikoshi, 1989; Cruden *et al.*, 1992; Weber *et al.*, 1993)

6.1. Adaptations at the level of the membrane

Several adaptive changes in the membrane composition have been observed in reaction to the accumulation of organic solvents in the membrane of microorganisms (Table 3). These changes will, as discussed below, counteract the effects induced by the accumulation of a solute. These modifications of the membrane composition can be explained either as a mechanism to restore the fluidity of the membrane (homeoviscous adaptation) (Sinensky, 1974) or as a method to restore the balance between bilayer and non-bilayer forming phospholipids. Changes in the membrane composition can also reduce the solute partitioning into the membrane, resulting in lower concentrations in the membrane, thus also reducing the toxicity (Antunes-Madeira & Madeira, 1985, 1989).

Table 3: Adaptive changes observed in various microorganisms in response to the presence of organic solvents.

Organism	Solvent	Effect on:			Reference
		Fatty acid composition	Headgroup composition	Others	
Acholeplasma laidlawii	Ethanol	-	MGDG:DGDG ↑		Wieslander *et al.*, 1986
	C6-C12 alkanols	-	MGDG:DGDG ↓		Wieslander *et al.*, 1986
	Benzene	-	MGDG:DGDG ↓		Wieslander *et al.*, 1986
	Toluene	Unsat:Sat ↑	PG↑	Cholesterol ↑; Carotenoids	Govorun *et al.*, 1989, 1993
Bacillus cereus	Ethanol, Propanol	Branched fatty acids ↑	-		Kates *et al.*, 1962
Bacillus subtilis	Ethanol	Branched fatty acids ↓			Rigomier *et al.*, 1980
Clostridium acetobutylicum	C2-C8 alkanols	Unsat:Sat↓			Vollherbst-Schneck *et al.*, 1984; Baer *et al.*, 1987; Lepage *et al.*, 1987
Clostridium thermocellum	Ethanol	Unsat:Sat ↑, Chain length ↓, Anteiso ↑			Herrero *et al.*, 1982
Escherichia coli	Ethanol	Unsat:Sat ↑, Chain length ↑	PE↓, DPG↑, PG↑	Lipid:Protein ↓	Ingram, 1976; Sullivan *et al.*, 1979; Dombek & Ingram, 1984
	C6-C10 Alkanols	Unsat:Sat ↓			Ingram, 1976; Sullivan *et al.*, 1979
	Phenol	Unsat:Sat ↓		Lipid:Protein ↓	Keweloh *et al.*, 1990; Helpieper *et al.*, 1991
Lactobacillus heterohiochii	Ethanol	Unsat:Sat ↑, ± 20% > C20			Uchida, 1975a
Lactobacillus homohiochii	C1-C7 Alkanol	Unsat:Sat ↑			Uchida, 1975b
Microsporum sp.	Ethanol	Unsat:Sat ↓	PE↑, PS↓		Bansal & Khuller, 1981

Table 3: Adaptive changes observed in various microorganisms in response to the presence of organic solvents (continued).

Organism	Solvent	Effect on:			Reference
		Fatty acid composition	Headgroup composition	Others	
Mycobacterium smegmatis 27°C	Ethanol	Unsat:Sat ↑	-	Total Phospholipid ↓	Taneja & Khuller, 1980
37°C	Ethanol	Unsat:Sat ↓	PE↓, PI↑	Total Phospholipid ↓	Taneja & Khuller, 1980
Pseudomonas putida	Ethanol	Unsat:Sat ↑, *Trans*:*Cis* ↑	PE↓, DPG↑, PG↑		Heipieper & de Bont, 1994
	Phenol	Unsat:Sat ↓, *Trans*:*Cis* ↑			Heipieper *et al.*, 1992
	Toluene	*Trans*:*Cis*↑	PE↓, DPG↑, PG↑	LPS-modifications	Weber *et al.*, 1994
Rhizobium sp.	2,4-dichlorophenoxy-acetic acid	Unsat:Sat ↓			Fabra de Peretti *et al.*, 1992
Saccharomyces cerevisiae	Ethanol	Unsat:Sat ↑; Chain length ↑		Ergosterol ↑ ; ATPase activity ↑; Heat-shock proteins	Beaven *et al.*, 1982; Mishra & Prasad, 1989; Watson & Cavichioli, 1983; Alexandre *et al.*, 1993
Sarcina ventriculi	Ethanol, butanol	Long chain fatty acids ↑			Jung *et al.*, 1993
Schizosaccharomyces pombe					
Anaerobic	Ethanol	Unsat:Sat ↓	PI ↑, PC ↓		Koukou *et al.*, 1990
Aerobic	Ethanol	Unsat:Sat ↑	PI ↑, PC ↓		Koukou *et al.*, 1990
Zymomonas mobilis	Ethanol	Unchanged; >70% 18:1 *cis*	DPG↑, PE↓	Lipid:Protein ↓; Hopanoids	Ohta *et al.*, 1981; Carey & Ingram, 1983; Barrow *et al.*, 1983; Buchholz *et al.*, 1987

6.1.1. *Lipid composition*

6.1.1.a. Adaptation to alkanols

The changes observed in lipid composition of microorganisms due to the presence of alkanols and especially ethanol, have been reviewed before (Casey & Ingledew, 1983; Ingram & Buttke, 1984; Ingram, 1986, 1990; Mishra & Kaur, 1991). One of the changes in membrane lipid composition in response to ethanol is an increase in the amount of unsaturated fatty acids observed in *Lactobacillus heterohiochii* (Uchida, 1975a), *Lactobacillus homohiochii* (Uchida, 1975b), *Clostridium thermocellum* (Herrero *et al.*, 1982), *Escherichia coli* (Ingram, 1976), and *Saccharomyces cerevisiae* (Beaven *et al.*, 1982). However, an opposite effect, in response to ethanol, is observed in several other microorganisms. An increased content of less fluid lipids is observed in *Bacillus subtilis* (Rigomier *et al.*, 1980; Bohin & Lubochinsky, 1982), *Clostridium acetobutylicum* (Vollherbst-Schneck *et al.*, 1984; Baer *et al.*, 1987; Lepage *et al.*, 1987) and two *Microsporum* species (Bansal & Khuller, 1981). The high levels of unusual long-chain fatty acids found in *L. heterohiochii* are also expected to reduce the fluidity (Uchida, 1975a).

Apparently, two modes of membrane adaptation occur: (1) The increased incorporation of unsaturated fatty acids which will counteract the effects of ethanol on the bilayer structure. (2) The increased incorporation of less fluid lipids which will compensate the increase in membrane fluidity caused by ethanol (Homeoviscous adaptation). As will be discussed below either of the two mechanisms emerged.

Adaptations to maintain the bilayer stability. Accumulation of ethanol in lipid bilayers has a pronounced effect on the headgroup area of the membrane phospholipids and results in an increase in the apparent phospholipid headgroup area 'a'. As a result of this increase ethanol can (1) induce the formation of the interdigitated gel phase, and (2) will increase the T_{LH} of the membrane.

The formation of the interdigitated phase, resulting in an increased permeability of the membrane, is expected to affect the viability of the cell. The observed incorporation of unsaturated fatty acids in various microorganisms in response to ethanol, will increase the lipid volume 'v' and is therefore expected to prevent the formation of the interdigitated lipid phase. As pointed out earlier, most of the lipids in microorganisms will be in the liquid-crystalline phase. The interdigitated phase, however, is only observed in the gel phase. It is therefore doubtful if this phase will occur in biological membranes under normal conditions.

Ethanol, and other short-chain alkanols ($C \leq 3$) increase the T_{LH} and consequently have a positive effect on the stability of the bilayer, unless they would be able to induce the formation of a normal hexagonal (H_I) or micellar lipid structure.

However, the induction of this lipid configuration by ethanol has not been observed. Apparently, ethanol has a stabilizing effect on the bilayer configuration. Several studies have shown that microorganisms have to maintain a certain balance between bilayer and non-bilayer phospholipids in order to remain viable (Wieslander *et al.*, 1980; Goldfine *et al.*, 1987; Rietveld *et al.*, 1993). As discussed before, growth of an *E. coli* mutant, defective in the synthesis of PE, is only observed when the T_{LH} of the phospholipids is about 10°C above the growth temperature (Rietveld *et al.*, 1993). Ethanol increased the T_{LH}, and will thus disturb the bilayer to non-bilayer phospholipid ratio, which is expected to affect the cell viability. The observed increase in unsaturated fatty acids in various microorganisms is expected to compensate for the ethanol induced increase in T_{LH}, as unsaturated fatty acids decrease the T_{LH}.
In *Lactobacillus heterohiochii* and *Lactobacillus homohiochii,* the most ethanol resistant microorganisms known that are capable of growing in concentrations up to 20%, an increase in the amount of unsaturated fatty acids is observed in response to ethanol (Uchida, 1975a, 1975b). Similar changes have been observed in *Clostridium thermocellum* (Herrero *et al.*, 1982), *Escherichia coli* (Ingram, 1976), and *Saccharomyces cerevisiae* (Beaven *et al.*, 1982). The membranes of *Zymomonas mobiles,* an obligate fermentative bacterium producing high concentrations of ethanol, are particularly rich in 18:1 *cis*. This fatty acid accounts for more than 70% of the total fatty acids (Carey & Ingram, 1983; Barrow *et al.*, 1983; Buchholz *et al.*, 1987). Only in continuous cultures where a low glucose feed results in a low concentration of ethanol a reduced content of 18:1 *cis* was observed (Bringer *et al.*, 1985).

E. coli grown in the presence of short-chain alkanols ($C \leq 3$) showed an increased incorporation of unsaturated fatty acids. Long-chain alkanols (C5 - C9) caused the opposite effect: an increase in the amount of saturated fatty acids. Decanol (C10) did not induce changes in the fatty acid composition (Ingram, 1976; Sullivan *et al.*, 1979). A very good agreement exists between the observed adaptive changes in the membrane composition and the effects of these alkanols on the T_{LH}. The short-chain alkanols ($C \leq 3$) will increase the headgroup area of the phospholipids in the membrane. The observed increase in the amount of unsaturated fatty acids will decrease '*l*' and increase '*v*' and compensate the increase in '*a*' caused by these alkanols. The opposite is observed with the long-chain alkanols (C5 - C9). Accumulation of long-chain alkanols promotes the formation of an inverted hexagonal (H_{II}) phase (increase in '*v*'). The higher amount of saturated fatty acids found (decrease in '*v*', increase in '*l*') compensates this and stabilizes the bilayer structure. Decanol will, just as the other long-chain alkanols, increase the lipid-volume '*v*'. However, no changes in the lipid

composition are observed in response to decanol (Ingram, 1976; Sullivan *et al.*, 1979). These changes were expected when the adaptation mechanisms was directed at restoring the balance between bilayer and non-bilayer forming phospholipids.

Experiments with mutants of *E. coli*, defective in the synthesis of 18:1 *cis*, have shown that an increase in 18:1 *cis* content is beneficial for ethanol tolerance. These mutants, lacking 18:1 *cis*, are hypersensitive to killing by ethanol (Ingram *et al.*, 1980). Supplementation of these mutants with 18:1 *cis* rendered these cells less sensitive to ethanol-killing (Ingram *et al.*, 1980; Ingram, 1990). The incorporation of 18:1 *cis* increases the fluidity of the membrane which apparently is beneficial to acquire ethanol resistance as it counteracts the increase in T_{LH} due to ethanol accumulation in the membrane. These results suggest that control of membrane fluidity is not important for cells as a defence mechanism against toxic concentrations of alkanols. Also for *A. laidlawii*, Wieslander and co-workers concluded from extensive studies that this microorganism only changes its membrane composition to maintain a certain balance between bilayer and non-bilayer promoting phospholipids (Rilfors *et al.*, 1984; Wieslander *et al.*, 1986). The observed changes in membrane compositions in response to hydrocarbons, alkanols and detergents could not be explained as a mechanism to maintain a constant membrane fluidity (Wieslander *et al.*, 1986). Furthermore the capacity of various microorganisms to grow at temperatures that are well above the gel to liquid crystalline phase transition temperature (T_M) of the membrane lipids, would also indicate that regulation of the membrane fluidity is less important for cell viability (Steim *et al.*, 1969; McElhaney, 1974). Similar conclusions might be drawn from work of Jackson and Cronan (1978) who studied the growth rate of *E. coli* in relation to the physical state of the membrane. The membrane lipids of wild-type *E. coli* were entirely fluid at both 25°C and 37°C. They showed that at least some part of the lipids must be in the fluid state for *E. coli* to grow, and that cells can tolerate large variations in the amount of fluid lipids without showing any major effects on vital processes (Jackson and Cronan, 1978).

Homeoviscous adaptation. Although the results presented in the previous paragraph indicate that a regulation of membrane fluidity is not important to acquire solvent tolerance, several other studies do show a regulation of membrane fluidity.

An interesting example in this respect is the reaction of *E. coli* to decanol. This alkanol has a strong effect on the packing of lipids in the membrane and can induce the formation of the H_{II} phase (Fig. 5). But *E. coli* does not change its lipid composition in response to decanol (Ingram, 1976; Sullivan *et al.*, 1979), which is in keeping with the homeoviscous adaptation theory, since decanol has no effect

on the membrane fluidity (Fig. 3). Ethanol in the same organism induces besides an increase in the amount of unsaturated fatty acids also an increase in chain length of the fatty acids. This increase in chain length reduces the membrane fluidity, while the increased unsaturation has the opposite effect. The net result of both effects apparently is a less fluid membrane. Further evidence that control of membrane fluidity is important comes from studies with *E. coli* mutants, defective in the synthesis of 18:1 *cis*. Supplementation of these mutants with *trans* fatty acids was more beneficial to acquire ethanol tolerance than supplementation of these mutants with *cis*-fatty acids (Ingram *et al.*, 1980; Ingram, 1990). *Trans* fatty acids have a higher T_M than the corresponding *cis* isomer and will thus reduce the membrane fluidity.

Further evidence that the regulation of membrane fluidity is important for cells to acquire ethanol-tolerance has been obtained in mutant-studies of *Clostridium thermocellum*. Accumulation of ethanol, similar as in *E. coli*, resulted in the synthesis of lipids with a reduced melting point (Herrero *et al.*, 1982). However, it was speculated that these changes are maladaptive, as in an ethanol-tolerant mutant strain the changes in fatty acid composition upon ethanol addition were not as dramatic as in the wild type (Herrero *et al.*, 1982).

Other information available on fatty acid composition as affected by alkanols in several other microorganisms also indicates that control of membrane fluidity might be important. In the extreme ethanol tolerant *L. heterohiochii* high levels of unusual long-chain fatty acids (C20-C24) have been found (Uchida, 1975a) which will reduce the membrane fluidity. Similarly, an ethanol-induced increase in the content of less fluid lipids was observed in *Bacillus subtilis* (Rigomier *et al.*, 1980; Bohin & Lubochinsky, 1982), *Clostridium acetobutylicum* (Vollherbst-Schneck *et al.*, 1984; Baer *et al.*, 1987; Lepage *et al.*, 1987) and two *Microsporum* species (Bansal & Khuller, 1981). Apparently in these microorganisms the membrane fluidity is compensated by this increase in the amount of rigid fatty acids. However, these membrane changes will not compensate for the effect which ethanol has on the T_{LH}.

In *Schizosaccharomyces pombe* and *Mycobacterium smegmatis* opposite adaptation mechanisms in response to ethanol were observed at various growth conditions. In aerobic-grown cells of *S. pombe*, similar to *E. coli*, an increase in the amount of unsaturated fatty acids was observed in response to ethanol (Koukou *et al.*, 1990). However, in anaerobically-grown cells an opposite effect is observed. In these cells an increase in saturated fatty acids was found (Koukou *et al.*, 1990). In *M. smegmatis* ethanol induced opposite effects on the saturation index of cells grown at either 27 and 37°C. At 37°C a decrease in the amount of saturated fatty acids was observed, whereas at 27°C the amount of saturated fatty acids increased (Taneja & Khuller, 1980).

6.1.1.b. Adaptation to other organic solvents

In contrast to the huge amount of literature concerning the adaptation mechanisms of microorganisms to alkanols, only a limited amount of information is available concerning the adaptation to other organic solvents. Ingram (1977) has studied the changes in lipid composition of *E. coli*, resulting from growth in the presence of several organic solvents. From this study it appears that *E. coli* dramatically alters its lipid composition in response to the presence of lipophilic compounds. Relatively polar solvents, like acetone, dimethyl sulfoxide, and tetrahydrofuran all caused an increase in the synthesis of lipids containing unsaturated fatty acids, analogous to the effect of short-chain alkanols. Incubation with more apolar solvents such as benzene, chloroform, or aniline caused an opposite effect, an increase in the amount of saturated fatty acids, similar as caused by the long-chain alkanols.

