Project 2.2 Exposure model for plasticizers

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Final report

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Appraisal of the program

The present Research Centre ”Environment and the Lung” was established in 1998 by funding from the subprogramme “Environmental and health damaging compounds” under the Danish Environmental Research Programme 1998-2001. The Centre is the result of integration and focusing of the following three originally independent Centre applications:

- Adjuvant effects of plasticizers and surfactants – Risk assessment and management (Otto M. Poulsen)
- Non Infectious Health Effects of Airborne Micro-organisms: NIHAM (Torben Sigsgaard)
- Human Health Effects of Exposure to Air pollution: HEAP (Ole Hertel)

The Centre was formally closed 15 April 2003.

The overall objective of the Centre has been to provide a scientific knowledge base for decision making, aiming at reduction of exposure to environmental pollutants being a substantial health risk to the population. To reach this objective the Centre has had particular emphasis on the development of new measurement methods or advanced models, which are generally applicable in risk assessment of environmental, airborne chemicals or microorganisms.

Even though many of the research activities of the Centre have the characteristics of basic research, we have developed a series of new, highly useful methods and models. Hence, the objective of the centre has to a large extend has been successfully met.

The publication list of the Centre clearly demonstrates, that the Centre has been highly productive in the dissemination of results to the scientific community as well as to decision makers and the public in general. More than 70 scientific papers and contributions to proceedings have originated from the Centre. In addition, 21 theses, reports or chapter contributions to books have been published, and participants of the Centre have made numerous oral presentations both in English and Danish.

Also, the Centre has been very active in the education of scientists. Nine Ph.D.-projects have fully or partially been carried out as part of the Centre projects.

During autumn 2001 an independent expert group carried out a midterm evaluation of the Centre. The expert group emphasized the novelty of the developed methods and models, but also suggested that spin-off and mutual benefits from collaboration between the three main research areas were limited. It is correct, that it has been difficult to obtain synergy between the three main research areas – they are simply too distant. However, it should be appreciated that the Centre emerged as a fusion of three independent Centre applications. To do this a highly restricted selection process was necessary since funding was only available for less than half of the projects of the three Centre applications. Projects were only selected if they could contribute to the establishment of necessary methods for risk assessment. In addition, the projects should also contain the potential for synergy with other projects within the particular main research areas.

A high number of researchers have been involved in the research activities of the Centre, and during the lifespan of the Centre there has been a very fruitful scientific dialog between the main research areas. Also, the Centre has collaborated with a multitude of researchers outside the Centre. Their

contribution with theoretical expert knowledge and advanced experimental procedures is gratefully acknowledged.

In conclusion, the Centre has provided the framework for highly productive and innovative research activities and strong collaborations within Denmark and internationally. A platform has been created for future research in adverse effects of the environment on the lung. We have the potential for research which is both of high relevance to the society and of outstanding scientific quality and impact. Hopefully, funding will be made available for this in near future.

We decided to prepare this final report in English to provide both national and international experts with detailed knowledge on the results and achievements of the Centre. For the Danish decision makers in governmental and private organisations we have in parallel presentedd the main results of the Centre in a special issue of "Miljø og Sundhed" (Research Centre for Environment and Health), and in a thematic issue of "Miljøforskning" (Danish Environmental Research Programme).

April, 2003

Otto Melchior Poulsen
Centre leader

Participants of the Centre

Centre leader was research director Otto Melchior Poulsen (OMP), National Institute of Occupational Health (AMI).

The board of governors:

- Research director Otto Melchior Poulsen, National Institute of Occupational Health
- Associate professor Jesper Bælum, Odense University Hospital, Department of Occupational and Environmental Medicine
- Senior scientist Ole Hertel, National Environmental Research Institute**,** Department of Atmospheric Environment
- Professor Steffen Loft, University of Copenhagen, Institute of Public Health
- Research director, Lars K Poulsen, National University Hospital, Allergy Unit
- Associate professor Torben Sigsgaard, University of Aarhus, Institute of Environmental and Occupational Medicine
- Professor Erling H Stenby, Technical University of Denmark, Department of Chemical Engineering
- During the last year, senior scientist Thomas Schneider has been scientific and administrative coordinator of the Centre, including coordination of inputs for the preparation of the final report.

The participating institutions and project leaders:

- Aarhus University Hospital, associate professor Martin Iversen, laboratory chief Hans Jürgen Hoffmann, Department of Pulmonary Medicine
- Danish Building Research Institute, senior scientist Lars Gunnarsen
- Technical University of Denmark, professor Erling H. Stenby, Department of Chemical Engineering
- Danish Veterinary and Food Administration, senior scientist Ole Ladefoged, senior scientist Elsa Nielsen, Institute of Food Safety and Nutrition
- EnPro APS, M.Sc. Eva Margareta Wallström
- National Environmental Research Institute**,** senior scientist Ole Hertel, Department of Atmospheric Environment
- National Institute of Occupational Health, senior scientist Mari-Ann Flyvholm, programme leader Uffe Midtgård, senior scientist Gunnar Damgård Nielsen, senior scientist Erik Olsen, senior scientist Håkan Wallin, senior scientist Per Axel Clausen
- National University Hospital, research director Lars K. Poulsen, Allergy Unit
- Odense University Hospital, associate professor Jesper Bælum, Department of Occupational and Environmental Medicine
- University of Aarhus, associate professor Torben Sigsgaard, associate professor Lars Mølhave, associate professor Eva C Bonefeld-Jørgensen, Department of Environmental and Occupational Medicine
- University of Copenhagen, professor Steffen Loft, Institute of Public Health

Project participants:

Traffic emission

- Helle Vibeke Andersen, National Environmental Research Institute

- Herman Autrup, Aarhus University
- Ruwim Berkowicz, National Environmental Research Institute
- Jette Bornholdt, National Institute of Occupational Health
- Lars Dragsted, Institute of Food Safety and Nutrition
- Marianne Dybdahl, National Institute of Occupational Health
- Martin Hvidberg, National Environmental Research Institute
- Lisbeth E Knudsen, Institute of Public Health, University of Copenhagen
- Peter Møller, National Institute of Occupational Health
- Finn Palmgren, National Environmental Research Institute
- Ole Raaschou-Nielsen, Institute of Cancer Epidemiology, Danish Cancer Society (DCS)
- Henrik Skov, National Environmental Research Institute
- Steen Solvang Jensen, National Environmental Research Institute
- Mette Sørensen, Institute of Public Health, University of Copenhagen
- Peter Vinzents, Institute of Public Health, University of Copenhagen
- Ulla Vogel, National Institute of Occupational Health
- Peter Wåhlin, National Environmental Research Institute

Micro-organisms and their biologically active components

- Kurt Andersson, Department of Occupational and Environmental Medicine, Örebro Medical Centre
- JJ Bønløkke, Department of Environmental and Occupational Medicine, Aarhus Universitet
- Gert Doekes, Department of Occupational and Environmental Medicine, Odense University Hospital
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- T Karlsson, Department of Occupational and Environmental Medicine, Örebro Medical Centre
- Søren K Kjærgaard, Department of Environmental and Occupational Medicine, Aarhus Universitet
- Tanja Krüger, Department of Environmental and Occupational Medicine, Aarhus Universitet
- Preben Larsen, Department of Occupational and Environmental Medicine, Odense University Hospital
- Håkan Lövsted, Department of Occupational and Environmental Medicine, Örebro Medical Centre Hospital
- Göran Stridh, Department of Occupational and Environmental Medicine, Örebro Medical Centre Hospital.

Plasticizers and surfactants

- Ali Afshari, Danish Building Research Institute
- Rikke L Lindeberg Bille, National Institute of Occupational Health and DHI Water and Environment
- Søren Bøwadt, European Commision, Research Directorate (B7, 3/12)
- Hongyuan Cheng, IVC-SEP, Department of Chemical Engineering, Technical University of Denmark
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- Lars Gunnarsen, Danish Building Research Institute
- Vivi Hansen, National Institute of Occupational Health

- Jim Hart, Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration
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- Søren Thor Larsen, National Institute of Occupational Health
- Anne Kirstine Müller, Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration
- Tobias Nilsson, Dept. of Analytical Chemistry, Lund University
- Bo Svensmark, Department of Chemistry, University of Copenhagen
- Grete Østergaard, Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration.

Collaboration with researchers outside the Centre
The Centre has cooperated with a large number of researchers in Denmark, with researchers engaged in EU research programs and with other researchers from Sweden, Germany, the Netherlands, and the UK. The most important contributors were:

Traffic emission
- Professor Henrik E Poulsen, Dept of Clinical Pharmacology, National Hospital
- Associate professor, DMSc Poul Kristjansen, Institute of Molecular Pathology

- Professor Jos Kleinjans, Maastricht University, The Netherlands
- Dr. Andrew Collins, Rowett Institute, Abeerdeen, Scotland
- Dr. Ryszard Olinski, Bydgodz University, Poland
- Dr. Frank Raes og Dr. Rita van Dingenen, Joint Research Centre ISPRA, Italy
- Dr. Paul Borm, Düsselsdorf University
- Dr. Roel Schins, Düsselsdorf University
- Dr. Jan Beyea, Consulting in the Public Interest, Box 220, Lambertville, NJ 08530, USA

Micro-organisms and their biologically active components
- MD, Dr.MSc. Ivan Brandslund, Department of Clinical Chemistry, Vejle County Hospital
- Professor Carsten Bindslev Jensen, Allergy Center, Odense University Hospital
- Professor Per Stahl Skov, Department of Allergy, National University Hospital
- Senior researcher Bent Løschenkohl, Danish Institute of Agricultural Sciences

- Dr. Jens Seedorf, Tierärtzliche Hochschule Hannover, Germany
- Professor Leif Bjermer, Trondhjems Universitet, Norway
- Environmental and Occupational Health Group, University of Utrecht, The Netherlands

Plasticizers and surfactants
- Professor Yves Alarie, University of Pittsburgh
- Professor C. C. Chen, Aspen Tech, Boston, USA
- Prof. Harald Renz, Dept. of Clinical Chemistry, University of Marburg, Germany
- Dr. Leif Øie, Dept. of Population Health Sciences, Nat. Inst. of Public Health, Oslo

Contents

Overall background and objectives

The overall objective of the Centre was to provide a scientific knowledge base for decision making, aiming at reducing exposure to environmental pollutants being a substantial health risk to the population. To achieve this objective the Centre had particular emphasis on the development of new measurement methods and advanced models, that are generally applicable in risk assessment of environmental, airborne chemicals or micro-organisms. The research activities of the Centre have focused on pulmonary effects of exposures within the three high priority areas:

- Traffic emission: Project 1.4 and 2.1
- Micro-organisms and their biologically active components: Project 1.1, 1.2, 1.5, 2.6, and 3.5
- Plasticizers and surfactants: Project 1.3, 1,6, 2.2, 2.3, 2.4, 2.5, 3.1, 3.2, 3.3, and 3.4

Due to resource and time constraints it was not possible to include all elements of risk assessment within all three areas. Thus, each area focused on selected elements of risk assessment. Of the 17 projects one covered literature review, six were within hazard identification, six within exposure assessment, and five within risk assessment and risk management.

Traffic emission is today the main source of air pollution in the cities of Denmark. It is well known that long term exposure to high concentrations of air pollution from traffic, industry and power plants can lead to increased risk of cancer and cardiovascular diseases in humans, whereas short term exposure may lead to exacerbation of bronchitis, asthma and other respiratory tract diseases. With respect to traffic regulation policy it is of great strategic importance to obtain knowledge on the impact of traffic emission on the health of the Danish population. Knowledge was limited particularly on health effects of exposure to small particles from traffic emission, and the possibility of obtaining such knowledge was hampered by the lack of valid high-resolution air pollution models. Consequently, a major objective of the Centre was to develop a high resolution street air pollution model (for Greater Copenhagen), both for the prediction of the effect of traffic regulation on air pollution levels, and for the estimation of short and long term exposure level for traffic generated pollution of each individual in epidemiological studies. Emphasis has been on models for small particles that are the result of combustion in diesel engines (DEP) and which are considered particularly hazardous, and the Centre has included hazard identification studies on DEP.

Thanks to substantial additional funding the Centre succeeded in conducting extensive measurements comprising four measurement campaigns that also provide data on annual variations etc.

Exposure to bio-aerosols containing micro-organisms or biologically active components thereof may pose a health risk not only in isolated occupational settings with high exposure (e.g. cotton mills, waste handling facilities, and swine confinements) but also more widespread in private houses, schools and Kindergartens. Knowledge was limited on the pulmonary health effects of exposure to non-infectious airborne micro-organisms and biologically active components thereof. Thus, the Centre has focused on the development of methods both for the identification of susceptible individuals and for hazard identification of biological agents. Overall, the focus has been on the study of mechanisms that are relevant for hazard identification.

Plasticizers and surfactants are high volume chemicals used in numerous products and spread to the environment in large quantities. Plasticizers, particularly phthalates, and surfactants are present on indoor air dust, and recent studies indicated that surfactants and plasticizers may enhance the health damaging potential of common allergens (possess adjuvant effects). The potential adjuvant effects of plasticizers and surfactants are of particular strategic interest because the prevalence of allergic diseases and symptoms is very high and increasing in the Danish population. The Centre has identified exposure routes, which has also included development of exposure assessment models. No methods existed for hazard identification of adjuvant effects related to the development of pulmonary allergy. Thus, the Centre has to a large extent developed new methods.

The quality of research at the Centre is documented in the large number of scientific publications and presentations originating from the Centre. In accordance with the aims in the Danish Environmental Research Programme the Centre also has been very active in disseminating the knowledge at a national level to administrative and political decision makers in e.g. agencies and governmental departments.

Finally, the Center has contributed substantially to education of researchers (PhD and Master students). Thereby, the Centre has contributed to the recruitment of environmental researchers, and the participating research institutions have created the necessary platform for continuing the research activities of the Centre after termination of the program.

Overall achievements of the Centre

To reach the overall objective the Centre has had particular emphasis on the development of new measurement methods or advanced models, which are generally applicable in risk assessment of environmental, airborne chemicals or microorganisms. In this respect the Centre has been very successful, and we have developed a series of new methods and models.

The publication list of the Centre clearly demonstrates, that the Centre has been highly productive in the dissemination of results to the scientific community as well as to decision makers and the public in general. More than 70 scientific papers and contributions to proceedings have originated from the Centre. In addition, 21 theses, reports or chapter contributions to books have been published, and participants of the Centre have made numerous oral presentations both in English and Danish.

Also, the Centre has been very active in the education of scientists. Nine Ph.D.-projects have fully or partially been carried out as part of the Centre projects.

Traffic emission

One major specified objective of this part of the Centre was to provide a platform for the preparation of a large Centre application for research funding from the subsequent research programme for "Transport Research on environmental and health Impacts and Policy" (TRIP) under the Danish Environmental Research Programme 2000. This was successfully achieved, and the application for the large inter institutional research centre was granted 19.5 mio. DKK, of which research on traffic and health and on exposure modelling received 8.5 mio DKK. In addition, the Centre research group on health effects of traffic emission has obtained a series of other grants.

Hazard identification models

In relation to hazard identification we have successfully established an inhalation model in mice. The model has been used to study the genotoxicity and inflammatory potency of diesel exhaust particles (DEP) and other fine particulate matters.

Exposure models

One of the main objectives of the Centre was to develop and validate a model for personal exposure assessment to ambient air pollution, including gaseous compounds and particles for the population of Greater Copenhagen. This has been achieved, and the results on traffic emission have been presented in several scientific and popular papers; see the list of publications for further details. The further validation of the model is a part of the TRIP project. The project has been extremely valuable in providing new knowledge of personal exposure to air pollution, which is applicable nationally as well as internationally. Moreover, the generated dataset is indispensable for the model development, which a whole new series of studies on health effects rely on (TRIP).

Data on biomarkers have been used to validate the personal exposure model. The collected biological samples are kept in a bio-bank for further measurement of biomarkers pending further funding. Such funding has been partly obtained from The Environmental Medicine Research Centre of the Health Ministry and more funds will be applied for in 6th Framework in EU and National funding including Cross Disciplinary Research Groups etc.

Microorganisms and their biologically active components

The main objective in this area has been to study mechanisms of relevance for hazard identification, including the importance of individual susceptibility and hazard identification of complex biological agents. This is a huge research area. The Centre has contributed significantly both by providing new methods and by increasing the current basic knowledge on the mechanisms. The second objective was risk assessment of the use of bio-pesticides in greenhouses.

Mechanisms and hazard identification

The study on previous farming students suggests that persons, who have left the occupation due to work related respiratory symptoms caused by inhalation of organic dust, have different pulmonary reactions compared with persons, who have left the occupation without symptoms. Hence, this study challenges the relevance of several previous exposure experiments with naïve subjects in this occupational setting. We hereby have set a new standard for this type of studies.

A series of experiments, aimed at hazard identification of β-(1,3)-D-glucan, indicate that β-(1,3)-D-glucan, when present on dust particles, may have a strong inflammatory potency. Hence, it may be that β-(1,3)-D-glucan pose a genuine health hazard when people are exposed to organic dust.

One particular achievement of the Centre has been the characterization of the full-blood assay for hazard identification purposes. We have highlighted the potential usefulness of this new method by clearly demonstrating the high inflammatory potency of LPS, β-(1,3)-D-glucan and swine confinement dust *in vitro.* This study also demonstrated that there is a difference in the gene activation (mRNA level) between atopics and non-atopics. Furthermore, it has been demonstrated that RT-PCR analyses of gene activation have the potential to detect weak responses at lower exposure levels than can be detected using direct analysis of the gene products. Finally, we have shown that there is a difference between the potential of agents to induce cytokine protein release and activation from the cells.

Risk assessment of the use of bio-pesticides in greenhouses

The Centre has performed a 3-year follow-up epidemiological study of the effects of exposure to three types of microbiological pesticides on symptoms and signs of inflammatory diseases in greenhouse workers. In conclusion the study shows a relatively high prevalence of symptoms, and the results indicate a relation to exposure to bio-pesticides. The results from the last follow up are presently being analysed and will be published in near future. This will give more reliable estimates of the risk related to occupational exposure to the different groups of microbiological pesticides.

Plasticizers and detergents

The overall aim of these research activities was to add new knowledge of relevance for the risk assessment of plasticizers (phthalates) and detergents (surfactants) in relation to adjuvant effects, i.e. the ability to induce the development of allergic sensitisation over the development on tolerance. However, at an early stage highly interesting results were obtained for adjuvant effects of phthalates. It thus was approved by the Danish Environmental Research Programme to strengthen research on phthalates within this priority area. A consequence of this decision is, of course, that the results obtained from the projects on structure-activity modelling of tensides cannot be integrated in the overall output from the Centre to the extent originally planned. Nevertheless, the structure-

activity modelling of tensides has been highly successful, and the Centre has added a new dimension to the art of structure-activity modelling.

Hazard identification

The Centre has developed two new murine *in vivo* models for hazard identification of phthalates with respect to adjuvant effects. The first model is a screening test based on subcutaneous injection of test compound. Adjuvant effect was demonstrated for one out of four different tensides tested. In addition, adjuvant effects were demonstrated among the six most important mono-phthalates, as well as the five most important phthalates. The adjuvant effect was dependent on the dose and the length of the lipophilic side chain.

The second model is an inhalation model for the study of allergic sensitisation via the airways. No such model was available, and the development of this model turned out to be a very difficult task. However, a novel and highly unique model has now been developed. Even though time did not allow for test of phthalates in this model, the model provides a very strong platform for future research in adjuvant effects of chemical compounds.

