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# Electron Paramagnetic Resonance as a Method to Monitor the Intrinsic Oxidant Activity of Ambient Particulate Matter(PM)

***Design, validation and application of the method in environmental settings***

## Dissertation

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# Abstract

Epidemiological studies have demonstrated a relationship between ambient particulate matter exposure and adverse health effects. There is still a fundamental lack of understanding on the causal constituents or possible mechanism through which they act. The free radical generating activity of particles, especially hydroxyl radical (•OH) generation, has been suggested as a unifying factor in their biological activity, but so far mostly indirect methods for assessment of oxidant activity have been used. Electron paramagnetic resonance (EPR) is one of the most sensitive and definite methods, especially for the detection of very reactive oxygen species, such as the hydroxyl radical. Here, we have developed a method using EPR to evaluate the capacity of ambient particulate matter to induce hydroxyl radical generation in different PM fractions, temporal as well as regional variations and related this activity to the effect of PM to cause DNA damage in target cells and nude DNA.

Various particles, such as residual oil fly ash (ROFA), total suspended particles (TSP), coarse and fine PM fractions have been shown to have the ability to generate hydroxyl radicals in the presence of hydrogen peroxide and this was related to particle mass, size and the source of particles. Hydroxyl radical generation was facilitated by exogenous hydrogen peroxide and was inhibited by the metal chelator desferoxamine. Carbon black particles coated with different soluble metal salts showed hydroxyl radical generation that varied with different metals, as well as their valency. A higher ability of free radical generation was found in those particles coated with $Cu^{2+}$, $V^{2+}$, $V^{5+}$, $Fe^{2+}$ and lower with $Fe^{3+}$, $Ni^{2+}$ and $Zn^{2+}$(Chapter II).

Samples that were collected over 6 weeks in summer and 6 weeks in autumn/winter at one sampling location were analysed for the hydroxyl radical generation by EPR, induction of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and transition metal content. An immuno-dotblot assay was developed and used for the measurement of 8-OHdG in calf thymus DNA; immunocytochemistry was used to determine 8-OHdG formation in A549 human epithelial lung cells. The content of leachable V, Cr, Fe, Ni, and Cu was determined by inductively coupled plasma mass spectrometry (ICP-MS). The ·OH generating ability of these weekly coarse and fine PM samples was correlated to the formation of 8-OHdG in calf thymus DNA. Both PM fractions elicited ·OH generation as well as 8-OHdG formation in calf thymus DNA and in A549 cells. The formation of 8-OHdG in naked DNA was

significantly related to ·OH generation, but not to metal concentrations except for copper. A significantly higher ·OH generation was observed for coarse PM, but not fine PM collected during autumn/winter season and this was not due to differences in sampled mass or metal content. Specific weather conditions were associated to ·OH formation by the coarse mode particles which suggests that other, yet unknown, anthropogenic components may affect the radical-generating capacity of PM (Chapter III).

Weekly samples of coarse and fine PM from 4 different sites were analysed for ·OH-formation using EPR, formation of 8-OHdG in calf thymus DNA using an immuno-dotblot assay, DNA strand breakage in A549 human lung epithelial cells using the alkaline comet assay and transition metals by ICP-MS. Both PM sizes elicited ·OH generation and 8-OHdG formation in calf thymus DNA. DNA strand breakage by fine PM was significantly related to ·OH generation and both DNA damage and ·OH generation were correlated to the concentration of several metals, such as Fe, Cu, Cr, Cd and Pb. A significantly higher ·OH generation was observed for PM sampled at urban/industrial areas as well as coarse fractions, however higher soluble metal contents were found in the fine fractions. When considered at equal mass, ·OH formation showed considerable variability with regard to the sampling places as well as the fraction of PM.

We conclude that our method of measuring ·OH by EPR with spin trap integrates bioavailability and redox activity of metals in the presence of particles. It can be used to measure reproducibly the intrinsic oxidant capacity of particulate matter and it may be an alternative metric to mass in evaluating effects of PM.

**Elektroparamagnetische resonanz spektroskopie als methode zum monitoring der intrinsischen oxidationsaktivität von umweltpartikeln**

Epidemiologische Studien haben einen Zusammenhang zwischen der Belastung mit Umwelt-partikeln (particulate matter, PM) und gesundheitsschädigenden Effekten gezeigt. Grundle-gende Mängel bestehen jedoch am Verständnis möglichen Wirkungsmechanismen. Die Eigen-schaft der Partikel, freie Radikale, insbesondere Hydroxylradikale (·OH) zu erzeugen, gilt als Ursache für ihre biologische Aktivität, die bis jetzt jedoch meist durch indirekte Messung der Oxidantien-Aktivität ermittelt wurde. Elektroparamagnetische Resonanz Spektroskopie (EPR) ist eine der empfindlichsten und spezifischsten Methoden zum Nachweis hoch reaktiver Sauerstoffspezies, wie des ·OH. Wir entwickelten eine Methode, um mit der EPR-Technik die Kapazität zur Erzeugung von ·OH in 3 unterschiedlichen PM- Fraktionen zu bestimmen, sowie zeitliche und regionale Variationen mit dem Effekt von PM auf DNA-Schädigung in Zielzellen und freier DNA in Beziehung zu setzen.

Alle untersuchten Partikel erzeugten ·OH. Dies wurde zu Masse, Größe und Quelle der Partikel in Beziehung gesetzt. Die ·OH-Erzeugung wurde durch exogenes Wasserstoff-peroxid eingeleitet und durch den Metallchelator Desferoxamin gehemmt. Die ·OH-Generierung von mit verschiedenen löslichen Metallsalzen beschichteten Russpartikeln variierte hinsichtlich Metallbeschichtung, sowie bei gleichem Metall hinsichtlich Oxidationsstatus. Eine stärkere Fähigkeit zur Erzeugung freier Radikale wurde bei $Cu^{2+}$-, $V^{2+}$-, $V^{5+}$- und $Fe^{2+}$-, eine geringere bei $Fe^{3+}$-, $Ni^{2+}$- und $Zn^{2+}$-Beschichtung ermittelt.

Staubproben von je 6-Wochen-Intervallen der Sommer- bzw. Herbst/Winter-Saison wurden auf die ·OH-Generierung , den Gehalt an den Übergangsmetallen V, Cr, Fe, Ni und Cu sowie die Induktion von 8-Hydroxy-2'-deoxyguanosine (8-OHdG) in Kälberthymus-DNA (Immuno-Dotblot-Assay) und menschlichen Lungenepithelzellen A549 (Immuncytochemie) gemessen. ·OH-Generierung der groben und feinen PM-Fraktionen wurde zur 8-OhdG Bildung in Kälberthymus-DNA in Bezug gesetzt. Beide PM-Fraktionen erzeugten ·OH sowie 8-OHdG in Kälber-thymus-DNA und in A549 Zellen. 8-OHdG Bildung in reiner DNA war von der ·OH-Generierung, nicht aber von den Metallkonzentrationen, mit Ausnahme von Kupfer, abhängig. Eine signifikant höhere ·OH-Produktion wurde für die grobe, nicht aber für die feine PM-Fraktion der Herbst/Winter-Saison ermittelt. Dies wurde nicht durch Unterschiede bezüglich Masse oder Metallgehalt verursacht und weist auf andere, noch unbekannte, anthropogene Komponenten hin, welche die Radikal-erzeugende Kapazität von PM beeinflussen könnten.

Untersuchungen von Proben grober und feiner Umweltpartikel von 4 unterschiedlichen Orten in Nordrhein-Westfalen ergaben, dass durch feine PM-Fraktionen verursachte DNA-Strangbrüche ursächlich mit der ·OH-Generierung zusammenhingen, und sowohl DNA-Schädigung als auch ·OH-Generierung mit der Konzentration einiger Metalle, wie Fe, Cu, Cr, Cd und Pb, korrelierten. Eine signifikant höhere ·OH-Generierung wurde für PM-Proben aus Industriegebieten sowie für grobe Fraktionen ermittelt, höhere lösliche Metallgehalte wurden jedoch in die feinen Fraktionen analysiert. Auf die gleiche Masse bezogen, zeigt die ·OH-Bildung eine beträchtliche Variabilität sowohl hinsichtlich Proben-sammelort als auch hinsichtlich PM-Fraktion.

Unsere Ergebnisse demonstrieren, dass die EPR-Methode unter Einsatz der Spin-trap Analyse die Bioverfügbarkeit und Redox-Aktivität von Metallen der Partikeln integriert und reproduzierbar die intrinsische Oxidationskapazität von Umweltpartikeln messen kann. Die Ergebnisse diese Doktorarbeit zeigen, dass die Oxidationsaktivität eine alternative Methode zur reproduzierbare Messung von PM-Effekten darstellt.

# Abbreviations:

| | |
|---|---|
| •OH | hydroxyl radical |
| 8-OHdG | 8-hydroxydeoxyguanosine |
| AAS | atomic absorption spectrometry |
| ANOVA | analysis of variance |
| BAL | bronchoalveolar lavage liquid |
| COPD | chronic obstructive pulmonary disease |
| CRP | C-reactive protein |
| DAB | diaminobenzidine-tetrahydrochloride |
| DCDHF | 2', 7'-dichlorodihydrofluorescin |
| DCF | dichlorofluoresin |
| DMPO | 5,5-dimethyl-1-pyrroline-N-oxide |
| DMSO | dimethylsulfoxide |
| DSB | double strand breakage |
| EDTA | ethylene diaminetetraacetic acid |
| EDX | energy dispersive X-ray analysis |
| EPR | electron paramagnetic resonance |
| ESR | electron spin resonance |
| G | Gauss |
| HVS | high volume sampler |
| ICP-MS | inductively coupled plasma mass spectrometry |
| NO | nitric oxide |
| $ONOO^-$ | peroxynitrite anion |
| PBS | phosphate buffered saline |
| PM | ambient particulate matter |
| $PM_{0.1}$ | particulate matter diameter less than 0.1 μm |
| $PM_{2.5}$ | particulate matter diameter less than 2.5 μm |
| $PM_{10}$ | particulate matter diameter less than 10 μm |
| RNS | reactive nitrogen species |
| ROFA | residual oil fly ash |
| ROS | reactive oxygen species |
| SOD | superoxide dismutase |

| | |
|---|---|
| SSB | single strand breakage |
| STEM | scanning /transmission electronic microscopy |
| TMTU | tetramethylthiourea |
| TSP | total suspended particles |

# Chapter I Introduction

The human lung is exposed to 10,000 to 20,000 L of ambient air daily. Apart from the necessary oxygen, the inhaled air contains a wide range and large number of particles originating from automobile exhaust, cooking, cigarette smoking, wood fire, mechanical wear processes and wind blown dust. Although the healthy lung is capable of dealing with particles deposited on to its surface, these defence mechanisms may be overwhelmed by either the particle mass/number or by the inherent toxicity of particles or during impaired defence. In the past decade, numerous epidemiological studies have demonstrated that particulate air pollution was associated to adverse health effects, such as an increase the morbidity and mortality (Daniels *et al*., 2000; Pope *et al*., 2002).

## 1.1 Adverse health effects of ambient particulate matter (PM)

Historical data have well documented air pollution episodes that led to adverse health effects, even to death. In December 1930, during a 5 day fog episode, 63 people died in the Meuse Valley in Belgium (Nemery *et al*, 2001). In Donora, Pennsylvania, 20 subjects died and 7000 experienced acute illness in October 1948, subjects 55 years of age and older were more severely affected. The occurrence of some 4000 excess deaths due to cardiorespiratory diseases during London Smog of December 1952 is probably the best-known early evidence of the acute health effects of air pollution. Due to the very strong correlation of black smoke and sulphur dioxide in air pollution, there was little prospect of disentangling the effects of the two pollutants through further epidemiological study. The effects were ascribed to the mixture, with the WHO in its 1987 Air Quality Guidelines for Europe (WHO, 1987) setting a joint standard for black smoke (Gravimetrically determined particulate matter) and sulphur dioxide. Although it has long been suspected that ambient particulate pollution may be connected to the mortality and morbidity outcomes, it took decades and a series of epidemiological studies to convince both scientists and lawmakers. It was not until the 1990s that, by using improved statistical methodologies Dockery (Dockery *et al*., 1993) and Schwartz (Schwartz *et al*., 1994) were able to demonstrate the effects of particulate matter on health at concentrations hitherto believed to be safe. Moreover, the effects were independent of the effects of other co-pollutants.

The first target of inhaled air particles is the respiratory system. Nasal inflammation is among the most frequent adverse effects of air pollution and a more specific indicator or response to inhaled substances is nasal mucus and cellular constitutes of the epithelial by washing of one or both nasal passages, a so called nasal lavage (Graham and House, 1988). Nasal lavage from urban children content higher levels of interleukin-8 (IL-8), uric acid, albumin, and nitric oxide metabolites when compare to suburban children. These nasal markers, as well as the peak expiratory flow, were associated with levels of particulate matter with diameters less than or equal to 10 micrometers ($PM_{10}$) (Steerenberg *et al*., 2001). However, in a recent study among 6 year old children and their mothers in Germany, no increased cells, cell types or IL-8 were observed in site with more air pollution (Polat *et al*., 2001). On the other hand, the observed associations between child mortality and PM were dose dependent with an estimated 7% proportion of respiratory deaths attributed to $PM_{10}$ in the city of Sao Paulo, Brazil, from 1994 to 1997 (Conceicao *et al*., 2001) and numerous studies have demonstrated that ambient particulate matter causes lung function decline, increased respiratory symptoms, worsening of chronic obstructive pulmonary disease (COPD), increasing hospital admissions as well as respiratory morbidity and mortality (Tozan *et al*., 1992; Nitta *et al*., 1993; Smirk *et al*., 1996; Schwartz *et al*., 1996; Brunet *et al*., 1997; Klein *et al*., 2000; Cifuentes *et al*., 2000; Pope *et al*., 2002). Recent research also suggests that PM is correlated to lung cancer mortality (Pope *et al*, 2002). The adverse health effects of PM on the respiratory system are summarized in Table 1.1.