In certain *Pseudomonas* strains a fully different adaptation mechanism has been observed. A conversion of *cis* fatty acids into the corresponding *trans*-isomer was found in a *P. putida* strain in reaction to phenol and chlorophenols (Diefenbach *et al.*, 1992; Heipieper *et al.*, 1992). A similar adaptation has been observed in *P. putida* strains capable of growing in the presence of supersaturating concentrations of toluene (Weber *et al.*, 1994). Cells grown in the presence of 1% (v/v) toluene possessed a membrane with an increased amount of *trans*-unsaturated fatty acids, and a decreased amount of the *cis*-isomers (Weber *et al.*, 1994). This adaptation not only occurred at supersaturating concentrations but also at subsaturating concentrations (< 640 mg/l) of toluene in water. No effects were seen in *P. putida* S12 below 300 mg/l toluene, but above 300 mg/l the *trans*:*cis* ratio of the cells increased dramatically. Cells having a high *trans*:*cis* ratio were well equipped to survive shock additions of toluene (1% (v/v)). The benefit of this isomerization reaction lies in the steric differences between *cis*- and *trans*-unsaturated fatty acids. *Trans* fatty acids have a steric configuration similar to saturated fatty acids. The *cis*-isomer, however, has a nick in the acyl chain of the fatty acids, which causes steric hindrance and a membrane with a higher fluidity (Fig. 8).

The observed increase in the *trans*:*cis* ratio will thus have a rigidifying effect on the membrane. In the toluene-adapted strain, possessing a high *trans*:*cis* ratio, the transition temperature of the membrane *in vivo* was 7-9°C higher compared to the non-adapted cells (Weber *et al.*, 1994).

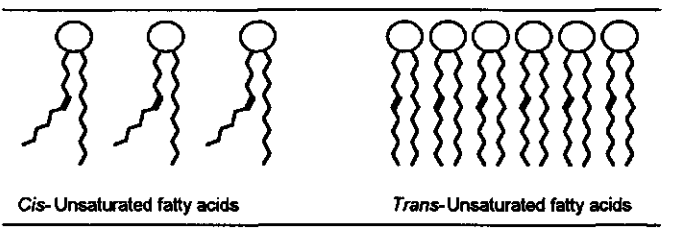

Figure 8: Configuration of *cis* and *trans* unsaturated fatty acids in a membrane

Toluene will, besides increasing the membrane fluidity, also promote the formation of an inverted hexagonal lipid phase. The observed conversion of *cis* unsaturated fatty acids into *trans* fatty acids in *P. putida* is expected to counteract the formation of a non-bilayer structure. This observation can be rationalized because the lipid volume 'v' of the *trans* fatty acids is smaller than the *cis* isomer. In studies with liposomes it was shown that 18:1 *trans* PE exhibits a lamellar to hexagonal (H_{II}) transition between 60°C and 63°C, whereas for 18:1 *cis* PE this transition occurs between 7 and 12°C (Tilcock & Cullis, 1982).
Interestingly, *P. putida* cells are still able to form *trans*-fatty acids when the fatty acid biosynthesis is inhibited by cerulenin (Diefenbach *et al.*, 1992; Heipieper *et al.*, 1992). This *cis:trans* isomerization is an adaptation mechanism which does not require *de novo* synthesis of lipids like the other adaptation mechanisms known (Diefenbach *et al.*, 1992) and provide the cell with a very quick mechanism to compensate for the increase in membrane fluidity and the risk of excessive formation of non-bilayer structures, caused by toluene.

In summary, it can be concluded that microorganisms change their lipid composition in the membrane in response to the accumulation of organic solvents. Apparently these changes are a mechanism to maintain a certain ratio between bilayer and non-bilayer phospholipids which prevent the formation of non-bilayer structures or are mechanisms to restore the fluidity of the membrane (homeoviscous adaptation). However, especially for ethanol, opposite adaptation mechanisms have been observed in various microorganisms. It is difficult to explain the adaptation of these various microorganisms on the basis of the observed changes in membrane composition only. As will be discussed hereafter other adaptive changes, which also have an effect on both the fluidity and the T_{LH}, have to be taken into account as well.

6.1.2. Headgroup composition

Besides the fatty acid composition, obviously the headgroups are also of major importance when considering the physico-chemical properties of lipid bilayers.

Considerably less data are, however, available on the headgroup composition than on fatty acid composition. Headgroups usually determined by TLC techniques include phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), diphosphatidylglycerol or cardiolipin (DPG), monogalactosyldiglyceride (MGDG) and digalactosyldiglyceride (DGDG) (Fig. 9).

Adaptive changes in the headgroup composition will have an effect on the physico-chemical properties of the membrane. The differences in T_M between phospholipids with different polar headgroups can be as great as 25-30°C (Boggs, 1984). At neutral pH, PG has the lowest transition temperature. Protonation increases the T_M of the acidic PG. Diphosphatidylglycerol has the highest T_M of all phospholipids at neutral pH (Boggs, 1984; Keough & Davis, 1984). The difference of 25-30°C in T_M of PG and DPG might be biologically important as a mechanism to compensate for changes in the membrane fluidity. However, extensive changes in the phospholipid composition should be necessary to control the fluidity, and such changes are not generally observed. Furthermore, changes in the fatty acid composition have a more pronounced effect on the membrane fluidity. For instance the introduction of a double bond in one fatty acid chain of a phospholipid reduces the T_M by 50-60°C (Keough & Davis, 1984).
Besides an effect on the membrane fluidity, adaptive changes in the headgroup composition will have a strong effect on the transition from a bilayer to a hexagonal phase (H_{II}) (Fig. 4). For instance, in *A. laidlawii* MGDG and DGDG occur as the two main phospholipid species. The ratio between these headgroups was affected by solvents. Accumulation of ethanol in the membrane resulted in an increase in the MGDG : DGDG phospholipid ratio. MGDG has a smaller lipid headgroup area than DGDG, and the changed ratio compensates for the increase in headgroup area caused by ethanol (Wieslander *et al.*, 1986). Benzene has an opposite effect on membranes and promotes the formation of a hexagonal phase, which was counteracted in *A. laidlawii* with a decrease in the MGDG : DGDG-ratio (Wieslander *et al.*, 1986).

We have been studying the adaptation mechanisms of *P. putida* S12, a Gram-negative bacterium which can grow in the presence of supersaturating amounts of toluene (Weber *et al.*, 1993). Apart from changes in fatty acid composition (Weber *et al.*, 1994) also changes in the phospholipid composition were observed when the organism was grown in the presence of toluene (Table 4).

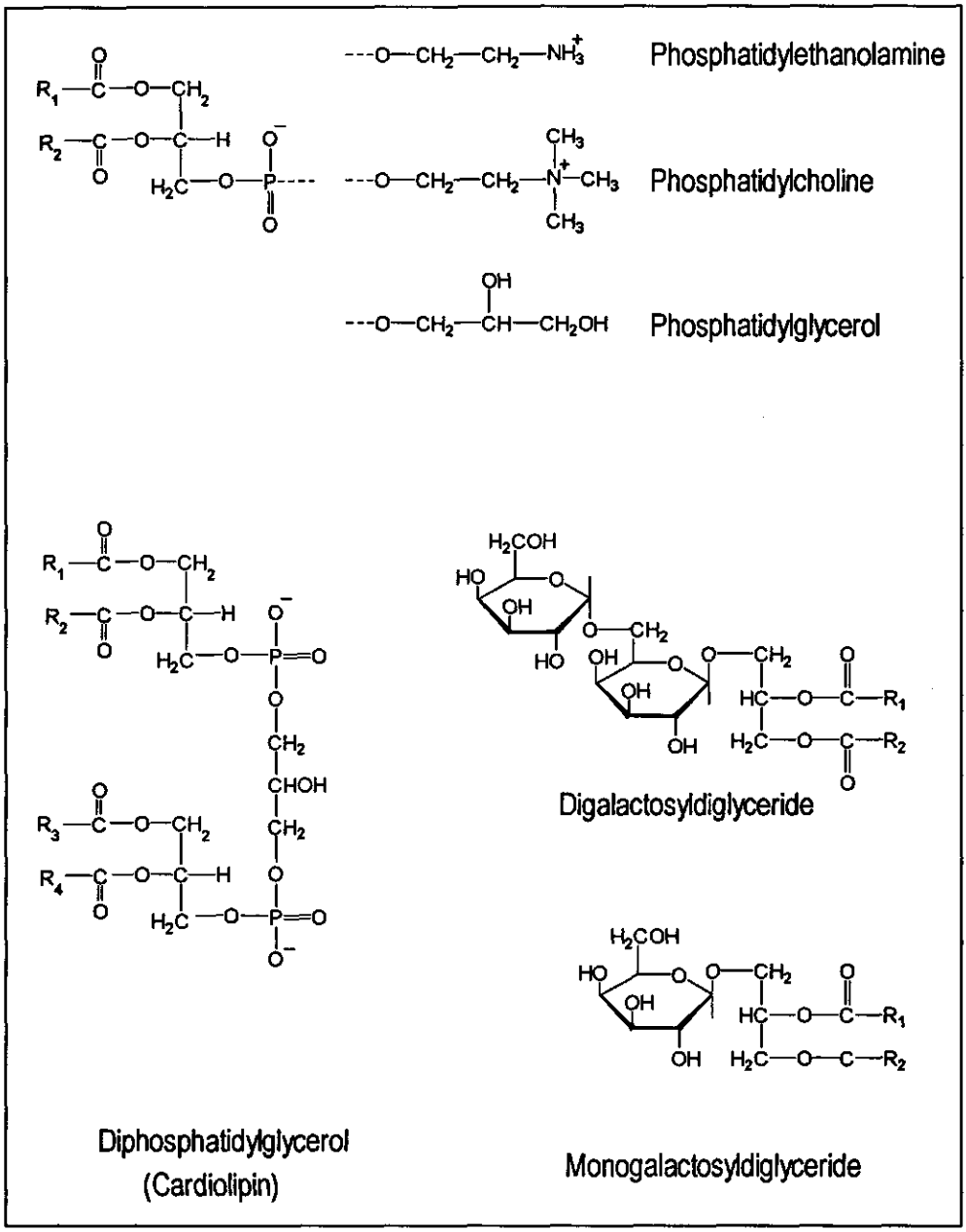

Figure 9: Structures of various membrane phospholipids, illustrating the variety of polar headgroups.

Table 4: Phospholipid composition of *P. putida* S12 grown in a chemostat on glucose medium in the presence of various concentrations of toluene. Phospholipids were identified by TLC (Skipski *et al.*, 1967) and quantified by a phosphate determination (Rouse *et al.*, 1966).

Toluene concentration (mg/l)	PE	DPG	PG	PE/(DPG+PG)
0	82.0 ± 3.7	9.6 ± 2.0	8.4 ± 1.4	4.6
185	74.9 ± 3.1	15.0 ± 0.5	10.1 ± 1.6	3.0
365	73.3 ± 0.7	14.5 ± 2.5	12.1 ± 2.0	2.8
550	68.1 ± 2.6	19.8 ± 2.3	12.2 ± 2.2	2.1
640	67.2 ± 3.1	26.0 ± 3.1	6.8 ± 4.0	2.0
1% (v/v)	70.3 ± 4.0	24.7 ± 2.6	4.9 ± 0.8	2.4

Toluene, as seen in Fig. 2, will accumulate in the vicinity of the headgroup area in between the lipid acyl chains. Consequently, it is expected to increase *(v/l)/a* and it will induce the formation of a hexagonal H_{II} lipid phase. As shown in Table 4, *P. putida* S12 reacts to the presence of toluene by decreasing the incorporation of PE in the membrane. PE is a phospholipid with a relatively small headgroup area '*a*' compared to PG and DPG. The decreased incorporation of PE and the increased incorporation of DPG will thus increase the average phospholipid headgroup area '*a*', which is expected to compensate for the increase in lipid volume '*v*' caused by the accumulation of toluene and thus stabilizes the bilayer structure of the membrane. As the T_M of DPG is about 10°C higher than PE (Keough & Davis, 1984), the increased incorporation of DPG will also have a rigidifying effect on the membrane and is expected to (partially) compensate for the toluene induced increase in membrane fluidity.

Regulation of the phospholipid headgroup composition not only controls the bilayer/non-bilayer phase preference of the lipids, but there is also evidence that the bilayer surface charge density is regulated. As shown before, accumulation of certain solvents in the lipid bilayer might increase the distance between the phospholipids in the lipid bilayer. These changes in the cross section area of the phospholipids will affect the overall surface potential or surface charge density of the membrane.

Growth of *A. laidlawii* in the presence of different amounts of palmitic acid (16:0) and oleic acid (18:1 *cis*) resulted in cell membranes with various degrees of unsaturation. *A. laidlawii* decreased the MGDG : DGDG ratio in response to an increase in unsaturation in order to maintain a proper balance between bilayer and non-bilayer forming phospholipids. However, an increasing degree of

unsaturation also resulted in an increased ratio of charged lipids (PG) to uncharged lipids (MGDG plus DGDG). An increase in oleic acid and the resulting decrease in the MGDG : DGDG ratio of the phospholipids results in an increase in the molecular area occupied by the lipids. The increased incorporation of anionic PG keeps the surface charge density constant. (Cristiansson *et al.*, 1985; McElhaney, 1989). Similarly, in *E. coli* the ratio between the anionic and zwitterionic phospholipids is kept almost constant. Studies with *E. coli* mutants, defective in the synthesis of various phospholipids, have shown that this ratio can not be varied without disturbing cell growth (Hawrot & Kennedy, 1975; Raetz, 1976, 1978).

Accumulation of ethanol in a lipid bilayer will increase the surface area of the lipids, and thus influences the surface charge of the lipids. Studies with *S. cerevisiae* have shown that PS-enriched cells are more ethanol resistant compared to PC or PE enriched cells. This increased ethanol tolerance has been attributed to the increased anion-zwitterionic phospholipid ratio of the phospholipids. (Mishra & Prasad, 1988). A similar correlation between ethanol-tolerance and an increased anionic:zwitterionic ratio has been observed in *E. coli* (Clark & Beard, 1979) and *Bacillus subtilis* (Bohin & Lubochinsky, 1982).

The mechanism and reason for this regulation of surface charge density are not well understood. It is expected that it plays a role in the bilayer stability. Due to the electrostatic repulsion between charged lipids, these are more pronounced to form lamellar phases. (Gennis, 1989). The activity of membrane-bound enzymes will also be influenced by an altered surface charge (Gennis, 1989).

6.1.3. Membrane protein content

Studies with fluorescent probes have shown that artificial membranes prepared of phospholipids extracted from ethanol-grown *E. coli* cells were more fluid than those from control cells. This increase in fluidity was expected from the observed changes in lipid composition. However, the intact plasma membrane of ethanol-grown *E. coli* cells is more rigid, indicating that a non-lipid component compensated for the increase in fluidity caused by ethanol (Dombek & Ingram, 1984). The ethanol-adapted cells had an increased protein-to-lipid ratio, which has an rigidifying effect on the membrane (Dombek & Ingram, 1984). In general proteins can be envisaged as a bulky rigid domain in the bilayer. The motion of the lipids in the vicinity is expected to be markedly hindered, resulting in an overall reduction in membrane fluidity (Shinitzky, 1984).

Similar to ethanol, phenol also induced an increase in the protein content of *E. coli* (Keweloh *et al.*, 1990). An ethanol dose-dependent increase in the protein content has also been observed in *Z. mobiles* (Carey & Ingram, 1983). A decrease in the total amount of phospholipids, probably caused by an increase in the

protein content, has been observed in ethanol-grown cells of *B. subtilis* (Rigomier *et al.*, 1980) and *Mycobacterium smegmatis* (Taneja & Khuller, 1980).

6.1.4. *Other membrane components*

In the previous paragraphs we have shown that microorganisms can change their phospholipid composition in response to the toxic action of organic solvents. Several other specific methods to counterbalance the effects of external factors on both the T_M and T_{LH} have been observed.

6.1.4.a. *Sterols and Hopanoids*

Sterols are an important constituent of the yeast plasma-membrane and can influence both the fluidity and the bilayer stability. A decrease in ergosterol content of the *Saccharomyces cerevisiae* cell membrane has been directly related to a decrease in cell viability in the presence of ethanol (Larue *et al.*, 1980; Lees *et al.*, 1980; Del Castillo Agudo, 1992). Similarly, supplementation of *Saccharomyces sake* and *Kluyveromyces fragilis* with sterols enhanced the ethanol tolerance (Hayashida & Ohta, 1980; Janssens *et al.*, 1983; Novotny *et al.*, 1992). The rigidifying effect of sterols on membrane lipids has been suggested to be crucial in enhancing the viability of cells in the presence of ethanol. (Hossack & Rose, 1976).