In addition, we have developed and applied new *in vitro* models for the study of inflammatory potency (i.e. the ability to induce cytokine production) and cytotoxicity of xenobiotics. These models were applied on the six water-soluble mono-phthalates, but not the insoluble phthalates. Together with the *in vitro* methods developed by the Centre as part of the study on biological active compounds of microorganisms, we have developed a very useful battery of *in vitro* methods for fast screening of the inflammatory potency of xenobiotics in future.

Exposure assessment

Qualitative data on the presence of phthalates and surfactants in different products were obtained from the product register PROBAS showing that these compounds are present in numerous products.

A large effort of the Centre has been to produce experimental data on emission of phthalates from building materials and other products, and data on the content of phthalates in dust from domestic dwellings and institutions. This has included development and application of new or modified methods for emission testing and chemical analysis.

EUSES was applied for modelling of consumer exposure to phthalates. The highest exposure of adults seemed to be the dietary (indirect) exposure followed by either the exposure via inhalation of indoor air or dermal contact to gloves and other phthalate-containing products (different for the various phthalates). For children, the exposure via phthalate-containing toys contributed most significantly to the total daily intake. For infants a major route of exposure was via intake of infant formulae.

Risk assessment and structure-activity relationship

EU expert groups have recently made a critical toxicological evaluation of phthalates. The critical effects identified for four of the five phthalates were toxicity to reproduction, the liver and kidney. Since awareness of adjuvant effects of phthalates first raised lately during this critical evaluation process, adjuvant effects were not included in this critical evaluation. Thus, the risk assessment of the adjuvant effects of phthalates is a major achievement of the Centre, indicating that the measured

concentrations of phthalates in dust from dwellings and institutions may pose a genuine risk of adjuvant effects in humans.

Several physicochemical parameters of phthalates were evaluated for structure-activity relationship (SAR) analyses regarding the adjuvant effects. However, none of the parameters seemed more efficient than the (simple) number of carbon atoms in the lipophilic side chain of the molecules.

A particular achievement of the Centre has been the development of models for the prediction of physical properties of groups of tensides based on their chemical structure. Such models have previously been developed for fairly simple molecules like organic solvents, but never for complex, surface-active molecules with a biphasic nature. The effort of the Centre included a comprehensive review on existing knowledge on physical properties of tensides. In future the models developed by the Centre have interesting technical applications both with respect to design of new tensides with predictable physical and technical properties, and with respect to more advanced modelling of SAR for biological effects of different groups of tensides.

Individual projects

In the following the results and achievements of the Centre are presented in detail.

Project 1.0 State of the science review

This project has been reported in "Oversigt over vidensgrundlaget for risikovurdering af luftforureninger i relation til lungekræft og luftvejsallergi. Trafikforurening - Mikroorganismer - Blødgørere og tensider", Arbejdsmiljøinstituttet, May 2000, with English summary.

Traffic emission

A major purpose of supporting these activities in the present programme was to enable the researchers to prepare a large research activity under the programme for research on traffic (Danish Environmental Research Program 2000). The research groups have been highly successful in obtaining grants from this programme and the projects are listed in section "Related and derived research programmes". The Centre projects described below are closely connected to and partly integrated in these projects.

Project 1.4 Pulmonary effects of diesel particles in vivo.

Project leader: Håkan Wallin

Project participants: Marianne Dybdahl, Jette Bornholdt, and Ulla Vogel, National Institute of Occupational health. Steffen Loft and Peter Møller, University of Copenhagen, Institute of Public Health. Herman Autrup, Aarhus University.

Background and objectives

The purpose of this project was to initiate the development of a fast, highly sensitive *in vivo* model, based on the use of transgenic mice for hazard identification of complex carcinogenic exposures. The project focused on diesel particles, and on the comparison of genotoxic effects observed in mice under controlled exposure condition and genotoxic effects observed in humans exposed to ambient air pollution in the city of Copenhagen (Project 2.1). Transgenic mice were used for a fast identification of target organ specific genotoxic effects, being indicative for an excess risk of cancer. In addition, genotoxic effects were studied *in vitro* on isolated human lung epithelium A549 cells (cell line assay) and compared with the *in vivo* genotoxic effects. Coal tar has a similar composition of polycyclic aromatic hydrocarbons as those that can be extracted from diesel exhaust particles. Therefore coal tar was used for establishing the mutagenic effect.

Ozone is a well-known oxidant pollutant present in photochemical smog. Although ozone is suspected to be a respiratory carcinogen it is not regulated as a carcinogen in most countries.

Methods

The animal experiments were conducted in 8-week old male Muta^{TM}mouse or non-transgenic BALB. Mutational activity was studied by isolation of genomic DNA from target organs, and after cloning into E. coli Lac Z mutations were identified by positive selection on P-galactose plates.

The dorsal skin of C3H/Tif/hr hairless mice was painted with coal tar, pharmacological grade. Epidermal cells and hepatocytes were isolated after 4, 24, 48, and 96 h and DNA strand breaks were determined as tail moment by the alkaline comet assay. The identity of mutations in the Lac Z gene was determined the mutations by DNA sequencing.

SRM 1650 Diesel emission particles, 0, 0.7, or 2.1 mg, were suspended is saline as installed intratracheally in three groups of each 12 Guinea pigs.

Diesel emission particles, SRM 1650, from the National Institute of Standards, USA, were dispersed and inhaled by groups of mice at four doses. The biomarker levels were determined in the relevant target organ and cells.

The genotoxic and inflammatory effects of ozone were investigated in female mice exposed to ozone for 90 min. The tail moment in bronchoalveolar lavage (BAL) cells from BALB/c mice was determined by the comet assay as a measure of DNA strand breaks.

Results

The tail moment in the Comet assay of epidermal cells was significantly greater at the time points 4, 24, 48, and 96 h after exposure compared to the controls, with the most DNA strand breaks at 24 h. The DNA strand breaks in epidermal cells increased linearly with the dose of coal tar. In hepatocytes, no difference in DNA strand breaks was found between exposed animals and controls. DNA adducts were determined by the 32P-postlabeling assay. For epidermal cells, the mean DNA adduct level was 12-fold greater in coal tar painted mice after 24 h than in controls. Again, a linear dose/response relationship was seen 24 h after painting. For liver DNA, the mean DNA adduct level was 3-fold greater than for controls. The mutation frequency in epidermal and liver cells was examined in lambdalacZ transgenic mice (MutaMouse). Thirty-two days after painting, the mutation frequency in epidermal cells was 16-fold greater in coal tar treated mice compared to controls. No effect was detected in hepatocytes. We found that a single painting of coal tar resulted in strong genotoxic effects in the murine epidermis, evidenced by induction of DNA strand breaks and DNA adducts in hairless mice and lambda lacZ mutations in the MutaMouse. This demonstrates that it is possible to detect genotoxic effects of mixtures with high sensitivity in mouse skin by these end-points. Coal tar was found to primarily induce G:C to T:A transversions and one-base pair deletions of G:C base pairs. More than half of the mutations were at CpG sites. The mutational spectrum is in agreement with that of benzo[a]pyrene and other polycyclic aromatic hydrocarbon mixtures.

Guinea pigs exposed to DEP had increased levels of bulky DNA adducts, DNA strand breaks, and 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG) in the lung five days after intratracheal installation of SRM 1650. A dose-dependent decrease of antioxidant enzyme activity was seen in red blood cells. There was no difference in DNA strand breaks in lymphocytes, or urinary excretion of 8-oxodG at the three doses tested. Protein oxidations in plasma and on hemoglobin were not altered by DEP exposure. There was an increased ratio of protein oxidation markers in the lungs of exposed animals, indicating a local increase in metal-catalyzed oxidation and/or a decrease in peroxidase-mediated protein oxidation. The ascorbate status in liver, lung, and plasma was unaltered by the DEP exposure. The results suggest that intratracheal instillation in addition to formation of bulky DNA adducts, induces a localized oxidative stress with accompanying oxidative DNA damage in the lung, and that depletion of ascorbate is not a prerequisite for the generation of oxidative DNA damage in lung of Guinea pigs.

Following inhalation of DEP for 1,5h mice showed signs of acute inflammation with a 7-fold increased induction of IL-6 in the lung and infiltration of neutrofilic granulocytes in the lung fluid. Inhalation of DEP caused DNA-damage (strand breaks) in cells in the lung fluid, but no induction of mutations was seen in the lung for these relatively short exposures. For comparison, ozone likewise causes DNA-damage in cells in the lung fluid and inflammation in the lung.

We exposed mice to 0, 5 and 20 mg DEP/m^3 by inhalation for 90 min on 4 consecutive days. One h and 22 h after the last inhalation inflammation and DNA damage by the comet assay were determined in bronchoalveolar lavage fluid (BAL) and lung tissue. Mice exposed to 20 mg DEP/m^3 had a 3-fold increase in the total cell count in the BAL with a marked increase in the fraction of neutrophile granulocytes compared to unexposed controls. The interleukin-6 mRNA expression in the lung tissue was induced 10-fold by inhalation of the 20 mg DEP/m^3 after 1 h, but the level returned to the background level at 22 h. DNA damage was higher in all DEP-exposed mice compared to control mice.

DEP caused DNA-damage and induced inflammatory mediators (IL-1α, IL-6, IL-8 and TNF-α) and DNA damage by the comet assay in the human lung epithelial cell line A 549 in culture.

Within the first 200 min after exposure, the BAL cells from the mice exposed to 1 or 2 ppm ozone had 1.6- and 2.6-fold greater tail moments than unexposed mice. After 200 min there was no effect. It could be ruled out that the effect during the first 200 min was due to major infiltration of lymphocytes or neutrophils. Unexpectedly, ozone had no effect on the content of 8-oxo-deoxyguanosine (8-oxo-dG) in nuclear DNA or on oxidised amino acids in the lung tissue. The mRNA level of the repair enzyme ERCC1 was not increased in the lung tissue. Inflammation was measured by the cytokine mRNA level in lung homogenates. An up to 150-fold induction of interleukin-6 (IL-6) mRNA was detected in the animals exposed to 2 ppm ozone compared to the air-exposed control mice. Also at 1 ppm ozone, the IL-6 mRNA was induced. The large induction of IL-6 mRNA in the lung took place after DNA strand breaks were induced in BAL. This does not support the notion that inflammatory reactions are the cause of DNA damage. To determine whether these exposures were mutagenic, Muta Mice were exposed to 2 ppm ozone, 90 min per day for 5 days. No treatment-related mutations could be detected in the cII transgene lung.

Conclusions

We have successfully established an inhalation system for mice. The system has been used for exposure to diesel exhaust particles (DEP) and other fine particulate matters, and the genotoxicity of DEP and inflammatory potency has been investigated. DEP is a potent inflammatory agent and it induces DNA damage in cells in the lumen of the lung. Similar effects have been detected in Guinea pigs after intrathacheal installation and in cells in culture.

Coal tar, as substitute for extractable DEP aromatic hydrocarbons, is potently mutagenic to mice.

A short episode of ozone exposure - at five times the threshold limit value (TLV) in US - induces lung inflammatory mediators and DNA damage in the cells in the lumen of the lung.

Project 2.1 Development of an air pollution model for traffic emission - Biomarkers and Air samplers for Assessment of Exposure and Effects of Urban Air Pollution – BIOAIRPEX

Project leaders Ole Hertel and Steffen Loft.
Project participants: Ole Raaschou-Nielsen, Danish Cancer Society (DCS), Institute of Cancer Epidemiology. Steen Solvang Jensen, Helle Vibeke Andersen, Henrik Skov, Ruwim Berkowicz, Finn Palmgren, Peter Wåhlin, Martin Hvidberg, National Environmental Research Institute. Herman Autrup, Aarhus University. Lars Dragsted, Institute of Food Safety and Toxicology

This project has been the largest individual project of the research Centre and has included numerous measurements of exposure and a range of biomarkers.

Background and objectives
The present levels of traffic generated air pollution in Copenhagen and other Danish cities may cause significant health risks in the general population. The budget for the Centre did not leave possibilities for supporting activities covering the total chain: traffic emissions - air pollution - exposure - dose - effect. Therefore, the emphasis was on the development and testing of air pollution exposure models. An important activity was the preparation of applications aiming at extending the originally planned activities. Such extensions included use of the developed air pollution exposure models for providing links to ongoing epidemiological studies as well as carrying out detailed animal and human dose - response experiments. As mentioned earlier, this was very succesful.

The objectives of this project were to develop and validate a model for personal exposure assessment to ambient air pollution, including gaseous compounds and particles for the population of Greater Copenhagen.

Methods
The personal exposure model based on the Operational Street Pollution Model (OSPM) as the central part has been developed. OSPM was originally developed in 1989 and improved and refined over the years enabling it to calculate hourly mean concentrations of nitrogen dioxide, nitrogen monoxide, carbon monoxide and benzene at fixed positions. A module for treating traffic-emitted particles in the OSPM has been developed and was funded from other sources. The present project has developed a separate route module for the OSPM model, which can calculate personal exposure based on time-activity data a person.

The models were validated by individual measurements of external dose in the breathing zone and internal and biologically effective doses in relation to individual susceptibility estimated by bio-markers.

Results
Determinants and biomarkers of internal and biologically effective dose and susceptibility of personal exposure to particulate matter (PM2.5) and NO_2 have been assessed in 50 healthy subjects in each of the 4 seasons over one year (1999-2000). Results obtained by individual samplers and samplers inside and outside the subjects' residence as well as in urban background and busy streets were compared. Measurements of PM2.5 and NO_2 worked satisfactory. A passive NOx sampler was

tested but did not work properly. A passive benzene sampler was also tested during a campaign but the results were too uncertain since the levels were too close to the detection limit.

The routes of the students during the 48-h monitoring periods were tracked by use of GPS and questionnaires including maps. The diary-based method provides routes for all participants whereas the GPS often failed due to technical and handling errors. All routes have been digitised for the calculation of the personal exposure.

For the exposure calculations a separate route module was developed for the OSPM model. This module can calculate personal exposure along a route of a study subject. Route data were collected using questionnaires and routes drawn on maps. Routes have manually been digitised on a road network from the drawn routes, but they have also been registered by means of GPS (Global Positioning System). A GIS based model able to generate route data for the OSPM route module has been developed. Route data is here represented as a series of points along a stay and transport route. The personal exposure model is part of the so-called AirGIS system, which is an air pollution exposure system based on the OSPM, digital maps, national registers and GIS. AirGIS was originally developed to estimate exposure levels at addresses using the address as exposure indicator for personal exposure. The present project has expanded the model system into a personal exposure model.

A preliminary validation of the personal exposure model has been performed based on data from campaign number 4 where modelled and measured NO_2 has been analysed. The validation will continue on the rest of the data set. Comparisons between model output and monitored pollution and biomarker levels will be published in international journals. A comparison between time-activity data obtained by questionnaires and maps, and GPS will also be published in an international journal.

Biomarker results
Determinants and biomarkers of internal and biologically effective dose and susceptibility of personal exposure to particulate matter (PM2.5) and NO_2 have been assessed in 50 healthy subjects in each of the 4 seasons over one year. Results from individual samplers and samplers inside and outside the subjects' residence as well as in urban background and busy streets were compared. Biomarkers include a battery of genotoxicity markers, including oxidative DNA damage by comet assay and 8-oxodG and PAH DNA adducts as well as markers of oxidative stress and hematology. Susceptibility markers include a battery of metabolism enzymes. So far, data under publication include significant correlations between biomarkers of oxidative stress, oxidative DNA damage and personal exposure to $PM_{2.5}$ and black smoke.

The BIOAIRPEX study will continue with measurement of personal exposure to ultrafine particles and relations to biomarkers of exposure in specified traffic scenarios. The generated data are also used for validation of modeling of exposure based on geographic information systems.

Bio-bank
The collected biological material consisting of whole blood, isolated and cryo-preserved lymphocytes, isolated lymphocyte DNA, plasma and urine is kept in a bio-bank for further measurement of biomarkers pending further funding. Such funding has been partly obtained from The Research Centre for Envvironmental Health of the Health Ministry and more funds will be applied for in 6th Framework in EU and National funding including Cross Disciplinary Research

Groups etc. Currently, expression of DNA repair and oxidative stress response genes is investigated by newly developed methods. The biobank is also used for assessment and possible beneficial effects in relation to air pollution of intake of substances in plants and fruits by measuring the levels in urine.

Ongoing and future studies
For a grant from Research Centre for Environmental Health (ISMF) the Centre for Environment and Cancer headed by Steffen Loft has been established and with participants of a major part of the project group of the present Centre. The new centre studies associations between environmental factors, including traffic emission, and cancer in lung and breast based on population studies in Copenhagen and Aarhus.

In the final Danish Environmental Research Programme centre TRIP the models developed by the present Centre are employed to study health outcomes in terms of acute cardiopulmonary morbidity and mortality, birth weight, airway symptoms in small children at risk of atopy, annoyance and selfreported symptoms, and obstructive lung disease. Studies in small children are also funded by the Danish EPA. Moreover, based on funds from the Research Centre for Environmental Health (ISMF) and the Danish EPA similar studies focused on measurement and modelling of personal exposure to ultrafine particles are ongoing. In this study an integrated GPS and mobile phone is applied for tracking individuals that promises very few technical and handling problems. Further funding will be applied for from the Health Effects Institute, USA.

Conclusions
The project has been extremely valuable in providing new knowledge of personal exposure to air pollution, which is applicable nationally as well as internationally. Moreover, the generated dataset is indispensable for the model development, which a whole new series of studies on health effects rely on. Analysis of the material in the biobank will provide further new important knowledge of oxidative stress and other biological effects of air pollution.

Micro-organisms and their biologically active components

Project 1.1 Acute airway inflammation during work in swine confinement buildings. AIBAL

Project leaders: Martin Iversen & Hans Jürgen Hoffmann, Department of Lung Disease, Aarhus University Hospital.

Background and objectives

Respiratory disease has emerged as an important occupational disease in farmers. Farming is a high-exposure and high-risk occupation for the development of respiratory symptoms related to work and probably also to the development of respiratory disease. This problem concerns 20.000 persons in Denmark and is one of the most important problems regarding occupational lung diseases.

The aim of the project was to investigate the acute bronchial inflammatory response in young farming students from a longitudinal survey. Some farming students develop respiratory symptoms after a few months in work in pig confinement buildings and leave the occupation while others do not develop symptoms and leave the occupation for other reasons. The specific aim of the study was to investigate the different susceptibility to organic dust in the two groups and elucidate the basic mechanisms of disease.

This is the first study to include persons from a longitudinal study known to have developed symptoms during work, whereas previous studies on acute effects of organic dust exposure have used students not previously exposed to farming. Consequently, the results can be used for focusing risk assessment on the susceptible sub-populations.

Methods

Measurement of lung function (Body-plethysmography with measurement of diffusion capacity for carbon monoxide with single-breath technique and measurement of bronchial reactivity by metacholine challenge) was made one week before exposure and the day after exposure. In addition, bronchial reactivity was measured one week and two weeks after exposure.

We were looking for a tendency, in persons more sensitive to pig dust, of the acute response to feed into a chronic response too early, or following the wrong stimuli. Experimentally we were looking for differences in the immune response of persons more and less sensitive to dust in pig confinement buildings. Thus we followed the onset of the acute and chronic responses in blood (plasma and serum), and in broncho-alveolar lavage (BAL). This included measurement of relevant cytokines in plasma and serum taken from one hour after the participants start working until 24 hrs later, but especially in the first 8 hours after start of exposure. Both plasma and serum were taken as serum giving an indication of the cytokines released during a clotting reaction, whereas plasma levels are the levels of cytokines actually found in the body at that time. They give an indication of the effective level of cytokine released in response to the exposure rather than a measure of the capacity of blood cells to synthesize these cytokines. IL-1b, IL-4, IL-6, IL-8, Interferon γ, TNF and GM-CSF were measured in blood and BAL. Complement and other acute phase proteins were measured in blood. Relevant surface markers on leukocytes in blood and BAL were measured.