During the past decade, it has also become clear that inhaled particulate matter causes adverse health effects outside the respiratory tract and that these effects may be more important than the respiratory effects. Studies have reported an association between ambient particulate matter and cardiovascular mortality and morbidity, especially among the elderly population (Dockery *et al*., 1993; Pope and Dockery, 1999; Goldberg *et al*., 2001; Magari *et al*., 2002). In the combined six-city analysis, $PM_{2.5}$ was associated with a 2.1% increase in ischemic heart disease deaths (Schwartz, 2000). For the estimated effects of $PM_{10}$ with regard to congestive heart failure and ischemic heart disease, an increase of approximately 0.8% and 0.7% respectively, were attributed to an increase of 10 $\mu g/m^3$ in $PM_{10}$ (Table 1.2). Furthermore, $PM_{10}$ has been demonstrated to significantly increase the heart rate (Pope *et al*., 1999) and decrease the heart rate variability (Magari *et al*., 2001; 2002). Rabbits instilled with high dose of ambient particulate collected from outdoor air in Ottawa experienced a systemic inflammatory response that included bone marrow

stimulation and progression of atherosclerotic lesions in the coronary arteries and aorta, as well as increased plaque with characteristics which are more likely to rupture and trigger coronary events (Suwa *et al*., 2002). Air particulate exposure has also been found to enhance the blood viscosity (Peters *et al*., 1997) and significantly raise the C-reactive protein (CRP) levels (Seaton *et al*., 1999), which has been proposed as a marker of unstable atheromatous plaques and underlying atherosclerosis.

Taking into account the attribution of cardiovascular diseases to all cause mortality, for instance, in the United States, respiratory deaths account for approximately 8.5% of all deaths while the cardiovascular deaths (heart, cerebrovascular, and arterial diseases) account for 39.5%. So it is obvious, although the relative effects of particulate air pollution for cardiovascular diseases are smaller, that the total numbers of deaths attributed to cardiovascular diseases are larger.

**Table 1.1. Relationship between PM and respiratory disease mortality and morbidity**

| Health outcomes (First author) | % increase per10μg/$m^3$ for $PM_{10}$ (95% C.I.) | % increase per10μg/$m^3$ for other fractions (95% C.I.) |
|---|---|---|
| All respiratory diseases admission | | |
| Atkinson, 2001 | 0.9% (0.6-1.3). | |
| COPD hospital admission | | |
| Zanobetti, 2000 | 1.5 (1.0-1.9) | |
| Atkison, 2001 | 1.0 (0.4-1.5) | |
| Braga, 2001 | 1.7 (0.1-3.3) | |
| Asthma | | |
| Atkinson, 2001 (0-14yr) | 1.2 (0.2-2.3) | |
| (15-64yr) | 1.1 (0.3-1.8) | |
| Yu, 2000 (5-13yr) | 10 (3-16) | 14 (4-26) ($PM_1$) |
| Sheppard, 1999 | 2.6 (1.1-4.2) | 4.3 (2.2-7.5) (PM2.5-10) |
| Pneumonia mortality | | |
| Braga, 2001 | 2.7(1.5-3.9) | |
| Cancer mortality | | |
| Pope, 2002 | | 8 (1-16) ($PM_{2.5}$) |

**Table 1.2. Relationship between PM and hospital admissions for specific cardiovascular disease**

| Health outcomes (First author) | % increase per10μg/$m^3$ for $PM_{10}$ (95% C.I.) | % increase per10μg/$m^3$ for other fractions (95% C.I.) |
|---|---|---|
| All cardiovascular diseases | | |
| Schwartz, 1997 | 0.93 (0.00-1.86) | |
| Burnett, 1997 | -0.28 (-2.64-2.14) | 2.81 (-0.25-5.97) ($PM_{2.5}$) |
| Zanobetti, 2000 | 1.1 (0.9-1.3) | |
| Atkison, 2001 | 0.5 (0.2-0.8) | |
| Braga, 2001 | 1.0 (0.6-1.4) | |
| Congestive heart failure | | |
| Schwartz, 1995 | 0.99 (0.37-1.61) | |
| Morris, 1997 | 0.77 (0.2-1.35) | |
| Burnett, 1999 | 1.87 (0.82-2.93) | 2.58 (0.76-2.58) ($PM_{2.5}$) |
| Morris, 2001(Pooled) | 0.83 (0.5-1.15) | |
| Ischemic heart disease | | |
| Burnett, 1999 | 1.62(1.04-2.2) | 3.14 (2.12-4.18) ($PM_{2.5}$) |
| Morris, 2001(Pooled) | 0.68 (0.41-0.96) | |
| Cerebrovascular accident | | |
| Moolgavkar, 2000 (L.A) | 0.59 (-1.22-2.43) | -0.3 (-1.47-0.88) ($PM_{2.5}$) |
| Dysrhythmia | | |
| Burnett, 1999 | 1.63 (0.57-2.70) | 2.38 (0.77-4.02) ($PM_{2.5}$) |

## 1.2 Particles Characteristics

### 1.2.1 Size distribution of PM

Particulate matter is the term used to define a complex mixture of solid particles and liquid droplets that may vary in mass, size and composition, depending on the sources and weather conditions. With respect to size, PM ranges from a few nanometers to several tens of micrometers and is generally described under total suspended particles (TSP), coarse ($PM_{2.5-10}$), fine ($PM_{2.5}$) and ultrafine ($PM_{0.1}$) particles. Coarse particles, fine particles and ultrafine particles are defined in terms of the modal structure of particle size distributions typically observed in the atmosphere. The coarse fractions are relatively large particles, the diameter between 2.5 to 10 μm, mainly derived from soil (road dust) and other crusted material and from mechanical wear processes, such as drilling, crushing and grinding. The fine fraction is defined as particles with an aerodynamic diameter of approximately 0.1 to 2.5 μm, and includes carbonaceous material such as soot and secondary aerosols. They are derived directly or indirectly from combustion of fossil fuel used in power generation, industry and automobile engines. Ultrafines are the particles with diameter less than 0.1μm, and consist of 50% carbon and 50% salts, mainly ammonium sulphate and ammonium nitrate primarily derived from the exhausts of automobile engines.

Most particles obtained from the ambient atmosphere lie in the range between 0.05 and 0.2 μm. Amongst various emission sources, diesel exhaust is the major contributor of particulate matter and more than 80% of its particles are in the size around 0.1μm and adsorbed onto which are an estimated 18,000 different high molecular organic compounds (Weisenberger, 1984; Fleming *et al*., 1996). The relative occurrence of the three particle modes in ambient air at various locations and during various air pollution circumstances is largely unknown. It is generally assumed that fine particles and even the ultrafine particles are more associated with the adverse health outcomes (Lipfert and Wyzga, 1997; Schwartz *et al*., 1999; Klemm *et al*., 2000; Cifuentes *et al*., 2000). Inhalation studies with rats exposed to highly insoluble particles of low intrinsic toxicity, such as $TiO_2$, result in significantly increased pulmonary inflammatory responses and lung tumours when their size is in the ultrafine particle range (Oberdorster, 2001). It has therefore been suggested that particle numbers and size appear to be more important than the mass in producing biological effects. One reason to explain this is that biological effects are related to the large surface available on the smaller particles. It is estimated that the mass of a 1 μm particles is equivalent to the mass of 1000 0.1μm particles, and the surface of the same

mass of a 0.1 μm particles is 10 times that of a 1 μm particles (Salvi and Holgate, 1999). Teflon fumes at ultrafine particle concentrations of approximately 50 $\mu g/m^3$ have been shown to be extremely toxic to rats when inhaled for only 15 minutes. Interesting, neither the ultrafine Teflon particles alone when generated in argon nor the Teflon fume gas-phase constituents when generated in air were toxic after 25 minutes of exposure. Only the combination of both phases when generated in air caused high toxicity, suggesting the existence of either radicals on the particle surface or a carrier mechanism of the ultrafine particles for adsorbed gas-phase compounds (Johnston *et al*., 2000). Other possible mechanisms may concern its easy infiltration through the cell membranes and escaping phagocytosis by the alveolar macrophage.

Evidence for a role of larger particles has also been presented in several studies. Ostro and colleagues (Ostro *et al*., 1999) found an association between $PM_{10}$ and daily mortality in the Coachella Valley, a desert resort and retirement area east of Los Angeles, where coarse particles of geologic original typically comprise approximately 50-60 % of $PM_{10}$ and can exceed 90% during wind events. In comparing the cytotoxicity and induction of pro-inflammatory cytokines of human monocytes exposed to fine and coarse particles in outdoor and indoor air, Monn and Becker (Monn and Becker, 1999) found a significant toxicity and cytokine production by the outdoor coarse fraction but not by outdoor fine particles or the particles collected indoors. Outdoor coarse PM induced 20 times the amounts of IL-6 and IL-8 than fine particles. However, *in vitro* studies can only evaluate the toxic properties of the particles and the outcome is also determined by the dosage, which again is related to particle size, but also to clearance, retention, translocation and dissolution in the respiratory tract.

### 1.2.2.Dosage of particles

A major factor that determines the outcome of particle inhalation is dosage, which include particle deposition, clearance, retention, translocation and dissolution within the different regions of lung.

Particle deposition

For assessing the ultimate injury induced by particles in the respiratory tract, consideration needs to be given to the mechanisms and patterns of deposition as well as to the clearance of particles respectively in and from the lung. The region and location where deposition has occurred is an important factor for the ultimate effect. Generally, the physiologic and

pathologic processes elicited by interaction of inhaled particles within the airway occur at the site where these particles are initially deposited. The predominant mechanisms by which particles deposit in the lung are A) settling, which means that particles, preferably those with a high density, move in the direction of gravity; B) impaction, when particles travel in the initial direction after sudden changes of the air stream direction; and C) diffusion, when particles deposit due to Brownian diffusion. Additionally, two other secondary mechanisms can be distinguished: electrostatic precipitation, driven by the electrostatic attraction between charged particles and the airway wall, and interception, which usually applies for fibrous particles (Clarke and Yeates, 1994; Foster, 1999). In the lung, roughly three regions of particle deposition can be found: the nasopharyngal, the tracheobronchial, and the pulmonary/alveolar region. Normally, particles with diameters less than 10μm are considered to be respirable by humans. The nose acts as the first line of defence against inhaled particles, and completely traps particles with a diameter above 30 μm. When the particle diameter becomes smaller, the chance of being deposited in the lower airways or alveoli increases (Foster, 1999). The exception are particles less than 0.001μm, which deposit by about 80% in the upper airways (Cheng *et al*., 1989).

**Table 1.3 Calculation of total deposited dose of PM fractions in human airways and lungs, using the ICRP model and expressed relative to the dose in a healthy adult male at light exercise and standardised to respiratory tract tissue mass** (Freijier *et al*., 1997).

| Subjects | Ambient PM fraction | | |
|---|---|---|---|
| | Ultrafine (<0.1μm) | Fine (0.1-2.5μm) | Coarse (2.5-10μm) |
| Adult male | 1.0 | 1.0 | 1.0 |
| Children,<10 years old | 1.2-1.7 | 1.1-2.5 | 1.4-1.7 |
| Adult male at: | | | |
| Rest or sleep | 0.3-0.4 | 0.2-0.4 | 0.3-0.4 |
| Heavy exercise | 1.9-2.0 | 1.4-1.7 | 2.0 |
| Adult male with COPD | 4.0-4.4 | 3.7-5.0 | 4.2-4.3 |

The local dose of inhaled particles and deposition sites within the lung vary widely and are mainly determined by their aerodynamic size and shape, breathing pattern and lung

structure. Generally, as the particle size and breathing rate increase, particles deposit more in the proximal area. Studies in humans using radioactive particles of varying sizes demonstrated that particles with median size 2.5 μm undergo 83% total lung deposition while particles of 8.2 μm and 11.5 μm diameters undergo 49% and 31% deposition respectively (Anderson *et al*., 1995). This suggests an inverse relationship between particle size and total lung deposition. Particles smaller than 2 μm in diameter achieve greater peripheral deposition than those greater than 2 μm. Similarly, smaller size particles tend to be retained in the lung for a longer duration. The deposition pattern of particles in compromised airways is changed compared to healthy airways (Menache *et al*., 1995; Kim and Kang, 1997) and the total deposition may be enhanced and shifted to the alveolar region.

Using the ICRP model, the estimated overall deposition of particles of different size in compromised airways is 4-5 times increased compared to healthy airways (Freijer *et al*., 1997) (Table 1.3). This partly explains why sub-populations with COPD and children are especially susceptible to ambient particulate matter pollution.

Particle clearance

To reduce retention of the inhaled particles, deposited particles are cleared from the lung. Clearance involves a series of mechanisms, including expelling by sneezing and coughing, solubilisation, mucociliary transport and phagocytosis by inflammatory cells. The ultimate fate of particles to be cleared is dependent upon several factors, including the solubility of the particles and the deposition site. Particles deposited onto ciliated surfaces of the conducting airways can be removed by the (rapid) action of the mucociliary escalator. Particles deposited in the alveolar region, where ciliated cells are sparse and the mucociliary escalator only has a minor function, are generally cleared by the action of the alveolar macrophages, which is a considerably slower process (Clarke and Yeates, 1994). Phagocytosis is the fate of most of the particles deposited in the alveoli. After phagocytosis, the macrophage loaded with particles will move to the ciliated airways, where they move further upwards via the mucociliary clearance. Alternatively, loaded macrophages can enter the interstitial space of the lymphatic system. However, clearance processes are not perfect and can be affected by different factors. For example, mucociliary transport is usually severely impaired in chronic bronchitis and COPD (Ericsson *et al*., 1995; Brand *et al*., 2002) and particles, especially ultrafine particles, can impair macrophage phagocytosis and slow down the processes of particle clearance

(Renwick *et al*., 2001). It has been suggested that a considerable part of the deposited particles are taken up by the respiratory tract epithelium and reach the interstitium (Churg, 1996).

### 1.2.3 Chemical composition of particles

Apart from dosage characteristics of inhaled particles, chemical composition is another important factor which may influence the toxicity of particles. Particulate matter, such as coarse and fine or even ultrafine, is not a single component, but a heterogeneous mixture, varying in its composition and dependent on location, weather conditions, and emission sources. For most ambient particulate matter, the major components include elemental carbon and organic carbon, sulphate, nitrate, chloride, ammonium, crust materials and /or biological materials (pollen fragment, allergens and endotoxins). Most notably road traffic emits soot particles containing solid elemental carbon black core coated on the surface with many compounds condensed from exhaust gases, including polycyclic aromatic organic compounds (Amann and siegla, 1982). A simplified impression of the complex chemical composition of PM is shown in figure 1.1.