Sterols are only present in eukaryotic organisms, but sterol-like structures have also been identified in prokaryotes. *Zymomonas mobilis* is a bacterium which can produce high concentrations of ethanol. The membranes of this obligate fermentative bacterium are particularly rich in 18:1 *cis* (>70%) (Carey & Ingram, 1983; Barrow *et al.*, 1983), presumably to counterbalance the formation a hexagonal H_{II} structure. This high amount of unsaturated fatty acids can not compensate for the ethanol-effect on the fluidity. However, in this organism hopanoids have been identified as another important membrane constituent. The amount of hopanoids (1,2,3,4-tetrahydroxypentane-29-hoptane) synthesized by *Z. mobilis* rises with increasing ethanol concentrations. Since these hopanoids reduce the membrane fluidity, accumulation of these compounds is apparently a mechanism to compensate for the increase in fluidity caused by ethanol (Buchholz *et al.*, 1987).

6.1.4.b. *Carotenoids*

Carotenoids may be present in some bacterial membranes and, similar to sterols and hopanoids, they have an influence on lipid fluidity. In *Staphylococcus aureus*, a direct correlation has been observed between carotenoid production, membrane fluidity, and resistance to oleic acid (Chamberlain *et al.*, 1991). Also *A. laidlawii* is known to produce carotenoids (Smith, 1963). The regulatory role of carotenoids in controlling the fluidity of *A. laidlawii* cell membranes was

suggested by showing that the carotenoids biosynthesis is regulated by membrane fluidity (Rottem & Markowitz, 1979). This finding suggests that the carotenoid content of the membrane might restore the fluidity of the membrane when affected by solvents. Changes in the carotenoid composition of *A. laidlawii* in response to the presence of toluene in the medium have indeed been observed (Govorun *et al.*, 1989).

6.1.4.c. Dicarboxylic acids

In *Sarcina ventriculi* very high percentages (≈45 %) of dicarboxylic acids are formed in response to ethanol or butanol (Jung *et al.*, 1993). Control cells only contained about 7% of these bipolar long chain fatty acids (C28-C36). These lipids are believed to be important for membrane integrity under stress conditions. The two polar groups on the dicarboxylic acids, which are present at each end suggests that this unusual lipid component spans the membrane (Jung *et al.*, 1993). Liposomes composed of these lipids, originating from the thermoacidophilic *Sulfolobus acidocaldarius*, were considerably more stable and had a much lower proton permeability than liposomes composed of (normal) diester phospholipids (Elferink *et al.*, 1994).

6.1.5. Bilayer fluidity versus bilayer stability

In the previous paragraphs we have seen that the accumulation of solutes in the membranes of microorganisms can be compensated for by several mechanisms. These mechanisms can be explained as either a mechanism to restore the fluidity (homeoviscous adaptation) or as a mechanisms to maintain a proper balance between bilayer and non-bilayer promoting phospholipids.

Wieslander and co-workers have postulated from their studies with *A. laidlawii* that all the observed changes in membrane composition are a mechanism to maintain an optimal ratio between the bilayer and non-bilayer forming phospholipids and are not a mechanism to control the membrane fluidity (homeoviscous adaptation). The ethanol induced increase in the amount of unsaturated fatty acids observed in *E. coli* and several microorganisms seems to support this theory. The increase can only be explained as a mechanism to control the bilayer to non-bilayer forming phospholipid ratio and not as a mechanism to compensate the fluidity. However, it seems unlikely that this hypothesis is valid in all cases: Firstly, *E. coli* does not change its lipid composition in response to decanol. Such changes would be expected since decanol decreases the T_{LH} (Fig. 5) of the phospholipids. The absence of membrane changes suggests the control of membrane fluidity is important since decanol has no effect on the membrane fluidity (Fig. 3). Secondly, the amount of unsaturated fatty acids decreased in response to ethanol in several microorganisms other than *E. coli* (Table 3). Thirdly, in *A. laidlawii* strain B a regulation of the phospholipid

composition in response to either changes in temperature or cholesterol levels was not observed (McElhaney *et al.*, 1970, 1973; McElhaney 1974, 1984). Finally, it is worth noting that long-chain alkanes, as for instance hexadecane strongly decrease the T_{LH} of phospholipids. However, these solvents are non-toxic for microbial cells, although small amounts of these alkanes have a large effect on the T_{LH} and destabilize the bilayer structure.

Caution should be taken to correlate, for instance, changes in fatty acid composition, with an increase in solvent tolerance when other important parameters have not been studied. These other parameters (e.g. headgroup composition, protein or sterol content) can have opposite effects on the membrane integrity. A clear example of this is the adaptation of *E. coli* to ethanol. The observed increase in 18:1 *cis* content will restore the ratio between bilayer and nonbilayer promoting phospholipids. As this increase in 18:1 *cis* content will further fluidize the membrane, it could be concluded that control of the membrane fluidity is not important to acquire solvent tolerance. However, further studies with *E. coli* have shown that the membrane fluidity is restored by an increased protein content in the membrane. The possibility that changes in the protein content of the *A. laidlawii* membranes play a role in maintaining a certain membrane fluidity can not be ruled out. And although mycoplasmas as *A. laidlawii* can not synthesize sterols, they are known to produce carotenoids (Smith, 1963). The regulatory role of carotenoids in controlling the fluidity of *A. laidlawii* cell membranes was suggested by showing that the carotenoid biosynthesis system senses membrane fluidity (Rottem & Markowitz, 1979). Changes in the carotenoid composition of *A. laidlawii* in response to the presence of toluene in the medium have indeed been observed (Govorun *et al.*, 1989).

6.2. Outer membrane

We have discussed adaptation mechanisms so far at the level of the cytoplasmic membrane under the assumption that it is the key component of the bacterial cell envelope in combating toxic organic solvents. The other elements of the envelope are quite different in Gram-positive and Gram-negative bacteria. The Gram-positive bacteria have a thick cell wall consisting of murein and other polymers which can not be expected to have a role in excluding solvents from the cell. Gram-negative organisms have only a very thin murein layer but have an outer membrane outside this layer. The inner leaflet of this membrane resembles the cytoplasmic membrane composition but the outer leaflet is composed of lipopolysaccharides (LPS) (Lugtenberg & van Alphen, 1983). These LPSs have an unusually low permeability for hydrophobic compounds (Yoshimura & Nikaido, 1982; Hiruma *et al.*, 1984; Nikaido & Vaara, 1985; Vaara *et al.*, 1990).

The question now arises whether the outer membrane has a specific function in the defence of an organism against solvents. From comparative studies, one might conclude that Gram-negative bacteria are in general less sensitive to hydrophobic organic solvents than Gram-positive organisms (Sheu & Freese, 1973; Harrop *et al.*, 1989; Inoue & Horikoshi, 1991), but the differences are not overwhelming. Consequently, the cytoplasmic membrane indeed is a very critical part of the cell envelope with respect to solvent toxicity.

On the other hand, certain organisms that are able to acquire resistance against very toxic solvents, as, for instance, toluene are all Gram-negative organisms. Furthermore, in several cases it has been observed that the resistance to these hydrophobic solutes is decreased drastically when the structure of the outer membrane is modified by mutation or (chemical or enzymatic) removal of parts of the LPS molecule (Sheu & Freese, 1973; Nikaido, 1976; Hancock, 1984; Okuyama *et al.*, 1991; Tamura *et al.*, 1993; Vaara, 1993). Deep-rough mutants (lacking most of the saccharides) of *E. coli* and *S. typhimurium* had a very high sensitivity to a variety of antibiotics, detergents and other drugs. In addition an enhanced uptake of the hydrophobic dye gentian violet was shown in these mutants (Newton, 1954; Stan-Lotter *et al.*, 1979). The growth and oxygen consumption of *E. coli* and *S. typhimurium* is inhibited by short-chain fatty acids (C2-C6) but not by medium or long chain fatty acids (C10-C18) (Sheu & Freese, 1973). Removal of a portion of the lipopolysaccharide layer by EDTA made the organisms sensitive to these longer-chain fatty acids (Sheu & Freese, 1973).

Another important component of lipopolysaccharide, besides the polysaccharide chain are fatty acids. Unlike phospholipids which have usually only two fatty acids linked to the backbone structure, the LPS molecule has six to seven fatty acids linked to the glucosamine dissaccharide backbone (Lugtenberg & van Alphen, 1983). Another important difference with fatty acids present in phospholipids is that the fatty acids present in LPS are all saturated (Nikaido & Vaara, 1985). The absence of unsaturated fatty acids is expected to make the interior of the LPS leaflet much less fluid. Also the large number of fatty acids chains linked to a single backbone will decrease the fluidity (Nikaido, 1994a). Indeed the fluidity of the LPS interior has experimentally been shown to be very low (Seydel *et al.*, 1993).

Due to the presence of LPS molecules in the outer membrane, this membrane has a very low permeability (Yoshimura & Nikaido, 1982; Hiruma *et al.*, 1984; Nikaido & Vaara, 1985; Vaara *et al.*, 1990). Nevertheless these Gram-negative bacteria must exchange nutrients and waste products with the environment. Most of this exchange takes place through protein channels in the outer membrane (Nikaido, 1994b). These channel-forming proteins include specific channels, which contain specific binding sites facilitating the transport of certain molecules, and

porins, which allow nonspecific and spontaneous diffusion of small solutes. These porins are water-filled channels that allow the influx of small hydrophilic solutes but exclude large and more hydrophobic molecules (Nikaido, 1994a, 1994b). The resistance of Gram-negative bacteria against small, rather hydrophilic antibiotics has been contributed to changes in the porins present in the outer membrane (Nikaido, 1989). For instance, spontaneous mutants of *E. coli* possessing an increased resistance towards carbenicillin lacked the OmpF porin, which produces relatively large pores, but retained the OmpC porin which gives a narrow channel (Harder *et al.*, 1981).

The specific role of the outer membrane in coping with organic solvents has not been investigated so far. Most likely, the complex structure of the lipopolysaccharide has discouraged researchers from dealing with this aspect of a possible response of cells to toxic organic solvents. For lipophilic antibiotics, which usually have a relatively high molecular weight, however, several adaptive changes in the outer membrane have been observed (Diver *et al.*, 1991; Leying *et al.*, 1991; Michea-Hamzehpour *et al.*, 1991). The carbapenem resistance in *Enterobacter aerogenes*, for instance, has been correlated with alterations in the lipopolysaccharide composition (Leying *et al.*, 1991). The isolated resistant strains possessed more carbohydrates (thus a longer saccharide chain) than the control cells (Leying *et al.*, 1991).

We have investigated the role of the outer membrane in the solvent-tolerant *P. putida* S12 at two levels. Both the effects of divalent cations on the organization of the LPS-layer and changes in the hydrophobicity of the outer membrane will be discussed.
Especially Mg^{2+} and Ca^{2+} ions are important in the organisation of the outer membrane (Nikaido & Vaara, 1985). Adjacent polyanionic LPS molecules are linked electrostatically by divalent cations to each other forming a stable "tiled roof' on the surface of the outer membrane (Labischinski *et al.*, 1985; Okuyama *et al.*, 1991). The outer membrane can be disorganized by removing these divalent cations with chelators such as EDTA (Lugtenberg & van Alphen, 1983; Nikaido & Vaara, 1985; Okuyama *et al.*, 1991). As a consequence, *Salmonella* and other Gram-negative bacteria were less sensitive to hydrophobic inhibitors upon the addition Mg^{2+} and Ca^{2+} (Stan-Lotter *et al.*, 1979).
When *P. putida* S12 was grown with acetate as carbon source, a shock load of toluene resulted in the killing of more than 99.99% of the cells (Table 5). The survivors eventually grew to a high cell density in the presence of a second-phase of toluene (Weber *et al.*, 1993). Supplementing the growth medium of *P. putida* S12 with 5 mM $MgSO_4$ or $CaCl_2$ resulted in a 70- to 350-times increase in cell

Table 5: Effect of the addition of Mg^{2+} and Ca^{2+} ions on the viable count of exponentially growing *P. putida* S12 on acetate after the addition of 1% (v/v) toluene.

Addition	Viable cells (ml^{-1})		Survival
	Before	1h After	
none	$3.8*10^7$	$7.8*10^2$	0.002%
5 mM $MgSO_4$	$1.1*10^8$	$1.5*10^5$	0.14%
5 mM $CaCl_2$	$9.0*10^7$	$6.2*10^5$	0.7%

survival after the addition of toluene (Table 5). This effect is even more remarkable when the destructive effects of these ions on the phospholipid bilayer are taken into account. Normally, these ions will promote the formation of a hexagonal H_{II} configuration by anionic phospholipids (Seddon, 1990). These results suggest that the outer-membrane might contribute to the resistance of *P. putida* S12 to toluene. Similar results were reported by Inoue *et al.* (1991) for the toluene resistance of *P. putida* IH-2000. Addition of Mg^{2+} and Ca^{2+} to the growth medium resulted in a two- to three-fold increase in cell yield when the organisms was grown in the presence of 30% (v/v) toluene (Inoue *et al.*, 1991).

P. putida S12 apparently also changed the composition of its outer-membrane in response to toluene, resulting in a reduced hydrophobicity of the cell. As a measure of the hydrophobicity the contact angle of a water droplet on a layer of cells was used (van Loosdrecht, 1987). Glucose-grown cells are very sensitive to toluene, and one hour after the addition of a shock load of toluene no viable cells could be observed (Weber *et al.*, 1993). These glucose-grown cells have quite a hydrophobic outer membrane, as indicated by the high water contact angle (Table 6). Acetate-grown cells, however, are less hydrophobic and are more toluene resistant. Cells grown on acetate in the presence of toluene were even less hydrophobic and are more toluene resistant (Table 6). The observed reduction in the hydrophobicity of the outer membrane is expected to reduce the permeability of the membrane for the lipophilic compound toluene.

6.3. Other responses of microorganisms to solvents

Besides changes in the cytoplasmic membrane and outer membrane composition several other responses have been observed in microorganisms in response to the effects of organic solvents.

6.3.1. Compatible solutes

Compatible solutes like trehalose, betaine and proline are found in high concentrations in a wide variety of microorganisms that survive osmotic stress, severe hydratation and temperature stress (van Laere, 1989; Wiemken, 1990).

Table 6: Hydrophobicity of *P. putida* S12 cells and the effect of the addition of 1% (v/v) toluene on the viable count of exponentially growing cells (from Weber *et al.*, 1993). Adapted cells had been grown in the presence of 1% (v/v) toluene.

Strain	Growth substrate	Viable cells (ml^{-1})		Survival	Water contact angle
		Before	1h After		
Wild Type	Glucose	$3.7*10^7$	<10	0%	49°
Wild Type	Acetate	$3.8*10^7$	$7.8*10^2$	0.002%	33°
Adapted	Acetate	$1.1*10^8$	$1.1*10^7$	10%	28°

These compatible solute have a protective effect on membranes subjected to desiccation or freezing by affecting both the lipid packing geometry and fluidity of the membrane (Crowe *et al.*, 1984a ,1984b; Rudolph *et al.*, 1986). As compatible solutes are generally found in microorganisms it is expected that they can also stabilize membrane structures against solvent stress. Indeed, an accumulation of intracellular trehalose has been reported in *Saccharomyces* strains in response to an ethanol shock (Odumera *et al.*, 1993). It is postulated that this accumulation of trehalose is a response to the decrease in water activity (a_w) caused by the high concentrations of ethanol (Jones & Greenfield, 1987). Studies of Mansure *et al* (1994) have shown that addition of trehalose to various PC liposomes significantly decreased the ethanol-induced leakage of the vesicles. Whether compatible solutes also have a protective effect against the action of other organic solvents still has to be established.