Measurement of acute and chronic immune response in BAL and in blood/serum included cytokines and surface markers for subpopulations of T-cells and macrophages. A number of the selected

cytokines were also measured in the *in vitro* studies in projects 1.5 and 1.6. This enabled us to compare the immune response *in vitro* with the human *in vivo* response to organic dust containing microbial agents. In addition, comparison was made with the results obtained *in vivo* in project 1.3.

Results

The project differs from earlier experimental studies by using persons who previously have worked in farming and of whom half developed airway symptoms during work. We have included smokers, because they particularly easy develop airway symptoms during work with swine production. Exposure also differs from previous experiments, by being considerably lower and being more realistic.

The results show low to average exposure to respirable dust: 0,16 mg/m^3 and 0,18 mg/m^3 in control respectively symptom group. The corresponding concentrations for total dust were 4,1 and 5,4 mg/m^3. Concentrations of respirable endotoxin was 1,1 ng/m^3 and 4,1 ng/m^3.

Contrary to previous experiments no acute changes were seen in spirometry (FEV_1 and FVC) or in bronchial reactivity following metacholine provocation (PD_{10} and PD_{20}). For the first time in such a study the diffusion capacity has been measured. We found considerable and significant changes in non-smoking cases (-10,5%), smoking cases (-7,5%) and smoking controls (-6,25%), but not in non-smoking controls (+0,5%). Thus there is a considerable physiologic reaction at low exposure that does not induce acute changes in spirometry. The cause is not yet known.

Investigation of cells in BAL did not show a difference in acute phase reactant proteins (alpha-1-antitrypsin, fibrinogen and orosomucoid) between the groups, but the cases had higher concentration and larger increase in hyaluronic acid. Cytokine response (IL-6 and IL-8) showed significant increase in BAL, but without difference between the groups. The immunological response was down-regulated for the persons previously exposed to organic dust compared with volunteers (used in other studies), who had not previously been exposed to such occupational environments.

Conclusions

There are differences in the acute immune response of cases and controls. The results suggest that persons with work related respiratory symptoms due to organic dust have different pulmonary reactions than persons without symptoms. Thus, the present study challenges the relevance of results obtained from exposure experiments with naïve subjects in this occupational setting.

Project 1.2 Cytokine release from mucosal membranes after experimental exposures to organic dust - DAMOS.

Project leaders: Torben Sigsgaard, Lars Mølhave, Department of Environmental and Occupational Medicine, University of Arhus
Participants: SK Kjærgaard, J Bønløkke, E. Bonefeld-Jørgensen, Department of Environmental and Occupational Medicine, University of Arhus. JE Juto, Huddinge Sjukhus, Öron, Näsa, Halskliniken, Huddinge, Sweden. KAndersson, G Stridh, , H Lövsted, T Karlsson, Department of Occupational and Environmental Medicine, Örebro Medical Centre Hospital, Sweden.

The project has been fused with a Swedish project (DAMM) concerning the effects of aldehydes on the respiratory mucosa, and the major part of the cost of the project including the medical doctor for the exposure studies were covered by that funding.

Background and objectives
A number of cases of respiratory illnesses and dysfunctions among employees in the occupational environment have been reported. The respiratory effects have been associated with airborne exposures of organic dust containing microbes or to compounds emitted from micro-organisms. Candidates are endotoxin (LPS) from Gram-negative bacteria, β-(1→3)-D-Glucan from mould, and allergens in the form of proteins from the micro-organisms or their spores. Also in the indoor environment micro-organisms have caused adverse respiratory and systemic effects in the exposed population. Furthermore, the severity of asthma has been associated with the LPS concentration in house dust. Previous investigations using cell cultures and dust from a garbage treatment plant show that such dust is cytotoxic, and that the active components of the dust may be associated with fungal growth in the dust.

The aim of the project was to investigate the hypotheses:
a. Inhalation of β-(1,3)-D-glucan provoke inflammatory effects and an increased release of cytokines from human respiratory epithelium.
b. Individuals respond differently to these exposures depending on personal susceptibility factors.

Methods
The study compared individuals with and without atopy. The subjects were exposure in an exposure chamber for clean air, standard dust, standard dust with β-(1,3)-D-glucan and standard dust with aldehydes. Before, during, and after exposure a range of parameters relevant for the reactions were investigated. Typically, the measurements were made immediately before and after exposure and the following day. The measurements included rhinostereometry, laser-Doppler flow measurements, acoustic rhinometry, discomfort, and symptoms. Lung function and diffusional capacity, nasal lavage (ECP, IL-1, IL-8, cells, Trolox Equivalent Antioxidant Capacity (TEAC), gene expression), induced sputum (ongoing) and measurements in the eye (break-up time (BUT), epithelial damage, and TEAC and cells in tear liquid).

Results
The experimental part including human exposure, collection of data and of biological samples has been finalized with a collection efficiency exceeding 98%. The following activities are ongoing now by funding from the DAMM project and other sources:
- Data transfer to computer (90 % finalized).
- Analysis of biological samples and data transfer to computer (90 % finalized).

- Proof reading and initial analysis for data validation and planning of statistical analysis (80% finalized).
- Statistical analysis (90% finalized).

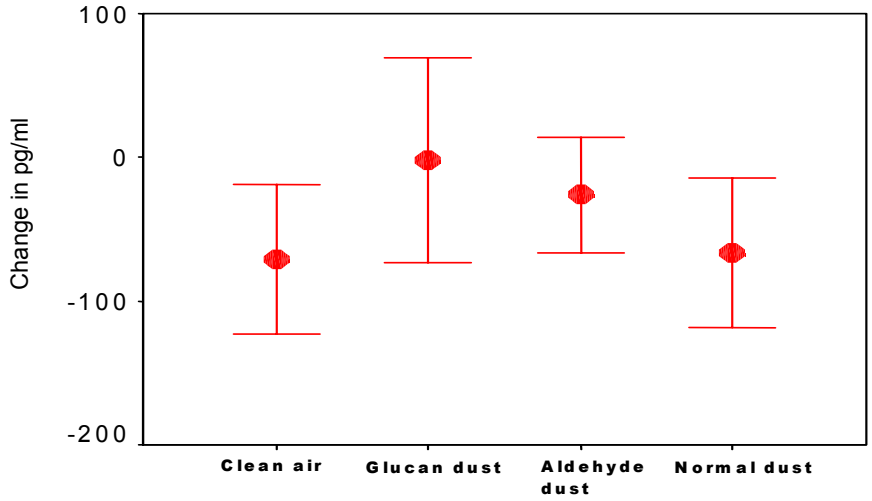


Figure 1. Mean and 95% confidence interval for interleukin-8 concentrations in nasal lavage among 36 persons exposed to different types of house dust (p = 0.042 for effect of exposure, paired data analysis of variance).

The initial analysis of relations between airway reactions (FEV1, FVC and DLCO), BUT, and epithelial damage has not shown an unequivocal relation with exposure and the various types of dust.

For nasal measurements an effect has been observed on cytokine concentration (IL-8) in nasal lavage immediately following exposure in the chamber, see Fig. 1. A paired analysis of variance showed an effect, and it can furthermore be seen that there is a borderline significant difference between the effects of glucan and aldehyde spiked dust relative to clean air. This indicates that there is an increased effect of exposures on cytokine release in the nasal mucosa when glucan and aldehydes have been added to the dust.

Table 1 shows the effect of exposures on changes in nasal volume. At 6 hours, just after the exposure, there is a significant effect of exposure on nasal volume. Furthermore, a paired analysis shows that glucan spiked dust is significantly different from both clean air and conventional house dust, and that aldehyde spiked dust is different from clean air. After 18 hours there is a borderline significant effect of the exposures, a paired analysis shows that glucan and aldehyde spiked dust is different form clean air. The result thus indicate that glucan spiked dust has the largest effect on the mucus membrane, and it can furthermore be seen that atopics have larger daily variations in nasal volume than non-atopics.

Figure 2 illustrates the relation between dust exposure and nasal volume. It is seen that non-spiked dust does not affect nasal volume in contrast to the dust spiked with glucan or aldehydes.

Table 1. Change in nasal dimensions right after and the day after exposures by three groups of subjects

Group	Nasal Histamine reactive		Normals		Allergy	
Exposure	Mean	SEE	Mean	SEE	Mean	SEE
Volume at 0-5 cm 6 hours, right after exposure (cm^3) [a]						
Clean air	-0.50	0.28	-0.81	0.25	-1.31	0.28
Glucan+dust[p]	-1.59	0.44	-0.86	0.33	-1.46	0.30
Aldehydes+dust[q]	-1.09	0.36	-0.99	0.29	-2.10	0.51
Normal dust	-0.60	0.36	-0.40	0.42	-1.61	0.34
Volume at 0-5 cm 18 hour after exposure (cm^3) [b]						
Clean air	-0.40	0.24	-0.26	0.34	-1.22	0.23
Glucan+dust[r]	-1.38	0.51	-0.65	0.55	-1.54	0.43
Aldehydes+dust[s]	-0.99	0.46	-0.85	0.23	-1.79	0.58
Normal dust	-0.85	0.37	-0.35	0.32	-1.61	0.47

A negative number means that the mucus membrane has swollen, and thereby that the volume has decreased relative to the initial value

a Difference between exposures (p=0.036) in a repeated type GLM
b Difference between exposures (p=0.083) in a repeated type GLM
p p = 0.020 compared to clean air and p=0.050 compared to normal dust
q p= 0.015 compared to clean air and p=0.056 compared to normal dust
r p = 0.029 compared to clean air
s p= 0.033 compared to clean air

Figure 3 shows that the change in Eosinofilic Cationic Protein (ECP) mainly was observed for the spiked dusts. This change was correlated with changes in nasal volume, table 1. This supports the hypothesis that the nasal effects are related to onset of inflammation. Figure 4 shows changes in nasal irritation at cessation of exposure. It is seen that the perceived irritation is the same for all types of dust.

Conclusions

The preliminary conclusions that can be drawn are that dust spiked with glucan (and aldehydes) leads to increased inflammatoy response as measured with two different parameters – with simultaneous 'individual' correlation, and that there is a general sensory response in the nose to dust exposure.

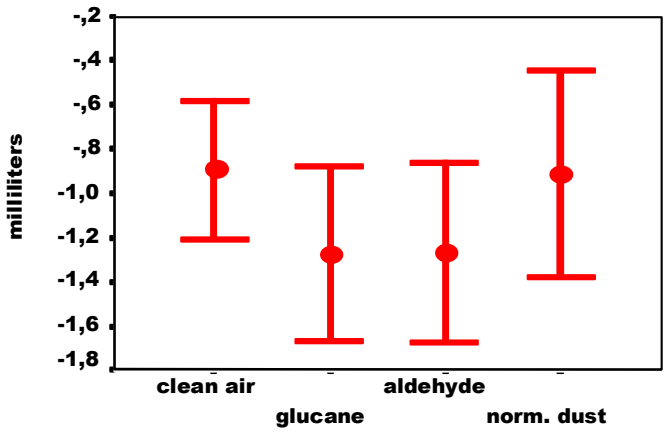


Figure 2 Change in nasal volume (2-5 cm) during dust exposure

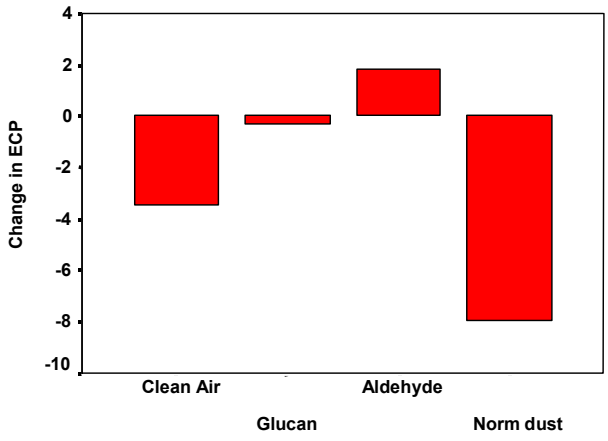


Figure 3 Changes in Eosinofil Cationic Protein (ECP) after exposure to different types of dust.

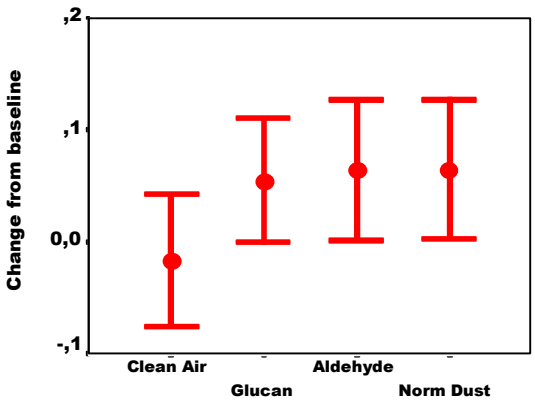


Figure 4. Changes in nasal irritation after cessation of exposure.

Project 1.5 Inflammatory and allergic reactions to micro-organisms in vitro. INFAL.

Project leaders: Torben Sigsgaard, Eva C. Bonefeld-Jørgensen
Participants: Tanja Krüger, Kirsten Østergaard, Department of Environmental and Occupational Medicine, Aarhus University.

Background and objectives
Several studies have shown that organic dust can lead to the development of symptoms and signs on skin and mucus membranes, including airways among exposed workers. Studies in water-damaged buildings have shown an association between symptoms and concentration of β-1,3-D-glucan in the air. During the last decade airway diseases have been observe following exposure to organic dust containing microorganisms. Several microbial components have been in focus of attention as possible causes of these occupational airway diseases. The components are endotoxins from Gram-negative bacteria (lipopolysaccharides, LPS), β -1,3-D-Glucan from molds, and allergens in the dust, e.g. proteins and mold spores. Experiments with exposures of cell cultures (human lung epithelial cells) have shown that extracts from this dust is cytotoxic. These experiments also indicate that the active component is associated with mold content in the dust.

The purpose of this project was to investigate whether a test method based on whole-blood and nasal lavage can be used for hazard identification of components of microbial origin. These components included LPS, β-(1→3)-D-Glucan and extracts of organic dust from greenhouses and swine confinements. Furthermore, we wanted to investigate whether differences in the inflammatory response could be used as a marker of individual susceptibility.
Also inheritance of the inflammatory response was studied among mono- and dizygote twins.

Methods
Nasal lavage
Exposure of study subjects and sampling has been optimized regarding different analytical parameters that affect reproducibility of the results such as the stability of cells and cytokines. We analyzed cytokine release into the nasal lavage fluid using the ELISA method and analyzed cytokine gene activation in the cells by determination of mRNA using on-line RT-PCR.

Whole-blood assay
The whole-blood assay (WBA) was performed for determination of induced cytokine gene expression (mRNA) and cytokine release from cells upon incubation with different organic materials. The mRNA was determined by RT-PCR and cytokine ELISA kits from the company R&D (London) was optimized and highly reproducible data was obtained.

RT-PCR analysis
For determination of mRNAs of selected cytokines (expression at the gene level) the high sensitive, semi-quantitative RT-PCR analysis was performed using the on-line *real-time PCR apparatus, Light Cycler,* from Roche.

Panel analysis and field analysis
We have used panel investigations to establish and evaluate our analyses. By the use of WBA, whole blood samples from volunteers (10 atopic and 10 non-atopic), we studied the ex *vivo* cytokine release and activation. WBA incubated with LPS, ß-1,3-D-Glucan and dust from swine confinements was performed to obtain the induced cytokine mRNA level and release of cytokine

from the cell. In the field analyses we have used WBA on symptomatic and asymptomatic workers, by *ex vivo* incubation with LPS and ß-1,3-D-Glucan. IL-8 and IL-1ß was determined by ELISA and RT-PCR analysis.

Results

Using WBA on blood samples from twins, we have shown a significantly lower difference in cytokine response after incubation with LPS between mono- than between dizygote twins for IL-1ß ($p < 0,05$). This result indicates, that there is a genetic component in sensitivity to stimulation with LPS. Such an association could not be demonstrated for ß-1,3-D-Glucan. For IL-8, no difference could be demonstrated between the reaction of mono- and dizygotic twins neither to stimulation with LPS nor to ß-1,3-D-Glucan.

For the panel studies of WBA we have demonstrated that both LPS, ß-1,3-D-Glucan, fish components and dust from swine confinements induce a strong cytokine release, Table 1 and 2. For all incubations IL-1 release was increased by a factor up to 200 relative to spontaneous background release. For IL-8 the release was approximately 10 and 30 fold increased after incubation with LPS and Glucan respectively. Upon incubation with dust from swine confinements an 80-fold increase of IL-8 was observed (Table 1). No significant difference was found between atopics and non-atopics neither for Il-1 nor for IL-8 (Table 1). The IL-1 mRNA level (gene-activation) was significantly higher for atopics than for non-atopics after incubation with LPS, whereas a significantly lower IL-8 gene activation was found among atopics after exposure to ß-1,3-D-Glucan (Table 2). It thus can be ascertained that different responses were observed between the cytokine release from cells and the corresponding gene activation within the cells.

Table 1, Induced IL-1 and IL-8 release (protein), A= Atopics, NA=Non Atopics

Incubation	IL-1NA	IL-1A	IL-1 ratio NA/A	IL-8NA	IL-8A	IL-8 ratio NA/A
LPS	100	95	1.1	100	100	1
Glucan	80	80	1	350	300	1.2
Swine confinement	95	95	1	780	800	1.0
Background	0.5	0.5	1	14	10	1.4

Table 2. Induced m-RNA level of IL-1 and IL-8 (gene activation)
A= Atopics, NA=Non Atopics* $p< 0,05$ t-test A vs NA

Incubation	IL-1NA	IL-1A	IL-1 ratio NA/A	IL-8NA	IL-8A	IL-8 ratio NA/A
LPS	100	180	*0.6	100	75	1.3
Glucan	125	50	2.5	160	45	*3.6
Swine confinement	125	50	2.5	120	48	2.5
Background	4	2	2	15	7	2.1

In connection with project 1.1, (see this project) we have performed the WBA using LPS, ß-1,3-D-Glucan and dust from swine confinements among all 16 participants. A significantly higher cell cytokine release was found for cases, that had developed asthma symptoms during farm work, than

for the controls, who did not have symptoms during farm work, after incubation with LPS and dust from swine confinements. We thus conclude, that asthmatic swine farmers react with a stronger IL-1 release after exposure to LPS and dust from swine confinements compared with healthy swine farmers. On the other hand, the very high level of IL-8 release after induction with dust from swine confinements did not differ between atopics and non-atopics.

Conclusions

Compared to the determination of cell cytokine release, we observed an increased sensitivity using the RT-PCR RNA assay. This supports our expectations that the RT-PCR analyses provides a potential to measure weaker responses at lower exposure levels, and the potential to discriminate between whether an exposure has effects at the gene level, and/or release of cytokine proteins from the cell.

Our studies also show that there is a difference in the gene activation (mRNA level) between atopics and non-atopics. Furthermore, the responses between atopics and non-atopics were contrary depending on the inducing agent. For LPS we found an increased IL-1 cytokine gene activation among atopics, whereas for ß-1,3-D-Glucan and dust from swine confinements we found a decreased IL-8 cytokine gene activation among atopic persons.

Finally, we found differences between the potential of agents to induce cytokine protein release from the cells especially for IL-8 where a greater release was observed after Swine confinement dust and ß-1,3-D-Glucan treatment compared to LPS.

