Inflammatory Potential of Dust from Waste Handling Facilities Measured as IL-8 Secretion from Lung Epithelial Cells In Vitro

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Objectives: Organic dust contains several different components which may cause pulmonary effects, and many health problems have been associated with the collection and recycling of organic waste. It is often difficult to obtain a precise measurement of the exposure to each component in dust, and organic dust samples obtained from different workplaces may vary profoundly in composition. The aim of this study was to evaluate the inflammatory potential of dust from different waste handling plants. Furthermore, we set out to investigate the role of endotoxin in the inflammatory potential of dust.

Methods: Dust samples were obtained from four incineration plants, three samples from a plant sorting household waste, five paper-sorting plants, two mail centres, four bottle-sorting plants, and two combined paper-sorting and composting plants. The samples were tested in a bioassay with the lung epithelial cell line A549. Cells were stimulated for 24 h with dust samples at six concentrations, and subsequently the interleukin 8 (IL-8) secretion into the growth medium was measured. The initial slope of the dose response curves was used to calculate the potency factor (PF) of the dust samples, and correction against positive control samples was used to reduce day-to-day variation. The concentration of endotoxin in the dust samples was measured by the limulus amebocyte lysate (LAL) assay.

Results: The inflammatory potential of the dust samples for dust from the paper- and mail-sorting plants showed a significantly lower PF as compared with dust from the plants handling mixed household waste. A significantly lower PF for the dust samples from the bottle-sorting plants (excluding one outlier plant) compared with dust from the plants handling mixed household waste was also found. No correlation was observed between the PF and the concentration of endotoxin in the samples.

Conclusion: The PFs obtained seem to reflect the material handled, with mixed household waste generating organic dust with the highest inflammatory potentials.

INTRODUCTION

Several occupational health problems have been described in relation to the collection and sorting of household waste for recycling and incineration. These problems are often related to exposure to organic dust, and range from slight irritation of the eye and the mucus membranes of the throat, to severe pulmonary diseases such as toxic pneumonitis (Organic Dust Toxic Syndrome = ODTS), allergic and toxic asthma, bronchitis, and allergic alveolitis (Sigsgaard et al., 1994a; Poulsen et al., 1995a, b). Also, itching of the skin, nausea, vomiting or diarrhoea are observed among workers handling waste materials (Lundholm and Rylander, 1980; Sigsgaard et al., 1994a; Poulsen et al., 1995b). It is well known that health symptoms differ among workers from different types of recycling plants.
The symptoms are often related to the handling of mixed household waste, and compost, while the sorting of more pure fractions such as paper seems less harmful (Lundholm and Rylander, 1980; Sigsgaard et al., 1990, 1994a, b; Malmros et al., 1992; Malmros and Jonsson, 1994).

Organic dust is characterized by a high content of both living and dead organic material of vegetable, animal or microbial origin (Chan-Yeung et al., 1994). Endotoxin, an integrated part of the outer membrane of Gram-negative bacteria, is often related to the observed symptoms caused by exposure to organic dust (Olenchock, 1994; Rylander, 1997b). Exposure to endotoxin may induce respiratory effects such as toxic pneumonitis, airway inflammation, bronchitis, asthma and systemic effects such as toxic pneumonitis, airway inflammation, bronchitis, asthma and systemic effects such as fever, pain in muscles and joints, and excessive fatigue (Rylander, 1997b). \( \beta-1,3-D-\text{Glucan} \), a component of the membrane of fungi and actinomycetes, is also an important constituent of dust (Fogelmark et al., 1991; Rylander et al., 1993). The symptoms related to exposure to glucans are not as clear and well documented, but a correlation between airway inflammation and exposure to glucans has been reported (Rylander, 1997a), and also "sick building syndrome" (SBS) may be related to exposure to glucans (Rylander et al., 1992). Fogelmark et al. (1994) found a synergistic effect of endotoxin and glucan on the influx of inflammatory cells to the alveolar space and beginning of granuloma formation in the lungs of guinea pigs. In addition, specific mycotoxins or toxins from bacteria may cause adverse pulmonary effect in some work environments (Dutkiewicz, 1997). Inorganic substances, such as metals, may also contribute to an inflammatory response in the lung (Carter et al., 1997).