Data on controlled human clinical and laboratory animal exposure has demonstrated that sulphuric acid and its neutralised salts might be related to health effects (Utell and Samet, 1993; Schlesinger, 1995; Chen *et al*., 1995a). Some other organic substances, such as PAH and semi-quinones have been suggested to be causative factors for free radical generation in diesel exhaust, carbonaceous particles, as well as ambient particles (Massolo *et al*., 2002; Dellinger *et al*., 2001). Trace metals are another important chemical composition, including some highly toxic metals, such as vanadium, lead, mercury and cadmium. For these highly toxic metals the exposures through inhalation of urban PM are likely to be insufficient to cause toxicity, especially in developed countries. On the other hand, in lung epithelial cells, the cytotoxicity of Utah-Valley dust has been linked to their transition metal content (Carter *et al*., 1997; Frampton *et al*., 1999). In macrophages, the toxicity of $PM_{10}$ has also been shown to involve transition metals, but interestingly, this effect was merely observed in its coarse (2.5-10μm) fraction and not in its fine (<2.5μm) fraction (Monn and Becker, 1999). Ghio and Devlin (Ghio and Devlin, 2001) demonstrated by exposure of volunteers to aqueous extracts of PM collected before closure and after reopening of a steel mill a greater inflammatory response relative to PM extract acquired during the plant shutdown suggesting that mass may not be the most appropriate measure

to use in assessing health effects after PM exposure but rather specific components such as transition metals must be identified and assessed. In human airway epithelial cells exposed to residual oil fly-ash (ROFA) *in vitro*, the production of increased levels of IL-6, IL-8 and TNFα, as well as mRNA coding for these cytokines is induced and these effects are inhibited by either metal chelators or free radical scavengers (Carter *et al*, 1997). This led to the suggestion that metals presented in particles produce an oxidative stress, which may be responsible for the production and release of inflammatory mediators.

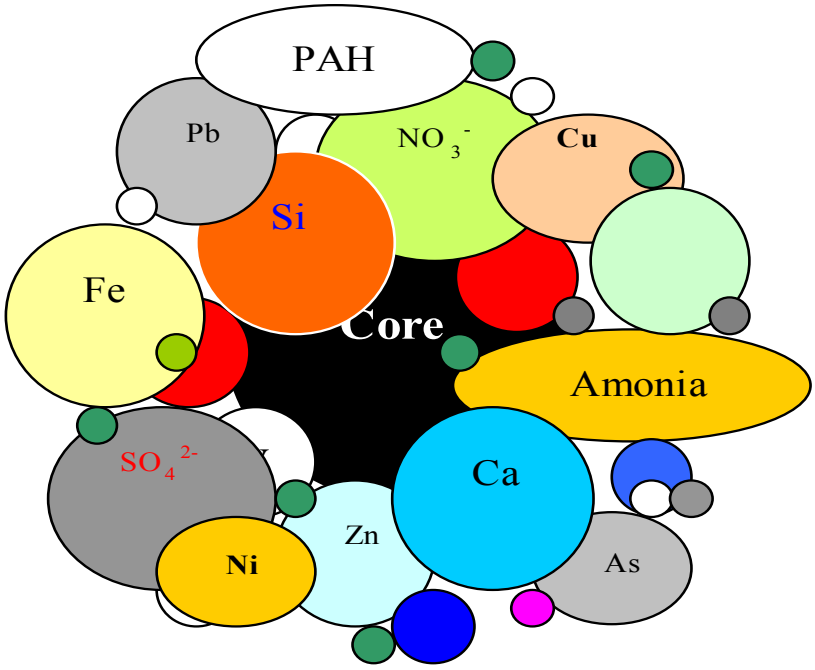


**Figure 1.1 A simplified artistic illustration of the chemical heterogeneity of ambient particulate matter and its suggested organic and inorganic components.**

## 1.3 PM related oxidative stress

### 1.3.1 Terminology of ROS and oxidative stress

Reactive oxygen species (ROS) is a collective term often used to describe oxygen radicals, including superoxide ($O_2\bullet^-$), hydroxyl radical (•OH), peroxy radicals ($RO_2\bullet$) and alkoxyl radicals (RO•). Additionally, the term is also used to describe certain non-radicals that are either oxidising species or that can easily be converted into radicals, such as hypochlorous acid (HOCL), ozone ($O_3$), peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$) and hydrogen peroxide ($H_2O_2$).

ROS can be derived from numerous sources *in vivo*. These include autooxidation, photochemical and enzymatic reactions of both endogenous compounds and various xenobiotics. The number of different enzymes shown to be capable of generating ROS is extensive, and includes cytochromes P450, various oxidases, peroxidases, lipoxygenases and dehydrogenases. The involvement of xenobiotics can be particularly important in the determination of the extent of ROS generated by these enzymes. One should note that the word 'reactive' is a relative term. The half-lives of these species range from minutes, such as hydrogen peroxide, seconds, such as peroxide radical to about a nanosecond, such as hydroxyl radical. The hydroxyl radical is by far the most reactive ROS and reacts with whatever biological molecule is in its vicinity.

At steady-state formation of prooxidants in cells and organs is balanced by a similar rate of their consumption by antioxidants that are enzymatic and/or non-enzymatic. The enzymatic processes in eukaryotic organisms mainly involve catalase, peroxidases, methionine sulphoxide reductase, superoxide dismutase (SOD), the non-enzymatic mechanisms involve small molecules, such as ascorbic acid, glutathione and uric acid. If the steady state between formation and consumption of oxidants is disturbed, oxidative stress may occur. Professor Sies first defined oxidative stress in 1991 (Sies, 1991) as a disruption of the prooxidant-antioxidant balance in favour of the former, leading to potential damage. Oxidative stress involves the process of ageing and contributes to many diseases including inflammation, autoimmune diseases, cancer, diabetes, neurodegenerative diseases, heart attack and stroke (Lehucher-Michel *et al.*, 2001; Hensley and Floyd, 2002; Sorescu and Griendling, 2002).

### 1.3.2 PM induced oxidative stress

It has become clear that environmental particles, including asbestos, crystalline silica, heavy metal containing dusts, oil fly ash, coal fly ash and ambient particles have the ability to generate ROS and cause oxidative stress in many experimental models (Hansen and Mossman, 1987; Hedenborg and Klockars, 1989; Leanderson and Tagesson, 1992; Berg *et al*., 1993; Beck *et al*, 1996; Hitzfeld *et al*, 1997; Prahalad *et al*., 1999; 2001; Dellinger *et al*., 2001; Keane *et al*., 2002). Sources of ROS include inflammatory cells and direct generation by particulate matter themselves and/or their constituents (Prahalad *et al*., 1999).

Several specific particle characteristics have been demonstrated to be involved in the ROS generation. For mineral dusts such as crystalline silica it has been shown that ROS release from inflammatory cells was related to the physical dimensions and the surface based radical generating properties of the particles (Vallyathan *et al*., 1992). Procedures used to modify the particle surface, such as grinding and coating of the surface by specific compounds clearly influence the ROS release by inflammatory cells (Klockars *et al*., 1990; Nyberg, 1991; Vallyathan *et al*., 1991; 1992, Knaapen *et al*., 2002). In chemically complex particles such as PM, ROFA and coal fly ash, the chemical composition was clearly related to the ability to activate ROS generation. Berg (Berg *et al*., 1993) demonstrated that in macrophages exposed to metal containing dusts, the release of hydrogen peroxide was associated with the metal content. By exposing different particles to 2'-deoxyguanosine (dG), calf thymus DNA and human airway epithelial cells, Prahalad and co-workers demonstrated that particle related oxidative DNA damage was mediated by hydroxyl radical generation, which was catalysed by metals, especially transition metals and the ability was associated with particle metal content, especially water soluble metals (Prahalad *et al*., 2001). However, by comparing ultrafine carbon black (ufCB) with non-ultrafine respirable carbon black (CB), Li has found that ultrafine carbon black caused more oxidative stress than the same mass of respirable carbon black (Li *et al*., 1996; 1999). Furthermore, treatment of ufCB with a metal chelator, a manoeuvre that decreases the oxidative activity of $PM_{10,}$ had no effects on ufCB caused inflammation in rat lungs. On the other hand, the same research group reported recently that those combinations of ufCB and metals showed a synergistic effect on inflammatory response (Wilson *et al*., 2002). Together, these findings suggest that some particles can cause inflammation via non-metal mediated pathways, such as surface free radicals, and that an interaction with

metal can occur. In addition, organic compounds adsorbed onto the particle surface of PM such as diesel exhaust particles, can reduce oxygen by semiquinones and produce ROS (superoxide, hydrogen peroxide and ultimately hydroxyl radical) (Dellinger *et al*., 2001). Proposed mechanisms of hydroxyl radical generation and DNA damage by PM are illustrated in figure 1.2.

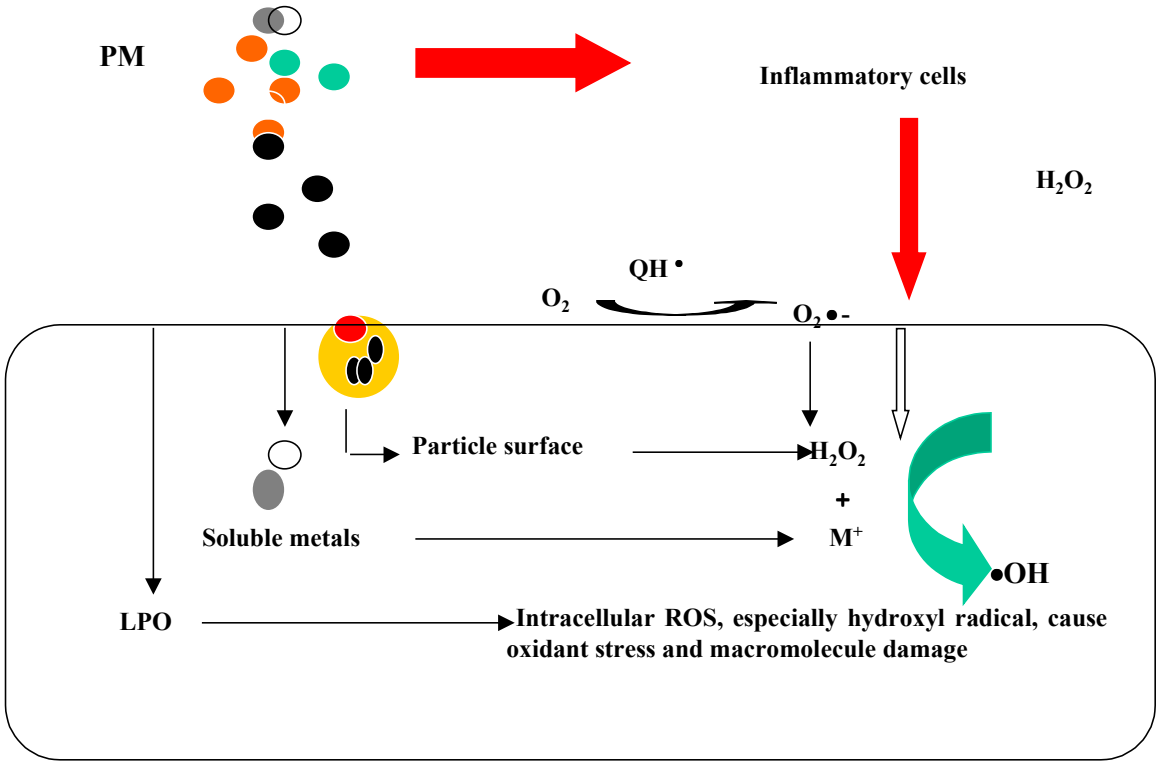


**Figure 1.2. Schematic of the proposed mechanism for the oxidant stress of particles.**

### 1.3.3 Measurement of ROS

The high reactivity of most ROS makes their detection difficult, especially in biological systems. Numerous assays have been developed to detect ROS *in vitro* or *in vivo*. Generally these methods have been divided into direct and indirect assays.

The indirect assays include the measurement of changes in endogenous antioxidant levels or an increase in biochemical markers of oxidative damage. Endogenous antioxidant changes include specific antioxidant enzymes such as catalase, superoxide dismutase, peroxidases and non-specific antioxidant molecules such as ascorbic acid, glutathione, uric acid and carotenoids. The measurement of biomarkers related to oxidant stress concern

membrane damage and formation of lipid peroxides (malondialdehyde), protein oxidation and oxidant DNA damage (dot-blot assay, immunocytochemistry, immunohistochemistry, Comet assay, etc).

Direct methods for ROS measurement introduced here mainly will focus on the method which has been currently used to measure very active ROS, such as free radicals and peroxynitrite anion.

**2', 7'-dichlorodihydrofluorescin (DCDHF or DCFH)** has been used to detect reactive oxygen species (ROS) and reactive nitrogen species (RNS) in a variety of biological systems. The commercially available compound 2',7'-dichlorodihydrofluorescin diacetate can be passively loaded into whole tissue or whole cells . After hydrolysis of the diacetate groups by cytosolic esterase or base-catalyzed cleavage of the diacetate group, DCDHF is oxidised to the highly fluorescent product dichlorofluoresin (DCF). DCDHF is directly oxidised by hydroxyl radical (Zhu *et al*., 1994) and peroxynitrite (Ischiropoulos *et al*., 1999). Neither nitric oxide (NO) and superoxide nor hydrogen peroxide alone appears to oxidise DCDHF. But peroxynitrite is the product of fast radical–radical reaction between nitric oxide and superoxide.

$$^{\bullet}NO + O_2^{-}\bullet \longrightarrow ONOO^{-}$$

DCDHF oxidation may serve to indicate either intra or extracellular formation of ROS or RNS. This important advantage provides information about oxidant regulation in an intact cell. Unfortunately, DCF fluorescence from intact cells is difficult to calibrate due to the associated artifacts, which include photooxidation, photobleaching and dye leak. The lack of specificity of DCDHF makes it difficult to identify the nature of oxidants detected by the probe. In addition to DCDHF, another fluorescence probe, dihydrorhodamine (DHR) has been widely used.

**Hydroxylation of aromatic** compounds as a method for detecting hydroxyl radical in biological systems was first described by Floyd (Floyd *et al*., 1984). This assay depends upon the reaction between reactive species (hydroxyl radical and peroxynitrite) and the aromatic ring of aromatic compounds such as benzoate, phenol, phenyalanine and salicylate to yield stable metabolites. These stable metabolites can be separated by high performance liquid chromatograph (HPLC) and identified and quantified. Salicylate is the most widely used compound. Specific radicals attack the phenolic ring of salicylate at the 3

or 5 position to yield stable dihydroxyl benzoic acid which can be separated and quantified. Salicylate does not react with hydrogen peroxide or superoxide but does react with peroxynitrite. The utility of this assay rests in the specificity of the two products in the presence of hydroxyl/peroxynitrite.