Further work will be needed to evaluate which of these responses are related to the clinical outcome. However, at the present state IL-8 seems better to distinguish between the inflammatory potential of different dust types than IL-1.

Project 2.6 Bio-pesticide work process-exposure matrix model. BIOGART.
Project 3.5 Risk assessment of work with bio-pesticides

The projects are integrated and reported as one

Project leader Jesper Bælum, Department of Occupational and Environmental Medicine, Odense University Hospital
Project participants: Preben Larsen, Gert Doekes, Department of Occupational and Environmental Medicine, Odense University Hospital

Background and objectives
Microbiological pest control has been introduced in greenhouses as a supplement or substitution for the chemical insecticides and fungicides. In Denmark the types most often used are the insecticides *Bacillus thuringiensis* and *Verticillium lecanii*. *Trichoderma harzianum* is used as a fungicide acting as a competitor to the plant pathogenic fungi. Development of this new technology gives a possibility for evaluation of the health risk.

Methods
The project is a 3-year follow-up epidemiological study of the effects of exposure to three types of microbiological pesticides on symptoms and signs of inflammatory diseases in greenhouse workers. The microbiological pesticides were strains of *Bacillus thüringiensis, Verticillium lecanii,* and *Trichoderma harzianum.* At the time the study was carried out a product based on *Paecilomyces fumosoroseus* was introduced and therefore included in the study.

At the annual examinations the persons were interviewed about work conditions with focus on exposure to microbiological pesticides, and health with focus on symptoms related to allergy and inflammatory upper and lower respiratory tract diseases. Spirometry and bronchial challenge test a.m. Yan using histamine chloride as well as skin prick test with standard inhalatory allergens. Blood samples were taken for analysis for IgE antibodies against the in all 8 products of the four types of pesticides.

Results
In the first round of the study in 1997-1998 456 persons were included. Of these were 316 reexamined in the follow up 1 year later at which time 123 persons were included. This gives a cross sectional material of 579 persons (32% males and 68% females) aged between 16 and 67 years with average seniority of 9,7 years in the trade. The group of persons was followed for three years until 2001 and the number of persons followed for three years were 262 while 344 persons were followed for at least two years.

In the cross sectional material of 579 persons the prevalence of in all 24 symptoms covering irritation in eyes and airways varied between 9% and 31% (itching in the eyes). 6.6% of the subjects reported they had asthma while about 20% reported on or more symptoms of rhinitis.

In greenhouses using *Bacillus thuringiensis* products increased prevalences of itching in the eyes at work and of itching more than once a week were seen. The persons who handled *Bacillus thuringiensis* product had more chest tightness than the rest while in the follow up an increased incidence of itching in the eyes was seen in greenhouses using *Bacillus thuringiensis. Trichoderma harzianum* in greenhouses was related to cough, difficulty of breathing, itching in the nose, and

more unspecific annoyance. The use of *Verticillium lecanii* was not related to any symptoms and the effects of *Paecilomyces fumosoroseus* could not be evaluated due to a low number of exposed persons.

There was no difference in the lung function and in histamine sensitivity between subjects exposed to the products and those not in contacts with the products.

There were measurable IgE antibodies against the different products. The prevalence of antibodies above the detection limits was highest for the *Verticillium lecanii* and the *Bacillus thuringiensis* products while antibodies against *Trichoderma harzianum* and *Paecilomyces fumosoroseus* were less frequent. There was no clear relation with the measures of individual exposure. Especially, no sensitization was seen in the highly exposed persons who had handled and sprayed out the products. During the one-year follow up the levels of antibodies for the individual were stable and there was no sign of increased sensitization.

The incidence of new symptoms was about 10% and only in a few symptoms the incidence was related to the measures of exposure or to sensitization. Due to the relatively low number of new symptoms in the short follow up period the power of this part of the study for detecting exposure-response relationship is low.

In the cross sectional material persons with one or more positive prick tests as an indicator of atopy showed higher prevalence of eye and nose symptoms as well as diagnosed asthma than the non-atopics. Furthermore, the incidence of new symptoms during follow-up in the group of atopic persons was considerably higher than among the non-atopic persons and persons with house dust mite allergy were over represented in the group that left the study between the first and second examination. This could indicate a "healthy worker effect" due to the different exposures in the greenhouses. The evaluation of health effects should therefore mainly be based on measures of incidence and the study has therefore been extended for three years to achieve a higher power.

Conclusions

In conclusion the study showed a relatively high prevalence of symptoms among greenhouse workers and there was a limited relation to estimates of exposure to *Bacillus thuringiensis* and *Trichoderma harzianum* but not to *Verticillium lecanii* products. A few effects in the follow up were seen, too, while there was no effect on physiological measurement. The power of the one-year study, however, was low and the "healthy worker effect" detected support that emphasis is laid on measures of incidence in a longer follow up.

A limited number of significant effects of the microbiological pesticides were seen in the present study. Nevertheless, altering procedures and using personal protection during handling and spraying may limit the exposure. The possible effects on persons exposed during re-entry activities need more investigation of both the real exposure and the dose response relationship.

The results from the second follow up in 2000 and the third follow up in 2001 are being analyzed and will be published during the next year. This will give better estimation of incidence rates related to exposure to the different groups of microbiological pesticides.

The three years comprehensive follow up of a cohort of greenhouse workers with a relatively high prevalence of symptoms gives the possibilities for testing the effects of several exposures. A study

of the allergenic effects of the different predatory animals (wasps, mites, nematodes) has been planned.

Plasticizers and surfactants

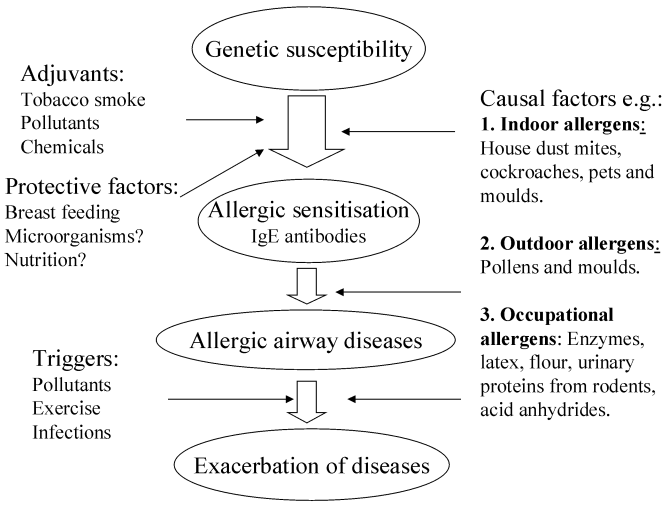
Project 1.3 Adjuvant effect of surfactants and plasticizers *in vivo*

Project leader Gunnar Damgård Nielsen, National Institute of Occupational Health.
Project participants: Søren T Larsen, Susanne K Clausen, National Institute of Occupational Health

Background and objectives

The airway diseases, asthma and rhinitis, are increasing in the westernized countries. The increase is mainly due to allergy, i.e. allergen specific IgE antibodies. There may be several reasons for the increase, for example, high allergen exposures, exposures to adjuvants that either may promote IgE formation or decrease IgE formation. Other reasons may be lack of breast-feeding, changed exposures to microorganisms and changed gut flora that may cause immune deviations. This study intends hazard identification (i.e. does a substance possess an inherent possibility to promote an adjuvant effect) and risk assessment (i.e. does a substance possesses a risk for an adjuvant effect in humans under normal exposure and use conditions).

Overall theoretical frame for the studies:



Hazard identification

Method

Hazard identification was performed by means of a subcutaneous injection (s.c.) model in BALB/c mice, i.e. the model can be used as a fast screening model. The model allergen, ovalbumin (OVA), in aqueous solution, was injected s.c. alone (control group), together with the test substance in four different concentrations (test groups), or together with $Al(OH)_3$, which was used as the control adjuvant. Afterwards animals were boosted (s.c.) once or twice with OVA alone. Antibody production (OVA specific IgE, IgG1 and IgG2a) was measured after one and two boosters. A Th2 cytokine dependent adjuvant effect was accepted if one of the concentrations of a test substance increased OVA specific IgE or IgG1 compared to the antibody production in mice in the OVA control group. Similarly, a Th1 cytokine dependent adjuvant effect was accepted if a test substance increased the OVA specific IgG2a production.

Surfactants

Two series of experiments were carried out with substances that were surfactants. The first series studied representative substances from the four commercially important groups of surfactants. In this study, only OVA specific IgE antibody production was determined. Test substances were the sodium salts of the anionic surfactants dodecylbenzene sulfonate, dodecyl sulfate, coconut oil fatty acids and the nonionic surfactant, dodecyl alcohol ethoxylate. For the anionic surfactants, the highest test concentration suppressed IgE formation. A lower concentration of sodium dodecyl sulfate showed adjuvant effect. As the four substances were commercially important surfactants, the study suggests that not all surfactants possess adjuvant effect and in this case was the effect observed with one of the four tested substances.

To obtain information on structure-activity relationships of adjuvant effects of substances with surfactant properties, it was decided to study adjuvant effects of monophthalates. They are important metabolites of the commercially important phthalate plasticizers and they are closely related chemicals. Monophthalates contain a carboxyl group, having hydrophilic properties, and a lipophilic part, the benzoic ester part, which cause the surfactants properties. We studied mono-n-butyl phthalate (MnBP), monobenzyl phthalate (MBnP), mono-n-octyl phthalate (MnOP), mono-2-ethylhexyl phthalate (MEHP), mono-iso-nonyl phthalate (MiNP), and mono-iso-decyl phthalate (MiDP). A suppressing effect on Th2 antibody production was observed and the no-observed effect level decreased with increasing size of the lipophilic part of the molecules. In contrast, the Th2 adjuvant effect showed a bell shaped relationship. Thus in relation to the size of the molecules, no adjuvant effect was detected with MnBP and MBnP whereas MEHP, MnOP and MiNP were adjuvants, but this was not the case with larger molecule MiDP. Overall, the suppressing effect was a direct function of the size/lipophilicity if the monophthalates whereas this was not the case with the adjuvant effect, suggesting that the adjuvant effect may not be a direct function of the toxicity, but rather is a specific effect of the molecules.

Additional studies were performed on the metabolites, phthalic acid and benzyl alcohol. None of the substances showed adjuvant effect, but benzyl alcohol suppressed the production of OVA specific IgG1.

Phthalate plasticizers

The phthalate plasticizers themselves (phthalic acid diesters) were also screened for adjuvant effects. These substances are not soluble in water and it was therefore necessary to develop the screening model for aqueous solutions to allow testing of the phthalates. It was shown that OVA alone and OVA together with the phthalates could be administered s.c. in the polyethylene glycol vehicle and the adjuvant effect determined as for the aqueous solutions. Several phthalates were tested: Di-n-butyl phthalate (DnBP), butyl benzyl phthalate (BBP), di-(2-ethylhexyl) phthalate (DEHP), di-n-octyl phthalate (DnOP), diisononyl phthalate (DINP), and diisodecyl phthalate (DIDP). In contrast to the monophthalates, the parent phthalates did not produce any suppression of antibody production. Similar to the monophthalates, the phthalates themselves showed a bell shaped relationship for the Th2 adjuvant effect with a maximum adjuvant effect with DEHP, DnOP and DINP. Another difference was apparent, the monophthalates mainly showed the Th2 adjuvant effect from the IgE antibody production whereas the phthalates mainly showed Th2 adjuvant effects from the IgG1 antibody production. The ratio between the antibody production in the test groups and the OVA control groups were higher for the phthalate than for the monophthalates, i.e. the main Th2 adjuvant effect of the phthalates were observed with the parent compounds.

Risk assessment
With inhalation, the lymphoid tissue in the respiratory tract plays an important role in processes of sensitization and development of tolerance. Tolerance is the predominant response with exposures to non-pathogenic soluble proteins. Therefore it is probably so that the question about reasons for increases in allergic airway diseases may be recast to "why is development of tolerance suppressed in the westernized societies"? As sensitization and tolerance appear from the respiratory lymphoid tissue, inhalation studies are mandatory in relation to risk assessment in humans. Unfortunately, no such method is available in the scientific literature.

Development of methods
Mice were exposed to different concentrations of OVA aerosols to study development of tolerance. The OVA concentration that caused the least degree of tolerance was determined and it was used in repeated studies with 10 consecutive aerosol exposures followed by one weekly exposure for six weeks. Each exposure lasted for 20 min. Similar exposures where the same OVA solution contained the $Al(OH)_3$ adjuvant were performed. The adjuvant strongly promoted development of OVA specific IgE and IgG1 antibodies. Thus, for aqueous solutions we were able to demonstrate one of the important steps in the development of allergic airway diseases, the promotion of development of Th2 dependent antibodies. This model can address the important question, is a substance an adjuvant by inhalation? However, the model has to be developed further as rhinitis and asthma in humans show additional characteristics as inflammation and airflow limitation. Also, a final model should be able to test substances that are not soluble in water.

To show that it is possible to determine irritation in the upper airways, which is characteristic for rhinitis, airflow limitation, which is characteristic for asthma, and pulmonary irritation, which is characteristic for allergic alveolitis (hypersensitivity pneumonitis), in conscious mice, we tested several airway irritants.

Conclusions
Overall, the developed models allow the study of substances for adjuvant effect, but the models have to be further developed it they have to be used as models of all aspects of human asthma and rhinitis.

Project 1.6 Inflammatory and allergic reactions to plasticizers *in vitro*

Project leader: Lars K Poulsen, Allergy Unit, National University Hospital
Project participants: Christian Glue, Allergy Unit, National University Hospital. Karen Frydendall Jepsen, National Institute of Occupational Health.

Background and objectives
The aim of this project was to develop and use *in vitro* models for screening xenobiotic substances like phthalates to elucidate any specific or unspecific proinflammatory or immune modulatory potential. A simple *in vitro* model cannot be used to identify adjuvant effects. Nevertheless, demonstrating that a xenobiotic substance can stimulate isolated cells to produce proinflammatory cytokines can contribute to a better understanding of how adjuvant effects can arise *in vivo*.

Methods
Two isolated cell lines were used as models for induction of early inflammation markers:

The human lung epithelial cell line A549 can be stimulated to release the proinflammtory cytokines IL-6 and IL-8, but doesn't release cytokines that can shift the balance of the immune system in direction of T-h1 (autoimmunity) or T-h2 response (allergy). Hence, a response in this cell line is considered rather unspecific. After incubation with monophthalates IL-6 and IL-8 was measured using ELISA.

The human monocytic cell line THP-1 shows macrophage-like properties and the capacity for production of a series of cytokines, which govern the T-h1/T-h2 balance of the immune system. The THP-1 cell line has been used for studies on environmental pollutants like diesel exhaust particles. A Quantative Competitive RT-PCR method for measuring IL-1β, IL-6 and IL-12α (p35) was developed. A linear correlation was found between output and amount of RNA for cDNA synthesis signifying that the final result of the analysis is linearly related to the amount of RNA or cDNA when operating within the range of 1-4 μg (RNA isolation) and within a dynamic range of 32-fold (cDNA synthesis procedure). After incubation with monophthalates or other test substances the IL-1β IL-6 and IL-12α (p35) cytokine gene expression levels were determined using this new method.

In addition, peripheral blood mononuclear cells (PBMC) from allergic patients and non-allergic controls were isolated and incubated for 48h with monophthalates and Tetanus toxoid (positive control). In this case, IL-4, IL-5 and IFNγ gene expression levels were determined using Real-time PCR.

In both cell lines and PBMC cell viability counting was performed using trypan blue.

Results
The studies on toxicity of the six monophthalates (mono-*n*-butyl phthalate (MBUP), monobenzyl phthalate (MBEP), mono-2-ethylhexyl phthalate (MEHP), mono-*n*-octyl phthalate (MOP), mono-*iso*-nonyl phthalate (MINP), and mono-*iso*-decyl phthalate (MIDP)), measured as cell viability using the trypan blue method, revealed a clear correlation between increased side chain length and toxicity both on lung epithelial cell line A549 and on the monocytic cell line THP-1 (see figure 1).

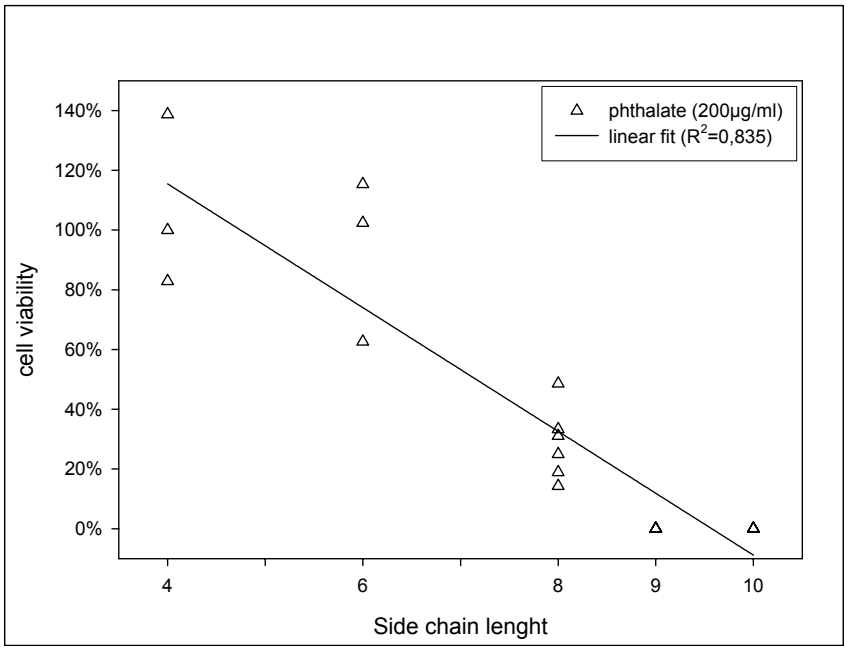


Figure 1. Relation between the alkyl/benzyl side chain length of the monophthalates and toxicity (R^2=0,835). $2 \cdot 10^5$ THP-1 cells/ml were incubated with monophthalates (200 µg/ml) for 24 h at 37 °C and 5% CO_2. The cells were collected and viability counting was performed using trypan blue.

In the lung epithelium cell line A549 clear dose-response curves for both immuno-stimulation and immuno-suppression were seen for all six monophtalates in the range 15 µg/mL – 2 mg/mL, and a good agreement was observed between the IL6- and IL-8 responses (see figure 2). Both immuno-stimulation and immuno-suppression were depending on the dose and the length of the lipophilic side chain: With increasing length of the side chain a decrease in the concentration of monophthalate needed to induce stimulation as well as suppression of cytokine production was observed. The concentration gab between these two opposite effects was very narrow, and suppression was typically observed at twice the concentration needed for optimal stimulation (Table 1). This result indicates that stimulation is unspecific and closely related to the cytotoxicity of the monophthalates.

In contrast to the lung epithelial cells, no cytokine induction was observed when the six monophthaltates was added to the growth medium of neither THP-1 cells nor PBMCs from allergic patients or from non-allergic controls. This result indicates that the adjuvant effects of monophthalates, demonstrated *in vivo* in the murine models, may not involve a specific mechanism acting on monocytes.

The sensitivity of the THP-1 cells was demonstrated used positive control substances. Upon stimulation of the monocytic cell line THP-1 with LPS or Actinomycin D the gene expression of early inflammation markers like IL-1β can be either up- or down regulated. The cytokine levels in the stimulated cells are almost 20 times higher than in unstimulated cells, leaving an advantageous difference between the positive and negative control for the system. Altogether the THP-1 assay is believed to be a reliable system by which it is possible to quantify and compare the expression of cytokines in different samples exposed to xenobiotic substances.