Consequently, organic dust contains several different components, and it is often difficult and very expensive to obtain a precise measurement of each relevant component in order to estimate the risk of developing airway problems. This is further complicated by the fact that organic dust samples obtained from different workplaces may vary profoundly in composition (Poulsen et al., 1995b). Alternatively, use of in vitro methods for the screening of the overall biological hazards of organic dust, regardless of the concentrations of the individual components, makes it possible to rank dust samples according to a given parameter, for example the inflammatory potential.

The epithelial lining of the lung is an important first line of defence against inhaled foreign particles. Although the lung epithelium is not a central part of the immune system, the lung epithelial cells are capable of cytokine production, especially IL-1, IL-6 and IL-8 (Devalia and Davies, 1993; Adler et al., 1994; Stadnyk, 1994). IL-8 is a proinflammatory cytokine with neutrophilic chemotactic capacity. IL-8 is often found in bronchoalveolar lavage (BAL) especially in patients with chronic airway inflammation, for example due to infection by the Gram-negative bacteria *Pseudomonas aeruginosa* (Oishi et al., 1994). IL-8 seems to be a universal indicator of pulmonary inflammation. In bioassays for the assessment of the potency of organic dust, the secretion of IL-8 from lung epithelial cells is measured after in vitro stimulation (Hansen et al., 1997, 1999; Palmberg et al., 1998; Allermann and Poulsen, 1999). The preferred cell line is the human A549 carcinoma lung epithelial cell, which displays many characteristics in common with the human type II lung epithelial cells. The A549 cell line secretes IL-8 when stimulated with a variety of biological and chemical agents (Sigsgaard et al., 1994c; Hansen et al., 1997; Van Wetering et al., 1997; Palmberg et al., 1998).

In the Danish research programme on waste collection and recycling (CORE) one of the aims was to see whether there is a correlation between health symptoms and the exposure to micro-organisms or microbial compounds in bioaerosols (Poulsen et al., 1995a, b). Different waste handling facilities were compared with respect to health symptoms and bioaerosol exposures. Some of the facilities handle waste containing a high concentration of micro-organisms, whereas the control plants sorted ordinary mail, known to contain very low concentrations of micro-organisms (Breum et al., 1997b; Würtz et al., 1998).

The present study was a part of the CORE programme, and the aim was to determine the extent to which a bioassay measuring the IL-8 secretion after in vitro stimulation of the lung epithelial cell line A549 with organic dust could distinguish between organic dust samples from plants handling different kinds of waste (paper, bottles and mixed household waste). Furthermore, we set out to investigate the role of endotoxin in the inflammatory potential of dust measured in the A549 bioassay.

**MATERIALS AND METHODS**

Dust samples were obtained from five paper-sorting plants, four bottle-sorting plants, two combined paper-sorting and composting plants, two mail centres, four incineration plants and three samples from a plant sorting household waste (Table 1). The samples were collected mainly from the floor and surfaces of the working hall with a dust brush and a dustpan. In addition, air samples from one of the incineration plants were collected on three CAMNEA filters with a Gilian AirCon2 high volume sampler. The three air samples were pooled into one sample before use. Further information about the waste handling plants is published in the report series "Occupational safety and health in..."
waste collection and recycling” (Breum et al., 1997b; Würtz et al., 1998).

Preparation of dust samples
The dust samples were divided as follows: 50 mg dust for the A549 bioassay and 5 mg for endotoxin analysis. The 50 mg sample was sterilized by gamma irradiation, with an absorbed dose of 1 \times 35 kGy (Risø, Denmark) or with an absorbed dose of 2 \times 32 kGy (LR-Plast, Denmark). All dust samples were stored at -20°C prior to analysis. The samples for the A549 bioassay were suspended in cell culture media, and sonicated three times 1 min immediately before use. For the endotoxin extraction a standard procedure was followed: each of the 5 mg samples was suspended in pyrogen-free water, and inverted for 2 h at room temperature. After centrifugation at 1000 g in 10 min the supernatant was stored at -80°C in triplicate aliquots until endotoxin analysis.

A549 bioassay
Lipopolysaccharides (LPS) from Escherichia coli O55:B5 chromatographically purified by gel filtration (Sigma, L 2637, Bie & Berntsen, Denmark), and human recombinant tumour necrosis factor-α (TNF-α) (Genzyme, Bie & Berntsen, Denmark), were used as positive controls in the A549 bioassay.