6.3.2. Plasma-membrane ATPase

Accumulation of ethanol in the lipid bilayer will increase the permeability of the membrane (Fig. 7). An increase in the permeability of the membrane for protons will result in a dissipation of the proton motive force and will affect the viability of the cell. In *S. cerevisiae* ethanol was shown to activate the membrane H^+-ATPase activity (Rosa & Sá-Correia, 1991; Alexandre *et al.*, 1993). It is expected that this increase in H^+-ATPase activity is a response to the increase in permeability. Similarly, in a *Rhizobium* sp. confronted with 2,4-dichlorophenoxyacetic acid, an increase in the H^+-ATPase activity was observed in response to the increased membrane fluidity (Fabra de Peretti *et al.*, 1992). Although, this increase in activity is merely a response to the increase in permeability, differences in the tolerance of the H^+-ATPase to solvents are expected to contribute to the solvent tolerance of the cell. The H^+-ATPase of *Kluyveromyces marxianus* was more sensitive to ethanol than the H^+-ATPase of *S. cerevisiae* (Rosa & Sá-Correia, 1992). Apparently, differences in these H^+-ATPases have contribute to the ethanol tolerance of

S. cerevisiae, which has a higher tolerance than *K. marxianus* (Rosa & Sá-Correia, 1992).

6.3.3. *Heat Shock Proteins*

Exposure of *E. coli* to benzene and other pollutants led to the induction of numerous proteins. Some of these proteins were identified as heat shock or carbon starvation proteins, but at least 50% were only induced by a given chemical (Blom *et al.*, 1992). Ethanol induced heat shock proteins in *S. cerevisiae* resulting in an increased viability after a heat shock (Plesset *et al.*, 1982; Curran & Khalawan, 1994). Similarly, a heat shock treatment resulted in a remarkable increased tolerance of the cell to a subsequent ethanol shock (Watson & Cavichioli, 1983; Odumera *et al.*, 1992).
The fact that a chemical stress such as ethanol can protect against heat shock suggests that these heat shock or ethanol induced proteins function in a general cellular response mechanism to stress (Mager & Ferreira, 1993).

6.4. Methods for preventing the accumulation of solvents

Toxicity of organic solvents due to their accumulation in membranes can be counterbalanced by cells by modifying the membrane to make it more suitable to cope with the solvents. As seen before, the fatty acid profile and the headgroup composition may be altered, specific molecules may be incorporated in membranes, and the protein/phospholipid ratio can be shifted. These reactions are meant to stabilize the physical structure of the membrane and to allow it to play its role as barrier also in the presence of solvents. These reactions all are very defensive in nature in the sense that they try to make the best of an unwanted situation already in existence.
A more aggressive approach of cells to cope with solvents would be to prevent or reduce accumulation of solvents in membranes. When it would be possible to keep the concentration of solvent in the membrane low, then the negative effects of solvents would be minimized. In principle, several options are open to an organism to exclude solvents from the membrane, but all methods will be difficult due to the intrinsic properties of lipophilic solvents and their tendency to accumulate in the lipid bilayer.

6.4.1. *Decreased affinity of membranes for solvents*

One method to counterbalance partitioning of solvents in membranes is to make the membrane less attractive for the dissolving molecule by altering its lipid composition. The scope for such adaptation has been shown by work of Antunes-Madeira and Madeira (1984, 1986, 1987, 1989) and by De Young and Dill (1988). These authors used artificial membranes and showed that the partitioning of hydrophobic compounds very strongly depended on the fatty acid composition

of the phospholipids. For instance, the partitioning of lindane is 50 times lower in liposomes of DSPC (C18:0) than in liposomes of DMPC (C14:0) (Antunes-Madeira & Madeira, 1989). By modifying the fatty acid composition microorganisms can thus not only counteract the effects of solvents on the membrane but can also reduce the partitioning of the compound in the membrane.

6.4.2. *Degradation or modification of the solvent*

Another method to prevent the accumulation of solvents in membranes is to modify the compound and make it less lipophilic or metabolize it all together. Modification of a compound is possible for instance in the case of aromatic hydrocarbons by partially oxidizing the compound or by making it more water-soluble by making conjugates from it. Complete mineralization of a compound may also be an effective method to overcome the toxic effect provided its concentration is not too high at any specific moment. Such situation may occur, for instance, in the case of organisms growing on aromatic hydrocarbons like toluene or styrene.

6.4.3. *Active efflux mechanisms*

A very interesting method to reduce the actual concentration of solvents in membranes would be to transport solvents molecules out of the membrane by an active efflux system. Such a mechanisms would be of great help to a cell because any passive defence mechanism based on a low-permeability barrier or based on a modified membrane will have its drawback on the cell for its normal functioning. With one possible exception to be discussed later, no such active efflux system for organic solvents has been detected in bacteria, but a great deal of information has become available on active efflux mechanisms for lipophilic antibiotics. These systems have been reviewed by Levy (1992), Leewis (1994) and Nikaido (1994a). In summary, it can be said that these multi-drug efflux systems are widespread in microorganisms and the most salient feature is their being nonspecific: a great variety of lipophilic substrates can be handled by these translocases. The efflux complexes are located in the cytoplasmic membrane of, for instance, *E. coli* where it captures drug molecules that have managed to pass the outer membrane. The drug is transported via an accessory protein to a channel in the outer membrane and thus is excluded from the bacterial cell envelope. The interesting question now arises whether such multi-drug resistance pumps play a role in solvent resistance in bacteria. Recently Aono and colleagues (1995) made the very interesting observation that indicates that this indeed may be the case. They isolated cyclohexane-tolerant mutants of *E. coli* and subsequently showed that these mutants were resistant to low levels of ampicillin, chloramphenicol, naladixic acid and tetracycline. The other way around, mutants of *E. coli* resistant to ampicillin and chloramphenicol had

resistance to cyclohexane. The relationship between organic solvent tolerance on the one hand and resistance to antibiotics on the other hand therefore appears obvious. From these results it might be speculated that a multi-drug efflux mechanism of *E. coli* contributes to the organic solvent tolerance. It will be of great interest to see if a correlation exists in other bacteria as well, and particularly in solvent-tolerant strains, between solvent tolerance and multi-drug resistance.

7. CONCLUDING REMARKS

In this review we have shown that accumulation of organic solvents in the membrane of microorganisms affects the physico-chemical properties of the lipid bilayer and consequently the biological functioning of the membrane. The insight in the various effects which especially alkanols have on the properties and functioning of the biological membrane is growing. Especially the effects of alkanols on both the fluidity (T_M) and bilayer stability (T_{LH}) of the membrane are now well characterized in model membrane systems like liposomes. However, the consequences of these effects on the functioning of the cell membrane and viability of microbial cells are less clear. Although the effects of alkanols on (model) membranes are quite well characterized, the effects of various other organic solvents (e.g. aromatics) are unfortunately not yet as extensively studied. In view of the extreme toxicity of these aromatics for microorganisms, further insight in the effects of these solvents would be desirable.

Although organic solvents can affect the properties and functioning of the cell membrane, various microorganisms have developed adaptation mechanisms to counteract these effects. In many microorganisms changes in the lipid composition have been observed in response to several organic solvents. These changes can generally be explained as a mechanism to restore the fluidity of the membrane or as a mechanism to control the required balance between bilayer and non-bilayer promoting phospholipids. However, especially for ethanol, apparently contradicting adaptation mechanisms have been observed, which makes interpretation of the results difficult. Further research into these changes in lipid composition as well as other possible adaptation mechanisms is therefore required.

In view of practical applications, it is important to gain a better understanding of the effects of organic solvents on the functioning and properties of the cell membrane as well as of the adaptation mechanisms of microorganisms. Potentially, knowledge about solvent toxicity and solvent resistant microorganisms might be useful in both biotransformations and environmental biotechnology. Biocatalytical processes performed in aqueous phases often result in low concentrations of the product, which must be recovered from the water phase.

Obviously, new biotechnological processes would become feasible if microorganisms could be engineered to tolerate toxic organic solvents. For instance, a mutant of the solvent tolerant *P. putida* S12 is able to produce optically pure styrene oxide (Nöthe & Hartmans, 1994). In environmental biotechnology, the application of solvent-tolerant microorganisms will enable the biodegradation of highly toxic pollutants. As the degradation capacity of a microorganism is frequently limited by the toxicity of the contaminant being degraded, the use of specific solvent tolerant strains would by very useful for the removal of various environmental pollutants.

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9. REFERENCES

Alexandre, H., I. Rousseaux and C. Charpentier. (1993). Ethanol adaptation mechanisms in *Saccharomyces cerevisiae*. *Biotechnol. Appl. Biochem.* **20:** 173-183.

Antunes-Madeira, M.C. and V.M.C. Madeira. (1984). Partition of parathion in synthetic and native membranes. *Biochim. Biophys. Acta* **778:** 49-56.

Antunes-Madeira, M.C. and V.M.C. Madeira. (1985). Partition of lindane in synthetic and native membranes. *Biochim. Biophys. Acta* **820:** 165-172.

Antunes-Madeira, M.C. and V.M.C. Madeira. (1986). Partition of DDT in synthetic and native membranes. *Biochim. Biophys. Acta* **861:** 159-164.

Antunes-Madeira, M.C. and V.M.C. Madeira. (1987). Partition of malathion in synthetic and native membranes. *Biochim. Biophys. Acta* **901:** 61-66.

Antunes-Madeira, M.C. and V.M.C. Madeira. (1989). Membrane fluidity as affected by the insecticide lindane. *Biochim. Biophys. Acta* **982:** 161-166.

Aono, R., M. Kobayashi, H. Nakajima and H. Kobayashi. (1995). A close correlation between improvement of organic solvent tolerance levels and alteration of resistance towards low levels of multiple antibiotics in *Escherichia coli*. *Biosci. Biotechnol. Biochem.* **59:** 213-218.

Baer, S.H., H.P. Blaschek and T.L. Smith. (1987). Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.* **53:** 2854-2861.

Bangham, A.D., M.M. Standish and N. Miller. (1965). Cation permeability of phospholipid model membranes: effect of narcotics. *Nature* **208:**1295-1297.

Bansal, V.S. and G.K. Khuller. (1981). Changes in phospholipids of *Microsporum* species in the presence of ethanol. *Arch. Microbiol.* **130:** 248-249.

Barrow, K.D., J.G. Collins, P.L. Rogers and G.M. Smith. (1983). Lipid composition of an ethanol-tolerant strain of *Zymomonas mobilis*. *Biochim. Biophys. Acta* **753:** 324-330.

Beaven, M.J., C. Charpentier and A.H. Rose. (1982). Production and tolerance of ethanol in relation to phospolipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC 431. *J. Gen. Microbiol.* **128:** 1447-1455.

Bertoli, E., M. Masserini, S. Sonnino, R. Ghidoni, B. Cestaro and G. Tettamanti. (1981). Electron paramagnetic resonance studies on the fluidity and surface dynamics of egg phosphatidylcholine vesicles containing gangliosides. *Biochim. Biophys. Acta* **467:** 196-202.

Blom, A., W. Harder and A. Matin. (1992). Unique and Overlapping stress proteins of *Escherichia coli*. *Appl. Environ. Microbiol.* **58:** 331-334.

Boden, N., S.A. Jones and F. Sixl. (1991). On the use of deuterium nuclear magnetic resonance as a probe of chain packing in lipid bilayers. *Biochem.* **30:** 2146-2155.

Boggs, J.M. (1984). Intermolecular hydrogen bonding between membrane lipids. *In:* M. Kates, and L.A. Manson (eds.), Biomembranes, Vol. 12: Membrane Fluidity, pp. 1-53, Plenum Press, New York.

Bohin, J.-P. and B. Lubochinsky. (1982). Alcohol-resistant sporulation mutants of *Bacillus subtilis*. *J. Bacteriol.* **150:** 944-955.

Bringer, S., T. Hartner, K. Poralla and H. Sahm. (1985). Influence of ethanol on the hopanoid content and fatty acid pattern in batch and continuous cultures of *Zymomonas mobilis*. *Arch. Microbiol.* **140:** 312-316.

Buchholz, S.E., M.M. Dooley and D.E. Eveleigh. (1987). *Zymomonas* - an alcoholic enigma. *Trends Biotechnol.* **5:** 199-204.

Carey, V.C. and L.O. Ingram. (1983). Lipid composition of *Zymomonas mobilis*: effects of ethanol and glucose. *J. Bacteriol.* **154:** 1291-1300.

Carruthers, A. and D.L. Melchior. (1986). How bilayer lipids affect membrane protein activity. *Trends Biochem. Sciences* **11:** 331-335.

Cartwright, C.P., J.R. Juroszek, M.J. Beavan, F.M.S. Ruby, S.M.F. de Morais and A.H. Rose. (1986). Ethanol dissipates the proton-motive force across the plasma memrbane of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **132:** 369-377.

Casey, G.P. and W.M. Ingledew. (1983). Ethanol tolerance in yeasts. *CRC Crit. Rev. Microbiol.* **13:** 219-280.

Centeno, J.A. and T.J. O'Leary. (1990). Interactions of short-chain alcohols with dimyristoylphosphatidylethanolamine bilayers: A calorimetric and infrared spectroscopic investigation. *Biochem.* **29:** 7289-7296.

Chamberlain, N.R., B.G. Mehrtens, Z. Xiong, F.A. Kapral, J.L. Boardman and J.I. Rearick. (1991). Correlation between carotenoid production, decreased membrane fluidity, and resistance to oleic acid killing in *Staphylococcus aureus* 18Z. *Infect. Immun.* **59:** 4332-4337.

Cheng, K.-H., J.R. Lepock, S.W. Hui and P.L. Yeagle. (1986). The role of cholesterol in the activity of reconstituted Ca-ATPase vesicles containing unsaturated phosphatidylethanolamine. *J. Biol. Chem.* **261:** 5081-5087.

Clark, D.P. and J.P. Beard. (1979). Altered phospolipid composition in mutants of *Escherichia coli* sensitive or resistant to organic solvents. *J. Gen. Microbiol.* **113:** 267-274.

Cristiansson, A., L.E.G. Erickson, J. Westman, R. Demel and A. Wieslander. (1985). Involvement of surface potential in regulation of polar membrane lipids in *Acholeplasma laidlawii*. *J. Biol. Chem.* **260:** 3984-3990.

Crowe, J.H., L.M. Crowe and D. Chapman. (1984a). Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science* **223:** 701-703.

Crowe, J.H., M.A. Whittam, D. Chapman and L.M. Crowe. (1984b). Interaction of phospholipid monolayers with carbohydrates. *Biochim. Biophys. Acta* **769:** 151-159.

Cruden, D.L., J.H. Wolfram, R.D. Rogers and D.T. Gibson. (1992). Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. *Appl. Environ. Microbiol.* **58:** 2723-2729.

Cullen, J., M.C. Phillips and G. G Shipley. (1971). The effects of temperature on the composition and physical properties of the lipids of *Pseudomonas flourescens*. *Biochem. J.* **125:** 733-742.

Cullis, P.R. and B. de Kruijff. (1979). Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta* **559:** 399-420.

Curran, B.P.G. and S.A. Khalawan. (1994). Alcohols lower the treshold temperature for the maximal activation of a heat shock expression vector in the yeast *Saccharomyces cerevisiae*. *Microbiol.* **140:** 2225-2228.

De Young, L.R. and K.A. Dill. (1988). Solute partitioning into lipid bilayers. *Biochem.* **27:** 5281-5289.

DeChavigny, A., P.N. Heacock and W. Dowhan. (1991). Sequence and inactivation of the *pss* gene of *Escherichia coli*. Phosphatidylethanolamine may not be essential for cell viability. *J. Biol. Chem.* **266:** 5323-5332.

Del Castillo Agudo, L. (1992). Lipid content of *Saccharomyces cerevisiae* strains with different degrees of ethanol tolerance. *Appl. Microbiol. Biotechnol.* **37:** 647-651.

Diefenbach, R., H.-J. Heipieper and H. Keweloh. (1992). The conversion of *cis* into *trans* unsaturated fatty acids in *Pseudomonas putida* P8: evidence for a role in the regulation of membrane fluidity. *Appl. Environ. Microbiol.* **38:** 382-387.

Diver, J.M., T. Schollaartd, H.R. Rabin, C. Thorson and L.E. Bryan. (1991). Persistence mechanisms in *Pseudomonas aeruginosa* from cystic fibrosis patients undergoing ciprofloxin therapy. *Antimicrob. Agents Chemother.* **35:** 1538-1546.

Dombek, K.M. and L.O. Ingram. (1984). Effects of ethanol on the *Escherichia coli* plasma membrane. *J. Bacteriol.* **157:** 233-239.

Elferink, M.G.L., J.G. de Wit, A.J.M. Driessen and W.N. Konings. (1994). Stability and proton-permeability of liposomes composed of archaeal tetraether lipids. *Biochim. Biophys. Acta* **1193:** 247-254.

Eliasz, A.W., D. Chapman and D.F. Ewing. (1976). Phospholipid phase transitions: Effects of *n*-alcohols, *n*-monocarboxylic acids, phenylalkylalcohols, and quartinary ammonium compounds. *Biochim. Biophys. Acta* **448:** 220-230.

Espand, R.M. (1985). Diacylglycerols, Lysolecithin, or hydrocarbons markedly alter the bilayer to hexagonal phase transition temperature of phosphatidylethanolamines. *Biochem.* **24:** 7092-7095.

Fabra de Peretti, A., R. Duffars and A.M. Evangelista de Duffard. (1992). Effects of 2,4-dicholorphenoxyacetic acid on *Rhizobium* sp. membrane fluidity. *Arch. Environ. Contam. Toxicol.* **23:** 301-306.