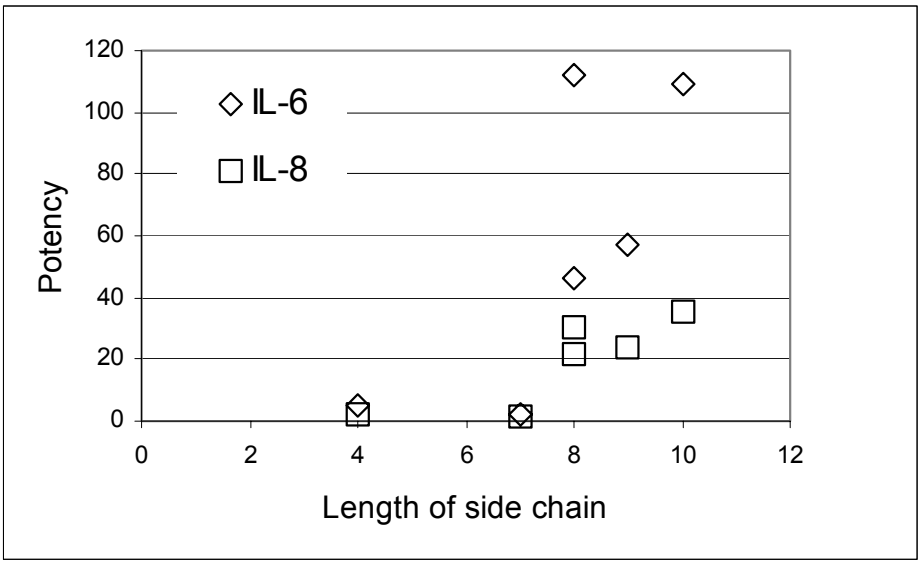


Figure 2. Association between length of alkyl side chain and potency of monophthalates to induce IL-6 and IL-8 secretion in lung epithelial cells A549.

Table 1. Rank of monophthalates with respect to stimulation and suppression of IL-6 and IL-8 secretion from lung epithelial cells A 549

Monophtalate	C_n*	Lowest conc. for suppression (µg/mL)	Lowest conc. for max. Stimulation (µg/mL)
MbuP	4	2000	1000
MbeP	7	2000	1000
MEHP	8	500	125
MOP	8	500	125
MINP	9	250	125
MIDP	10	125	62,5

*C-atoms in side chain

Conclusions

The Centre has succeeded in developing *in vitro* models, including new methods for measurements of cytokine m-RNA levels, which are useful for testing of immune modulating effects of xenobiotics. Moreover, a parallel model system has been developed employing primary human cells as targets. Cytotoxicity of the six monophthalates was demonstrated in all *in vitro* models, and non-specific immune modulating effects were observed in lung epithelial cells. However, no effects of monophthalates on cytokine production were seen neither in monocytic cell lines nor in primary human cells (PBMCs). Since various pharmacological agents clearly have effects in the monocytic cell line models, it would be interesting to test other classes of xenobiotics for possible *in vitro* immune modulating effects.

Project 2.2 Exposure model for plasticizers

Project leaders: Ole Ladefoged, Elsa Nielsen, Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration
Project participants: Anne Kirstine Müller, Grete Østergaard, Jim Hart, Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration

Background
Within the EU, specific programs on risk assessment for chemical substances are on-going. A directive (Commission Directive 93/67/EEC of 20 July 1993, laying down the principles for the assessment of risks to man and the environment of substances notified in accordance with Council Directive 67/548/67) provides the regulation on risk assessment of new notified chemical substances[1] and two Council regulations (Council Regulation 793/93/EEC of 23 March 1993 on the evaluation and control of the risks of existing substances), (Commission Regulation (EC) No 1488/94 of 28 June 1994 on risk assessment for existing substances) on risk assessment of existing substances. A Technical Guidance Document in support of the risk assessment legislation has been prepared (Technical Guidance Document in support of Commission Directive 93/67/EEC on risk assessment for new notified substances and Commission Regulation (EC) No 1488/94 on risk assessment for existing substances) and is currently under revision.

The risk assessment process, in relation to both human health and the environment, entails a sequence of actions: 1) Effect assessment comprising a) hazard identification (identification of the adverse effects which a substance has an inherent capacity to cause, and b) dose (concentration) - response (effects) assessment (estimation of the relationship between dose, or level of exposure to a substance, and the incidence and severity of an effect, where appropriate; 2) Exposure assessment (estimation of the concentrations/doses to which human populations or environmental compartments are or may be exposed); and 3) Risk characterisation (estimation of the incidence and severity of the adverse effects likely to occur in a human population or environmental compartment due to actual or predicted exposure to a substance).
The exposure assessment and risk characterisation are carried out separately for three subgroups of the human population: workers, consumers, and man exposed indirectly via the environment. The risk characterisation results in one or more of the following conclusions: (i) There is need for further information and/or testing; (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already; (iii) There is a need for limiting the risks, risk reduction measures which are already being applied shall be taken into account.

The consumer, i.e. a member of the general public who may be of any age, either sex, and in any stage of health may be exposed to a new or existing substance by using consumer products. A consumer product is one, which can be purchased from retail outlets by members of the general public and may be the substance itself, or a preparation, or an article containing the substance. To assess the exposure to substances present in consumer products, information is needed on two sets of parameters: 1) contact parameters (where, how long and how often contact with the consumer occurs) and 2) concentrations parameters (the concentration of as substance in a medium that might contact the body. Measured data on external exposure for specific scenarios may be available for a

[1] Substances not on the EU market in the 10 years prior to 18 September 1981 and therefore not appearing in the European INventory on Existing Commercial chemical Substances (EINECS).

number of substances; however, most often such data are not available. A number of computerised models have been developed; no particular model is recommended above any other. Whenever possible measured or estimated values should be used for each of the numerical parameters in a given model, but when this is not possible default values may be derived from available data sources.

Indirect exposure of humans via the environment may occur by consumption of food (fish, crops, meat and milk) and drinking water, inhalation of air, and ingestion of soil. The indirect exposure is assessed by estimating the total daily intake of a substance based on the predicted environmental concentrations for (surface) water, groundwater, soil and air. Indirect exposure is principally assessed on two spatial scales: locally near a point source of the substance, and regionally using averaged concentrations over a larger area. For existing substance, measured levels in various environmental compartments may be available; however, for new substances, usually no relevant measured data are available and concentrations of a substance in the environment must be estimated. The model 'EUSES' is recommended for estimation of indirect exposure.

The European Union System for the Evaluation of Substances (EUSES) has been developed for quantitative assessment of the risks posed by new and existing chemical substances to man and the environment. EUSES can specifically be used in the initial (or screening) and intermediate (or refined) stages of assessment. On the basis of the screening, it can be decided if more data need to be generated and if a more refined assessment is necessary. EUSES can also be applied for refined assessments by allowing the replacement of default values, estimated parameter values, or intermediate results by more accurately estimated values or by measured data. EUSES is not specifically designed for site-specific assessments, but adjustment of parameters may allow for insight into specific local or regional situations.

Aim of the project

The aim of the project is to refine EUSES to a "DK-USES" in order to carry out exposure assessments for the region Denmark. The five phthalates now under review under EU Regulation 793/93 (Council Regulation 793/93/EEC of 23 March 1993 on the evaluation and control of the risks of existing substances), dibutyl phthalate (DBP), bis(2-ethylhexyl) phthalate (DEHP), diisononyl phthalate (DINP), diisodecyl phthalate (DIDP) and benzyl butyl phthalate (BBP), were selected as model substances for the refinement of EUSES. Furthermore, the project shall also provide data (exposure and effects) for project 3.2 in which a risk characterisation of the five selected phthalates is carried out. Regarding exposure assessments, the main stress will be laid on the gathering of data for the five phthalates on measured concentrations and exposures in Denmark as well as on exposures estimated by EUSES according to the EU Risk Assessment Reports (EU-RAR) of these phthalates in order to evaluate if EUSES exposure estimates for the five phthalates seem reliable for the region Denmark. Regarding effect assessment data for project 3.2, summaries will be prepared based primarily on the data in the EU-RARS of the five phthalates.

Methods

<u>Indirect exposure of humans</u>

Input parameters for EUSES specific for Danish conditions have been proposed. Predicted environmental concentrations (PECs) for different compartments (air, water, soil, sediments, biota) have been estimated by the use of EUSES with the proposed set of parameters specific for Denmark. Measured concentrations in the Danish environment obtained by monitoring studies -

whenever they exist – have been compared with the estimated concentrations. Additional estimations on the exposure have been made by the use of monitoring data in EUSES. In the calculations made by EUSES, all substance specific information have been used as reported in the EU-RARs of each phthalate

Consumer exposure

Exposures have been estimated for the following selected exposure scenarios: toys, building materials etc., infant formulae and baby food, artificial leather and gloves, paints etc., and nail polish. The estimation of the exposures are different in the various scenarios depending on the available data, but exposures have generally been estimated based on e.g., total amounts in the consumer products, emission data from products (e.g., toys, building material) as well as measured concentrations (e.g., infant formulae and baby food, house dust). Furthermore, the computer exposure modeling program CONSEXPO has been used for estimations of dermal exposures to nail polish and paints. Also measured and/or estimated exposures from the EU-RARs have been included.

Effects

Toxicological summaries have been prepared based primarily on the data in the EU-RARs of each phthalate. The following end-points have been evaluated: toxicokinetics, acute toxicity, irritation, sensitization, repeated dose toxicity, mutagenicity, carcinogenicity and toxicity to reproduction. Based on an evaluation of the toxicological data, the critical effect(s) of each phthalate have been identified and the no observed adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL) have been established for the critical effect(s).

Results

This project is very much delayed due to the fact that the discussions in the EU Working Group have taken much more time than expected. Final drafts for DBP, DEHP, DINP and DIDP have been released from the EU Working Group in the second half of 2001 for commenting in the EU Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) who has responded during year 2002. Final drafts for decision on the conclusions by the EU Competent Authorities are now under preparation. Thus, none of the EU-RARs on these four phthalates have been finalised yet. Data on BBP from the EU-RAR have not been included as discussions are still on going in the EU Working Group and the report is not yet public available.

Indirect exposure of humans

The use of a specific Danish profile in EUSES only changed the exposure assessment of DEHP slightly when compared to the estimations based on EUSES with the standard profile.

DEHP and DBP have been estimated to occur in highest concentrations in the environmental compartments and therefore also resulted in the highest total daily intake. In general, the predicted regional concentrations (PECs) of DEHP and DBP in many of the environmental compartments were within the range of available measured concentrations. However, the PECs were not always worst-case concentrations; e.g., in soil the estimated concentration was in the lower end of the range of measured values. Only for DBP in surface water, the estimated concentration exceeded all measured concentrations.

Comparisons of estimated and measured concentrations of DIDP and DINP were only possible for a limited number of the environmental compartments, since for most of the compartments no measurements exist.

The estimated concentrations of DEHP in crops and milk were much lower than the few available measurements for Denmark. Also, the total daily intake of DEHP and DBP estimated by EUSES appear to be underestimated compared to measured values, whereas for DEHP in fish the estimated concentration seemed to be reliable and the estimated concentration of DBP in drinking water equaled the single available measurement.

Comparisons of estimated and measured concentrations of DINP and DIDP were not possible, since no Danish measurements of the concentration of DINP and DIDP in food exist.

Several reasons for the discrepancies between estimated and measured concentrations can be suggested. EUSES might be an inappropriate model in order to estimate the exposure to the phthalates due to their special properties such as low water solubility, low vapour pressure etc. It could also be due to poor data input into the model as both the physico-chemical data and the data regarding the Danish tonnages of the substances and specific usages are subjected to uncertainties. Furthermore, measurements of the concentrations in the environment and especially in foods are scarce and may not be representative for the actual situation.

The measured concentrations in some foods and the total daily intake also include phthalates entering the food during processing, by contact to packaging materials etc. Food contact materials can lead to a substantial contamination of the food; however, this contribution is only included in some of the measured concentrations in foods, not in the EUSES estimations.

When PECs were replaced by measured environmental concentrations in EUSES, the estimated total daily intake increased. Furthermore, when estimated concentrations in foods were replaced with the few available measured concentrations in foods, this resulted in a further increase in the total daily intake.

Consumer exposure

Children are exposed to phthalates during their playing with toys. The potential exposure via mouthing phthalate-containing toys (worst case scenario) has been estimated to be relatively high. Furthermore, dermal exposure to phthalates also occurs during their handling of toys; however, this contribution seemed to be low compared to the oral exposure. In Denmark, the use of phthalates in toys and other childcare articles intended for 0-3 years old children are restricted (max. concentration 0.05% (w/w)). However, investigations of toys on the Danish market has suggested that exposure via toys still can occur.

Children are also exposed to phthalates via infant formulae and baby food. For infants specifically, it has been estimated that the main source was infant formulae.

The exposure via indoor air to phthalates emitted from building materials, furniture, cables etc. has been estimated based on measured vapour phase concentrations of the phthalates and the concentrations of the phthalates adsorbed to house dust. The present assessment indicated that the vapour phase and the house dust contributed equally to the exposure via indoor air.

Consumers can also be exposed to phthalates via dermal contact with phthalate-containing products such as water proofs, boots, gloves and artificial leather. The exposure seemed in some cases to be relatively high.

The dermal and inhalatory exposures via use of paints etc and nail polish were estimated to be very low.

Effects
The critical effect(s) identified for DBP, DEHP, DINP and DIDP were: testicular effects (DBP, DEHP, DINP), effects on fertility (DEHP), embryotoxicity (DBP), developmental toxicity (DBP, DEHP, DIDP), and effect on the liver (DINP, DIDP) and kidney (DEHP, DINP).

For DBP, the lowest dose-level tested (250 mg/kg b.w. per day) appeared to be an effect level (changes in testicular enzymes associated with degeneration of spermatogenic cells). In a two-generation study, DBP appeared to be a reproductive toxicant in rats; the lowest dose-level in this study, 0.1% in the diet (52 mg/kg b.w. per day for males; 80 mg/kg b.w. per day for females) is a LOAEL for embryotoxicity in the absence of maternal toxicity. Several of the recent developmental studies have revealed delayed preputial separation and reproductive tract malformations in male offspring at oral doses from 250 mg/kg b.w. per day; a NOAEL of 50 mg/kg b.w. per day can be derived for developmental toxicity in rats. In conclusion, an overall LOAEL of 52 mg/kg b.w./day has been established for embryotoxic effects observed in rats administered DBP by oral exposure; a NOAEL could not be established based on the available data.

For DEHP, severe and irreversible testicular injury was induced in rats exposed to low oral dose levels of DEHP in two different studies with a NOAEL of 3.7 mg/kg b.w./day in one study and a LOAEL of 3.5 mg/kg b.w./day in the other study. Also severe developmental effects were observed in rats and mice in the absence of maternal toxicity with a LOAEL of 3.5 mg/kg b.w./day for rats and a NOAEL of 20 mg/kg b.w./day for mice. Effects on male fertility have been observed in mice and rats. In mice, DEHP adversely affected the number of fertile matings. In a continuous breeding study in mice, an oral NOAEL of 0.01% in the diet (20 mg/kg b.w./day) was identified for fertility. In rats, the oral NOAEL for body weight, testis, epididymis, and prostate weights and for endocrine and gonadal effects in male rats was considered to be 69 mg/kg b.w. per day in a 60 day study. The effects on the kidneys included reduced creatinine clearance, increased absolute and relative kidney weights, increased incidence and severity of mineralisation of the renal papilla, increased incidence and/or severity of tubule cell pigment, and increased incidence and/or severity of chronic progressive nephropathy. The NOAEL for kidney toxicity is considered to be 500 ppm DEHP in the diet (corresponding to 28.9 mg/kg b.w./day in the males and 36.1 mg/kg/day in the females) derived from a well-performed 104-week-study in rats and based on increased absolute and relative kidney weight in both sexes at the next higher dose level (LOAEL: 2500 ppm corresponding to 146.6 mg/kg b.w./day in the males and 181,7 mg/kg b.w./day in the females). In conclusion, an overall LOAEL of 3.5 mg/kg b.w./day has been established for testicular damage observed in rats administered DEHP by oral exposure; a NOAEL could not be established based on the available data.

For DINP, effects on the liver doses consisting of hepatic biochemical changes (increased ALT, AST) and of increased liver weights in both sexes (increase of absolute and relative liver weights) concurrently with histopathological findings have been observed in rats with NOAEL of 88 mg/kg b.w./day established from a 2-year study. This NOAEL is established for liver effects, which are not

related to peroxisome proliferation. For kidney effects, a NOAEL of 88 mg/kg b.w./day can be established from the same study based on increased kidney weights in both sexes (increase of absolute and relative kidney weights) at higher dose levels. For effect on reproductive organs (decreased testicular weight without histological changes), a NOAEL of 276 mg/kg b.w./day can be derived from a 2-year mouse study. In conclusion, an overall NOAEL of 88 mg/kg b.w./day has been established for effect observed in the liver and kidneys in rats administered DINP by oral exposure for 2 years.

For DIDP, a NOAEL of 15 mg/kg b.w./day has been identified for effects in the liver observed in a 90-day dietary study in dogs (liver weight increase accompanied by swollen and vacuolated hepatocytes at higher doses, from 75 mg/kg b.w./day). A NOAEL of 60 mg/kg b.w./day has been identified for liver effects in rats from a standard 90-day study based on increased relative liver weight in female rats at higher dose levels (from 120 mg/kg b.w./day); in males, the NOAEL for liver effects (increased weight) was 200 mg/kg b.w./day. A NOAEL of 33 mg/kg b.w./day has been derived for developmental toxicity from a two-generation study in rats as a decrease of survival indices was observed at higher dose levels (from 114 mg/kg b.w./day). In conclusion, a NOAEL of 15 mg/kg b.w./day has been identified for effects in the liver observed in a 90-day dietary study in dogs.

Conclusions

For all the selected phthalates, the highest exposure of adults seemed to be the dietary (indirect) exposure followed by either the exposure via inhalation of indoor air or dermal contact to gloves and other phthalate-containing products (different for the various phthalates). For children, the exposure via phthalate-containing toys contributed most significantly to the total daily intake. As for adults, the dietary exposure also seemed to be a major route of exposure for infants, especially via intake of infant formulae.

The critical effect(s) identified for DBP, DEHP, DINP and DIDP toxicity to reproduction as well as effects on the liver and kidney. For DBP and DEHP, the overall LOAEL has been established for reproductive toxicity; NOAELs could not be established based on the available data. For DINP and DIDP, the overall NOAEL has been established for effect observed in the liver (DINP, DIDP) and kidneys (DINP).

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Project 2.3 Exposure information based on product composition and use

Project leader: Mari-Ann Flyvholm, National Institute of Occupational Health

Background and objectives
This project surveys occurrence of surfactants and phthalates in products registered in the Danish Product Register (PROBAS). For phthalates the purpose was to generate data of relevance for assessing exposure to five phthalates. For surfactants the purpose was to obtain a preliminary overview of exposure pathways and concentrations. The results also form the basis for selecting the surfactants for hazard identification in project 1.3 and 1.6.