The human lung epithelial cell line A549 (ATCC No. 185-CCL), was grown in Ham’s F12 medium supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin, 2 mM l-glutamine and 10% foetal bovine serum (FBS) (all reagents from GIBCO BRL, Life Technologies, Denmark). The cells were grown at 36.5°C and 5% CO2 in tissue culture flasks (GIBCO BRL, Life Technologies, Denmark). After treatment of the monolayer with trypsin (GIBCO BRL, Life Technologies, Denmark), 1 × 10^5 cells were transferred to each well in a 24 well multi-dish (GIBCO BRL, Life Technologies, Denmark) and incubated for 48 h at 36.5°C and 5% CO2 with a change of medium after 24 h. Six concentrations (0, 0.1, 0.5, 1, 3 and 5 mg/ml) of each dust sample were tested in triplicate. At the same time the two positive control samples were tested in triplicate (100 μg LPS/ml and 10 ng TNF/ml). After 24 h of incubation, IL-8 secretion was measured using the ELISA technique (Hansen et al., 1997, 1999).

From the dose response curves obtained, the initial slope was used to calculate the inflammatory potential, expressed as the potency factor (PF), of the dust sample. PF represents the IL-8 released per mg dust.

Scanning electron microscopy (SEM)
Scanning electron microscopy (SEM) of dust samples from the four bottle-sorting plants was performed on the JEOL JEM 1200EX II scanning transmission electron microscope (STEM), with an acceleration potential of 30 keV and a magnification of 2500 and 5000 times. About 1 g of dust was suspended in 25 ml 1% NaCl and large particles were allowed to sediment overnight. The suspension was then filtered through a 13 mm nucleopore polycarbonate membrane filter with 0.2 μm pores (Dust extract). For one dust sample (Bottle 2), a thin film of dust emerged on the surface of the extract after 24 h, and only this fraction was filtered through the polycarbonate filter (Surface extract of dust). The

<table>
<thead>
<tr>
<th>Plant sorting mixed household waste (3 samples from the same plant)</th>
<th>House 1a</th>
<th>Floor at the ventilation function of the floor pit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paper- and bottle-sorting plant</td>
<td>House 1b</td>
<td>Top of the ventilation of the floor pit</td>
</tr>
<tr>
<td></td>
<td>House 1c</td>
<td>Top of electric box by the floor pit</td>
</tr>
<tr>
<td>Paper and compost plant</td>
<td>Papbot 1</td>
<td>Floor and surfaces</td>
</tr>
<tr>
<td>(2 plants)</td>
<td>Papcom 1</td>
<td>Floor</td>
</tr>
<tr>
<td></td>
<td>Papcom 2</td>
<td>Floor in the room of compost storage</td>
</tr>
</tbody>
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Table 1. Dust collection sites at the different plants
for the LPS controls and 12 measurements was used to correct for this variation in the chart, the mean of the three LPS or TNF control samples in the X-R-chart was used to control the uncertainty of measurement (ISO, 1998). Each series of measurements was accepted only if the range was lower than the decision limit, which is twice the standard deviation obtained in the linear regression analysis. The interference of dust particles on the IL-8 ELISA results was routinely analysed by testing dust suspended in 5 mg/ml in cell culture media in duplicates in the ELISA test. The interference of dust particles with the measured IL-8 concentration was studied by adding increasing amounts of dust to samples with a known concentration of IL-8 in duplicates. Significant interference was only observed at the highest dust concentration. At a concentration of 1 mg/ml of dust, a 38% reduction in the measured IL-8 concentration was observed. The reduction increased to 80–90% at concentrations of 3–5 mg/ml of dust. This interference may be due to either binding of IL-8 to the dust particles or to quenching of the ELISA results. Since the PF was calculated from the initial linear part of the dose response curve, i.e. the samples with less than 1 mg/ml of dust, this interference had limited impact on the calculation of the PF, at the most a small underestimation of the PF. No attempts were made to reduce the interference.