Fernández, M.Z. and E. Calderón. (1980). Formation of micelles and membrane action of the local anesthetic tetrachain hydrochloride. *Biochim. Biophys. Acta* **597:** 83-91.

Gennis, R.B. (1989). Biomembranes: Molecular structure and function. Springer advanced texts in chemistry, Springer-Verlag, New York.

de Gier, J., J.G. Mandersloot and L.L.M. van Deenen. (1968). Lipid composition and permeability of liposomes. *Biochim. Biophys. Acta* **150:** 666-675.

Goldfine, H., N.C. Johnston, J. Mattai and G.G. Shipley. (1987). Regulation of bilayer stability in *Clostridium butyricum*: Studies on the polymorphic phase behavior of ester lipids. *Biochem.* **26:** 2814-2822.

Gordon, L.M., R.D. Sauerheber, J.A. Esgate, I. Dipple, R.J. Marchmont and M.D. Houslay. (1980). The increase in bilayer fluidity of rat liver plasma membranes achieved by the local anesthetic benzyl alcohol affects the activity of intrinsic membrane enzymes. *J. Biol. Chem.* **255:** 4519-4527.

Govorun, V.M., E.T. Mambetisaeve, L.A. Arthyushina and A.B. Kapitanov. (1989). *Acholeplasma laidlawii* culture adaptation to toluene. *Mikrobiologiya (English translation)* **58:** 614-618.

Govorun, V.M., E.V. Bondareva, S.N. Kulakova and A.B. Kapitanov. (1993). Toluene effect on lipid composition of *Acholeplasma laidlawii* cells. *Mikrobiologiya (English Translation)* **62:** 49-52.

Gruner, S.M., P.R. Cullis, M.J. Hope and C.P.S. Tilcock. (1985). Lipid polymorphism: The molecular basis of nonbilayer phases. *Ann. Rev. Biophys. Biophys. Chem.* **14:** 211-238.

Hancock, R.E.W. (1984). Alterations in outer membrane permeability. *Ann. Rev. Microbiol.* **38:** 237-264.

Harder, K.J., H. Nikaido and M. Matsuhashi. (1981). Mutants of *Escherichia coli* that are resistant to certain beta-lactam compounds lack the *ompF* porin. *Antimicrob. Agents Chemother.* **20:** 549-552.

Harrop, A.J., M.D. Hocknull and M.D. Lilly. (1989). Biotransformations in organic solvents: a difference between Gram-positive and Gram-negative bacteria. *Biotechnol. Lett.* **11:** 807-810.

Hawrot, E. and E.P. Kennedy. (1975). Biogenesis of membrane lipids: mutants of *Escherichia coli* with temperature-sensitive phosphatidylserine decarboxylase. *Proc. Natl. Acad. Sci. USA* **72:** 1112-1116.

Hayashida, S. and K. Ohta. (1980). Effects of phosphatidylcholine or ergosteryl oleate on physiological properties of *Saccharomyces sake*. *Agric. Biol. Chem.* **44:** 2561-2567.

Heipieper, H.J., H. Keweloh and H.J. Rehm. (1991). Influence of phenols on growth and membrane permeability of free and immobilized *Escherichia coli*. *Appl. Environ. Microbiol.* **58:** 1847-1852.

Heipieper, H.J., R. Diefenbach and H. Keweloh. (1992). Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. *Appl. Environ. Microbiol.* **58:** 1847-1852.

Heipieper, H.J. and J.A.M. de Bont. (1994). Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. *Appl. Environ. Microbiol.* **60:** 4440-4444.

Heipieper, H.J., F.J. Weber, J. Sikkema, H. Keweloh and J.A.M. de Bont. (1994). Mechanisms behind resistance of whole cells to toxic organis solvents. *Trends Biotechnol.* **12:** 409-415.

Herrero, A.A., R.F. Gomez and M.F. Roberts. (1982). Ethanol-induced changes in the membrane lipid composition of *Clostridium thermocellum*. *Biochim. Biophys. Acta* **693:** 195-204.

Hiruma, R., A. Yamaguchi and T. Sawai. (1984). The effect of lipopolysaccharide on lipid bilayer permeability of ß-lactam antibiotics. *FEBS Letters* **170:** 268-272.

Hornby, A.P. and P.R. Cullis. (1981). Influence of local and neutral anaesthetics on the polymorphic phase preferences of egg yolk phosphatidylethanolamine. *Biochim. Biophys. Acta* **647:** 285-292.

Hossack J.A. and A.H. Rose. (1976). Fragility of plasma membranes in *Saccharomyces cerevisiae* enriched with different sterols. *J. Bacteriol.* **127:** 67-75.

Ingram, L.O. (1976). Adaption of membrane lipids to alcohols. *J. Bacteriol.* **125:** 670-678.

Ingram, L.O. (1977). Changes in lipid composition of *Escherichia coli* resulting from growth with organic solvents and with food additives. *Appl. Environ. Microbiol.* **33:** 1233-1236.

Ingram, L.O., N.S. Vreeland and L.C. Eaton. (1980). Alcohol tolerance in *Escherichia coli*. *Pharmac. Biochem. Behav.* **13:** Suppl. 1: 191-195.

Ingram, L.O. and T.M. Buttke. (1984). Effects of alcohols on micro-organisms. *Adv. Microbiol. Physiol.* **25:**254-300.

Ingram, L.O. (1986). Microbial tolerance to alcohols: role of the cell membrane. *Trends Biotechnol.* **4:** 40-44.

Ingram, L.O. (1990). Ethanol tolerance in bacteria. *Crit. Rev. Biotechnol.* **9:** 305-319.

Inoue, A. and K. Horikoshi. (1989). A *Pseudomonas* thrives in high concentrations of toluene. *Nature* **338:** 264-266.

Inoue, A. and K. Horikoshi. (1991). Estimation of solvent tolerance of bacteria by the solvent parameter Log P. *J. Ferm. Bioeng.* **71:** 194-196.

Inoue, A., M. Yamamoto and K. Horikoshi. (1991). *Pseudomonas putida* which can grow in the presence of toluene. *Appl. Environ. Microbiol.* **57:** 1560-1562.

Israelachvili, J.N., D.J. Mitchell and B.W. Ninham. (1976). Theory of self-assembly of hydrocarbon amphiles into micelles and bilayers. *J. Chem. Soc. Faraday. Trans.* **72:** 1525-1568.

Israelachvili, J.N., D.J. Mitchell and B.W. Ninham. (1977). Theory of self-assembly of lipid bilayers and vesicles. *Biochim. Biophys. Acta* **470:** 185-201.

Jackson, M.B. and J.E. Cronan. (1978). An estimate of the minimum amount of fluid lipid required for growth of *Escherichai coli*. *Biochim. Biophys. Acta* **512:** 472-479.

Jain, M.K. and N.M. Wu. (1977). Effect of small molecules on the dipalmoitoly lecithin liposomal bilayer: III Phase transition in lipid bilayer. *J. Membr. Biol.* **34:** 157-201.

Jain, M.K. (1988). Introduction to biological membranes. Second edition, John Wiley & Sons, New York.

Janssens, J.M., N. Burris, A. Woodward and R.B. Bailey. (1983). Lipid-enhanched ethanol production by *Kluyveromyces fragilis*. *Appl. Environ. Microbiol.* **45:** 598-602.

Johnson, S.M. and A.D. Bangham. (1969). The action of anaesthetics on phospholipid membranes. *Biochim. Biophys. Acta* **193:** 92-104.

Jones, R.P. and P.F. Greenfield. (1987). Specific and non-specific inhibitory effects of ethanol on yeast growth. *Enzyme Microbiol. Biotechnol.* **9:** 334-338.

Jung, S.J., S.E. Lowe, R.I. Hollingsworth and J.G. Zeikus. (1993). *Sarcina ventriculi* synthesizes very long chain dicarboxylic acids in response to different forms of environmental stress. *J. Biol. Chem.* **268:** 2828-2835.

Kates, M., D.J. Kushner and A.T. James. (1962). The lipid composition of *Bacillus cereus* as influenced by the presence of alcohols in the culture medium. *Can. J. Biochem. Biophys.* **40:** 83-93.

Keough, K.M.W. and P.J. Davis. (1984). Thermal analysis of membranes. *In:* M. Kates, and L.A. Manson (eds.), Biomembranes, Vol 12: Membrane Fluidity, pp. 55-97, Plenum Press, New York.

Keweloh, H., G. Weyrauch and H.J. Rehm. (1990). Phenol-induced membrane changes in free and immobilized *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **33:** 66-71.

King, G.E., R.E. Jacobs and S.H. White. (1985). Hexane dissolved in dioleoyllecithin bilayers has a partial molar volume of approximately zero. *Biochem* **24:** 4637-4645.

Koukou, A.I., D. Tsoukatos and C. Drainas. (1990). Effect of ethanol on the phospholipid and fatty acid content of *Schizosaccharomyces pompe* membranes. *J. Gen. Microbiol.* **136:** 1271-1277.

de Kruijff, B., P.R. Cullis, A.J. Verkleij, M.J. Hope, C.J.A. van Echteld and T.F. Taraschi. (1985). Lipid polymorphism and membrane function. *In:* A.N. Martonosi (ed.), The enzymes of biological membranes, Vol 1: Membrane structure and dynamics, second edition, pp. 131-205, Plenum Press, New York.

Laane, C., S. Boeren, K. Vos and C. Veeger. (1987). Rules for optimization of biocatalysis in organic solvents. *Biotech. Bioeng.* **30:** 81-87.

Labischinski, H., G. Barnickel, H. Bradaczek, D. Naumann, E.T. Rietschel and P. Giesbrecht. (1985). High state of order of isolated bacterial lipopolysaccharide and its possible contributions to the permeation barrier property of the outer membrane. *J. Bacteriol.* **162:** 9-20.

van Laere, A. (1989). Trehalose, reserve and/or stress metabolite. *FEMS Microbiol. Rev.* **63:** 201-210.

Larue, F., S. Lafon-Lafourcade and R. Ribereau-Gayon. (1980). Relationship between the sterol content of yeast cells and their fermentation activity in grape must. *Appl. Environ. Microbiol.* **39:** 808-811.

Lees, N.D., S.L. Lofton, R.A. Woods and M. Bard. (1980). The effects of varied energy source and detergent on the growth of sterol mutants of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **118:** 209-214.

Lepage, C., F. Fayolle, M. Hermann and J.-P. Vandecasteele. (1987). Changes in lipid composition of *Clostridium acetobutylicum* during acetone-butanol fermentation: Effect of solvents, growth temperature and pH. *J. Gen. Microbiol.* **133:** 103-110.

Levy, S.B. (1992). Active efflux mechanisms for antimicrobial resistance. *Antimicrob. Agents Chemotherp.* **36:** 695-703.

Lewis, K. (1994). Multidrug resistance pumps in bacteria: variations on a theme. *Trends Biochem. Sciences* **19:** 119-123.

Leying, H., W. Cullmann and W. Dick. (1991). Carbapenem resistance in *Entrobacter aerogenes* is due to lipopolysaccahride alterations. *Chemother.* **37:** 106-113.

Lin, T.-L., S.-H. Chen, N.E. Gabriel and M.F. Roberts. (1987). Small-angel neutron scattering techniques applied to the study of polysisperse rodlike diheptanoylcholine micelles. *J. Phys. Chem.* **91:** 406-413.

Lindblom, G. and L. Rilfors. (1989). Cubic phases and isotropic structures formed by membrane lipids. Possible biological relevance. *Biochim. Biophys. Acta* **988:** 221-256.

Lohner, K. (1991). Effects of small organic molecules on phospholipid phase transitions. *Chem. Phys. Lipids* **57:** 341-362.

van Loosdrecht, M.C.M. (1987). The role of bacterial cell wall hydrophobicity in adhesion. *Appl. Environ. Microbiol.* **53:**1893-1897.

Lugtenberg, B. and L. van Alphen. (1983). Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. *Biochim. Biophys. Acta* **737:** 51-115.

Mager, W.H. and M. Ferreira. (1993). Stress response of yeast. *Biochem. J.* **290:** 1-13.

Mandersloot, J.G., W.J. Gerritsen, J. Leunissen-Bijvelt, C.J.A. van Echteld, P.C. Noordam and J. de Gier. (1980). Ca-induced changes in the barrier properties of cardiolipin/phosphatidylcholine bilayers. *Biochim. Biophys. Acta* **640:** 106-113.

Mansure, J.J.C., A.D. Panek, L.M. Crowe and J.H. Crowe. (1994). Trehalose inhibits ethanol effects on intact yeast cells and liposomes. *Biochim. Biophys. Acta* **1191:** 309-316.

Mason, J.T. and F.A. Stephenson. (1990). Thermotropic properties of saturated mixed acyl phosphatidylethanolamines. *Biochem.* **29:** 590-598.

McDaniel, R.V., S.A. Simon, T.J. McInosh and V. Borovyagin. (1982). Interaction of benzene with bilayers. Thermal and structural studies. *Biochem.* **21:** 4116-4126.

McElhaney, R.N., J. de Gier and L.L.M. van Deenen. (1970). The effect of alterations in fatty acid composition and cholesterol content on the permeabiltiy of *Mycoplasma laidlawii* B cells and derived liposomes. *Biochim. Biophys. Acta* **219:** 245-247.

McElhaney, R.N., J. de Gier and E.C.M. der Neut-Kok. (1973). The effect of alterations in fatty acid composition and cholesterol content on the nonelectrolyte permeabiltiy of *Acholeplasma laidlawii* B cells and derived liposomes. *Biochim. Biophys. Acta* **298:** 500-512.

McElhaney, R.N. (1974). The effect of alterations in the physical state of the membrane lipids on the ability of *Acholeplasma laidlawii* B to grow at various temperatures. *J. Mol. Biol.* **84:** 145-157.

McElhaney, R.N. (1984). The structure and function of the *Acholeplasma laidlawii* plasma membrane. *Biochim. Biophys. Acta* **779:** 1-42.

McElhaney, R.N. (1989). The influence of membrane lipid composition and physical properties of membrane structure and function in Acholeplasma laidlawii. *Crit. Rev. Microbiol.* **17:** 1-32.

McIntosh, T.J., S.A. Simon and R.C. MacDonald. (1980). The organization of *n*-alkanes in lipid bilayers. *Biochim. Biophys. Acta* **597:** 445-463.

McIntosh, T.J., R.V. McDaniel and S.A. Simon. (1983). Induction of an interdigitated gel phase in fully hydrated phosphatidylcholine bilayers. *Biochim. Biophys. Acta* **731:** 109-114.

Melchior, D.L. (1982). Lipid phase transition and regulation of membrane fluidity in prokaryotes. *Cur. Top. Membr. Transp.* **17:** 263-316.

Michea-Hamzehpour, M., C. Lucian and J.-C. Pechere. (1991). Resistance to pefloxacin in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **35:** 512-518.

Mishra, P. and S. Kaur. (1991). Lipids as modulator of ethanol tolerance in yeast. *Appl. Microbiol. Biotechnol.* **34:** 697-702.

Mishra, P. and R. Prasad. (1988). Role of phospholipid head groups in ethanol tolerance of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **134:** 3205-3211.

Mishhra, P. and R. Prasad. (1989). Relationship between ethanol tolerance and fatty acyl composition of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **30:** 294-298

Mitchell, D.J., G.J.T. Tiddy, L. Waring, T. Bostock and M.P. McDonald. (1983). Phase behavior of polyoxyethylene surfactants with water. Mesophase structures and partial miscibility (cloud points). *J. Chem. Soc., Faraday Trans. I* **79:** 975-1000.

Navarro, J., M. Toivio-Kinnucan and E. Racker. (1984). Effect of lipid composition on the calcium/adenosine 5'-triphosphate coupling ratio of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *Biochem.* **23:** 130-135.

Newton, B.A. (1954). Site of action of polymixin on *Pseudomonas aeruginosa*: antigonism by cations. *J. Gen. Microbiol.* **10:** 491-499.

Nicholls, D.G. (1982). Bioenergetics. Academic Press, New York.

Nikaido, H. (1976). Outer membrane of *Salmonella typhimurium*: trans membrane diffusion of some hydrophobic substances. *Biochim. Biophys. Acta* **433:** 118-132.

Nikaido, H. and M. Vaara. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* **49:** 1-32.

Nikaido, H. (1989). Outer membrane barrier as a mechanism of antimicrobial resistance. *Antimicrob. Agents Chemother.* **33:** 1831-1836.

Nikaido, H. (1994a). Prevention of drug access to bacterial targets: Permeability barriers and active efflux. *Science* **264:** 382-388.

Nikaido, H. (1994b). Porins and specific diffusion channels in bacterial outer mmebranes. *J. Biol. Chem.* **269:** 3905-3908.

Nöthe, C. and S. Hartmans. (1994). Formation and degradation of styrene oxide stereoisomers by different microorganisms. *Biocatalysis* **10:** 219-225.