Methods
PROBAS is the short name for the Danish Product Register Database. It is a common register for both external environmental and work environment authorities, and it is located at the Danish Working Environment Service. Registration in PROBAS is based on obligatory notification rules, product data submitted to the authorities for various reasons, surveys and research projects on chemical products and other relevant sources. The database includes mainly hazardous products for occupational use or products containing specific types of ingredients, e.g. epoxy- and isocyanates or substances considered to be carcinogenic. Other product categories are included, but often not fully covering the products marketed. The registered information on products includes ingredients, industrial area of use, product category, quantities used or marketed, physical properties, labelling etc., and administrative information. The most frequently registered product categories are cleaning agents, paint/lacquers and toiletries/cosmetics. Some product categories relevant for the present project are not included in the registration, e.g. building materials such as insulating materials, linoleum for flooring and wallpaper. All searches were based on products registered or updated within the last 5 years. January 1999 PROBAS had computerized information on about 74,000 chemical products registered or updated within the last 5 years (data on surfactants); January 2001 the corresponding figure was 80,000 products (data on phthalates).

For phthalates the identification of substances was defined in the project plan; alternative CAS RN (Chemical Abstract Service Registry Numbers) were included for two of the phthalates. For surfactants the selection of substances was part of the project. Available list and experiences from other projects (especially input by EnPro from Project 3.4) were used for this part.

Results
All investigated phthalates were registered in PROBAS, most frequently DBP followed by BBP, DEHP, DINP, and DIDP. The concentrations were mostly below 50%. The most frequently product categories were printing inks (DBP, BBP, DEHP, DINP), paint/lacquers (DBP, BBP, DEHP), and filling agents (DBP, BBP, DEHP, DINP) and to a less extent adhesives/glue (DBP, BBP, DEHP). One of the substances (DBP) was registered in polish and in cosmetics. Information on yearly quantity (amount) for the registered products is missing for a considerable number of products registered with phthalates. This and the fact that these data are not regularly updated, limit the scope of the present project to a qualitative screening of the prevalence of phthalates in registered products (Ref.: CML working document “Eksponeringsdata baseret på produktsammensætning og anvendelse: Phthalater”, M-A Flyvholm, 2001). The results will be included and published in papers from other projects.

Conclusions
Qualitative data on the occurrence of phthalates and surfactants were obtained from PROBAS. The current limitations regarding information on yearly quantity (amount) for the registered products had made PROBAS-data less suitable for generating input data for the EUSES model. Surfactants were not studied in details due to changes in the focus of the overall project.

Project 2.4 Emission of plasticizers from construction products

Project leader: Lars Gunnarsen, Danish Building Research Institute
Project participants: Alireza Afshari, Danish Building Research Institute. Per Axel Clausen, Vivi Hansen, Peder Wolkoff, National Institute of Occupational Health

Background and objectives
The objective of the project is to modify existing emission testing procedures into a validated method for simplified emission testing of plasticizers from construction products, and to test some products suspected to contribute significantly to exposures indoors in order to generate basic input to models for total exposure to plasticizers.
Most plasticizers belong to the group of semi-volatile compounds (SVOC) with boiling points in the range 240-400 °C. At room temperature their emission rates are low and once vaporized they have a high affinity for adsorption to other surfaces. Subsequently, the products containing plasticizers may contaminate both room air and all other surfaces and dust in a room, and indoors the airways of people may be exposed through inhalation of both vapours and dust with adsorbed plasticizers.

Methods
The existing Climpaq-based procedure for emission testing of construction products was modified to get a simple method for the measurement of emissions of plasticizers from PVC and other materials. Sampling and analysis methods were optimized to measure plasticizers. The modified method was applied to a range of products. Some of them were suspected of contributing to the indoor concentration of plasticizers.

The emission from the PVC flooring in the Climpaq was compared with results from the ultra-small chamber FLEC Samples were taken in exhaust air from the chambers after 6, 35, 62, 105 and 150 days from the start of the experiment. PVC flooring was tested for an additional 100 days.

Results
Tests have lead to the optimization of the experimental procedure for sampling and analysis of phthalates from construction products. The duration of the measurements in the chambers is required to be 150 days for stable concentrations. For estimates with less precision it may be sufficient to measure after 35 days in the chambers and multiply measured concentrations with a factor in the range 1-2.5.

The following products have been tested: PVC flooring (2 types), polyolefin flooring, a refrigerator list, two electric cables, PVC skirting and floor wax (Figure 1,2).

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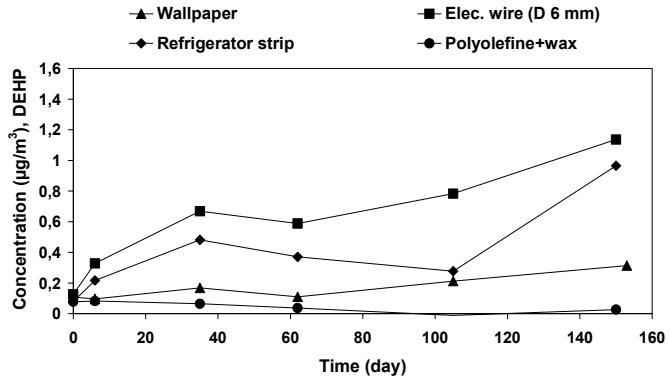


Figure 1. Concentration versus time data for emission of DEHP from some construction products in a Climpaq.

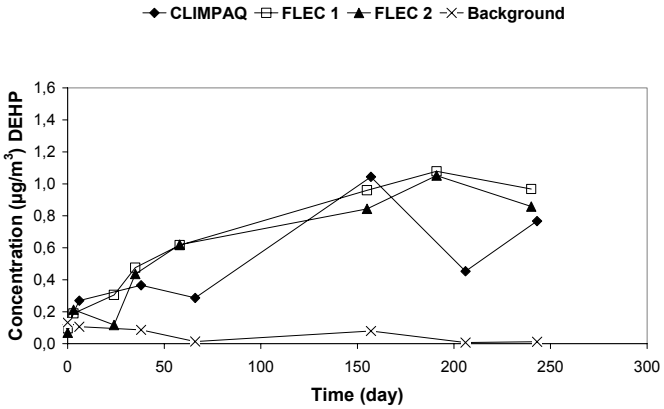


Figure 2. Concentration versus time data for emission of DEHP from PVC flooring in Climpaq and FLEC and additional background for an empty Climpaq

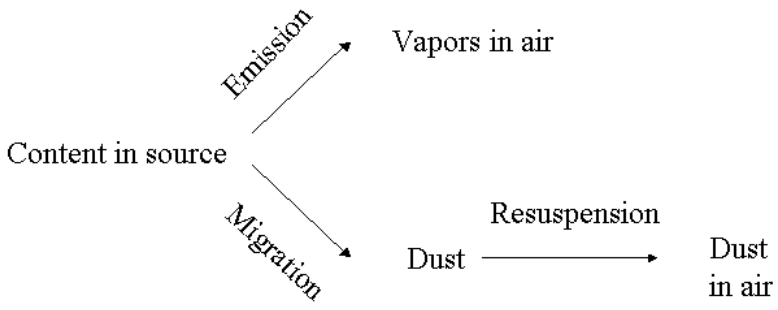
Conclusions

The tested floor wax resulted in a high concentration of DBP – far higher than for the other tested products. The other products emitted DEHP and only trace amounts of other phthalates. For these tested products the resulting concentrations in different chambers were quite similar.

Project 2.5 Plasticizers and surfactants in indoor dust.

Project leader: Per Axel Clausen, National Institute of Occupational Health
Project participants: Ali Afshari, Lars Gunnarsen, Danish Building Research Institute. Vivi Hansen, National Institute of Occupational Health. Rikke L. Lindeberg Bille, National Institute of Occupational Health and DHI Water and Environment. Tobias Nilsson, Dept. of Analytical Chemistry, Lund University. Bo Svensmark, Department of Chemistry, University of Copenhagen, Søren Bøwadt, European Commision, Research Directorate (B7, 3/12)

Background and objectives
Previous investigations have shown that house dust is likely to always contain plasticizers and surfactants, but in highly variable amounts. The purpose of this project was to measure the concentrations of plasticizers (selected phthalates) and surfactants (selected an- and non-ionic surfactants) in indoor dust samples. To allow developing an exposure model for plasticizers the project should generate data on background concentrations and knowledge on dissipation:



Methods
The same PVC flooring was tested in two very different types of chambers, the CLIMPAQ (Chamber for Laboratory Investigations of Materials, Pollution and Air Quality) and the FLEC (Field and Laboratory Emission Cell), respectively, in order to obtain very different test conditions.

The migration or sorption experiments were conducted in three CLIMPAQs in which the five test pieces were placed horizontally in contrast to the emission experiments. The three upper test pieces in each chamber were soiled on the upper side with ca. 0.5 g of the particle fraction of homogenized house dust with known content of DEHP. All other test chamber conditions were identical to the emission experiment. After five days, one month and two months the chambers were opened and the one of the test pieces was vacuumed with a specially designed stainless steel dust sampler using cleaned glass fibre filters.

The selection of 25 homes was based on a visual inspection of PVC. This showed that 11 had PVC flooring mainly in the kitchen and the bathroom and 14 had no PVC flooring. A standard household vacuum cleaner with adjustable speed equipped with the specially designed stainless steel dust sampler was used for the dust sampling. During 2 min 2 m^2 of the floor near the centre of the living room was vacuumed with each sampler. Four steel frames (1 m x 1 m) were used for that purpose. They were placed on the floor either in a square or rectangle. Each sampler was used either in diagonal frames or each second frame to compensate for inhomogeniety. The living room was the only room where it was possible to use this procedure.

In the 15 schools floor dust sampling was done with a specially designed vacuum cleaner HVS3 (Cascade Stack Sampling Systems, Oregon, USA). The HSV3 was modified to ensure a more constant suction pressure and volume. In 15 Danish schools dust was collected from 3 – 10 m^2 before the daily floor cleaning in each of 2 – 5 similar classrooms with identical floor covering. Shortly after collection the samples were divided into subsamples and stored in small glass vials at -18 °C. Ca. 10 % of each sample was used for analysis of DEHP and non-ionic surfactants. Before the extraction the dust samples were pooled to one sample for each school.

Chamber air was sampled on Tenax TA with a pump. Indoor air samples were collected with a filter/adsobent (Tenax TA) sampler designed for thermal desorption. Filters and sampled dust were extracted with pressurized liquid extraction. Extracts, filters and Tenax tubes were all analyzed by thermal desorption combined with GC-MS/FID. Dust sample extracts were analyzed for non-ionic surfactants with LC-MS.

Results

Chamber experiments showed that phthalates were emitted from PVC materials containing phthalates as plasticizers. However, the observed emission rates were in the order of magnitude of only 1 $\mu g/m^2/h$. It was found that the observed emission rates were strongly dependent on the test conditions and could not be used as estimates of emission rates in real indoor settings.

Studies published in the peer-reviewed literature indicated that compounds with physical/chemical properties similar to phthalates to a high degree (up to ~100%) are adsorbed to particles in the indoor air. Thus the relevance of pure vapor phase experiments was in question. Chamber experiments showed that phthalates from PVC soiled with house dust migrated to the dust in significant amounts.

Dust from 25 Danish homes had average concentrations of phthalates of the same level as 275 German homes and 38 Norwegian homes. The amount of PVC found in the 25 Danish homes appeared to play a minor role for the content of phthalates in dust than other sources. However, other materials such as carpets were suspected also to contain significant amounts of phthalates. A study of 15 Danish schools showed three times higher average concentrations of phthalates in the floor dust than found in the homes. This might be due to more extensive use of PVC as flooring material and a stronger physical degradation of the floor and footwear in the schools.

In a study of four offices, one classroom and a room in a daycare centre in Denmark concentration of phthalates in the air was found to be at least as high as the median concentration measured in 125 American homes.

Conclusions
The mechanisms for emission of phthalates from PVC are not fully understood. Some hypotheses concerning these mechanisms are being tested in ongoing experiments.

The phthalates in the indoor environment appear to concentrate in the dust.

The airway exposure is probably due to phthalates mainly adsorbed to particles. The high boiling phthalates may be 100% adsorbed to particles, the lower boiling less.

Removal of the dust should be an effective way to minimize airway exposure to phthalates in the indoor environment.

Project 3.1 Hazard assessment models for adjuvant effects based on structure activity

Project leader: Erik Olsen. National Institute of Occupational Health.

This project has been integrated with project Project 1.3 “Adjuvant effect of surfactants and plasticizers *in vivo*”

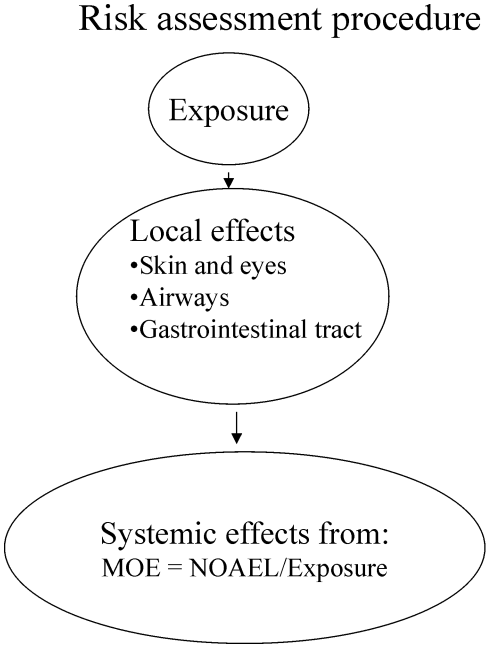
Conclusions
Several physicochemical parameters were evaluated for structure-activity relationship (SAR) analyses for Project 1.3. However, none of the parameters were more efficient than the (simple) number of carbon atoms in the side chain of the molecules. Due to parsimoniousness, this parameter was selected for the SAR analyses in Project 1.3. The efficiency of the number of carbon atoms as predictor of physicochemical parameters was further confirmed in Project 3.3.

Project 3.2 Risk assessment models for adjuvant effects

Project leader Gunnar Damgård Nielsen. National Institute of Occupational Health.
Project participants: Ole Ladefoged, Elsa Nielsen, Anne Kristine Müller, Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration

Background and objectives
The lack of toxicological data has increased the need to assess to which degree existing knowledge of toxicological properties of substances can be used for predicting toxicological properties of substances that have not been investigated. This has resulted in extended efforts to use computer models or other extrapolation models. The objective thus was to group existing toxicological data across groups of substances.

Methods
The integrated model with the different types of effect of each phthalate will use each exposure route, exposure level, local effects and systemic effects for the evaluations. The systemic effect will be assessed from the margin-of-exposure (MOE) approach, which compares the no-observed-adverse-effect level (NOAEL) and the exposure level (Exposure). The overall model is:



Results
The toxicity of the phthalates are currently being reviewed, which are based on the EU evaluations. It is expected that the review process will be finished in May this year and after that time, the last data are available for the construction of the model that will be compiled in a scientific paper and submitted to a scientific journal. The local effects in the respiratory tract and the exposure data from Clausen and co-workers allow hazard identification.

Table 1. Adjuvant effect of phthalates in a murine injection model [a)].

Substance	Number of carbon atoms in side chains	Presence of concentration-response relationship	Ratio between IgG1 in the test and corresponding ovalbumin group)[b)]	Adjuvant concentration (µg/mL)	Phthalate concentration in house dust (µg/g)		
					Germany [c)]	Norway	Denmark
DBP	4	Possibly (IgG1)	4	200	240	100 (10-1030)[d)] 370 (130-690) [e)]	
BBP	4 og 7	Not present	1	-	320	110 (0-440) [d)] 140 (0-750) [e)]	
D*n*OP	8	Yes (IgE, IgG1)	10	20 and 2000	-	-	
DEHP	8	Yes (IgG1)	10	2000	2600	640 (100-1610)[d)] 600 (240-940) [e)]	3214 (7063) [f)]
DINP	9	Yes (IgE, IgG1)	7	200 and 2000		≤ 100 [d)] 0 [e)]	
DIDP	10	Not present	1	-	-	-	-

a) Data from project 1.3

b) The maximum adjuvant effect taken from the median serum concentration in a test that has been divided by the corresponding concentration in the group that only has been treated with ovalbumin. Both IgE and IgG1 are Th2 cytokine dependent antibodies (Corry & Kheradmand 1999)

c) The 95% percentile for content in dust from 286 representative homes in the northern part of Germany (Butte et al. 2001).

d)) Mean phthalate concentration (range in parenthesis) in mixed dust from children's bed and bedroom, and floor in central living rooms and from top of shelves in central living rooms in 38 Norwegian dwellings (Øie et al. 1997).

e) Mean phthalate concentration, range in parenthesis, in suspended dust in 6 Norwegian dwellings (Øie et al. 1997).

f) Mean value and 95% percentile from floor dust in 15 Danish schools, project 2.5.

Conclusions

Within the area of adjuvant effects an association was found between the structure of substances and their toxicological effects, as shown in the Table 1.

Project 3.3 Modeling of Surfactant's Chemical Structure

Project leader: Erling H Stenby, IVC-SEP, Department of Chemical Engineering, Building 229, Technical University of Denmark, DK-2800 Lyngby, Denmark.
Project participants: Hongyuan Cheng, Georgios M. Kontogeorgis. IVC-SEP, Department of Chemical Engineering, Building 229, Technical University of Denmark, DK-2800 Lyngby, Denmark.

Background and objectives

In order to ensure that surface-active compounds (surfactants) are used in an optimal manner from an environmental, technical and economical point of view, it is necessary to have thermodynamic models that can predict the properties of surfactants such as critical micelle concentration (CMC), octanol-water partition coefficient (Kow), etc. Hence, the objective of the project is to develop predictive tools that can describe the physical properties and phase behaviour of surfactants in both hydrophilic and hydrophobic environments. The project will provide knowledge regarding the chemical structure of surfactants, and thereby create the basis for the development of structure-activity models for surfactants.

Results

Experimental Data Collection

Experimental data of physical properties of surfactants and surfactant solutions have been collected and reviewed from open literatures. The work was done in collaborating with experts in project 3.4. Some of the data, e.g. octanol-water partition coefficient (Kow) and critical micelle concentration (CMC), were collected in Microsoft excel format. All data are available in regular papers.

During a project meeting on 9, November, 1999 at CML, it was decided that priority should be given to the following surfactant families: alkylsulphates (AS), alkylethersulphates (AES), alkylbenzylsuphates (LAS) and alcoholethoxylates with AS and alcoholethoxylates being of major important.

Model Development

Many empirical correlations have been found for the physical properties of surfactants, e.g. critical micelle concentration (CMC), aggregate number, hydrophilic-lipophilic balance (HLB) and Krafft point. By using these empirical relations, the aggregate number, HLB and Krafft point can be obtained from a few key properties, especially CMC and Kow. Therefore, the structure-activity model development has been focused on these key properties (CMC, Kow).

Models for Critical Micelle Concentration (CMC)

Various theoretical and empirical methods exist in the literature for the critical micelle concentration of surfactant solutions. But no method can really predict CMC. In this work, a predictive group-contribution method (UNIFAC) has been developed to predict and correlate CMC for non-ionic surfactant (alcoholethoxylates, $Cn(OC_2H_4)nOH$) and ionic surfactants (alkylsulphates, $CnOSO_3Na$) based on the chemical structures of surfactant molecules (Ref. Fredenslund, A., J. Gmehling, and P. Rasmussen, *Vapor-Liquid Equilibria Using UNIFAC-a Group-Contribution Method*, Elsevier: Amsterdam, 1977). Using the UNIFAC model, the critical micelle concentration of alcoholethoxylates in aqueous solutions can be predicted according to chemical structure of surfactants shown in Figure 1, where alcoholethoxylates are abbreviated as C*i*E*j*; *i* is the number of alkylcarbon; *j* is the number of oxyethylene group OCH_2CH_2. The critical micelle concentration of

sodium alkylsulphates ($CnOSO_3Na$), sodium alkylsulphonates ($CnSO_3Na$) and potassium carboxylates (CnCOOK) can be correlated and extrapolated with their chemical structures (shown in Figures 2-7).