**The cytochrome C assay** is based on the reaction between ferricytochrome c with •OH; $O_2^{-\bullet}$ or NO (Bell and Ferguson, 1991; Kelm *et al*., 1997; Sharp and Cooper, 1998) to form ferrocytochrome c. Associated with this reduction reaction is an increase in cytochrome C absorbance at 550 nm. The magnitude of the absorbance change reflects the amount of cytochrome c reduction which is proportional to the amount of reducing agent in the test medium. Thus this system can be calibrated and used to quantify reactive species generated by biological systems. Cytochrome C also can be oxidized by hydrogen peroxide and peroxynitrite (Thomson *et al*., 1995), therefore the production of ROS estimated by cytochrome c reduction may be underestimated in biological systems that contain these species.

**Chemiluminescence** is defined as the production of light as the direct result of chemical reaction. One of the most widely used chemiluminescence probes for detection of ROS, such as hydroxyl radical, superoxide and peroxynitrite is luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione).
The general mechanism includes the production of an unstable endoperoxide intermediate which decays to ground state with the emission of light which can be monitored. Chemiluminescence makes it possible to follow the time course of ROS formation in biological systems. Another previously used probe, lucigenin, has been questioned for its validity of being used to detect superoxide, as lucigenin has been demonstrated to enhance the superoxide production (Vasquez-Vivar *et al*., 1999).

**Electron spin resonance (ESR) or electron paramagnetic resonance (EPR)** is one of the few techniques that can directly detect molecules with unpaired electrons making it uniquely suited for the measurement of free radicals. The basic principle of EPR involves the exposure of paramagnetic molecules to a magnetic field. This field aligns the magnetic moments of the electron spins of the unpaired electrons in free radicals (Swartz and Wiesner, 1972). A microwave energy field is then applied. Energy absorption promotes the unpaired electron to a higher energy level. It is the net absorption of electromagnetic

energy that is detected and results in a characteristic EPR spectrum as shown in chapter II, figure 2.3. The magnitude of the absorbance change corresponds to the amount of free radicals in the samples. The specific hyperfine splitting constant (hfsc) and g-factor are valuable in the partial identification of the signal origin. Free radicals can be centred on different atomic nuclei. The hfsc and g-factor provides information about the nuclei of the radicals being detected, e.g. oxygen-, nitrogen-, carbon-, or sulfur-centred molecules.

Spin traps or spin probes can be used in conjunction with EPR to identify individual free radical species, enhance the detection of a weak signal, or detect previously undetectable radicals (Mason *et al*., 1994; Valgimigli *et al*., 2001). Spin traps are compounds that bind to specific free radicals producing a more stable radical product (adduct) that can be detected with EPR. Furthermore, individual spin traps usually display a marked selectivity for particular species, thereby permitting modulation of the sensitivity toward a given radicals. The spin probe technique can usually prove the involvement of free radicals species in a process or condition and it provides a relative safety way of identifying species actually present in the system.

The methods widely used for ROS detection are summarised in table 1.4.

**Table 1.4 Methods used to detect ROS *in vitro* and *in vivo***.

| **Assay** | **ROS detected** | **Advantages** | **Disadvantages** |
|---|---|---|---|
| EPR | Free radicals | Widely used (*in vitro*, *in vivo*), Structural information, Quantitative | Not be able to calibrate *in vivo*. |
| Cytochrome C | ROS and RNS | Simple, Quantitative | Only *in vitro*, no information about nature of ROS |
| DCDHF | ROS | Intra-extra cellular ROS formation, Visualisation | No information about nature of ROS, Auto catalytic degradation |
| Salicylate | •OH and $ONOO^-$ | Quantitative | Limited to •OH and $ONOO^-$ |
| Chemiluminescence | Oxygen radicals | Time course of ROS generation, Quantitative | Specifity |

### 1.3.4 ROS and DNA damage

A major development of carcinogenesis research in the past 20 years has been the realisation that DNA damage and mutations are induced by ROS derived from both endogenous and exogenous sources (Marnett, 2000). More than 20 different modifications of DNA are formed under oxidative stress (Haliwell and Aruoma, 1991), including modification of DNA bases or deoxyribose residues to produce damaged bases or strand breaks. Alternatively ROS can oxidise lipid or protein molecules to generate intermediates that react with DNA to form adducts. The reactions of ROS which contribute to DNA damage are oxidation, nitration, depurination, methylation and deamination and the oxidation of DNA base, possibly leading to structure alterations in DNA, such as base pair mutations, deletions, or insertions, which are all commonly observed in mutated oncogenes and tumour suppressor genes (Wiseman and Halliwell, 1996). It should be noted that the reactivity of various ROS towards DNA is extremely different. For instance whereas superoxide and hydrogen peroxide are thought not to react with DNA at all (Haliwell and Aruoma, 1991; Wiseman and Halliwell, 1996), singlet oxygen selectively reacts with guanine bases (Van den Akker *et al*., 1994). However, the most potent ROS by far to react with DNA is the hydroxyl radical, which generates a multiplicity of products from all four bases (Pryor, 1988; Spencer *et al*., 1995). Among the major products of oxidant DNA damage, 8-hydroxy-2'-deoxyguanosine (8-OHdG) has received considerable attention. The formation of 8-OHdG by ROS was first reported in 1984 by Kasai (Kasai *et al*., 1984). Its demonstrated mutagenic potential (Kuchino *et al*, 1987; Moriya and Grollman, 1993) and easy detection, made it the most abundantly studied oxidative DNA adduct in carcinogenesis research. These studies have ultimately culminated to a consensus that the premutagenic 8-OHdG that can be formed by a wide variety of agents with different mechanism of action (Kasai, 1997) is an excellent marker of oxidant DNA damage and the presence of 8-OHdG in cellular DNA is closely related to carcinogenesis (Shibutani *et al*., 1991; Floyd *et al*., 1990).

Many approaches have been developed to identify and quantitate 8-OHdG in DNA, including HPLC, post-labelling assay and GC/MS. These methods are known to be cumbersome, require specialised technical processing and expensive instrumentation and are incompatible for the detection of 8-OHdG in intact cellular DNA. Other widely used methods include immunological techniques, which use specific antibodies and can be used for numerous specific damaged DNA bases. The major advantage of these methods is the

relative speed by which results can be obtained, and the fact that no complicated analytical equipment is needed. Moreover, such methods provide the possibility to investigate DNA damage on a single cell level, or to specifically locate DNA damage in tissue sections. However, in contrast to analytical chemical methods a quantitative analysis is not possible.

DNA strand breakage may result from a variety of reactions. The most obvious way is direct scission of the DNA backbone by chemical or radical attack. There are very few agents that directly break DNA. The best known is ionising radiation, which induces strand breaks by the direct deposition of energy in the ribose-phosphate backbone. Additionally, direct DNA strand breakage can also be induced upon reaction of hydroxyl radical with the sugars of the DNA backbone (Eastman and Barry, 1992). DNA strand breakage can also be induced in an indirect manner, via the induction of DNA base damage by ROS. Such DNA strand breakage will trigger DNA repair mechanism. However, during their action, DNA strand breaks are transiently introduced into the DNA due to the action of endonucleases, which cleave the phosphodiester backbone of the DNA molecule. Moreover, it should be noted that fragmentation of the DNA might also be a result of apoptotic processes (Stewart, 1994).

DNA strand breaks can be categorised as either single-strand break (SSB) or double-strand break (DSB). SSB normally represent reparable lesions, because the opposite strand holds both ends close together. In contrast, however, double strand breaks are usually considered to be lethal, because they are not easy repairable. Anyhow, detection of DNA strand breaks, either directly induced, or transiently induced during repair processes can be considered as a helpful tool to test the genotoxic properties of chemicals and particles.

A number of techniques for detecting DNA damage as opposed to the biological effects, for instance micronuclei, mutation, chromosomal aberrations, have been used to identify substances with genotoxic activities. The most frequently used methods have the shortage either in its limited sensitivity or the need of lots of cells. In past decades, a more useful approach for the detection of DNA damage is the single cell gel electrophoresis (SCG) or so called ‘Comet’ assay. This method was introduced in 1988 by Singh (Singh *et al*., 1988) and involves electrophoresis under alkaline (pH>13) condition for detecting DNA damage in single cells. At this pH, increased DNA migration is associated with increased levels of frank SSB, SSB associated with incomplete excision repair sites, and alkali-labile sites (ALS). As almost all genotoxic agents induce orders of magnitude more SSB and /or ALS

than DSB, this version of the assay offered greatly increased sensitivity for identifying genotoxic agents.

## 1.4 Aims of this research

Epidemiological studies have demonstrated that increased exposure to ambient particulate matter (PM) is associated with respiratory, cardiovascular and malignant lung disease, through increasing the morbidity and mortality (Dockery *et al*., 1993; Cohen *et al*., 1995). It is by no means clear how exposure to PM, typically as low as 30 $\mu g/m^3$, can produce these health effects observed in epidemiological studies or which components of PM mediate these effects. The wide number of endpoints suggests that more than one component may be driving the health effects (Donaldson *et al*., 1998; Dreher, 2000). Leading hypotheses regarding the agents or particle properties mediating the pulmonary effects include transition metal content (Dreher, 2000), particle size and surface (Donaldson *et al*., 1998) or endotoxin contamination (Monn and Becker, 1999; Monn *et al*., 2002). In addition particles can carry or present other compounds such as polycyclic aromatic hydrocarbons (PAHs) or proteins which could lead to an increased allergic or inflammatory response (Van Zijverden and Granum, 2000; Nel *et al*., 1998)

The free radical generating activity of particles has been suggested as a unifying factor in biological activity, causing acute and chronic lung inflammation (Mark *et al*., 2001; Kodavanti *et al*., 1998) and systemic disorders (Donaldson *et al*., 2001). From a body of studies we know that both surface-area and release of redox active metals influence the direct as well as the indirect (through inflammation) capacity of particles to generate ROS, among which the formation of hydroxyl radicals seems to be important (Donaldson *et al*., 1998). Intracellular hydroxyl radicals can randomly damage macro-molecules including DNA, and previously we noticed that acellular formation of hydroxyl radicals by various coal-fly ashes was highly correlated to its capacity to cause cellular oxidative DNA damage in lung epithelial cells (Van Maanen *et al*., 1999). On the other hand, •OH can perturb redox-balance in the cell leading to activation of transcription factors, such as NF-κB which has been demonstrated to cause histone acetylation by particle related oxidative stress (Jimenez *et al*., 2000, Gilmour *et al*., 2003) or AP-1 (Timblin *et al*., 1998). The most common way for hydroxyl radical generation is via the Fenton reaction which involves the reduction of hydrogen peroxide by a transition metal ion (Halliwell and Gutteridge, 1989;

Lloyd *et al*., 1997). Hydrogen peroxide which was produced during oxidative phosphorylation in living cells as endogenous ROS and has been observed in breath condensate and lavage fluid of patients with COPD (Dekhuizen *et al*., 1996). The lavage fluid of COPD patients has clastogenic activity that could be blocked by oxygen radical scavengers (Pinamonti *et al*., 1996). In addition, BAL cells of patients with lung diseases are known to produce exaggerated amounts of oxidants (Sybille and Reynolds, 1990). It has been suggested that increased oxidative stress which is associated to the release of $H_2O_2$ partly explains why those patients with pre-existing inflammatory disease in the airways such as COPD, are susceptible to ambient particulate matter pollution (Repine *et al*., 1998; Donaldson *et al*., 2002).

We hypothesised therefore that the acellular generation of hydroxyl radicals by sampled ambient particles possibly integrates the activity of Fenton active-metals, their size distribution and surface (Donaldson *et al*., 1998; Dreher, 2000) and therefore might better reflect its biological activity then simply mass or particle number. Based on this hypothesis, the purpose of this research was to measure hydroxyl radical generation of PM and relate its activity to several *in vitro* biological outcomes as well as its chemical composition. Electron paramagnetic resonance (EPR) has been suggested as one of the most sensitive and definitive method for free radical research (Halliwell and Gutteridge, 1985; Goldstein *et al*., 1993, Dellinger *et al*., 2001). The various chapters to come describe:

(i) The development, application and validation of the Electron Paramagnetic Resonance (EPR) method for free radical measurement (chapter II),

(ii) Temporal variation in coarse and fine PM in weekly samples were analysed for $H_2O_2$-dependent ·OH formation using EPR, formation of 8-OHdG in calf thymus DNA using an immuno-dot blot assay and 8-OHdG formation in A549 human lung epithelial cells using immunocytochemistry. Temporal effects of samples from 6 weeks in summer and 6 weeks in autumn/winter were compared using EPR and the dot blot assay and leachable transition metals (Chapter III),

(iii) Regional variations of weekly samples of coarse and fine PM from 4 different places were analysed for $H_2O_2$-dependent ·OH-formation using EPR, formation of 8-OHdG in calf thymus DNA using an immuno-dot blot assay and DNA strand break using Comet assay in A549 human lung epithelial cells and related to leachable metal content (Chapter IV),

(iv) At the end of this thesis, a brief summary and discussion.

# Chapter II

# HYDROXYL RADICAL GENERATION BY ELECTRON PARAMAGNETIC RESONANCE AS A NEW METHOD TO MONITOR AMBIENT PARTICULATE MATTER COMPOSITION

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**ABSTRACT**

Epidemiological studies have demonstrated the relationship between exposure to ambient particulate matter (PM) and health effects in those with cardiopulmonary diseases. The free radical generating activity of particles has been suggested as a unifying factor in the biological activity of PM in toxicological studies but so far has not been applied as a method for environmental monitoring of PM. The purpose of this study was to characterize hydroxyl radical (•OH) production by different size fractions of PM, to use as an alternative method for monitoring of PM composition and activity. We have developed a method, using electron paramagnetic resonance (EPR), to measure •OH formation in suspensions of particles in the presence of hydrogen peroxide and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a specific spin-trap. Samples of ambient particulate matter (PM) of different size fractions were collected from various sites on various filters. PM deposited on filters as well as suspensions in water retains its ability to generate •OH and this generation is determined by concentration of hydrogen peroxide and soluble metals. However, large variations in •OH formation and kinetics were found with different soluble metals and within metals (Fe, V) with different valences. The method was applied to environmental monitoring in Hettstedt-Zerbst, situated in South-Eastern Germany, where it showed a relation to Cu-content of PM. The method was also applied in Duisburg, where the $PM_1$ fraction showed the highest DMPO-OH generation but was not linked to particle counts. The method integrates metal bioavailability and reactivity and can provide a better understanding of the effect of small variations in mass concentrations on health.