Novotny, C., M. Flieger, J. Panos and F. Karst. (1992). Effect of 5,7-unsaturated sterols on ethanol tolerance in *Saccharomyces cerevisiae*. *Biotechnol. Appl. Biochem.* **15:** 314-320.

Odumera, J.A., T. D'Amore, I. Russell and G.G. Stewart. (1992). Changes in protein compostion of *Saccharomyces* brewing strains in response to heat shock and ethanol stress. *J. Ind. Microbiol.* **9:** 229-234.

Odumera, J.A., T. D'Amore, I. Russell and G.G. Stewart. (1993). Alterations in fatty acid composition and trehalose concentration of *Saccharomyces* brewing strains in response to heat and ethanol shock. *J. Ind. Microbiol.* **11:** 113-119.

Ohta, K., K. Supanwong and S. Hayashida. (1981). Environmental effects on ethanol tolerance of *Zymomonas mobilis*. *J. Ferment. Technol.* **59:** 435-439.

Okuyama, H., N. Okajima, S. Sasaki, S. Higashi and N. Murata. (1991). The *cis/trans* isomerization of the double bond of a fatty acid as a strategy for adaption to changes in ambient temperature in the psychrophilic bacterium, *Vibrio* sp. strain ABE-1. *Biochim. Biophys. Acta* **1084:** 13-20.

Osborne, S.J., J. Leaver, M.K. Turner and P. Dunnill. (1990). Correlation of biocatalytic activity in an organic-aqueous two-liquid phase sytem with solvent concentration in the cell membrane. *Enzyme Microbiol. Technol.* **12:** 281-291.

Piesset, J., C. Palm and C.S. McLaughlin. (1982). Induction of heat shock proteins and thermotolerance by ethanol in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Comm.* **108:** 1340-1345.

Pope, J.M., L.W. Walker and D. Dubro. (1984). On the ordering of *n*-alkane and *n*-alkanol solutes in phospholipid bilayer model membrane systems. *Chem. Phys. Lipids* **35:** 259-277.

Pope, J.M. and D.W. Dubro. (1986). The interaction of *n*-alkanes and *n*-alcohols with lipid bilayer membranes: a ^{2}H-NMR study. *Biochim. Biophys. Acta* **858:** 243-253.

Pope, J.M., D. Dubro, J.W. Doane and P.W. Westerman. (1986). The ordering of benzyl alcohol and its influence on phospholipid order in bilayer membranes. *J. Am. Chem. Soc.* **108:** 5426-5433.

Pope, J.M., L.A. Littlemore and P.W. Westerman. (1989). Chain-length dependence of *n*-alkane solubility in phosphatidylcholine bilayers: a H-NMR study. *Biochim. Biophys. Acta* **980:** 69-76.

Raetz, C.R.H. (1976). Phosphatidylserine synthetase mutants of *Escherichia coli*: genetic mapping and membrane phospholipid composition. *J. Biol. Chem.* **251:** 3242-3249.

Raetz, C.R.H. (1978). Enzymology, genetics, and regulation of membrane phospholipid synthesis in *Escherichia coli*. *Microbiol. Rev.* **42:** 614-659.

Rekker, R.F. and H.M. de Kort. (1979). The hydrophobic fragmental constant; an extension to a 1000 data point set. *Eur. J. Med. Chem.* **14:** 479-488.

Riddick, J.A., W.B. Bunger and T.K. Sakano. (1986). Organic solvents: physical properties and methods of purification. *In:* Techniques of chemistry, Vol. 2, 4th edition, Wiley, New York.

Rietveld, A.G., J.A. Killian, W. Dowhan and B. de Kruijff. (1993). Polymorphic regulation of membrane phospholipid composition in *Escherichia coli*. *J. Biol. Chem.* **268:** 12427-12433.

Rigomier, D., J.-P. Bohin and B. Lubochinsky. (1980). Effects of ethanol and methanol on lipid metabolism in *Bacillus subtilis*. *J. Gen. Microbiol.* **121:** 139-149.

Rilfors, L., G. Lindblom, A. Wieslander and A. Christiansson. (1984). Lipid bilayer stability in biological membranes. *In:* M. Kates, and L.A. Manson (eds.), Biomembranes, Vol 12: Membrane Fluidity, pp. 205-245, Plenum Press, New York.

Romijn, J.C., L.M.G. van Golde, R.N. McElhaney and L.L.M. van Deenen. (1972). Some studies on the fatty acid composition of total lipids and phosphatidylglycerol from *Acholeplasma laidlawii* B and their relation to the permeabiltiy of intact cells of this organism. *Biochim. Biophys. Acta* **280:** 22-32.

Rosa, M.F. and I. Sá-Correia. (1991). In vivo activation by ethanol of plasma membrane ATPase of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **57:**830-835.

Rosa, M.F. and I. Sá-Correia. (1992). Ethanol tolerance and activity of plasma membrane ATPase in *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. *Enzyme Microbiol. Technol.* **14:** 23-27.

Rottem, S. and O. Markowitz. (1979). Carotenoids act as reinforces of the *Acholeplasma laidlawii* lipid bilayer. *J. Bacteriol.* **140:** 944-948.

Rouse, G., A.N. Siakotos and S. Fleischer. (1966). Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. *Lipids* **1:** 85-86.

Rudolph, A.S., J.H. Crowe and L.M. Crowe. (1986). Effects of three stabilizing agents - proline, betaine, and trehalose - on membrane phospholipids. *Arch. Biochem. Biophys.* **245:** 134-143.

Saha, J., D. Papahadjopoulos and C.E. Wenner. (1970). Studies of model membranes. I. Effects of Ca^{2+} and antibiotics of cardiolipin liquid-crystalline vesicles. *Biochim. Biophys. Acta* **196:** 10-19.

Sandermann, H. (1978). Regulation of membrane enzymes by lipids. *Biochim. Biophys. Acta* **515:** 209-237.

Sandermann, H. (1993). Induction of lipid-protein mismatch by xenobiotics with general membrane targets. *Biochim. Biophys. Acta* **1150:** 130-133.

Sarti, P., G. Antonini, F. Malatesta, B. Vallone, M. Brunori, M. Masserini, P. Palstini and G. Tettamanti. (1990). Effect of gangliosides on membrane permeability studied by enzymic and fluorescence-spectroscopy techniques. *Biochem. J.* **267:** 413-416.

Seddon, J.M. (1990). Structure of the inverted hexagonal (HII) phase, and non-lamellar phase transitions of lipids. *Biochim. Biophys. Acta* **1031:** 1-69.

Seydel, U., H. Labischinski, M. Kastowsky and K. Brandenburg. (1993). Phase behavior, supramolecular structure, and molecular conformation of lipopolysaccharide. *Immunobiol.* **87:** 191-211.

Sheu, C.W. and E. Freese. (1973). Lipopolysaccharide layer protection of Gram-negative bacteria against inhibition by long-chain fatty acids. *J. Bacteriol.* **115:** 869-875.

Shinitzky, M. (1984). Membrane fluidity and cellular functions. *In:* M. Shinitzky (ed.), Physiology of membrane fluidity, Vol. 1, pp. 1-52, CRC Press, Boca Raton.

Sierra-Alvarez, R. and G. Lettinga. (1991). The effect of the aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. *Appl. Microbiol. Biotechnol.* **34:** 544-550.

Sikkema, J., B. Poolman, W.N. Konings and J.A.M. de Bont. (1992). Effects of the membrane action of tetralin on the functional and structural properties of artificial and bacterial membranes. *J. Bacteriol.* **174:** 2986-2992.

Sikkema, J., J.A.M. de Bont and B. Poolman. (1994a). Interaction of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* **269:** 8022-8028.

Sikkema, J., F.J. Weber, H.J. Heipieper and J.A.M. de Bont. (1994b). Cellular toxicity of lipophilic compounds: Mechanisms, implications, and adaptations. *Biocatalysis* **10:** 113-122.

Sikkema, J., J.A.M. de Bont and B. Poolman. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbial. Rev.* **59:** 201-222.

Silver, S. and L. Wendt. (1967). Mechanism of action of phenethyl alcohol: breakdown of the cellular permeability barrier. *J. Bacteriol.* **93:** 560-566.

Simon, S.A., R.V. McDaniel and T.J. McIntosh. (1982). Interaction of benzene with micelles and bilayers. *J. Phys. Chem.* **86:** 1449-1456.

Simon, S.A. and T.J. McIntosh. (1984). Interdigitated hydrocarbon chain packing causes the biphasic transition behavior in lipid/alcohol suspensions. *Biochim. Biophys. Acta* **773:** 169-172.

Sinensky, M. (1974). Homeoviscous adaption - A homeostatic process that regulates the viscocity of membrane lipids in *Escherichia coli*. *Proc. Nat. Acad. Sci. USA* **71:** 522-525.

Skipski, V.P., M. Barclay, E.S. Reichman and J.J. Good. (1967). Separation of acidic phospholipids by one-dimensional thin-layer chromatography. *Biochim. Biophys. Acta* **137:** 80-89.

Slater, J.L. and C. Huang. (1988). Interdigitated bilayer membranes. *Prog. Lipid. Res.* **27:** 325-359.

Smith, P.F. (1963). The carotenoid pigments of *Mycoplasma*. *J. Gen. Microbiol.* **32:** 307-319.

Stan-Lotter, H., M. Gupta and K.E. Sanderson. (1979). The influence of cations on the permeability of the outer membrane of *Salmonella typhimurium* and other gram-negative bacteria. *Can. J. Microbiol.* **25:** 475-485.

Steim, J.M., M.E. Tourtellotte, J.C. Reinert, R.N. McElhaney and R.L. Rader. (1969). Calorimetric evidence for the liquid-crystalline state of lipids in a biomembrane. *Proc. Natl. Acad. Sci. USA.* **63:** 104-109.

Sullivan, K.N., M.K. Jain and A.L. Koch. (1974). Activiation of the ß-galactoside transport system in *Escherichia coli* ML-308 by *n*-alkanols. Modification of lipid-protein interaction by a change in bilayer fluidity. *Biochim. Biophys. Acta* **352:** 287-297.

Sullivan, K.H., G.D. Hegeman and E.H. Cordes. (1979). Alteration of the fatty acid composition of *Escherichia coli* by growth in the presence of normal alcohols. *J. Bacteriol.* **138:** 133-138.

Szogyi, M., T. Cserhati and T. Tolgyesi. (1993). Effect of some potassium selective crown ethers on the permeability and structure of a phospholipid membrane. *Lipids* **28:** 847-851.

Tamura, K., Y. Muramoto and H. Kourai. (1993). Hydrophobicity of the cell surface and drug susceptibility of *Escherichia coli* cultivated at high pressures up to 30 MPa. *Biotechnol. Lett.* **15:** 1189-1194.

Taneja, R. and G.K. Khuller. (1980). Ethanol-induced alterations in phospholipids and fatty acids of *Mycobacterium smegmatis* ATCC 607. *FEMS Microbiol. Lett.* **8:** 83-85.

Tanford, C. (1973). The hydrophobic effect: formation of micelles and biological membranes. Wiley, New York.

Tilcock, C.P.S. and P.R. Cullis. (1982). The polymorphic phase behaviour and miscibility properties of synthetic phosphatidylethanolamines. *Biochim. Biophys. Acta* **684:** 212-218.

Uchida, K. (1975a). Effects of cultural conditions on the cellular fatty acid composition of *Lactobacillus heterohiochii*, an alcoholophilic bacterium. *Agr. Biol. Chem.* **39:** 837-842.

Uchida, K. (1975b). Alteration of the unsaturated to saturated ratio of fatty acids in bacterial lipids by alcohols. *Agr. Biol. Chem.* **39:** 1515-1516.

Vaara, M., W.Z. Plachy and H. Nikaido. (1990). Partitioning of hydrophobic probes into lipopolysaccharide bilayers. *Biochim. Biophys. Acta* **1024:** 152-158.

Vaara, M. (1993). Antibiotic-supersusceptible mutants of *Escherichia coli* and *Salmonella typhimurium*. *Antimicrobiol. Agents. Chemother.* **37:** 2255-2260.

In 't Veld, G., A.J.M. Driessen, J.A.F. Op den Kamp and W.N. Konings. (1991). Hydrophobic membrane thickness and lipid-protein interactions of the leucine transport system of *Lactococcus lactis*. *Biochim. Biophys. Acta* **1065:** 203-212.

Vermuë, M., J. Sikkema, A. Verheul, R. Bakker and J. Tramper. (1993). Toxicity of homologous series of organic solvents for the Gram-positive bacteria *Arthrobacter* and *Nocardia* sp. and the Gram-negative bacteria *Acinetobacter* and *Pseudomonas* sp. *Biotechnol. Bioeng.* **42:** 747-758.

Vierl, U., L. Löbbecke, N. Nagel and G. Cevc. (1994). Solute effects on the colloidal and phase behaviour of lipid bilayer membranes: Ethanol-dipalmitoylphosphatidylcholine mixtures. *Biophys. J.* **67:** 1067-1079.

Vighi, M. and D. Calamari. (1987). A triparametric equation to describe QSARs for hetrogeneous chemical substances. *Chemospere* **16:** 1043-1051.

Vollherbst-Schneck, K., J.A. Sands and B.S. Montenecourt. (1984). Effect of butanol on lipid composition and fluidity of *Clostridium acetobutylicum* ATCC 824. *Appl. Environ. Microbiol.* **47:** 193-194.

Ward, A.J.L., S.B. Rananavare and S.E. Friberg. (1986). Solvation changes in a lyotropic lamellar liquid crystal containing solubilized benzene. *Langmuir* **2:** 373-375.

Watson, K. and R. Cavichioli. (1983). Acquisition of ethanol tolerance in yeast cells by heat shock. *Biotechnol. Lett.* **5:** 683-688.

Weber, F.J., L.P. Ooijkaas, R.M.W. Schemen, S. Hartmans and J.A.M. de Bont. (1993). Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. *Appl. Environ. Microbiol.* **59:** 3502-3504.

Weber, F.J., S. Isken and J.A.M. de Bont. (1994). *Cis/trans* isomerization of fatty acids as a defence mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. *Microbiol.* **140:** 2013-2017.

Westerman, P.W., J.M. Pope, N. Phonphok, J.W. Doane and D.W. Dubro. (1988). The interaction of *n*-alkanols with lipid bilayer membranes: a ^{2}H-NMR study. *Biochim. Biophys. Acta* **939:** 64-78.

White, S.H., G.I. King and J.E. Cain. (1981). Location of hexane in lipid bilayers determined by neutron diffraction. *Nature* **290:** 161-163.

Wiemken, A. (1990). Trehalose in yeast, stress protectant rather than reserve carbohydrate. *Antonie van Leeuwenhoek* **59:** 209-217.

Wieslander, A., A. Christiansson, L. Rilfors and G. Lindblom. (1980). Lipid bilayer stability in membranes. Regulation in *Acholeplasma laidlawii* as governed by molecular shape. *Biochem.* **19:** 3650-3655.

Wieslander, A., L. Rilfors and G. Lindblom. (1986). Metabolic changes of membrane lipid composition in *Acholeplasma laidlawii* by hydrocarbons, alcohols, and detergents: Arguments for effects on lipid packing. *Biochem.* **25:** 7511-7517.

Yang, D,-P., T. Mavromoustakos, K. Beshah and A. Makriyannis. (1992). Amphiphathic interactions of cannabinoids with membranes. A comparison between Δ8-THC and its o-methyl analog using differential scanning calorimetry, X-ray diffraction and solid state H-NMR. *Biochim. Biophys. Acta* **1103:** 25-36.

Yeagle, P.L. (1989). Lipid regulation of cell membrane structure and function. *FASEB J.* **3:** 1833-1842.

Yoshimura, F. and H. Nikaido. (1982). Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. *J. Bacteriol.* **152:** 636-642.

Zeng, J., K.E. Smith and P.L.-G. Chong. (1993). Effects of alcohol-induced lipid interdigitation on proton permeability in l-α-dipalmitoylphosphatidylcholine vesicles. *Biophys. J.* **65:** 1404-1414.

9

Concluding remarks

Several fundamental aspects concerning the use of microorganisms in biological waste-gas treatment, as well as the growth of specific microorganisms in the presence of highly toxic solvents have been addressed in this thesis. In this final chapter I will discuss the results presented in this thesis in view of their possible impact on biotechnological processes.

POTENTIAL OF TRICKLE-BED REACTORS FOR WASTE-GAS TREATMENT

For the biological removal of pollutants from waste gases several types of bioreactors have been developed. Biofilters are already used since 1920 for the treatment of odorous waste-gas streams. Nowadays there is a growing interest to apply biological waste-gas treatment techniques for the removal of higher concentrations of specific compounds. A potential problem, however, could be the toxicity of temporary high contaminant concentrations in the waste gas. Especially compounds with a high affinity for water can already become toxic at relatively low concentrations in the gas-phase (Chapter 5). By using an adsorbent, toxic peak concentrations can be buffered and consequently toxic levels can be avoided (Chapter 2).