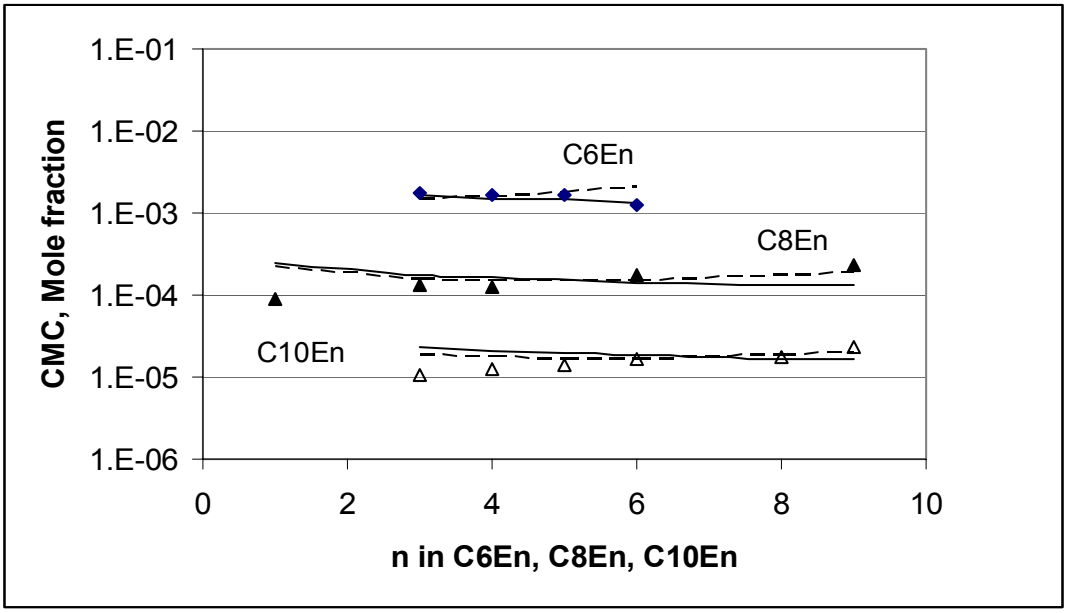


Figure 1. Predicted and fitted CMC values for alcohol ethoxylates ($Cn(OC_2H_4)nOH$) at 25ºC. all points are experimental CMC data; solid lines are prediction results of UNIFAC model; dash lines are fitting results of UNIFAC model.

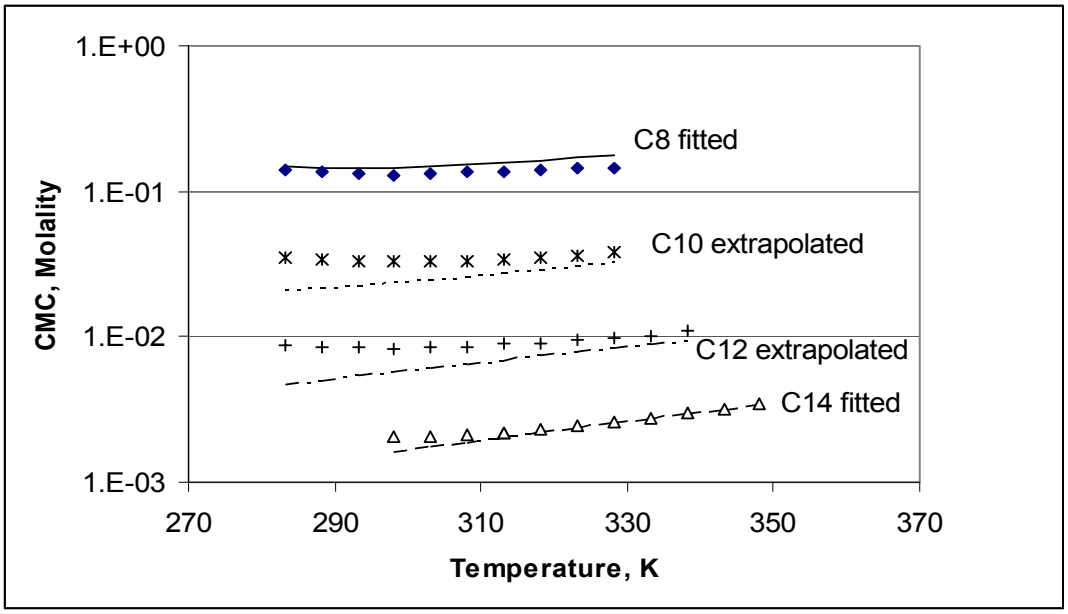


Figure 2. Correlation and extrapolation results for sodium alkyl sulphates. All points are experimental CMC data. "C8 fitted" and "C14 fitted" are model-fitting results for C_8OSO_3Na and $C_{14}OSO_3Na$. "C10 extrapolated" and "C12 extrapolated" are model extrapolation results for $C_{10}OSO_3Na$ and $C_{12}OSO_3Na$.

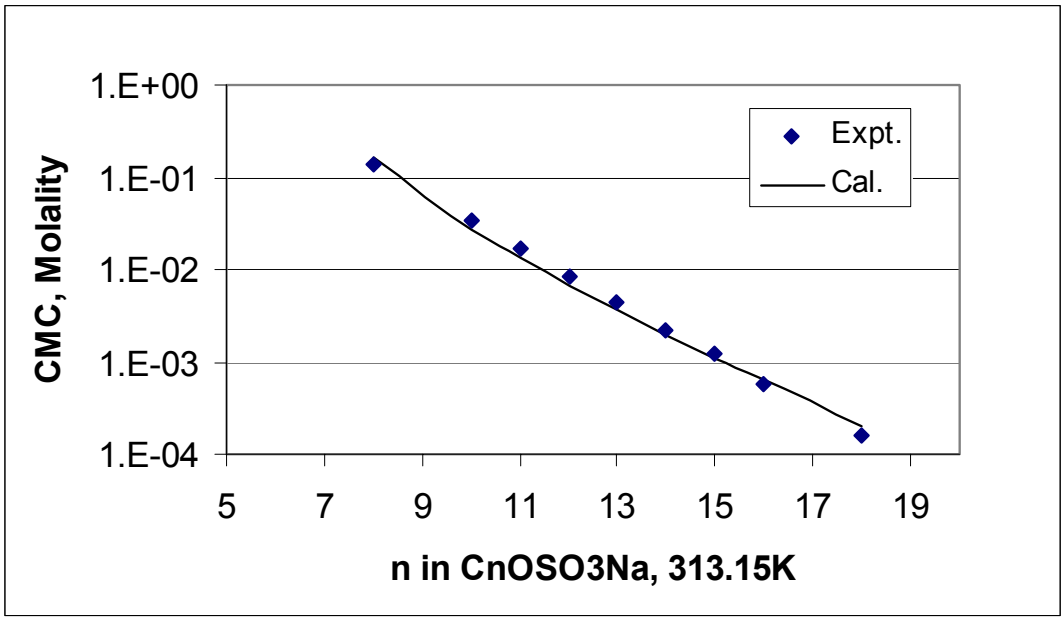


Figure 3. Model extrapolation results for sodium alkyl sulphates. Points are experimental CMC data. Solid line is model extrapolation results for $CnOSO_3Na$, n=8 to 18 at 313.15K.

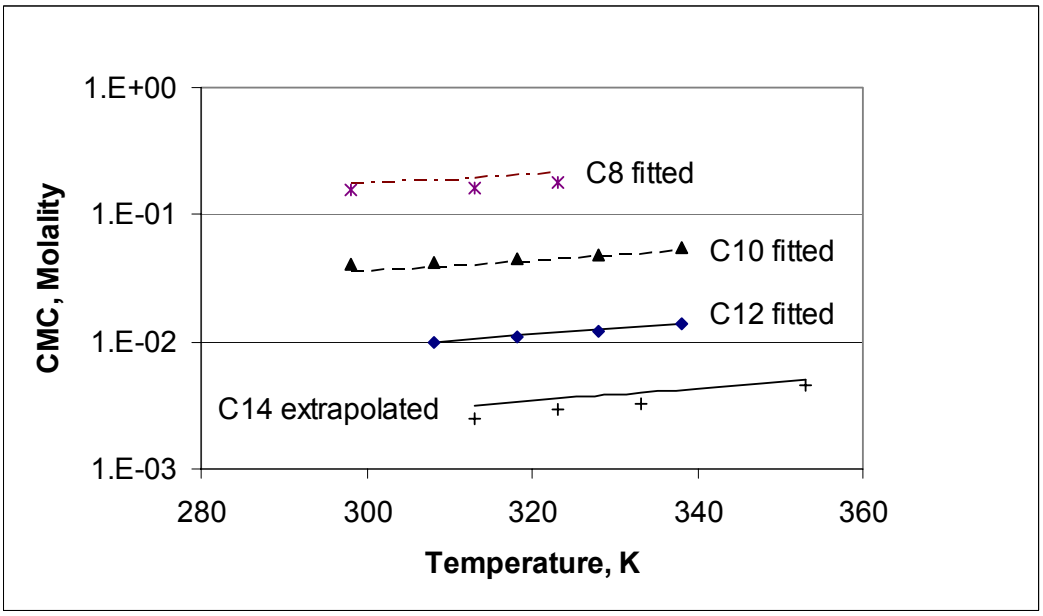


Figure 4. Correlation and extrapolation results for sodium alkyl sulfonates. All points are experimental CMC data. "C8 fitted", "C10 fitted" and "C12 fitted" are model-fitting results for C_8SO_3Na, $C_{10}SO_3Na$ and $C_{12}SO_3Na$. "C14 extrapolated" is model extrapolation results for $C_{14}SO_3Na$.

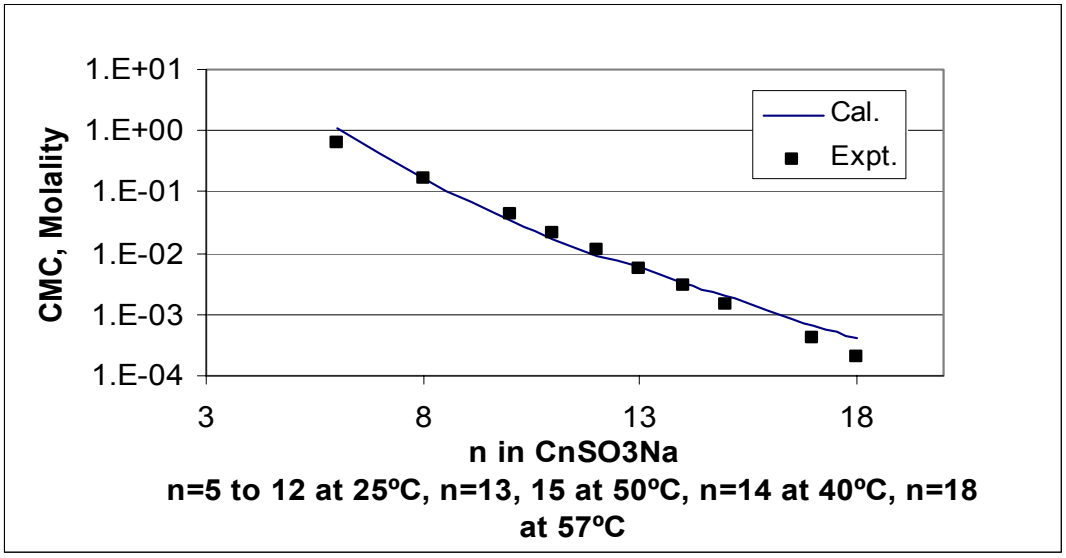


Figure 5. Model extrapolation results for sodium alkyl sulfonates. Points are experimental CMC data. Solid line is model extrapolation results for $CnSO_3Na$, n=5 to 18 at different temperatures.

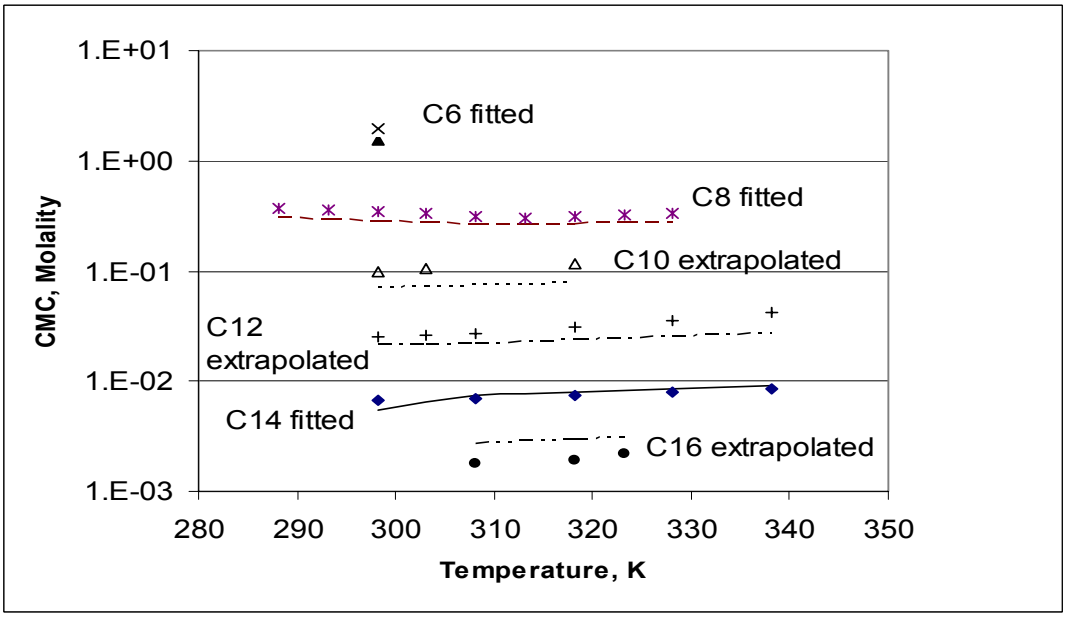


Figure 6. Correlation and extrapolation results for potassium carboxylates. All points are experimental CMC data. "C6 fitted", "C8 fitted", and "C14 fitted" are model fitting results for C_5COOK, C_7COOK and $C_{13}COOK$. "C10 extrapolated", "C12 extrapolated" and "C16 extrapolated" are model extrapolation results for C_9COOK, $C_{11}COOK$ and $C_{15}COOK$.

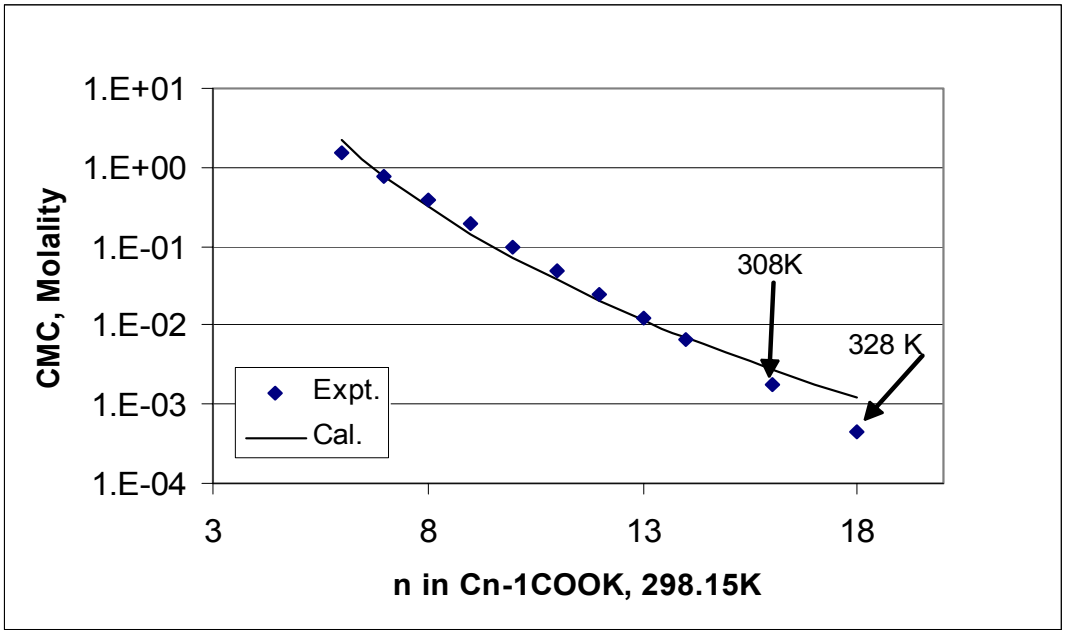


Figure 7. Model extrapolation results for potassium carboxylates. Points are experimental CMC data. Solid line is model extrapolation results for $C_{n-1}COOK$, n=6 to 18 at 298.16 K except two points marked on the figure.

Models for the Octanol-Water Partition Coefficient (Kow)

It is difficult to measure the octanol-water partition coefficients of surfactants because the surfactants adsorb on the surface (interface) of liquids or solids. An octanol-water partition coefficient database-*LOGKOW* (*LOGKOW*, Sangster Research Laboratories, Montreal, 2001) includes more than 20000 chemicals and is available in electric format. About 100 chemicals in the database are surfactants. The UNIFAC model was used to predict octanol-water partition coefficient of phthalates and alcoholethoxylates. The prediction results have been compared with a few experimental data and three commercial softwares (ACD/LogP, version 4.5, Advanced Chemistry Development, Inc. 2001; ClogP, Daylight Chemical Information systems, Inc. (www.daylight.com), 2001; KowWin, Syracuse Research Corporation (esc.syrres.com), 2001.). The UNIFAC method is as accurate as these commercial programs that are developed specially for Kow (figures 8-9).

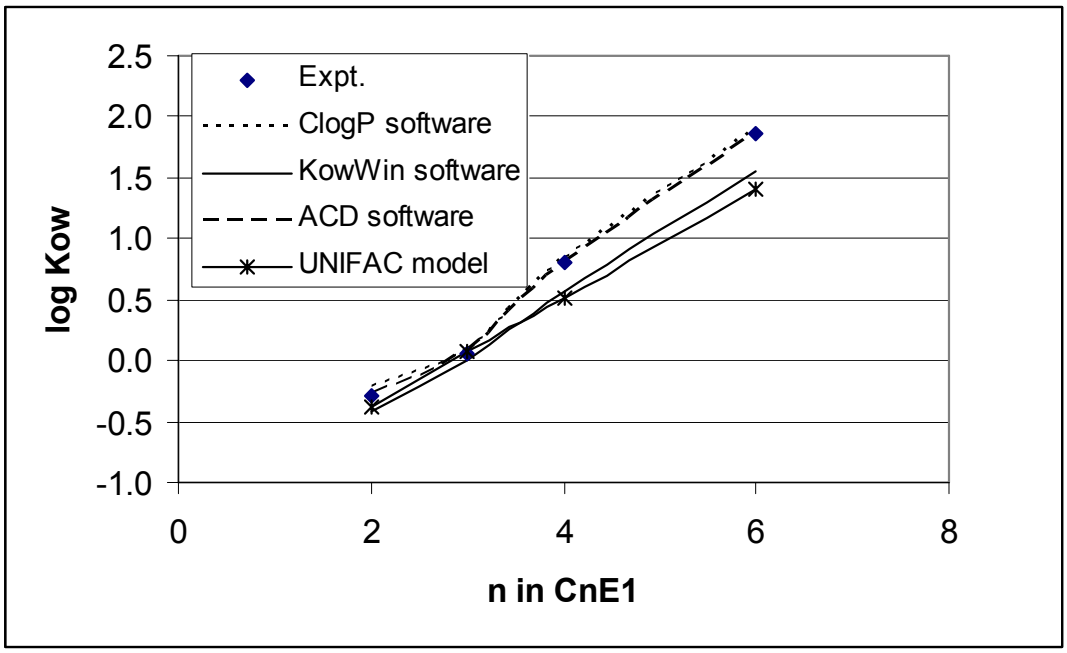


Figure 8. Experimental and predicted Kow values for CnE_1. Experimental data are from *LOGKOW* database.