# INTRODUCTION

Ambient particulate matter (PM) is the term used to define a complex mixture of anthropogenic and naturally occurring airborne particles, described under total suspended particles (TSP), $PM_{10}$, fine ($PM_{2.5}$) and ultrafine ($PM_{0.1}$) particles. For example, the $PM_{10}$ size fraction measures the mass concentration of particles which pass through a size selective inlet with 50 % efficiency at 10 μm aerodynamic diameter, with an upper cut-off of about 30 μm. Thereby $PM_{10}$ roughly corresponds to the thoracic fraction of particles, while $PM_{2.5}$ (with a 50 % efficiency at 2.5 μm) and a cut-off at 7 μm roughly corresponds to the respirable fraction. However, whereas ambient PM standards are based on sampling efficiency at a specific size, the thoracic and respirable fractions are based on physiological deposition curves and set in ISO standards. European and US regulatory agencies are considering more stringent air quality standards for airborne PM, largely based on epidemiological studies showing associations between mortality from respiratory and cardiovascular diseases and PM (Dockery *et al*., 1993; WHO, 1999; Samet *et al*., 2000). From these studies it is estimated that per 10 $\mu g/m^3$ increase in the annual concentration of $PM_{2.5}$, mortality increases with 1.4 %, while respiratory disease such as bronchitis or asthma exacerbations increase by as much as 4 % (WHO, 1999). It is by no means clear how exposure to PM, typically as low as 30 $\mu g/m^3$, can produce the health effects observed in epidemiological studies and furthermore, which components of PM mediate these effects. Although epidemiological and toxicological evidence suggest that it is the fine ($PM_{2.5}$) and even the ultrafine ($PM_{0.1}$) fraction that is responsible there is no general agreement (Oberdorster *et al*., 1994; Wichmann *et al*., 2000). The wide number of endpoints suggests that more than one component may be driving the observed health effects (Donaldson *et al*., 1998; Dreher, 2000). Leading hypotheses regarding the agents or particle properties mediating the pulmonary effects include transition metal content (Dreher, 2000), particle size and surface (Donaldson *et al*., 1998) or endotoxin contamination (Monn and Becker, 1999; Monn *et al*., 2002). In addition, particles can carry or present other compounds such as polycyclic aromatic hydrocarbons (PAHs) or proteins which could lead to an increased allergic or inflammatory response (Van Zijverden and Granum, 2000; Nel *et al*., 1996). Local effects in the lung appear to be driven by increased production of inflammatory mediators through oxidative stress-sensitive pathways. The free radical generation of particles, including $PM_{10}$, has been

suggested as a unifying factor in biological activity (Donaldson *et al*., 1996; Prahalad *et al*., 2000; Dellinger *et al*., 2001; Keane *et al*., 2002). A number of studies have shown that both surface-area and release of redox active metals influence the direct as well as the indirect (through inflammation) capacity of particles to generate oxidative stress. In the induction of oxidative stress, defined as any local imbalance between generated oxidants and the anti-oxidant system, the formation of hydroxyl radicals seems to be a very crucial event (Donaldson *et al*., 1998). On the one hand, intracellular hydroxyl radicals can randomly damage macro-molecules, such as DNA, and previously we noticed that the acellular formation of hydroxyl radicals by various coal-fly ashes was highly correlated to its capacity to cause cellular oxidative DNA-damage in lung epithelial cells (Van Maanen *et al*., 1999). On the other hand, •OH can upset the redox balance withing the cell, leading to activation of transcription factors, such as NF-κB (Jimenez *et al*., 2000) or AP-1 (Timblin *et al*., 1998). In summary, separate effects both qualitative and quantitative maybe associated with different particle fractions, which in turn are related to their different deposition and composition.

We hypothesised therefore that the acellular generation of •OH by sampled ambient particles, possibly integrates a combination of the activity of Fenton active metals, their size distribution and surface (Donaldson *et al*., 1998; Dreher, 2000). Furthermore, this may better reflect the particles biological activity rath than mass or particle number alone. Amongst the methods that have been developed for the measurement of hydroxyl radicals, electron paramagnetic resonance (EPR) is one of the most sensitive and definitive methods. Based on this, we set out to use EPR to measure hydroxyl radical generation of different PM fractions obtained by environmental sampling to develop a new monitoring method for PM.

# METHODS

## Chemicals

DMPO (5,5-dimethyl-1pyrroline-N-oxide), sodium formate, phosphate-buffered saline, desferoxamine and DMSO (dimethyl sulfoxide) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Ethanol, active coal, ferrous sulphate, copper sulphate and ferric chloride were purchased from Merck (Germany). Catalase, hydrogen peroxide ($H_2O_2$) and nickel sulphate were purchased from Fluka (Seelze, Germany). For EPR experiments, double-distilled de-ionised water was used.

## Sampling

Total suspended particles (TSP), coarse ($PM_{10–2.5}$) and fine ($PM_{2.5}$) were recovered from filters taken directly from various sampling programs. The TSP used in this study was sampled in Duisburg (Germany) during 1995 on nitro-cellulose filters (Pore size: 3 µm, Sartorius, Germany) using a high volume sampler (HVS 150, Ströhlein Instruments, Germany). The coarse and fine fractions were sampled on Teflon filters (37 mm, with support ring, pore size: 2 µm) in the summer in Hettstedt-Zerbst (2001) and spring and autumn in Düsseldorf (1999, 2000) using Andersen Dichotomous Samplers, model 241 (Andersen Inc, Atlanta, GA) at a flow-rate of 16.7 liters/min. In order to obtain about 1 mg mass on Teflon filters with low volume samplers, sampling intervals of 7 days were taken. Three manual high volume samplers (30 m³/h, Digitel DHA 80, Switzerland) were used to sample $PM_1$, $PM_{2.5}$ and $PM_{10}$ on a daily basis, in parallel during 10 days in July 2001 on Teflon filters. The $PM_{10}$ inlet used is in accordance to the European standard EN 12341. The $PM_{2.5}$ inlet of the DIGITEL DHA 80 was used in several comparison studies and was found to be comparable to other common $PM_{2.5}$ samplers such as the LVS 3 (Derenda, Berlin, Germany). The $PM_1$ inlet was calibrated with monodisperse particles and positively evaluated in field trials (Kuhlbusch, personal communication).The sampled PMX-mass was determined in accordance to the weighing procedure given in EN12341 with the determination of the mass difference after equilibration of the filters at 20 ± 1°C and 50 ± 5 rH. Particle size distributions were measured with a Scanning Mobility Particle Sizer (TSI SMPS Platform 3080, DMA 3081, CPC 3025, Neutralizer 3077) determining the particle number size distributions in the size range of 14-737 nm (dst; Stokes diameter) Particle size distributions were measured with a Scanning Mobility Particle Sizer (TSI SMPS;

Platform 3080, DMA 3081, CPC 3025, Neutralizer 3077) measuring size ranges of 14-737 nm (dst; Stokes diameter) by fractionating particles by their electrical mobility and subsequent counting and calculation of the concentration. All particles of the channels from 14,1 to 737 nm were summed and considered as total particle count. Loaded filters were stored at room temperature in the dark until further analysis. PM was recovered from the 30 filters and ·OH generation was assessed at mass concentrations between 57 and 337 µg/ml using EPR (mean: 125 µg/ml).

**Preparation and calibration of particle suspensions**

To prepare TSP suspensions, nitrocellulose filters were cut into pieces and immersed in double-distilled water and vorted for 5 minutes. Following removal of the filter, samples were sonicated in water bath (Sonorex TK52; 60 Watt, 35 kHz) for 5 minutes, as described previously (Knaapen AM *et al*., 2000). To prepare PM (coarse and fine) suspensions from Teflon filters, the support ring was removed, the filter placed into double-distilled water and agitated (5 min) before being sonicated in a water bath (5 min). Following sonication, a further 5 minutes agitation was performed. Blank filters were treated in the same way and used as a control in all experiments. Since it is difficult to quantify the weight of the particulates recovered from the filters, several methods were used to estimate concentration per ml, i.e. (i) comparative turbidometry at 405 nm against a carbon black standard (Huber 990, 260 nm), (ii) weighing of a large set of Teflon filters before and after removal of particles, and (iii) for TSP by filtering 500 µl of the suspension through a 0.2 µm syringe filters (Minisart RC15, Sartorius AG, Göttingen, Germany) and weighing the filter after drying. Filtrates were prepared by passing the suspensions through a 0.1µm filter (Acrodisc 25 mm syringe filter, Pall Gelman Laboratory, Ann Arbor, USA). Electron microscopy demonstrated that all the particles were removed upon this filtration procedure. Four pooled PM suspensions (2 coarse PM samples and 2 fine PM samples) which had been sampled and prepared as mentioned above, were used to investigate the stability of •OH generation of particle suspensions over prolonged storage periods.

Model carbon particles with an average size of about 1.5-2µm were prepared by coating carbon black with different metal ions, i.e. Cu (II), Fe (II), Fe (III), In (II), Zen (II), V (II) and V (V), with each sample containing about 20 µg/mg metal (Daniels *et al*., 2001). Two oil fly ash (OFA) samples as described previously (Kodavanti *et al*., 1998) were also used. The content of Fe, Ni, V and sulphate for these OFA are 21.2, 13.9, 18 and 320 µg/mg

(sample A) and 0, 0.5, 35 and 13.7 µg/mg (sample B) respectively. Both the model particles and ROFA samples were prepared in de-ionised water and subsequently agitated and solicited.

**Measurement of •OH generation using EPR**

Generation of hydroxyl radicals by particle suspensions was studied in the presence of hydrogen peroxide and the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). Prior to use, DMPO was purified with activated charcoal. DMPO was dissolved in deionized water and activated charcoal was added (30mg/ml). The suspension was shaken continuously for 20 minutes at 35°C prior to centrifugation 2000g for 10 minutes. This procedure was repeated once and the clear supernatant was filtered through a 0.45µm pore filter. The final concentration of DMPO was measured by absorption at 234 nm, using de-ionised water and its concentration adjusted to 1 M. Aliquots were stored at –20°C. For hydroxyl radical measurement, 50 µl of the particle suspension was mixed with 50 µl $H_2O_2$ (0.5 M in PBS) and 100 µl DMPO (0.05 M in PBS). The mixture was incubated in the dark and shaken continuously at 37ºC beforebeing filtered through a 0.1µm filter (Acrodisc 25 mm syringe filter, Pall Gelman Laboratory, Ann Arbor, USA). The clear filtrate was transferred immediately to a 100 µl glass capillary and measured with a Miniscope MS100 EPR spectrometer (Magnettech, Berlin, Germany).

The EPR-spectra were recorded at room temperature using the following instrumental conditions: Microwave frequency: 9.39 GHz, Magnetic field: 3360 G, sweep width: 100 G, scan time: 30 sec, number of scans: 3, modulation amplitude: 1.8 G, receiver gain: 1000. Quantification was carried out on first derivation of EPR signal of DMPO-OH quartet as the sum of total amplitudes, and outcomes are expressed as the total amplitude in arbitrary units (A.U). Comparative analysis of double integration of these signal to amplitude showed a high correlation (n=30, Pearson's, r=0.99, $p<0.001$). As a positive particle control a coal-fly ash (EVA-91) was used with a known iron release (30 nmoles/mg in 24hr) and described previously (Van Maanen JM *et al*., 1999). As a negative control, a mixture of water (or desferoxamine or catalase solution), $H_2O_2$ and DMPO were used.
To investigate the role of metals in the formation of hydroxyl radicals, three different types of experiments were performed. The differences of metals in hydroxyl radical generation

were measured using (i) different soluble metal salts, (ii) carbon black particles artificially coated with metal salts and (iii) various OFA samples. Other experiments were performed by mixing the metal chelator desferoxamine at a final concentration 0-200 μM with ambient particle (TSP) suspension for 10 minutes before DMPO and hydrogen peroxide were added.

The specific role of hydrogen peroxide was investigated by mixing particle suspension with different concentrations of $H_2O_2$ (0, 5, 25 and 125 mM) to a mixture of PM and DMPO or by the addition of catalase (0-1000 U/ml) to a mixture of PM, DMPO and $H_2O_2$ (125 mM). Inactivated catalase was prepared by heating of catalase (1000 U/ml) at 95ºC for 10 minutes.

**Statistical methods**

A paired sample T-test was used to investigate the variation of PM suspensions to induce •OH after 10 months freezing and the comparison •OH generation ability of particle from Hettstedt and Zerbst. Pearson correlation was used to determine the correlation between amplitude and double integration of EPR spectrum. All statistics were performed by using SPSS 9.0 for Windows NT. Differences were considered to be statistically significant at $P<0.05$.

# RESULTS

## Hydroxyl radical generation by PM

When different particle suspensions or filtrates (TSP, $PM_{10}$, coarse and fine PM) were incubated with $H_2O_2$ and DMPO, all samples exhibited similar EPR spectra, with only differences in peak intensities. Typical 1:2:2:1 EPR spectra were observed confirming the DMPO-OH adduct with a split centre at 3400 Gaussand suggesting the formation of hydroxyl radicals. A similar spectrum was also obtained from a mixture of DMPO with $Fe^{2+}$ in the presence of $H_2O_2$. DMPO-OH adducts can arise from either direct trapping of hydroxyl radical (eqn.1) or the decomposition of DMPO-OOH (eqn.2), the half life of which in neutral media is about 1 minutes:

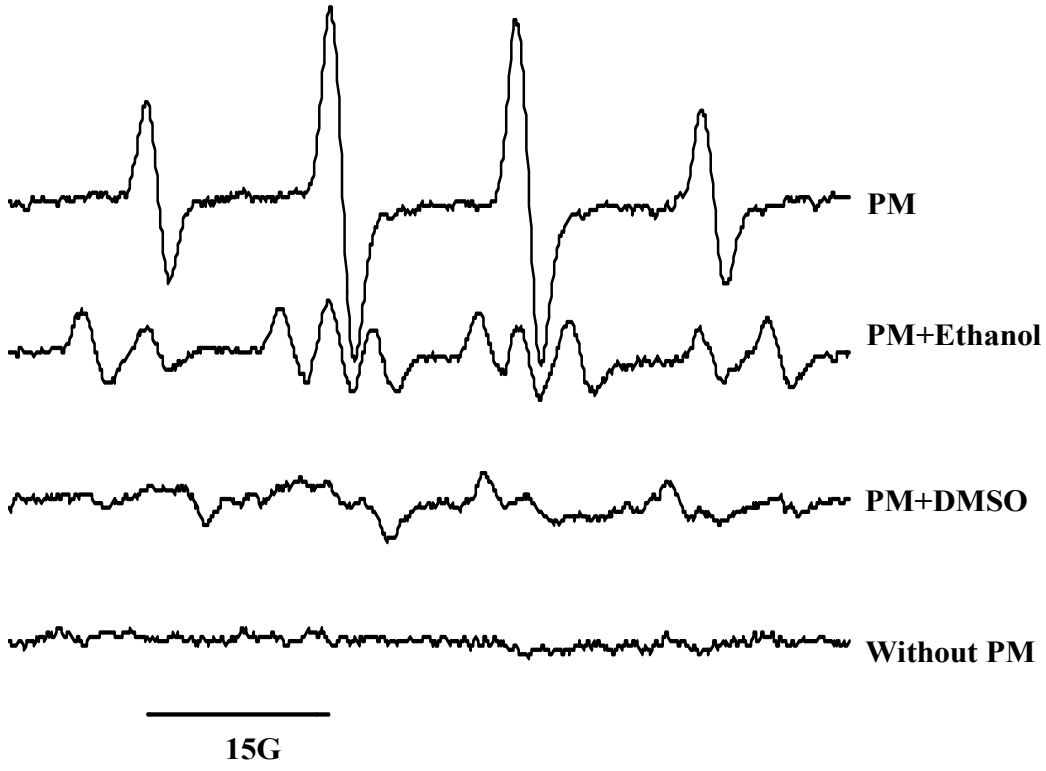


**Fig 1**. **EPR spectra of DMPO spin trapping adducts of TSP.** 125 μg/ml of PM suspension was incubated with 25mM DMPO and 125 mM $H_2O_2$ in the absence or presence of ethanol (10% of volume), hydroxyl radical scavenger DMSO (10% of volume) for 10 minutes at 37°C water bath.