Toxicity problems are not readily expected for several other solvents, toluene, for instance, becomes toxic only at concentrations above 70-80 g/m^3. When a waste-gas stream should contain these high contaminant concentrations, it would be advisable and relatively easy to recover the solvent with other techniques. Although for most compounds toxic levels in the bioreactor are not

expected it can be advantageous to buffer fluctuating concentrations in view of the required reactor volume. For a reliable operation the design of the bioreactor should be based on peak concentrations in the waste gas. By using a buffer it is possible to buffer these peak concentrations and to treat the same waste-gas stream with a smaller reactor, which of course results in a reduction of the costs.

Until recently mainly biofilters were used for the treatment of waste gases. A disadvantage of biofilters is that the pH is difficult to control. Due to the formation of HCl a biofilter can not be used for the treatment of chlorinated compounds. Chlorinated compounds can, however, be effectively degraded in a trickle-bed reactor, since in this reactor type the pH can be controlled by the circulating water phase. A problem which could limit the use of trickle-bed reactors for biological waste-gas treatment is the formation of an excessive amount of biomass resulting in clogging of the reactor (Diks, 1992). By limiting the amount of nitrogen available for biomass formation or by using regular NaOH-washes of the reactor the biomass content of the trickle-bed reactor can be controlled (Chapter 3). Using these methods stable toluene removal rates of 35 g-C/(m^3 h) could be maintained over a period of up to one year. Although, in literature often much higher activities have been reported for the removal of organic compounds in bioreactors (e.g. Kirchner *et al.*, 1991), it is unclear if these high activities can be maintained during longer periods of time. Compared to biofilters, which are used to treat waste gases from industry the observed activities in the trickle-bed reactor are good. In industrial biofilters the removal rate is often less than 15 g-C/(m^3 h) (Mildenberger, 1992). In trickle-bed reactors only the removal of dichloromethane has been studied over an longer period of time. In these reactors an average dichloromethane elimination capacity of 7 g-C/(m^3 h) was obtained (Diks & Ottengraf, 1991; Hartmans & Tramper, 1991).

Ecosens bv have shown with pilot studies performed with their cross-flow trickle-bed reactor "Biostack" that trickle-bed reactors can be used effectively to treat an industrial waste gas. These pilot studies were performed at the Lycra factory of Du Pont de Nemours & Co in Dordrecht, The Netherlands. During a period of 6 months the removal efficiency of *N,N*-dimethylacetamide (DMAc), at an organic load between 10.4 and 19.2 g-C/(m^3 h), was more than 95% (Waalewijn *et al.*, 1994). Surprisingly, no specific precautions were necessary to control the biomass content of the reactor, no clogging occurred and the pressure drop over the reactor remained below 60 Pa. Apparently in this case enough biomass was removed by the liquid drain to prevent clogging. From our experiments where we studied the removal of toluene with a trickle-bed reactor, it was concluded that clogging could only be prevented by applying regular NaOH-washes (Chapter

3). Apparently in the Biostack used for the removal of DMAc, biomass was removed from the reactor without the requirement of a NaOH wash. During the degradation of DMAc NH_3 is formed. Although the formed NH_3 is neutralized by titration with HCl, it is expected that in the biofilm locally high pH values exist. This high pH would, similar to the NaOH-wash used in the toluene trickle-bed reactor, cause lysis and remove biomass from the packing. A similar mechanism could also explain why clogging was not observed in the studies of dichloromethane removal with trickle-bed reactors, although the organic load in these studies was also much lower (Diks & Ottengraf, 1991; Hartmans & Tramper, 1991).

Although biofilm reactors are applied successfully in waste-water treatment, the microbial processes in biofilms are still poorly understood. Until recently most research has been done with biofilms of only one or two species, whereas in practice the situation is far more complex. From our studies on the removal of toluene under nutrient-limited conditions which restricted the growth of cells, it emerged that a large amount of the degraded toluene was converted into biopolymers like glycogen and extracellular polysaccharides (Chapter 3). Although the production of these biopolymers is generally observed under nutrient-limited conditions, this subject has only scarcely been studied in relation to biofilms. Since in practice nutrient limitations are expected to exist more research on the formation of biofilms under nutrient-limited conditions would be desirable.

For the application of biofilm reactors for waste-gas treatment the biofilm should ideally be thin and consist only of actively respiring microorganisms. In practice, however, a very large fraction of the total biomass present in biofilm reactors is expected to be dead. In order to maintain a thin, active, biofilm a high turnover of these dead cells should take place. Unfortunately, only a few papers deal with microbial processes concerning cell death, lysis and the subsequent metabolism of these lysis products (Mason *et al.*, 1986; Bryers & Mason, 1987). Consequently, knowledge and methods which could increase the mineralization rate of inactive biomass, resulting in thinner active biofilms, are absent. In compost biofilters, used for waste-gas treatment, almost no biomass can be seen, whereas in trickle-bed reactors thick biofilms are observed. The reason for this difference is still unclear, however, this clearly implicates the existence of microbial methods to maintain a thin biofilm.

Many waste gases contain solvents which are only poorly soluble in water. Hexane, for instance is a very important component of waste gases from the food and drink industry in the EC (Swannell *et al.*, 1991; Groenestijn & Hesselink, 1993).

With the current techniques the high mass-transfer resistance of poorly soluble compounds from the gas to the water phase limits the application of biological methods. The mass-transfer resistance can be reduced when the boundary water-layer of the biofilm is almost completely absent. By using a membrane bioreactor this could be achieved, since the biofilm growing on the membrane is in direct contact with the gas phase (Hartmans *et al.*, 1992; Reij *et al.*, 1995). However, due to the high costs of the membranes, the use of this reactor type will probably only be feasible for a few specific applications. Another approach would be to use a reactor with the biomass immobilized on a packed bed, to which water is only sparsely supplied (Wolff, 1992; Deheyder *et al.*, 1994). A further increase in mass transfer can perhaps also be expected when instead of hydrophilic, hydrophobic microorganisms are used. However, a very stringent humidity control should be maintained, as the mass-transfer resistance will increase when the humidity becomes too high, and a too low humidity will inactivate the microorganisms. As fungi generally require a less stringent control of the water activity, the application of hydrocarbon degrading fungi in these reactors might have advantages (Majcherczyk *et al.*, 1990; Cox *et al.*, 1993b). Although not yet extensively studied many solvents can be degraded by fungi. Besides oxygenated aromatic compounds (Cain *et al.*, 1968; Middelhoven, 1993; Wright, 1993), fungi have also recently been shown to degrade non-oxygenated compounds such as styrene (Cox *et al.*, 1993a) and toluene (Chapter 4).

SOLVENT-TOLERANT MICROORGANISMS

During our studies on toxicity we surprisingly observed that *P. putida* S12 could, after a lag-phase, grow in the presence of supersaturating amounts of toluene (Chapter 6). Similar solvent-tolerant microorganisms have been described by Inoue and Horikosi (1989) and Cruden *et al.* (1992).

Applications

Microorganisms with a high tolerance to organic solvents could be useful in both environmental biotechnology and biocatalysis. The potential of these solvent-tolerant microorganisms for practical applications is illustrated by the patents which have been deposited for various applications (Inoue & Horikoshi, 1988a, 1988b; Doi & Horikoshi, 1989; Wolfram & Rogers, 1991). Obviously the application of solvent-tolerant microorganisms could be very useful for the treatment of polluted waste waters containing high concentrations of contaminants. Also in polluted soils saturating amounts of contaminants can be present. Due to the toxicity of these high concentrations of xenobiotics the *in situ* bioremediation of these soils is difficult (Blackburn & Hafker, 1993; Heitzer & Sayler, 1993). However,

the existence of solvent-tolerant microorganisms could also make *in situ* bioremediation feasible in these situations.

***Pseudomonas putida* S12**

Although *P. putida* S12 can tolerate and grow in the presence of toxic concentrations of organic solvents like toluene, these solvents do affect the cell. A hugh reduction in the biomass yield is observed when *P. putida* S12 is grown in the presence of a supersaturating amount of toluene. On 15 mM glucose normally about 1.0 g/l biomass is formed, whereas in the presence of 1% toluene only 0.05 g/l of cells can be obtained (Isken and Weber, unpublished results). Furthermore, also in toluene-adapted cells an extensive leakage of potassium was observed up on the addition of toluene (Diender and Weber, unpublished results). These results clearly show that, despite the observed adaptations, high toluene concentrations still have a damaging effect on the cell.

In Chapter 7 and 8 we have shown that changes in the membrane composition of *P. putida* S12 compensate for the toxic effects of toluene. As almost all microorganisms change their membrane composition in response to changes in their environment (e.g. temperature) it is surprising that only a very few microorganisms have a similar solvent tolerance as *P. putida* S12. It is therefore expected that also other mechanisms are involved. This is supported by the finding that *P. putida* S12 cells grown on 200 mM acetate medium had a similar membrane composition as toluene-adapted cells, but possessed a much lower resistance towards toluene (Weber, unpublished results). An effective way to reduce the toxicity of organic solvents would be a reduction of the actual concentration of these solvents in the inner membrane. A very interesting method to achieve this would be an outer membrane possessing a low permeability for these solvents in combination with an active efflux system which transports solvents molecules out of the membrane. Such a mechanism has been observed in several Gram-negative bacteria which are resistant towards various lipophilic antibiotics (Chapter 8). These active efflux mechanisms are nonspecific, and can handle a great variety of lipophilic substrates. From the observation that toluene-adapted *P. putida* S12 cells also possess an enhanced resistance towards various antibiotics it might be speculated that a multi-drug efflux mechanism contributes to the organic solvent tolerance of this strain (Isken, personal communication). It will be of great interest to see if such an efflux mechanism is indeed present.

REFERENCES

Blackburn, J.W. and W.R. Hafker. (1993). The impact of biochemistry, bioavailability and bioactivity on the selection of bioremediation techniques. *Trends Biotechnol.* **11:** 328-333.

Bryers, J.D. and C.A. Mason. (1987). Biopolymer particulate turnover in biological waste treatment systems: a review. *Bioproc. Eng.* **2:** 95-109.

Cain, R.B., R.F. Bilton and J.A. Darrah. (1968). The metabolism of aromatic acids by micro-organisms. *Biochem. J.* **108:** 797-828.

Cox, H.H.J., J.H.M. Houtman, H.J. Doddema and W. Harder. (1993a). Growth of the black yeast *Exophiala jeanselmei* on styrene and styrene-related compounds. *Appl. Microbiol. Biotechnol.* **39:** 372-376.

Cox, H.H.J., J.H.M. Houtman, H.J. Doddema and W. Harder. (1993b). Enrichment of fungi and degradation of styrene in biofilters. *Biotechnol. Lett.* **15:** 737-742.

Cruden, D.L., J.H. Wolfram, R.D. Rogers and D.T. Gibson. (1992). Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. *Appl. Environ. Microbiol.* **58:** 2723-2729.

Deheyder, B., A. Overmeire, H. Van Langehove, and W. Verstraete. (1994). Ethene removal from a synthetic waste gas using a dry biobed. *Biotechnol. Bioeng.* **44:** 642-648.

Diks, R.M.M. and S.P.P. Ottengraf. (1991). Verification studies of a simplified model for the removal of dichloromethane from waste gases using a biological trickling filter. (Part II). *Bioproc. Eng.* **6:** 131-140.

Diks, R.M.M. (1992). The removal of dichloromethane from waste gases in a biological trickling filter. PhD-Thesis, Technical University, Eindhoven, The Netherlands.

Doi, S. and K. Horikoshi. (1989). A method for microbiological reaction. European Patent Application 0 315 949 A2.

Groenestijn, J.W. and P.G.M. Hesselink. (1993). Biotechniques for air pollution control. *Biodegradation* **4:** 283-301.

Hartmans, S. and J. Tramper. (1991). Dichloromethane removal from waste gases with a trickle-bed bioreactor. *Bioproc. Eng.* **6:** 83-92.

Hartmans, S., E.J.T.M. Leenen and G.T.H. Voskuilen. (1992). Membrane bioreactor with hydrophobic membranes for waste-gas treatment. *In:* A.J. Dragt, and J. van Ham (eds.), Biotechniques for air pollution abatement and odour control policies, pp. 103-106, Elsevier, Amsterdam.

Heitzer, A. and G.S. Sayler. (1993). Monitoring the efficacy of bioremediation. *Trends Biotechnol.* **11:** 334-343.

Inoue, A. and K. Horikoshi. (1988a). Pseudomonas. European Patent Application 0 281 523 A2.

Inoue, A. and K. Horikoshi. (1988b). Method for culturing microorganisms. European Patent Application 0 285 569 A2.

Inoue, A. and K. Horikoshi. (1989). A *Pseudomonas* thrives in high concentrations of toluene. *Nature* **338:** 264-266.

Kirchner, K., C.A. Gossen and H.-J. Rehm. (1991). Purification of exhaust air containing organic pollutants in a trickle-bed bioreactor. *Appl. Microbiol. Biotechnol.* **35:** 396-400.

Majcherczyk, A., A. Braun-Lülleman and A. Hüttermann. (1990). Biofiltration of polluted air by a complex filter based on white-rot fungi growing on lignocellulosic substrates. *In:* M.P. Coughlan and M.T. Amaral Collaço (eds.), Advances in biological treatment of lignocellulosic materials, Commission of the european communities, Bruxelles.

Mason, C.A., G. Hamer and J.D. Bryers. (1986). The death and lysis of microorganisms in environmental processes. *FEMS Microbiol. Rev.* **39:** 373-401.

Middelhoven, W.J. (1993). Catabolism of benzene compounds by ascomycetous and basidiomycetous yeast and yeastlike fungi. *Antonie van Leeuwenhoek* **63:** 125-144.

Mildenberger, H.-J. (1992). Biofiltersysteme zur Geruchsbeseitigung und zur Reduzierung von Organika-Emissionen auf Kläranlagen und in der chemischen Industrie. *In:* A.J. Dragt, and J. van Ham (eds.), Biotechniques for air pollution abatement and odour control policies, pp. 187-196, Elsevier, Amsterdam.

Reij, M.W., K.D. de Gooijer, J.A.M. de Bont and S. Hartmans. (1995). Membrane bioreactor with a porous hydrophobic membrane as a gas-liquid contactor for waste gas treatment. *Biotechnol. Bioeng.* **45:** 107-115.

Swannell, R.P.J., S.J. Richardson, N.R. Passant, M. Woodfiels, J.P. van der Lugt, J.H. Wolsink, P.G.M. Hesselink, V. Hecht, D. Brebbermann and L. Bischoff. (1991). Biodegradability and emissions of volatile organic compounds form the food, drink and metal degreasing industries. EC-STEP project No 900431.

Waalewijn, E., C.D. Meijer, F.J. Weber, U.C. Duursma and P. van Rijs. (1994). Practice runs of a bio-trickling filter in the exhaust gases from a fibre factory. *In:* VDI Berichte 1104, Biologische Abgasreiniging, pp. 545-550, VDI Verlag, Düsseldorf.

Wolff, F. (1992). Biologische Abluftreinigung mit einem intermittierend befeuchteten Tropfkörper. *In:* A.J. Dragt, and J. van Ham (eds.), Biotechniques for air pollution abatement and odour control policies, pp. 49-62, Elsevier, Amsterdam.

Wolfram, J.H. and R.D. Rogers. (1991). Biodegradation of liquid scintillation cocktails. United States Patent 5,036,012.

Wright, J.D. (1993). Fungal degradation of benzoic acid and related compounds. *World J. Microbiol. Biotechnol.* **9:** 9-16.

Summary

Due to the increasing stringent legislation concerning the emission of volatile organic compounds, there is nowadays a growing interest to apply biological waste-gas treatment techniques for the removal of higher concentrations of specific contaminants from waste gases. Fluctuations in the contaminant concentrations can strongly affect the performance of bioreactors used for the treatment of waste-gas streams. Temporary high concentrations can be toxic for the microorganisms in the reactor, resulting in inactivation of the system. Furthermore, for a reliable operation the design of the reactor should be based on the peak concentrations in the waste gas, which is not an economically favourable situation. Therefore, it would be desirable to buffer the fluctuations in the contaminant concentration by means of an adsorbent, so that a constant supply of contaminants to the bioreactor can be achieved. The buffer capacity of a number of activated carbons and other adsorbents was tested (Chapter 2). Using one selected type of activated carbon it was demonstrated that fluctuations between 0 and 1000 mg toluene per m^3 could be transformed to an average value of about 300 mg/m^3, which was subsequently completely degraded in a biofilter. Without an activated-carbon column significant amounts of toluene were not degraded in the biofilter when the inlet concentration was 1000 mg/m^3.