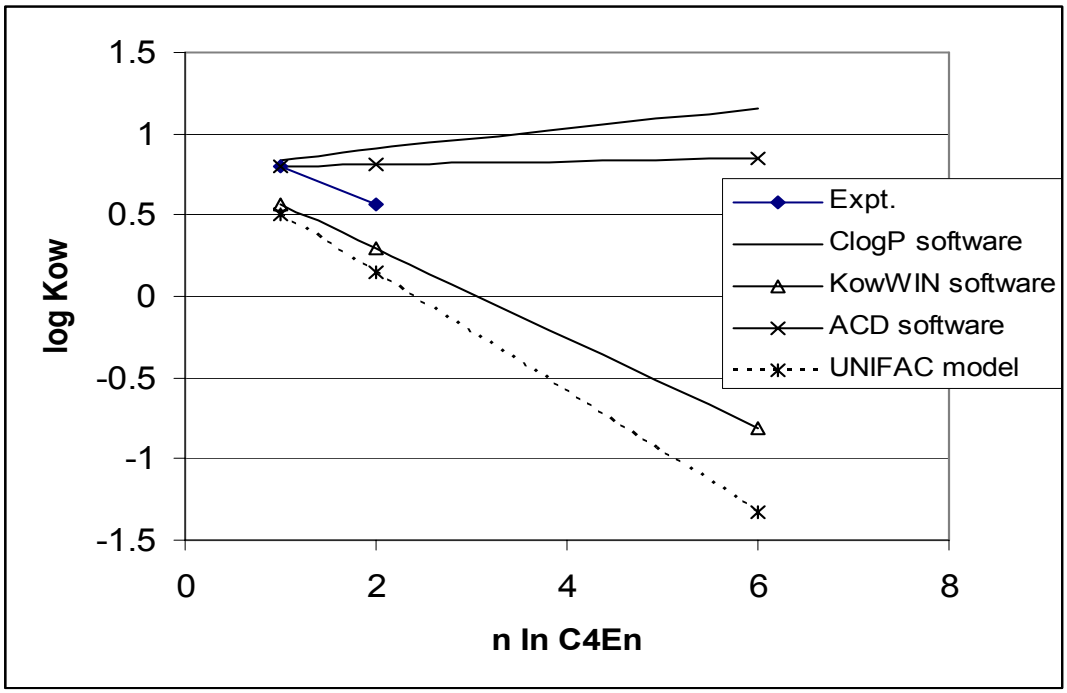


Figure 9. Experimental and predicted Kow values for C_4En. Experimental data for C_4E_1 and C_4E_2 are from *LOGKOW* database

Physical Properties Based on CMC

Based on CMC and Kow obtained by the UNIFAC model, other properties, e.g. HLB, Krafft point and phase inversion temperature, can be calculated from empirical relations considered in this work. Some calculation examples are shown in figures 10-11.

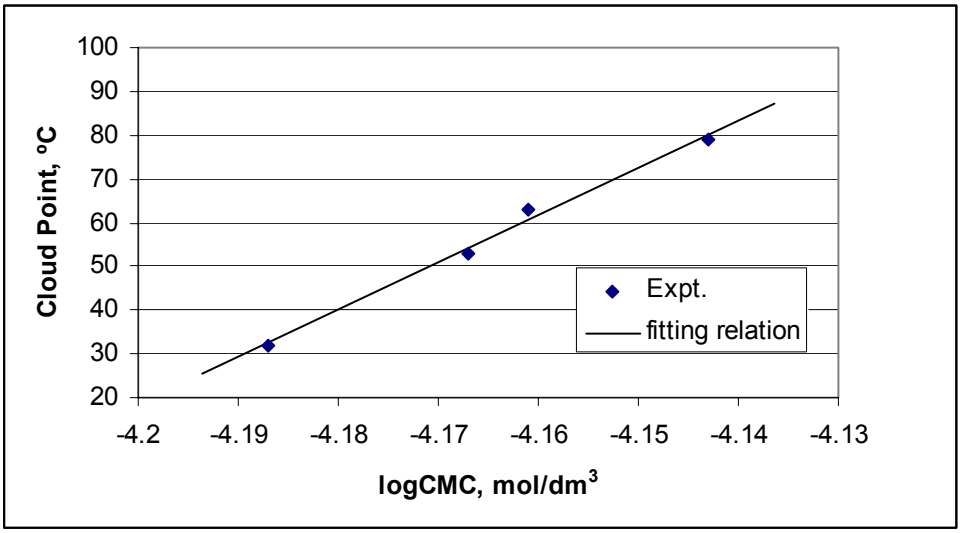


Figure 10. Cloud point versus CMC for non-ionic surfactant $C_{12}H_{25}(OC_2H_4)_mOH$.

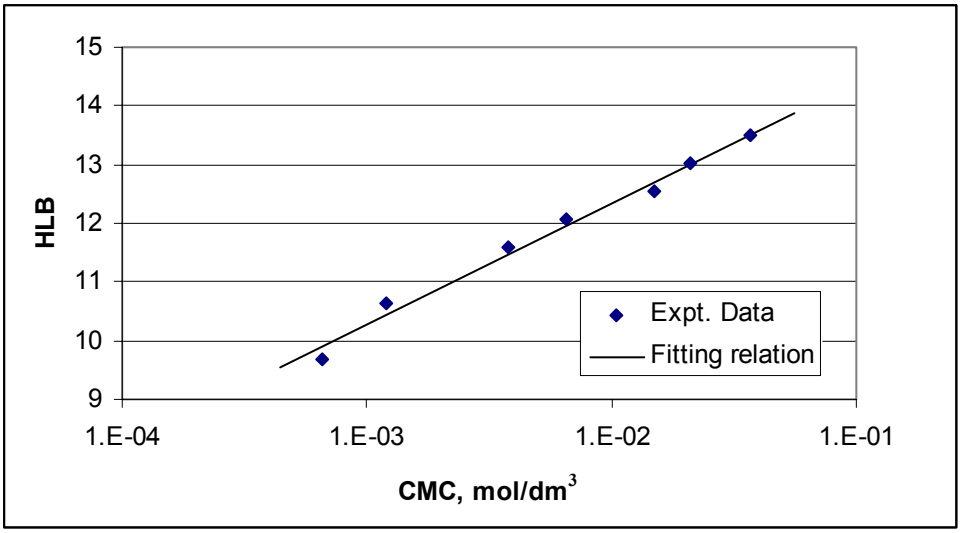


Figure 11. HLB versus CMC for $C_nH_{2n+1}C_6H_4SO_4Na$ surfactant

Project 3.4 Models for substitution of surfactants

Project leader Eva M Wallström, EnPro APS
Project participants: Charlotte Pilemand, EnPro APS

Background and objectives
Surfactants are used in almost any household product and personal care product as well as in nearly every industrial process. Contact with products containing surfactants is therefore inevitable. Furthermore, some surfactants only degrade slowly in the environment. Surfactants may have ecotoxic or adjuvant effects. The purpose of the project thus was to demonstrate to industry, that similar products can be produced by using alternative, less hazardous surfactants.

Methods
A literature study has been performed regarding surfactants from a technical and formulation viewpoint.

Results
Surfactants are described in general terms by discussion of chemical structure, types and abilities. The four different main types of surfactants: anionic, cationic, non-ionic and amphoteric surfactants are shortly presented and examples of compounds belonging to each type are listed. The most important technical abilities of surfactants and surfactant solutions like emulsification, solubility, wetting, dispersing, foaming and detergency are discussed and these abilities are related to relevant physico-chemical properties. It is discussed which physico-chemical properties are important for surfactants from a technical point of view and therefore useful to know if substitution is the issue. The results have been reported in: Charlotte Pilemand. Surfactants. Their abilities and important physico-chemical properties, by, EnPro ApS, May 2000.

Conclusions
The results have created an overview of the multitude of surfactants used. The report provides a tool for industry for identifying proper technical alternatives for a given surfactant.

Dissemination

Centre days
All participants in the Centre have participated once a year in a Centre day for discussing in plenum and in workshops content, problem formulations, and overall progress of the projects.

At each of the Centre days in 1999, 2000 three internationally acknowledged researchers outside Denmark gave a scientific presentation and participated in the discussions in workshops. These researchers contributed significantly to heighten the professional level and to strengthen the international network of the Centre. In 1999 it was

- *Dick Heederik, Dept. of Environmental Sciences, Agricultural University Waageningen*
- *Matti Jantunen, National Public Health Institute, Kuopio*
- *Leif Øie, Dept. of Population Health Sciences, Nat. Inst. of Public Health, Oslo*

and in 2000 it was

- *Lena Palmberg, National Institute of Working Life, Solna, Sweden*
- *S.A. Kyrtopoulos, National Hellenic Research Foundation, Inst. of Biological Research & Biotechnology, Athens, Greece*
- *Harald Renz, Department of Clinical Chemistry, University of Marburg, Germany*

The Centre day in 2001 was arranged as a two-day residential meeting to provide the frame for in-depth discussion of how to continue the Centre activities and the established cooperation after end of the programme.

Meetings within each of the three main topics
Members of the board have organized meetings within each of the three main topics, where the participants coordinated their research activities, exchanged ideas, and discussed scientific problems, joint publications etc.

The contact group
The Centre has established a larger contact group consisting of representatives from various agencies, trades/industries, and NGO's. At the annual contact group meetings, project leaders presented and discussed results obtained by the Centre and how to disseminate the knowledge.

Centre work-shop and scientific meeting
The Centre organizes the Workshop "Pollution in ambient and indoor air –Risk assessment in relation to pulmonary effects" December 5, 2002. The purpose was to bring together - in the final stage of the research activities of the Centre - invited experts and the project leaders of the Centre for discussion of results and future challenges in the research field of the Centre. Three invited speakers addressed the ambient air

- *Jan Beyea, USA. "Historical reconstruction of exposure to traffic-generated, polycyclic aromatic hydrocarbons: model validation and calibration for epidemiologic studies"*
- *Kirsi Timonen, Finland. "Epidemiology of health effects related to ultrafine particles"*
- *Roels Schins, Germany. "Mechanisms of action of air pollution particles"*

and three invited speakers the indoor air

- *Dick Heederick, The Netherlands. "Risk assessment of microbial contaminants in the indoor air. Future trends and implications for air pollution regulation"*
- *Martinus Løvik, Norway. "Particulate air pollution, allergy and asthma"*

- *James Brewer, Scotland. "Adjuvants and mechanisms of immune stimulation"*

Project leaders of the Centre presented results of the research on traffic generated particulate pollutants, micro-organisms, plasicizers, and surfactants obtained by the Centre with emphasis on their practical implications for risk assessment.

The following day a scientific meeting organized jointly by the Research Centre for Environment and Health and the present Centre presenting the results to a broader audience.

Special issues of national journals
A summary of all the projects achievements will be presented in a special issue of "Miljø og Sundhed" (Research Centre for Environment and Health), and in a thematic issue of "Miljøforskning" (Danish Environmental Research Programme).

Related and derived research activities

Traffic emission

A large part of the Centre research group also takes part in the TRIP programme (Transport Research on environmental and health Impacts and Policy), financed by the Danish Environmental Research Program by 19.5 mio DKK. The exposure models developed in TRIP are employed to study health outcomes in terms of acute cardiopulmonary morbidity and mortality, birth weight, airway symptoms in small children at risk of atopy, annoyance and self-reported symptoms, and obstructive lung disease. Studies in small children are also funded by the Danish EPA. Moreover, based on funds from the Research Centre for Environmental Health (ISMF) and the Danish EPA similar studies focused on measurement and modelling of personal exposure to ultrafine particles are ongoing. In this study an integrated GPS and mobile phone is applied for tracking individuals that promises very few technical and handling problems. The National Budget for the fiscal year 2001 has assigned 5 mio DKK/year for four years for the study of air pollution from fine particles. Further funding will be applied for from the Health Effects Institute, USA.

In parallel with the Centre, professor Steffen Loft, University of Copenhagen has coordinated a four year long programme financed by the Danish Research Council with a total of 14 mio. DKK. This programme elucidates how the oxidative damage caused by air pollution can be modified by intake of food. For a grant from the Research Centre for Environmental Health (ISMF) the Centre for Environment and Cancer, headed by Steffen Loft, has been established. This Centre has participants of a major part of the project group of the present Centre. The new centre studies associations between environmental factors, including traffic emission, and cancer in lung and breast based on population studies in Copenhagen and Aarhus.

Participants from the Institute of Public Health and University of Aarhus participate in a EU project on biomarkers and exposure to air contaminants in an Estonian mine.

A Ph.D. grant funded by the Danish Environmental Research Program.

Micro-organisms and their biologically active components

Project 1.2, DAMOS, obtained additional was supported from the Swedish Building Research Foundation, FORMAS (Sweden), “Statens Miljøforsknings Projekter” (Denmark), and The National Danish Research Foundation for Health Sciences (SSVF).

One Ph.D. grant was obtained from the University of Aarhus Research Foundation and one Ph.D. project received additional funding from the Danish Pesticide Research Program.

Synergy has been achieved with the research programme ”Molds in Buildings” funded by the Danish Research Council and headed by professor Finn Gyntelberg, Bispebjerg Hospital.

Plasticizers and surfactants

A project on adjuvant effects of quaternary ammonium compounds has been granted by the Danish Working Environment Fund to extend the activities of the Centre.

A total of five Ph.D. grants were obtained from the Danish Environmental Research Program, the Danish Research Training Council, and the Danish Working Environment Fund.

Synerggy has been achieved with the Danish Allergy Research Centre (DARC), headed by research director Lars K Poulsen.

Education of researchers

The following PhD students have been associated full or part time with the Centre:

Traffic emission

- Mette Sørensen, Copenhagen University, Institute of Public Health. "Biomarkers and Air Samplers for Assessment of Exposure and Effects of Urban Air Pollution". Was a central part of Project 2.1. Thesis submitted April 2002 and defended 24. January 2003. Funded by the Danish Environmental Research Program.

Micro-organisms and their biologically active components

- Jakob Bønløkke. Aarhus University, Department of Environmental and Occupational Medicine. Related to project 1.1 and 1.2. Funded by University of Aarhus Research Foundation.
- Zhiwei Pan. University of Aarhus, Institute of Environmental and Occupational Medicine, submitted (2002). Paper III in the Thesis "Ocular effects in humans following experimental exposures to different types of dust" was part of project 1.2
- Preben Larsen. Odense University, Department of Occupational and Environmental Medicine. Thesis in preparation. Additional grant from Danish Pesticide Research Program.

Plasticizers and surfactants

- Søren Thor Larsen. Royal Danish School of Pharmacy, Department of Pharmacology. "Adjuvant effect of phthalates and monophthalates in a murine injection model". Defended in 2002. Funded by the Danish Environmental Research Program (SMP 98).
- Susanne Knoth Clausen. University of Copenhagen, Faculty of Health Sciences. Accepted for defense (2003). Funded by the Danish Research Training Council.
- Christian Glue. University of Copenhagen, Faculty of Health Sciences."Inflammatory and allergic reactions to plasticizers *in vitro*" accepted for defense May 2, 2003. Funded by the Danish Environmental Research Program.
- Hongyuan Cheng. Technical University of Denmark, Department of Chemical Engineering. "Modelling of Surfactant Solutions". To be submitted in 2003. Funded by the Danish Environmental Research Program.
- Jitka Stilund Hansen started in 2002 and continues the research on adjuvant effects of surfactants. Funded by the Danish Working Environment Fund.

Publications by the Centre

The total number of publications and presentations are shown in the table.

Project No.	Scientific papers and proceedings			Reports and book chapters			Scientific presenta-tions in English	Scientific presenta-tions in Danish	Dissemi-nation to users
	P	S	I	P	S	I			
Traffic emission (1.4 and 2.1)	42	1	3	9			41	24	23
Microorganisms (1.1, 1.2, 1.5, 2.6, 3.5)	4	4	13	3		1	23	9	7
Plasticizers & surfactants (1.3, 1.6, 2.2, 2.3, 2.4, 2.5, 3.1, 3.2, 3.3, 3.4)	23	2	3	9	1		13	23	15

P Published, in press or accepted.
S Submittted.
I In preparation

Scientific papers and contributions to proceedings

2.4 Afshari A, Gunnarsen L, Clausen PA, Hansen V. Emission of phthalate esters from PVC and other plasticized materials. *Indoor Air*. Submitted.

2.1. Berkowicz R. Modelling street canyon pollution: model requirements and expectations. *Int. J. Environment and Pollution* 1997;8:3-6.

1.5 Bonefeld-Jørgensen EC, Sigsgaard T. Optimisation of the ex vivo whole blood assay. In preparation.

1.5 Bonefeld-Jørgensen EC, Sigsgaard T. Whole-blood assay. Methodological aspects. In preparation.

1.5 Bonefeld-Jørgensen EC, Thomassen M, Bønløkke JH, Sigsgaard T. Cytokine mRNA relase in symptomatic fish filetting workers. In preparation.

1.4 Bornholdt J, Dybdahl M, Vogel U, Dragsted LO, Loft S, Wallin H. Inhalation of ozone induces DNA strand breaks and inflammation in mice. *Mutation Res* 2002;520:63-68.

1.5 Bønløkke JH, Thomassen M, Viskum S, Omland Ø, Bonefeld-Jørgensen EC, Sigsgaard T. Respiratory symptoms and ex vivo cytokine release are associated in the whole blood from employees processing herring. Submitted.

1.5 Bønløkke J, Bonefeld-Jørgensen EC, Sigsgaard T. Whole-blood assay. The inflammatory potential of components in organic dust. In preparation.

3.3 Cheng HY, Kontogeorgis GM, Stenby EH. Prediction of Micelle Formation for Non-ionic Surfactants through UNIFAC Method. *Ind Eng Chem Res* 2002;41:892.

3.3 Cheng HY, Kontogeorgis GM, Stenby EH. Prediction of Critical Micelle Concentration of Surfactant Solutions Using the UNIFAC Model. International Symposium, Colloid and Interface Technology Fundamentals and Applications, November 6-8, 2002, Lund, Sweden.

2.5. Clausen PA, Wolkoff P, Svensmark B. Preliminary study of semivolatile organic compounds in some danish indoor environments. *Indoor Air 99, Proceedings of the 8th International conference on Indoor Air Quality and Climate*, Edinburgh, Scotland, 8-13 August 1999;2:434-439.

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2.5 Clausen PA, Bille RLL, Nilsson T, Hansen V, Svensmark B, Bøwadt S. Simultaneous extraction of di(2-ethylhexyl)phthalate and non-ionic surfactants from house dust. Concentrations in floor dust from 15 Danish schools. *J Chromatogr A* 2003;986(2):179-190.

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1.3. Clausen SK, Sophani S, Poulsen OM, Poulsen LK, Nielsen GD. Adjuvant Effect of Model Surfactants from the groups of Alkyl Sulphates, Alkylbenzene Sulphonates, Alcohol Ethoxylates and Soaps. *Food Chem Toxicol* 2000; 38:1065-1074.

1.3 Clausen SK, Bergqvist M, Poulsen LK, Poulsen OM, Nielsen GD. Development of sensitisation or tolerance following repeated OVA inhalation in BALB/cJ mice. Dose-dependency and modulation by the $Al(OH)_3$ adjuvant. *Toxicology* 2003;183:51-68.

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