DMPO+•OH→DMPO-OH (1)

DMPO+$O_2^{\bullet-}$→ DMPO-OOH→ DMPO-OH (2)

If the EPR signal of DMPO-OH is due to direct trapping of •OH, then scavengers of •OH would yield the corresponding DMPO adducts instead of the DMPO-OH adduct. In order to confirm the hydroxyl radical formation, experiments were carried out by adding DMSO and ethanol. The results are shown in Fig. 1. Below, a series of chemical equations are shown which explain the specific reaction of these compounds with the OH-radical and formation of the specific spin-trap adducts:

•OH+$(CH_3)_2SO$→$CH_3SO_2H$+•$CH_3$ (3)

DMPO+•$CH_3$→DMPO-$CH_3$ (4)

•OH+$CH_3CH_2OH$→•$CHCH_3OH$+$H_2O$ (5)

DMPO+•$CHCH_3OH$→DMPO-$C_2H_4OH$ (6)

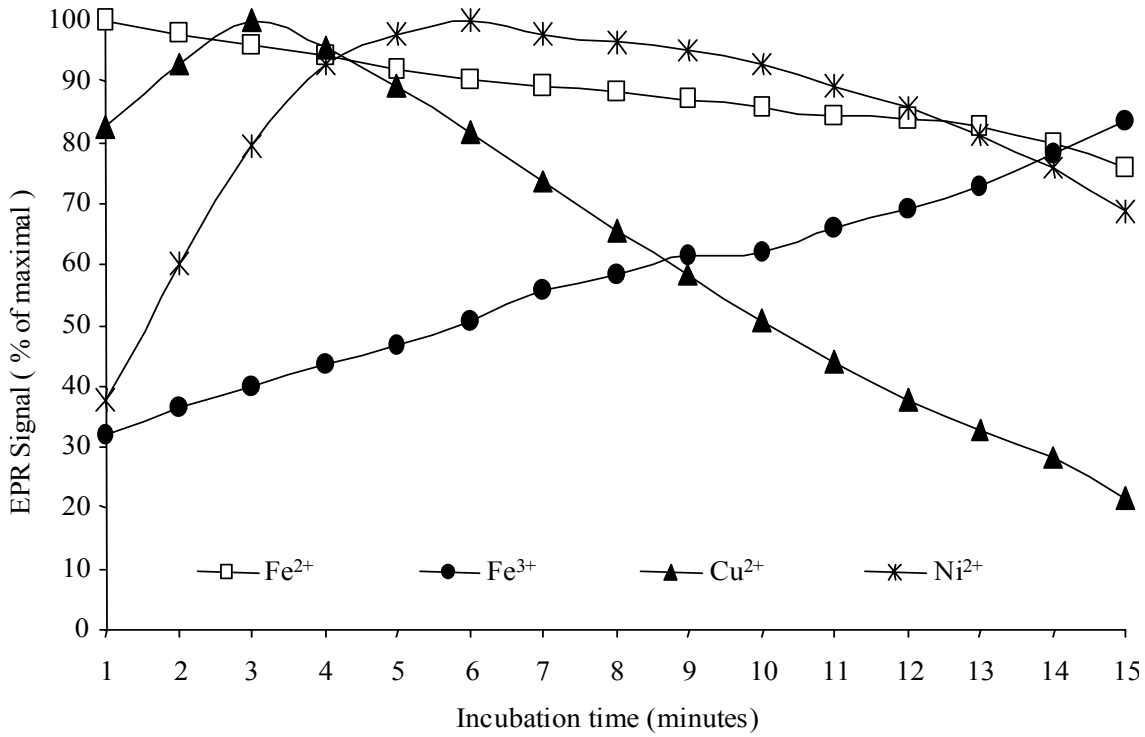


**Fig 2. The time course of the evolution of the EPR signal by several soluble metals**. 25mM of DMPO and $H_2O_2$ 125mM were mixed with 5µM of different metal sulfate and measured at different time point. $Fe^{2+}$ (□), $Fe^{3+}$ (●), $Cu^{2+}$ (▲) and $Ni^{2+}$ (*).

Addition of hydroxyl radical scavenger DMSO (10% of volume) abolished the signal and lead to a new DMPO adduct which is formed according to reactions in eqns (3) and (4). The addition of ethanol (10% of volume) yields the DMPO-$C_2H_4OH$ radical adduct according to the eqns (5) and (6), exhibiting a distinctive six–line EPR spectrum (Fig. 1). Without particle suspension or particle filtrate, no signal was observed. Taken together these results provide evidence that ambient particulate matter can generate •OH in the presence of $H_2O_2$.

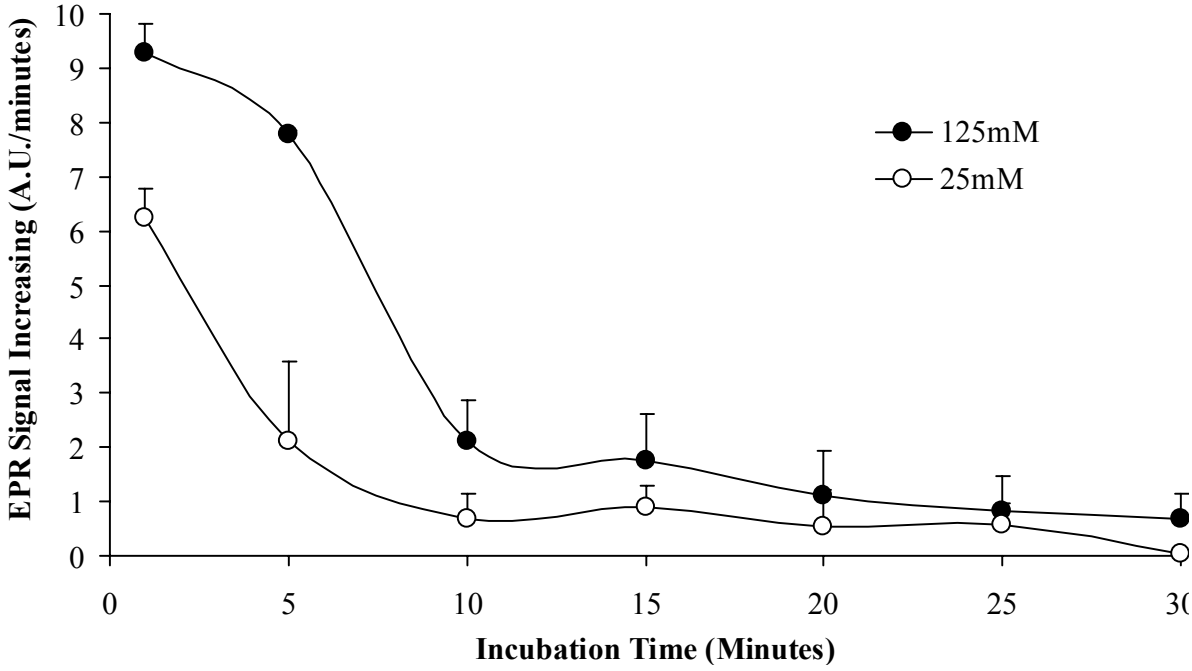


**Fig 3. The kinetics of DMPO-OH formation by TSP suspension**. 125 μg/ml of TSP suspension mixed with 25mM of DMPO and $H_2O_2$ 25mM or 125mM at 37°C. The DMPO-OH signal was measured at different time points after mixing.

**Kinetics of DMPO-OH adduct formation**

Since spin trapping is an integrative technique, it invariably takes time to reach sufficiently high levels of spin trap adducts that can be measured by EPR. In the presence of $H_2O_2$, different soluble metals such as $Fe^{2+}$, $Fe^{3+}$ and $Cu^{2+}$ showed different times to reach the maximal intensity of DMPO-OH. For example, the maximal concentration of the DMPO-OH adduct from the $Fe^{2+}$ catalyzed Fenton reaction is achieved immediately after $H_2O_2$ was added while for $Cu^{2+}$, about 3 minutes is needed to reach the maximum (Fig. 2). This finding also implicates that to measure the effect of a metal mixture, as occurring in

PM, one invariable needs a time that integrates and adds the redox activity of all metals. To study this in real life PM in more detail, the rate of formation of DMPO-OH adducts generated by PM was studied. This is shown in Fig. 3. Although the EPR signal increased with the incubation time over 30 minutes, the rate of formation decreased rapidly during the first 10 minutes, and after that the rate of formation is very low. To further investigate the reaction kinetics from particle-associated metals, a series of carbon black particles coated with different metals were tested for their abilities to generate hydroxyl radicals. At concentration of 125 μg/ml of these metal coated particles in measuring system, higher ability of free radical generation was found in those particles coated with $Cu^{2+}$, $V^{2+}$, $V^{v+}$, $Fe^{2+}$ and lower with $Fe^{3+}$, $Ni^{2+}$ and $Zn^{2+}$ (Fig. 4).

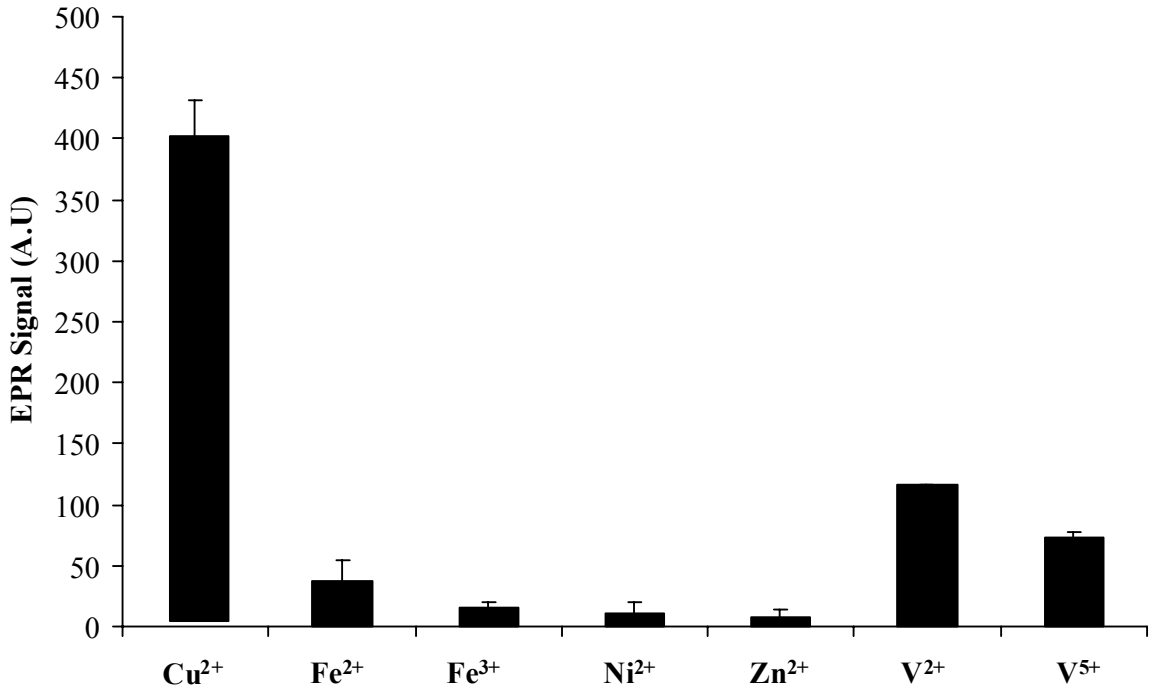


**Fig 4. Hydroxyl radical generation of metal-coated particles.** 25mM DMPO and 125 mM $H_2O_2$ was incubated at 37°C for 10 minutes with different metal-coated particles with the metal content of soluble Cu (II), Fe (II), Fe (III), Ni (II), Zn (II), V (II) and V (V) each 4 μg/ml (final concentration). EPR results were expressed as arbitrary units.