**RESULTS**

Secrecion of IL-8 from the A549 lung epithelial cells stimulated with dust from plants sorting paper and mail is shown in Fig. 1. The dose response curves generally show an increase up to stimulation with 1 or 3 mg dust. At higher doses, IL-8 secretion is at about the same levels as the maximum value or lower, some even as low as the background level. An exception is plant Paper 4, where the dose response curve increases over the whole stimulation range.

Figure 2 depicts the stimulation of the A549 lung epithelial cells with dust from bottle-sorting plants. Dust samples from the plants Bottle 1 and 3 show dose response curves with an increase in IL-8 secretion up to concentrations of 1 mg dust per ml, and then the curve levels off. The dose response curve of dust from the plant Bottle 2 shows a clear bell shape with an increase of the IL-8 secretion up to stimulation with 1 mg of dust per ml. The IL-8 secretion at higher concentrations of dust is in the same range as the background level. Dust from plant Bottle 4 stimulates the secretion of IL-8 over the whole concentration range.

The four incineration plants (Fig. 3) display dose response curves with a typical bell shape. The four
floor dust samples have maximum values at stimulation with 1 mg of dust per ml. The air sample, Incin 4 air, stimulates the epithelial cells to a greater extent. A linear increase is observed upon stimulation with up to 0.5 mg dust, after which the curve levels off and falls to the background level. Three dust samples collected at different places in the same plant sorting mixed household waste all
show the same dose response pattern (Fig. 4), with an increase up to concentrations of 0.5 mg of dust per ml and a more gradual increase until maximum IL-8 secretion is reached at a concentration of 1 mg of dust per ml.

No interference of dust particles was observed on the ELISA as all the results were below the limit of detection of the ELISA.

In order to estimate the inflammatory potential of the dust samples from the different waste handling facilities we calculate a PF based on the initial linear slope of the dose response curves. This reveals large differences in potency between the different types of plants (Fig. 5). Dust samples from the paper-sorting plants and mail-sorting centres have practically the same potency in the A549 bioassay, except for sample Paper 4, which has a lower PF. The potency of dust from the bottle-sorting plants is generally a bit lower than the potency of dust samples from the paper-sorting plants and mail-sorting centres, except for the Bottle 2 plant, which exhibits a very high potency. Dust from the mixed paper- and bottle-sorting plant (Papbot 1) and the two mixed paper-sorting and composting plants (Papcom 1 and 2) have a PF similar to that of the paper-sorting plants.

SEM on the dust samples from the four bottle-sorting plants revealed a high content of microorganisms in the sample Bottle 2, whereas the other samples had no or low contents of micro-organisms (Fig. 6).

Dust samples from the incineration plants and the three dust samples from the plant sorting mixed household waste show a potency of the dust samples, which is generally higher than the other waste handling plants. A one-factor ANOVA reveals statistically significant differences at $\alpha = 0.05$ in the LPS-corrected PF for dust from the group of paper- and mail-sorting plants versus dust from the group of incineration plants and the plant sorting mixed household waste. Significant differences are also observed in the LPS-corrected PF for the bottle-sorting plants (when excluding the outlier, plant Bottle 2) versus the incineration plants and the plant sorting mixed household waste. Furthermore, a significant difference in the LPS-corrected PF is observed for the group of paper- and mail-sorting plants versus the bottle-sorting plants (when excluding the outlier, plant Bottle 2).

The concentrations of endotoxin in the dust samples range from 1.5 EU/mg dust to 752.8 EU/mg dust. We found no correlation between the PF

![Fig. 3. Dose response curves of IL-8 secretion from the A549 lung epithelial cells stimulated with dust from incineration plants. Each point represents the mean of a triplet, shown with the standard deviation.](image-url)
of the dust in the A549 bioassay and the endotoxin concentration in the dust samples ($r_{PF} = 0.0006$).

**DISCUSSION**

Lung epithelial cells have the capacity to secrete a range of mediators although they are not directly part of the immune system (Kunkel et al., 1991; Devalia and Davies, 1993). The proinflammatory cytokine interleukin 8 (IL-8) is produced especially when the epithelial cells are stimulated with IL-1 and tumour necrosis factor (TNF) or with con-

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**Fig. 4.** Dose response curves of IL-8 secretion from the A549 lung epithelial cells stimulated with dust from a plant sorting mixed household waste. Each point represents the mean of a triplet, shown with the standard deviation.