A disadvantage of trickle-bed reactors for biological waste-gas treatment is the reduction in reactor performance which is sometimed observed due to the formation of an excessive amount of biomass resulting in clogging. By limiting the amount of nutrients available for biomass formation it was attempted to prevent clogging of the reactor (Chapter 3). As a consequence of this nutrient limitation a reduced removal rate of toluene was observed. However, when a fungal culture

was used to inoculate the reactor, the toluene removal rate under nutrient limiting conditions was almost twice as high. Over a period of 375 days an average removal rate of 27 g-C/(m^3 h) was obtained with this fungal culture. These results clearly show that, even under non-sterile conditions, inoculation of the reactor with a specific starter culture can influence the reactor performance over a prolonged period of time. From the carbon balance over the reactor and the nitrogen availability it was concluded that under these nutrient-limited conditions large amounts of carbohydrates are formed. Even under nutrient-limited conditions the biomass content (including extracellular polysaccharides and other polymers) of the reactor increased, which eventually can result in clogging of the reactor. In order to prevent clogging eventually biomass has to be removed regularly from the reactor. We therefore studied the application of a NaOH-wash to remove excess biomass. Using regular NaOH-washes an average toluene removal rate of 35 g-C/(m^3 h) was obtained with a mixed culture of bacteria. After about 50 days there was no longer a net increase in the biomass content of the reactor. The amount of biomass which was formed in the reactor equalled the amount removed by the NaOH-wash. Under these conditions it should be possible to maintain a high toluene removal rate without clogging of the reactor taking place for a long period of time.

From a biofilter used for the removal of toluene from waste gases we have isolated the fungus, *Cladosporium sphaerospermum*, which is able to grow on toluene as the sole source of carbon and energy (Chapter 4). To our knowledge this is the first report of toluene catabolism by an eukaryotic microorganism. The oxygen-consumption rates as well as the measured enzyme activities of toluene-grown *C. sphaerospermum* indicate that toluene is degraded by an initial attack on the methyl group.

The toxicity of various pollutants in a waste-gas stream for microorganisms could limit the application of biological waste-gas treatment techniques. Especially compounds, with a good solubility in water can be expected to accumulate in the water-phase of the reactor during the start-up period. This accumulation can result in the inactivation of the biomass in the reactor as the contaminant concentrations reach toxic levels. The toxicity of various volatile organic compounds frequently present as contaminants in waste gases has been determined (Chapter 5). For both the Gram-positive *Rhodococcus* S5 and the Gram-negative *Pseudomonas* S12 the toxicity was assessed as the concentration which reduced the growth rate of the bacterium with 50%. No significant differences were observed between the IC50% values for these two bacteria. A

relationship between the toxicity and hydrophobicity of various substituted benzene compounds was observed.
Surprisingly, one of the selected bacteria, *Pseudomonas putida* S12 was able to adapt to the presence of high concentrations of contaminants. This adaptation resulted in the capacity of this strain to grow in the presence of supersaturating amounts of toluene (Chapter 6).

In general, the toxicity of organic solvents is caused by the accumulation of these lipophilic solvents in the membrane lipid bilayer, affecting structural and functional properties of the membrane. The accumulation of solvents in the cell membrane can affect both the membrane fluidity and the bilayer stability. The physico-chemical effects of alkanols, alkanes and other hydrocarbons on biomembranes are summarized in Chapter 8. Although organic solvents can be highly toxic for microorganisms, some microorganisms are able to grow in the presence of concentrations of these solvents which are generally toxic (Chapter 8).

P. putida S12 and two other solvent tolerant *P. putida* strains reacted to toxic concentrations of toluene by accumulating *trans* unsaturated fatty acids in the membrane instead of the *cis* isomers. This higher *trans/cis* ratio of the unsaturated fatty acids in toluene-adapted cells resulted a higher lipid-ordering since the gel to liquid-crystalline transition temperature was about 7-9 °C higher compared to the non-adapted cells. This *cis/trans* isomerization of fatty acids is probably a mechanism to very quickly compensate for the increase in membrane fluidity, and destabilization of the bilayer structure caused by toluene accumulating in the membrane (Chapter 7 & 8). Apart from changes in the fatty acid composition also changes in the phospholipid composition were observed when the organism was grown in the presence of toluene. The decreased incorporation of phosphatidylethanolamine and the increased incorporation of diphosphatidylethanolamine (cardiolipin) are expected to prevent the formation of non-bilayer phospholipids configurations which might be caused by toluene (Chapter 8).
In Chapter 9 the results presented in this thesis are discussed in relation to biotechnological applications.

Samenvatting

Door de strengere wetgeving, die de emissie van vluchtige verbindingen regelt, is er een groeiende belangstelling om afvalgassen langs biotechnologische weg te zuiveren. Deze afvalgasstromen bevatten vaak een zeer beperkt aantal verontreinigingen in hogere concentraties. De concentraties van deze verbindingen in een afvalgasstroom kunnen sterk fluctueren en daardoor kan de efficientie van een biologische reactor sterk beinvloedt worden. Zo kunnen hoge concentraties toxisch zijn voor de micro-organismen in de reactor, en daardoor het systeem inactiveren. Daarnaast is het noodzakelijk om de dimensionering van de reactor te baseren op de te verwachten maximale belasting. Economisch gezien is dit zeer ongunstig omdat de grootte van de reactor is afgestemd op een situatie die maar gedurende korte periodes zal plaats vinden. Daarom is het wenselijk om de fluctuaties te bufferen, zodanig dat een constante hoeveelheid aan de reactor kan worden aangeboden. Hiervoor is de bufferende werking van een aantal typen actieve kool en andere adsorberende verbindingen getest (Hoofdstuk 2). Met een geselecteerd type actieve kool werd aangetoond dat fluctuaties van 0 tot 1000 mg tolueen per m^3 lucht gebufferd konden worden tot een constant nivo van ongeveer 300 mg/m^3, dat vervolgens volledig werd afgebroken in een biofilter. Indien geen actieve kool werd gebruikt, was de capaciteit van de reactor niet toereikend om de met tolueen verontreinigde lucht volledig te reinigen.

In trickle-bed reactoren die worden gebruikt voor het reinigen van verontreinigde lucht, vermindert de efficientie van de reactor vaak doordat er verstoppingen ontstaan. Deze verstoppingen worden veroorzaakt door de overvloedige groei van biomassa in de reactor. Door minder nutrienten voor de groei van biomassa toe te voegen is geprobeerd deze verstoppingen te voorkomen (Hoofdstuk 3). Als

gevolg van deze nutrient-limitatie werd een verminderde verwijderingsefficientie waargenomen. Echter wanneer een schimmelcultuur werd gebruikt om de reactor op te starten, werd de verwijderingsefficientie onder deze nutrient gelimiteerde condities bijna tweemaal verdubbeld. Met behulp van deze schimmelcultuur werd gedurende 375 dagen een gemiddelde tolueen verwijderingssnelheid van 27 g-C/(m^3 h) bereikt. Deze resultaten tonen duidelijk aan dat ook onder niet steriele condities de werking van de reactor gedurende lange tijd bepaald kan worden door beënting met een specifieke cultuur. Uit de koolstofbalans blijkt dat onder de nutrient gelimiteerde omstandigheden aanzienlijke hoeveelheden polysacchariden en andere polymeren worden gevormd. Door deze ophoping van polysacchariden neemt de biofilmdikte toe, en kunnen uiteindelijk verstoppingen ontstaan. Om deze verstoppingen te voorkomen zal er regelmatig biomassa uit de reactor verwijderd moeten worden. Om dit te bereiken werd geprobeerd de reactor met loog te spoelen om het teveel aan biomassa te verwijderen (Hoofdstuk 3). Door regelmatig te spoelen met loog werd na 50 dagen geen netto toename in het biomassagehalte van de reactor gevonden en werd gemiddelde 35 g-C/(m^3 h) tolueen verwijderd. Onder deze condities lijkt het mogelijk om gedurende lange tijd een hoge verwijderingssnelheid te handhaven, zonder dat er verstoppingen ontstaan.

Uit een biofilter welke gebruikt werd om met tolueen verontreinigde lucht te zuiveren, hebben we een schimmel (*Cladosporium sphaeropermum*) geïsoleerd. Deze schimmel is instaat om op tolueen als enige koolstof- en energiebron te groeien (Hoofdstuk 4). Dit is het eerste eukaryotische micro-organisme waarvoor dit is aangetoond. Zowel de gemeten zuurstofconsumptiesnelheden als de gemeten enzymaktiviteiten wijzen erop dat tolueen in *C. sphaerospermum* wordt afgebroken via een aanval op de methylgroep.

Vele verbindingen die voorkomen in afvalgassen kunnen toxisch zijn voor micro-organismen. Deze giftigheid kan de toepasbaarheid van biotechnologische methoden om afvalgassen te reinigen verminderen. Vooral verontreinigingen die goed in water oplosbaar zijn, zouden bij het opstarten van een reactor kunnen ophopen in de water-fase, en uiteindelijk resulteren in inactivatie van het systeem. De giftigheid van verschillende verbindingen, die veel in afvalgas kunnen voorkomen, is bepaald (Hoofdstuk 5). Voor zowel de Gram-positieve bacterie *Rhodococcus* S5 als de Gram-negatieve bacterie *Pseudomonas* S12 is de giftigheid bepaald als die concentratie waarbij de groeisnelheid van de bacterie is gehalveerd (IC50%). Tussen beide bacterien werden geen significante verschillen gevonden in de gemeten IC50% waarden. Voor de getestte

gesubstitueerde benzeenverbindingen bestond er een verband tussen de giftigheid en de hydrofobiciteit van de verbinding.
Geheel onverwachts bleek één van de gebruikte bacterien (*Pseudomonas putida* S12) zich zodanig te kunnen aanpassen dat deze uiteindelijk in staat was om te groeien in de aanwezigheid van zeer hoge concentraties tolueen (Hoofdstuk 6).

In het algemeen wordt de giftige werking van een verbinding veroorzaakt door een ophoping in het celmembraan, waarbij zowel de structuur als de werking van het membraan wordt beïnvloed. Zowel de vloeibaarheid als de stabiliteit kunnen worden verstoord. In hoofdstuk 8 worden de mogelijke effecten van alcoholen, alkanen en ander koolwaterstoffen op het membraan besproken. Tevens worden verschillende methoden behandeld, die sommige micro-organismen hebben ontwikkeld om genoemde effecten op het membraan tegen te gaan.

Pseudomonas putida S12 en twee andere *P. putida* stammen met een vergelijkbare resistentie, zetten in de aanwezigheid van tolueen de *cis*-vetzuren in het membraan om in *trans*-vetzuren. Hierdoor nam de transitie temperatuur van de starre naar de vloeibare-kristallijne fase met ongeveer 7-9 °C toe. Dit duidt op een toename in de ordening van de vetzuren in het membraan (Hoofdstuk 7). Door deze isomerisatie van *cis*-vetzuren in *trans*-vetzuren beschikt *P. putida* S12 over een mechanisme dat zeer snel het effect van tolueen op zowel de vloeibaarheid als stabiliteit van het membraan kan compenseren (Hoofdstuk 7 en 8). Naast de gevonden veranderingen in de vetzuursamenstelling werden ook aanpassingen in de kopgroep van de fosfolipiden aangetoond (Hoofdstuk 8). De toename in difosfatidylglycerol en de afname in fosfatidylethanolamine voorkomt waarschijnlijk de vorming van andere membraanstructuren die door tolueen geïnduceerd kunnen worden.
Voor verschillende biotechnologische toepassingen worden in hoofdstuk 9 de mogelijkheden van de in dit proefschrift gepresenteerde resultaten besproken.

Curriculum vitae

Frans Weber werd geboren op 26 maart 1967 te Schiedam. Na het behalen van het HAVO diploma aan de scholengemeenschap "Spieringshoek" te Schiedam begon hij in 1984 met de HLO studie Biotechnologie aan het "Van Leeuwenhoek Instituut" te Delft. Deze studie met als hoofdvakken Biochemie, Microbiologie en Proceskunde werd in 1988 met succes afgesloten. Als onderdeel van de opleiding werd tijdens een stage van 9 maanden, uitgevoerd bij de sectie Erfelijkheidsleer van de Landbouwuniversiteit te Wageningen, het pentose metabolisme van *Aspergillus niger* bestudeerd. Van november 1988 tot mei 1990 was hij werkzaam bij de sectie Industriële Microbiologie van de Landbouwuniversiteit te Wageningen, waar hij aan de zuivering en karakterisering van het alkeen-monooxygenase uit *Mycobacterium* E3 heeft gewerkt. Tussen juni 1990 en november 1990 heeft de auteur gewerkt aan de produktie van (bio)emulgatoren met behulp van micro-organismen bij de divisie Industriële Produkten van DMV Campina te Veghel. In december 1990 startte hij als assistent in opleiding (AIO) bij de sectie Industriële Microbiologie van de Landbouwuniversiteit te Wageningen aan een vierjarig promotieonderzoek. De resultaten van dit onderzoek, dat werd uitgevoerd in samenwerking met Ecosens bv te Moerkapelle, zijn beschreven in dit proefschrift.

Bibliography

Hartmans, S., F.J. Weber, D.P.M. Somhorst, and J.A.M. de Bont. (1991). Alkene monooxygenase from *Mycobacterium*: a multicomponent enzyme. *Journal of General Microbiology* **137:** 2555-2560.

Weber, F.J., W.J.H. van Berkel, S. Hartmans, and J.A.M. de Bont. (1992). Purification and properties of the NADH reductase component of alkene monooxygenase from *Mycobacterium* strain E3. *Journal of Bacteriology* **174:** 3275-3281.

Weber, F.J. and S. Hartmans. (1992). Biological waste-gas treatment with integrated adsorption for the treatment of fluctuating concentrations. *In:* A.J. Dragt, and J. van Ham (eds.) Biotechniques for air pollution abatement and odour control policies. pp. 125-130. Elsevier Science Publishers, Amsterdam.

Weber, F.J., L.P. Ooijkaas, R.M.W. Schemen, S. Hartmans, and J.A.M. de Bont. (1993). Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. *Applied and Environmental Microbiology* **59:** 3502-3504.

Weber, F.J. and S. Hartmans. (1994). Toluene degradation in a trickle-bed reactor. - Prevention of clogging. *In:* VDI Berichte 1104, Biologische Abgasreinigung, pp. 161-168, VDI Verlag, Düsseldorf.

Waalewijn, E., C.D. Meijer, F.J. Weber, U.C. Duursma, and P. van Rijs. (1994). Practice runs of a bio-trickling filter in the exhaust gases from a fibre factory. *In:* VDI Berichte 1104, Biologische Abgasreinigung, pp. 545-550, VDI Verlag, Düsseldorf.

Sikkema, J., F.J. Weber, H.J. Heipieper, and J.A.M. de Bont. (1994). Cellular toxicity of lipophilic compounds: Mechanisms, implications, and adaptations. *Biocatalysis* **10:** 113-122.

Witteveen, C.F.B., F.J. Weber, R. Bussink, and J. Visser. (1994). Isolation and characterization of two xylitol dehydrogenases from *Aspergillus niger*. *Microbiology* **140:** 1679-1685.

Weber, F.J., S. Isken, and J.A.M. de Bont. (1994). *Cis/trans* isomerization of fatty acids as a defence mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. *Microbiology* **140:** 2013-2017.

Heipieper, H.J., F.J. Weber, J. Sikkema, H. Keweloh, and J.A.M. de Bont. (1994). Mechanisms of resistance of whole cells to toxic organic solvents. *Trends in Biotechnology* **12:** 409-415.

Weber, F.J. and J.A.M. de Bont. (1994). Adaptive changes in the membrane composition of *Pseudomonas putida* S12 in response to growth in the presence of toxic concentrations of toluene. *Med. Fac. Landbouww. Univ. Gent.* **59:** 2295-2302.

Weber, F.J. and S. Hartmans. (1995). Use of activated carbon as a buffer in biofiltration of waste gases with fluctuating concentrations of toluene. *Applied Microbiology and Biotechnology* **43:** 365-369

Weber, F.J., K.C. Hage, and J.A.M. de Bont. (1995). Growth of the fungus *Cladosporium sphaerospermum* with toluene as the sole carbon and energy source. *Applied and Environmental Microbiology* **61:** in press.

Weber, F.J. and S. Hartmans. (1995). Removal of toluene from contaminated air with a biological trickle-bed reactor. Submitted for publication.

Weber, F.J. and J.A.M. de Bont. (1995) Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Submitted for publication.