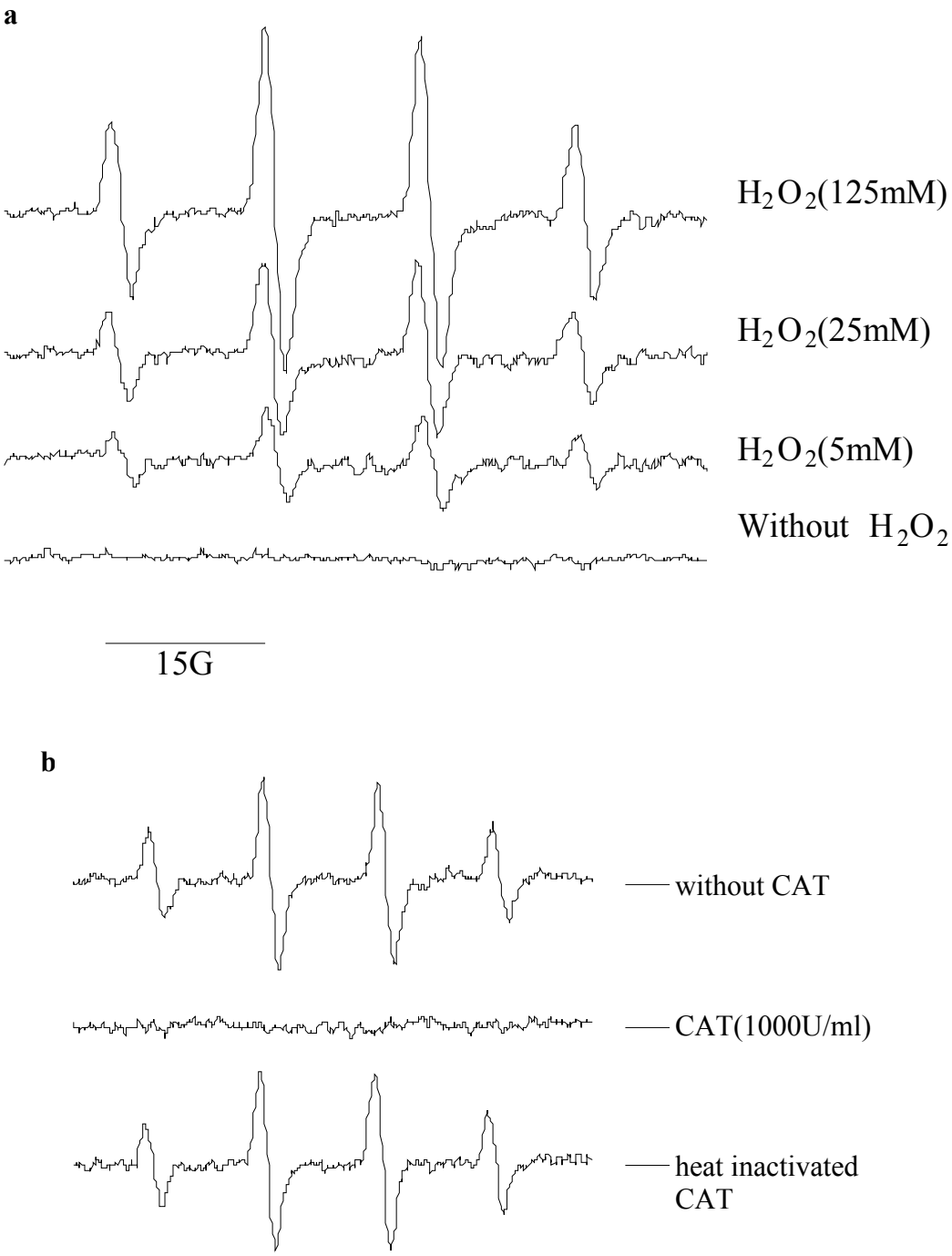


**Fig. 5** Panel a shows the effects of hydrogen peroxide on PM induced hydroxyl radical formation. TSP (125μg/ml) was mixed with 25mM DMPO and different concentrations of $H_2O_2$ for 10 minutes in a 37°C water bath. Panel b shows the effects of catalase on hydroxyl radical formation of PM. TSP (125 μg/ml) was mixed with catalase (1000U/ml) and inactivated catalase (95°C heat for 5 minutes) as well as 25 mM DMPO incubation 10 minutes at 37°C .

Previous studies in our laboratory (Knaapen *et al*, 2002) demonstrated that micromolar levels of $H_2O_2$ can cause significant increase of hydroxyl radical and also intracellular formation of ·OH in the presence of PM. The importance of the concentration of $H_2O_2$ for the formation of DMPO-OH by PM was further investigated in the following experiments. First, increasing the concentration of $H_2O_2$ leads to an increase of DMPO-OH adduct formation at equal PM concentration. The dose response effects of $H_2O_2$ on PM induced DMPO-OH adduct is shown in Fig. 5a. Secondly, experiments in the presence of catalase (1000 IU/ml) showed that catalase was able to prevent the formation of DMPO-OH by degrading of $H_2O_2$, since heat inactivated catalase (Fig. 5b) did not cause any inhibition. Studies comparing vortexing and water bath sonication at different time intervals and combinations showed that all transition metals were readily leachable. Within 1 minute of vortexing the EPR signal and iron leaching were already maximal (data not shown) and therefore the additional incubation time of the suspension (10 minutes) should be considered as sufficient to allow complete metal leaching. In order to determine the contribution of the metals in water-leachable versus the particle-fractions of the PM suspension to the formation of •OH, we compare the oxidant capacity of filtrates as well as the original suspensions of two contrasting oil fly ashes (OFA). For one sample, which is an OFA rich in Fe, the EPR signal of the suspension was similar to that in its filtrate (respectively 79 and 77 a.u.). In contrast, for the other OFA sample, which is rich in Vanadium, the particle suspension give a much higher signal than its corresponding filtrate (31 and 3 a.u. respectively). This indicates that also metals bound on particles can catalyze the formation of DMPO-OH in this reaction mixture. The inhibition of PM induced •OH formation with the metal chelator, desferoxamine was dose dependent; In the presence of 30μM desferoxamine, •OH generation was inhibited by 50% and a complete inhibition was achieved with 100 μM. This shows that transition metals, and especially Fe, are crucial in the formation of OH-radicals .

**Concentration dependency and time of storage**

The ability of freshly prepared suspensions of TSP in water following sonication to generate hydroxyl radicals remains stable over 3 hours. Similarly, when these suspensions were frozen at -20°C for 10 months, their ability to generate hydroxyl radicals upon thawing did not change (Table 1). The concentration dependency of the •OH formation was studied using different dilutions of solutions of sampled ambient PM, which was

assessed for mass concentration using comparative turbidometry. The latter method is based on the comparison of the 'blackness' of the particle suspension to standard carbon black (size 260 nm) by spectroscopic absorption at 405 nm. Although part of the PM is water-soluble, our comparative experiments using gravimetric analysis of PM before and after filter extraction confirmed the correlation between turbidometry and gravimetric analysis (data not shown). The DMPO-OH signal upon dilution of PM sampled during three different weeks at a site in Dusseldorf is shown in Fig. 6. Although the •OH formation is PM concentration dependent, the curves vary between different sampling time and PM fractions. These curves show that coarse PM usually has a higher •OH generation than fine PM, and that DMPO-OH generation has a different relation to mass concentration for each sample.

**Table 1. Comparison of PM suspension in hydroxyl radical generation after 10 months frozen**.

| Samples | DMPO-OH Signal (A.U) | |
|---|---|---|
| | Fresh Suspension | 10 Months Frozen |
| A | 29.12±1.53 | 22.70± 1.02 |
| B | 31.97±1.23 | 30.77 ± 0.74 |
| C | 41.79 ±1.20 | 41.64 ±1.80 |
| D | 51.09 ±0.95 | 51.76 ± 0.37 |

All values are the mean of 3 determinations and the SD. 80µg of PM suspension was mixed with 25 mM DMPO and 125 mM $H_2O_2$ for 10 minutes at 37°C. Then the PM suspensions were immediately frozen at –20°C and remeasured after 10 months after thawing, vortexing and 5 minutes water bath sonication which was used to break up agglomeration of particles. No significant difference was found (Paired Student T-test, P=0.41).

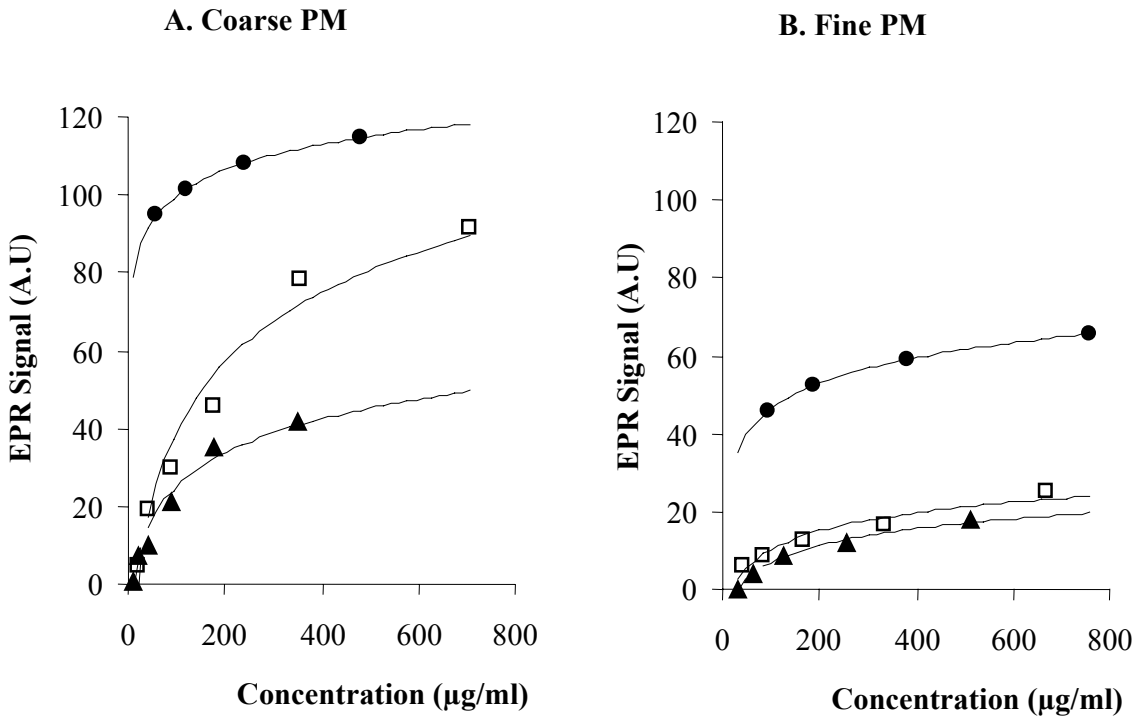


**Fig. 6 The particle concentration dependency of hydroxyl radical generation by coarse and fine PM, sampled in three different weeks.** PM suspension were serially diluted with water from original suspension, PM was randomised from three sampling weeks, week 1 (■), week 2 (□) and week 3 (▲). 2.10A is the EPR results of three coarse particle samples and 2.10B is the results of fine particle samples. Data are expressed as the intensity of the resulting DMPO-OH signal in arbitrary units.

**Hydroxyl radical formation in different size fractions of $PM_{10}$**

As already illustrated in Fig. 7, a difference in oxidant activity and its relation to particle number was noted in the coarse ($PM_{10-2.5}$) and fine ($PM_{2.5}$) fractions sampled with dichotomous samplers. To validate this finding, we sampled different fractions of $PM_{10}$ with three parallel samplers in an urban sampling site with high traffic and industrial load. The data (Figure 2.11) show that $PM_1$ contains by far the highest activity, which correlates with its high metal content (Fe, r=0.867; Cu, r=0.95, both n= 9, $P < 0.001$). Interestingly, the highest ratio between soluble metal content between $PM_1$ and $PM_{10}$ was found for Pb (= 8.7) and Platinum (= 3.0), which indicates that the fine particles originate from traffic

sources. The DMPO-OH formation by the $PM_{10}$ is only slightly higher on a mass basis compared to $PM_{2.5}$, suggesting that the coarse fraction ($PM_{10-2.5}$) in the $PM_{10}$ sample of this site contributes little extra. This study also allowed for the evaluation of the Fenton-reactivity of the different particulate fractions trapped on the filter versus the particle counts between 0.8 µm and 15 nm performed parallel. DMPO-OH formation by deposited $PM_1$ correlated with mass concentrations ($r=0.80$, $P<0.001$) but less so and not significantly with particle numbers ($r=0.53$, NS). $PM_{2.5}$ also showed no significant correlation but in contrast, the ESR activity in $PM_{10}$ was significantly associated to total particle number ($R = 0.70$, $n = 10$, $P< 0.05$). Correlations with metal content in $PM_{10}$ and $PM_{2.5}$ were much lower and confined to leachable Cu-content ($r= 0.86$, $N=10$, $P< 0.001$).

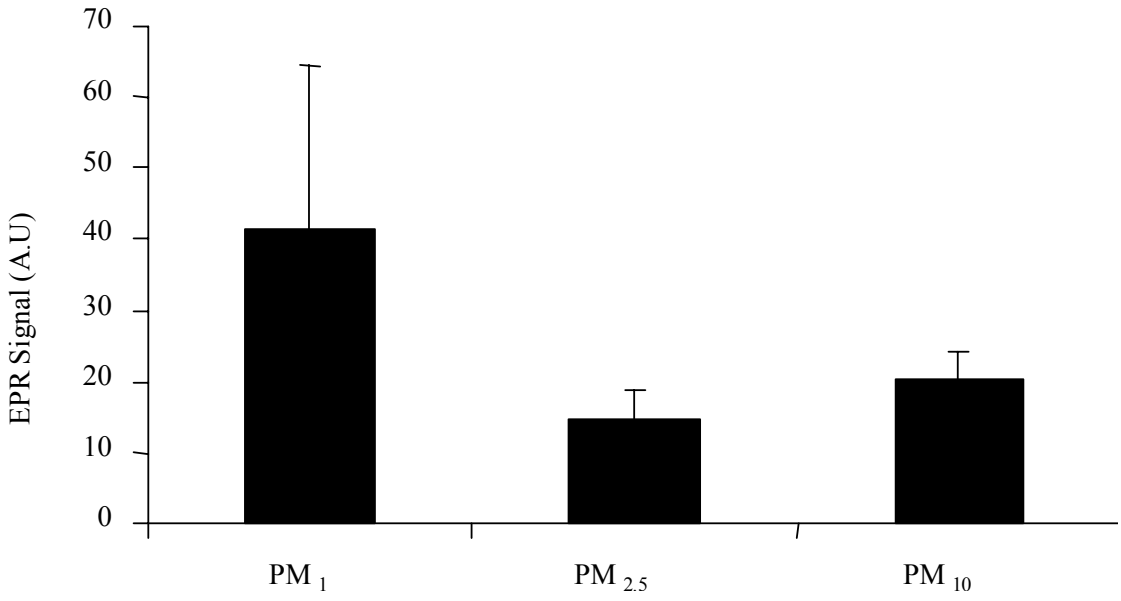


**Fig. 7 DMPO-OH generation per mass equivalent by different particle size fractions.** PM1, $PM_{2.5}$ and $PM_{10}$ were sampled parallel on 10 consecutive days in July-August 2001 in Duisburg (Germany) in the centre of the city. Data are the mean and standard deviations of n=9 ($PM_1$) or n=10 ($PM_{2.5}$, $PM_{10}$) samples.