**Fig. 5.** Potency factors (PFs) of surface dust from waste handling plants and mail-sorting centres. The PF is computed from the initial linear part of the dose response curve from the A549 bioassay. The PF corrected relative to the mean of the *E. coli* O55:B5 (LPS) is shown. Table 1 gives a description of the samples.
ditioned media from LPS-stimulated macrophages (Standiford et al., 1990; Nakamura et al., 1991). The *Haemophilus influenzae* endotoxin also has the capacity to induce increased levels of IL-6, IL-8 and TNF in human bronchial epithelia cells after 24 h of incubation *in vitro* (Khair et al., 1994). Recently, it has been shown that the A549 lung epithelial cell line produces and secretes IL-8 when stimulated not only with pure endotoxin but also when stimulated with complex samples of organic dust containing several microbial components (Hansen et al., 1997; Palmberg et al., 1998).

Fig. 6. Scanning electron microscopy (SEM) pictures. (a) Micro-organisms in surface extract of dust from the plant Bottle 2 magnified 5000 times. (b) Agglomerates of small particles of different types from dust extract from the plant Bottle 4, magnified 2500 times.
In contrast to other researchers, who stimulate the A549 cells with liquid extracts of dust (Roepstorff and Sigsgaard, 1997), sterilized dust is used in this study for stimulation. A pilot study has shown that the stimulation of IL-8 secretion is closely related to the insoluble particulate fraction of the dust, and the aqueous extracts only account for a small part of the total inflammatory potential (unpublished results).

In other studies, the secretion of IL-8 or IL-6 from 10^6 A549 lung epithelial cells has been measured after stimulation with up to 100 µg/ml of unsterilized dust from swine confinement buildings, grain dust or pure LPS (Wang, 1997; Palmberg et al., 1998). The doses of dust employed are lower than those used in the present study. However, the effect of live and growing micro-organisms is not yet clear, and maximal cytokine secretion or a cytotoxic response is not obtained. Another measure of potency was obtained by evaluating the cytotoxic potential of the dust. Roepstorff and Sigsgaard (1997) have developed an in vitro method with A549 epithelial cells and VERO (monkey kidney) cells using the metabolism of tetrazolium salt as an indicator of the physiological status of the cells after stimulation with aqueous or alkaline extracts of dust. This cytotoxic measure can be viewed as a late state measurement of the effect on the cells, whereas the dose response pattern of cytokine release, as used in the present study, can be viewed as a continuo measure from no inflammation to clear inflammation and eventually to cytotoxic response.

The observed dose response curves of IL-8 secretion from the A549 lung epithelial cells as a function of the concentration of dust often show a characteristic bell shape as seen very clearly in Fig. 2. The decline in IL-8 secretion down to the background level at the highest dust concentrations can be explained merely as being the result of interference from dust in the IL-8 measurements. In the dose response experiments producing such bell shaped curves, the cells at the higher concentrations, when examined by light microscopy, have altered morphology and tend to detach from the bottom of the well, indicating cytotoxic properties of the applied dust sample. However, the cytotoxicity of the samples was not studied further.

The problems with decreasing concentration of IL-8 at high dust concentration due to cytotoxicity or interference in the IL-8 ELISA, e.g. the binding of IL-8 to dust particles is solved using the initial linear slope of the dose response curves (PF) as an estimate of the potency of the dust. The maximum IL-8 secretion from the lung epithelial cells stimulated with different dust samples range from 3- to 24-fold above the background level. The highest PF is obtained from the sample Bottle 2 most likely caused by a high content of micro-organisms, not found in the dust samples from the other bottle-sorted plants. The PF shows significant difference between the groups of plants. The dust samples from paper- and mail-sorting plants are less potent than the dust from incineration plants and the plant sorting mixed household waste. In the CORE programme the mail-sorting plants are used as control plants, known to have a low content of micro-organisms (Breum et al., 1997b; Würtz et al., 1998).

Also dust samples from the bottle sorting plants (after exclusion of outlier Bottle 2) are less potent than the dust from incineration plants and the plant sorting mixed household waste. This indicates a difference in potency of the dust related to the waste material handled. The highest PF is observed for dust from plants handling mixed household waste and consistent with this are, epidemiological studies revealing the highest incidence of pulmonary symptoms among workers handling mixed household waste (Poulsen et al., 1995b). The PF therefore seems to be a suitable method for distinguishing between different groups of plants, and the PF of the dust samples may be useful in ranking the individual plants with respect to potential hazard. Differences within a plant were studied using the samples House 1a–1c. The PF values decrease with increasing distance of sampling site from the floor pit. This indicates that the source of particulate agents causing the inflammatory potency of the dust is emitted from the floor pit. Even the lowest PF value from House 1c is still high compared with the PF values for the dust samples from paper- and bottle-sorting plants. However, identification of particular hazardous work situations and processes within a plant requires the collection of airborne dust samples and air samples.

In one incineration plant (Incin 4) handling mixed household waste, both a sample of airborne dust and a sample of surface dust were collected. The air sample results in a higher maximum IL-8 secretion and PF than the surface sample from the same plant. This may be the result of a higher content of relatively inert particles (earth, sand or glass) in the surface dust sample giving weight to the sample, but not adding to the potency of the sample. We have obtained similar results for dust samples from the indoor environment (Allermann and Poulsen, 1999).

In mixed household waste, the high content of organic materials and long storage periods, sometimes at high temperatures, provides a good basis for the growth of micro-organisms. In a controlled experiment with the storage of mixed household waste, micro-organisms are found in high concentrations in the waste and in the fluid leaking from the waste (Breum et al., 1997a; Nielsen et al., 1998). But also volatile organic compounds (VOC) such as alcohol, carboxylic acid and sulphurous compounds...
are found in the air above the waste (Wilkins and Larsen, 1995; Breum et al., 1997a).

In the present study we observe no correlation between the PF values of the dust samples and the content of endotoxin in the sample. Previous analysis of pure endotoxins from the Gram-negative bacteria Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, and Salmonella enteritidis did not show a clear-cut correlation when tested in the A549 bioassay and in the LAL assay (Hansen et al., 1999).

The method evaluation of the A549 bioassay with LPS from E. coli showed that the limit of detection of the A549 bioassay is 17 µg of LPS (Hansen et al., 1999). The concentration of endotoxin in the dust samples tested ranged from 0.1 to 79 ng/mg dust, which is below the limit of detection of the A549 bioassay, implying that the contribution of endotoxin to the observed PF is low. This type of organic dust contains many other biological agents, besides endotoxin that may, contribute significantly to the potency of dust, for example glucans, mycotoxins or other toxins from bacteria and fungi.

A general correlation is seen when comparing the rank of the plants according to the PF with ranks based on data on the content of moulds and bacteria in dust generated from the same fractions of waste using a rotating drum technique (Breum et al., 1998a, b). This indicates that testing of surface dust samples in the A549 bioassay may provide useful knowledge on the potential sources of bioaerosol contamination in waste handling facilities, but surface dust samples provide no information on actual airborne concentrations.

CONCLUSION

In conclusion, we have tested 22 dust samples from different waste handling facilities and calculated the potency factor (PF) for the release of IL-8 in the A549 bioassay. Furthermore, endotoxin is measured in all the samples. No correlation between the PF and the concentration of endotoxin in the sample is found. Correlation is observed between the PF and the content of moulds and bacteria in dust generated from the same waste fractions using a rotating drum. The results obtained in the A549 bioassay reveal that the PF of the dust samples reflects the type of waste handled at the particular plants, with the plants handling mixed household waste giving the highest PF. An overall ranking based on the PF is therefore: Plants handling mixed household waste > Plants handling more pure and dry fractions of waste (paper, bottles, etc.). Further evaluation of the A549 bioassay needs to be done, for example measurement of inflammatory potential of airborne organic dust and correlation with the health symptoms of the exposed workers.

Acknowledgements—We wish to thank laboratory technicians Dorte Nørn, Frank Selmark, and Signe Hjortkjær Nielsen for the collection of dust samples. Laboratory technicians Anne-Karin Jensen and Mirella Simkus are thanked for good assistance with the analysis. We also wish to thank Ole Jørgensen for help and support in making the SEM. This project is supported by the 1993–98 research programme Waste Collection and Recycling, which is supported jointly by the Danish Ministry of the Environment and the Ministry of Labour.

REFERENCES


Inflammatory potential of dust