## Temporal and regional variations of hydroxyl radical formation by fine and coarse particles

Hettstedt and Zerbst are two neighbouring towns located in the eastern part of Germany. A number of epidemiological studies have described a consistent difference in morbidity

between these two places, despite minor differences in particulate mass. Hettstedt, with its industrial pollution caused by metallurgical industry having a higher copper emission has a very high morbidity of inflammatory respiratory disease, while its neighboring town Zerbst without any sources of industrial pollution, has low morbidity (Heinrich *et al*., 1999). Samples from a six week sampling campaign during 2001 in these two locations showed considerable differences in the ability to generate hydroxyl radicals at similar mass concentration (Fig. 8.). Samples from Hettstedt showed significantly higher ($P<0.01$) oxidant activity than its rural neighboring-town Zerbst, despite similar $PM_{10}$ mass levels in the air. The mean value of DMPO-OH formation in fine PM was 19.3±5.8 (n=6) in Zerbst versus 86.8±40.1 (n=6) in Hettstedt. This 4.5 - fold difference in DMPO-OH generation between Hettstedt and Zerbst was also seen in the coarse fraction (Hettstedt: 87.8 versus Zerbst: 17.1), and is readily explained by a significant difference in Cu-content in PM from both locations. Leachable copper content of coarse PM was 3.59 µg/mg in Hettstedt versus 0.43 µg/mg in Zerbst, while in the fine fraction these values were 2,63 and 0,36 µg/mg respectively. Furthermore, the time variation in the ability to generate ·OH was related to copper content in both fine ($r=0.95$, $n=12$, $P < 0.001$) and coarse ($r=0.91$, $n=12$, $P < 0.001$) mode particles.

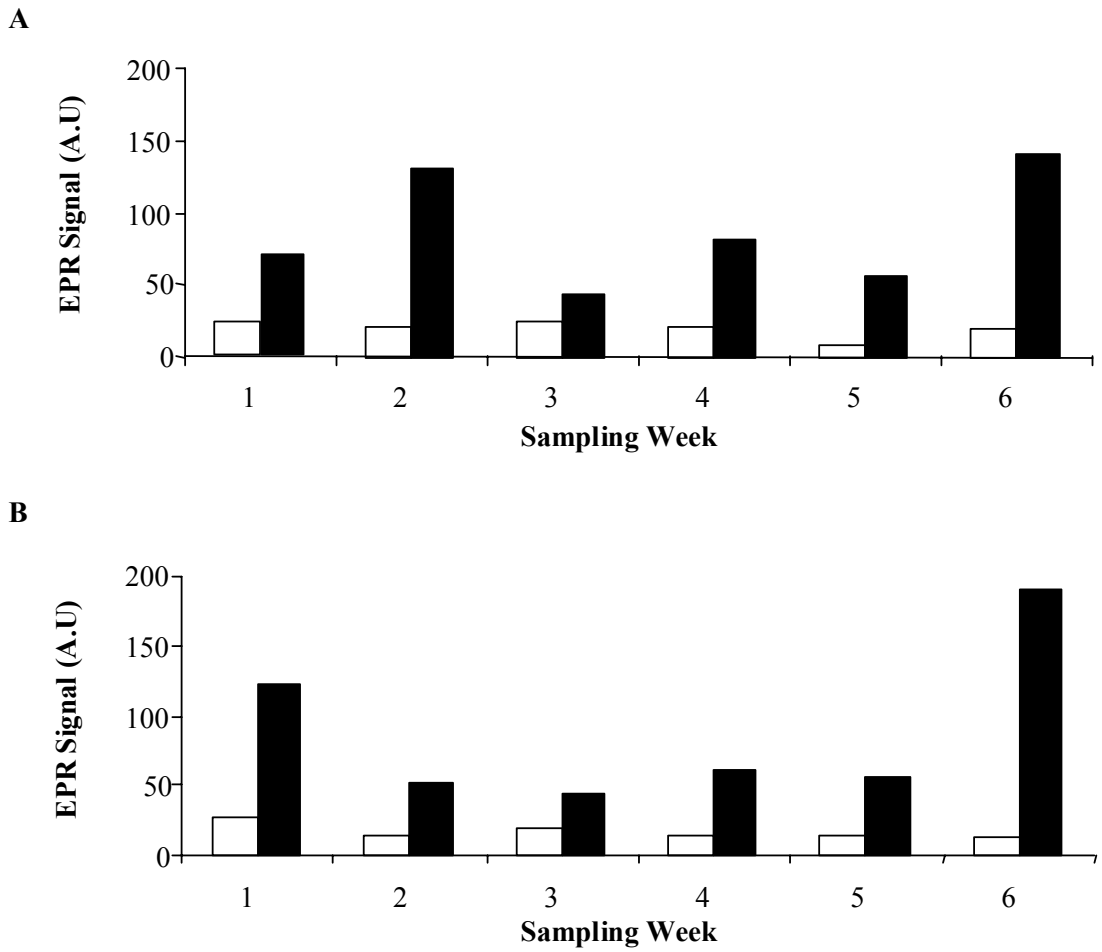


**Fig. 8 Comparison of hydroxyl radical generation by fine (A) and coarse (B) PM sampled at different places during a similar interval.** All PM suspensions were adjusted to the original concentration of 120μg/ml and 50 μl of these suspensions were incubated with 25mM DMPO and 125 mM $H_2O_2$ for 10 minutes at 37°C water bath. EPR results were expressed as arbitrary units for Hettstedt (■) and Zerbst (□).

# DISCUSSION

The results presented here strongly suggest that all size fractions of particulate matter recovered after sampling on filters are able to generate hydroxyl radicals in the presence of hydrogen peroxide. This was shown by direct spin trapping and detection of the •OH by EPR. Earlier work with both model particles or filter deposited PM have suggested that hydroxyl radicals are formed and responsible for strand breaks in plasmid DNA (Donaldson *et al*., 1997), or oxidation of the fluorescence probe DCFH (Stringer and Kobzik, 1998), vitamin C or salicylate (Pritchard *et al*., 1996; Ghio *et al*., 1996). Our previous studies have shown that acellular hydroxyl radical formation by various coal-fly ashes (Van Maanen *et al*., 1999) and PM (Knaapen *et al*., 2002; Shi *et al*., 2003) is highly correlated to its capacity to cause cellular oxidative DNA-damage in lung epithelial cells.

It is now generally accepted that the ability of PM to generate reactive oxygen species (ROS) plays a pivotal role in PM-induced adverse health effects (Donaldson *et al*., 2001; Gilmour *et al*., 1996; Li *et al*., 1997; Prahalad *et al*., 1999) and among these ROS, the •OH is of greatest concern as it is a highly reactive electrophilic species, known for its ability to attack DNA (Pryor, 1988). The most common mechanism for hydroxyl radical generation is via the Fenton reaction (eqn 7), which involves the reduction of hydrogen peroxide by a transition metal ion (Halliwell and Gutteridge, 1989; Lloyd *et al*., 1997).

$$Fe^{2+}+H_2O_2+H^+ \rightarrow Fe^{3+}+H_2O+\bullet OH \quad (7)$$

Hydrogen peroxide has been shown to be present endogenously in living cells. Beyond this, also the oxidative burst of human neutrophils can be activated by PM and cause an increase in the release of $H_2O_2$(Knaapen *et al*., 2002). However, cells are able to metabolise certain amounts of $H_2O_2$, mainly through catalase and GSH-dependent peroxidases to minimize the amount of $H_2O_2$ that can react with transition metals to generate very reactive hydroxyl radical (Haber and Weiss, 1934; Lloyd *et al*., 1997).

In our system, transition metals clearly are the major determinant of •OH formation in most fractions. This can be derived from the abrogation of the EPR signal by addition of deferoxamine. In agreement with these observations, others have shown that deferoxiamine is also effective in preventing transition metal-induced oxidation of ascorbate or salicylic acid (Pritchard *et al*., 1996; Ghio *et al*., 1996). Despite the lower content of water-leachable metals recovered on a mass-basis from our filters, a consistently higher •OH generation was found using suspensions of the coarse fraction (Shi *et al*., 2003). Several

explanations are available for this paradoxical finding. First, it has been confirmed that the chemical form of metals is important for its activity. When comparing the intrinsic ability of Fenton active metals to cause oxidative DNA damage in nude DNA, Lloyd (Lloyd *et al*., 1998) found the highest activity by Cr (III), Fe (II), V (III) and Cu (II). Unfortunately, ICP-MS analysis does not assess the valence of the leachable metals, which has been shown of crucial importance for its Fenton chemistry (Lloyd *et al*., 1998). Secondly, our present data with OFA indicate thatit is not only the soluble metals which are involved in the free radical generation but also insoluble metals present on the surface of particle may generate hydroxyl radical (Knaapen *et al*., 2002; Ghio and Samet, 1999). Thirdly, we suggest that other organic and inorganic components, such as sulphate (Ghio and Samet, 1999) and semiquinones (Dellinger *et al*., 2001) may affect the oxidant activity of the particulate. Finally metal-to-metal interactions certainly influence the oxidant activity of PM fractions with considerable variation of compositions. It has been well demonstrated that when mixing tungsten carbide with inert metal cobalt, its activity in radical generation is great enhanced (Fenoglio *et al*., 2000). Furthermore, when Ni, V and Fe were mixed together, the pathology and cytokine gene induction caused by these three metal were less severe than that caused by Ni along (Dreher *et al*., 1997). Our data certainly suggest that the particle size and composition have profound effects on their oxidant activity *in vitro* as measured by our assay and that this may be achieved by affecting solubilisation, offering reducing elements and catalysing surface and other redox active metals. This supports the concept of using an assay that integrates bioavailability and redox activity of metals in the presence of particles that are able to catalyze radical formation. The •OH generation measured by our protocol in water upon addition of $H_2O_2$ shows oxidant activity in different fractions, which can be assessed in particles retrieved by water extraction from different filter types, including Teflon, biosamplers and polyurethane foam.

Studies with Hettstedt-Zerbst samples showed that variations of •OH generation of PM sampled over time and in different sites were larger than similar variations in mass. In addition, samples from the heavily polluted industrial city (Hettstedt) showed a higher generation of hydroxyl radicals than Zerbst at equal mass measured by EPR. Studies in different sites show a variable ratio between DMPO-OH generation in coarse and fine PM. The samples taken with dichotomous low-volume samplers in Dusseldorf, Hettstedt and Zerbst all show a higher activity in the coarse particles than in the fine fraction. In agreement with this, $PM_{10}$ samples from high volume sampling in Duisburg show higher EPR signals than fine PM. Obviously considering that coarse PM has stronger oxidant

activity than fine, the difference between coarse ($PM_{10-2.5}$) and fine is anticipated to be larger than the difference between $PM_{10}$ and fine since the coarse fraction in $PM_{10}$ is 'diluted' with fine and ultrafine particles.
However, compared to PM10 and $PM_{2.5}$, the highest activity by far is found in $PM_1$. The relative proportion of ultrafine (i.e. <100nm) and metal-rich particles is highest in $PM_1$. This again suggests that this method integrates the reactivity of different metals in different size fractions and that ultrafine particles in $PM_{10}$ samples can have another reactivity than that in $PM_{2.5}$. Surprisingly, the ESR activity in the $PM_1$ fraction was related to mass concentration and not to particle counts, while the $PM_{10}$ activity was related to particle number. This suggests that the recovery of ultrafine particles from the filters is different from those loaded with only ultrafines versus a particle size mix. It is conceivable that upon deposition in the $PM_{10}$ sampler, many ultrafine particles are scavenged by coarse mode particles that are recovered well from the Teflon filters. The interaction between a relatively pure ultrafine particle fraction in $PM_1$ with the hydrophobic Teflon filter obviously does not allow full recovery of the ultrafine particles that drive the total particle number. This data suggest that standardization of the method with regard to filter type and fraction is necessary to compare data.

In conclusion, the data presented here, indicate that ambient particulate matter has the ability to generate hydroxyl radicals in the presence of hydrogen peroxide. Both soluble metals and insoluble metals on the particle surface are involved in •OH formation. Hydroxyl radical generation by sampled PM measured by EPR provides a simple method for environmental monitoring that can be applied to low-mass samples of PM. It remains to be investigated how this oxidant activity correlates with to biological effects measured *in vivo* and *in vitro* or epidemiological results. These studies are currently performed in our laboratory and preliminary analysis have demonstrated a link between •OH formation measured by EPR and depletion of anti-oxidants in synthetic lung lining fluid (data not shown), as well as the induction of DNA damage in naked and cellular DNA (Shi *et al*., 2003). Ongoing studies in collaboration with larger epidemiological programs in Europe (ECRHSII, PAMCHAR, HEPMEAP) will reveal how particle oxidant activity is related to different sources (traffic, industry, rural) and various health endpoints.

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# Chapter III

# TEMPORAL VARIATION OF HYDROXYL RADICAL GENERATION AND 8-HYDROXY-2'-DEOXYGUANOSINE FORMATION BY COARSE AND FINE PARTICULATE MATTER.

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## ABSTRACT

To determine the induction of 8-hydroxy-2'-deoxyguanosine (8-OHdG) by fine (<2.5µm) and coarse (10-2.5µm) particulate matter (PM) sampled over time at one sampling location, and to relate the observed effects to the hydroxyl radical (·OH) generating activities and transition metal content of these samples, and to meteorological parameters. Weekly samples of coarse and fine PM were analysed for $H_2O_2$-dependent ·OH-formation using Electron Paramagnetic Resonance (EPR) and formation of 8-OHdG in calf thymus DNA using an immuno-dotblot assay. Immunocytochemistry was used to determine 8-OHdG formations in A549 human epithelial lung cells. To determine temporal effects, samples from 6 weeks in summer and 6 weeks in autumn/winter were compared using EPR and the dotblot assay. Concentrations of leachable V, Cr, Fe, Ni, and Cu were determined by inductively coupled plasma mass spectrometry. Both PM fractions elicited ·OH generation as well as 8-OHdG formations in calf thymus DNA and in A549 cells. 8-OHdG formations in the naked DNA were significantly related to ·OH generation, but not to metal concentrations except for copper. A significantly higher ·OH generation was observed for coarse PM, but not fine PM collected during autumn/winter season; this was not due to differences in sampled mass or metal content. Specific weather conditions under which the increased ·OH formation in the coarse mode was observed suggests that other, yet unknown, anthropogenic components might affect the radical-generating capacity of PM. Both coarse and fine PM are able to generate ·OH, and induce formation of 8-OHdG. When considered at equal mass, ·OH formation shows considerable variability with regard to the fraction of PM, as well as the sampling season. The toxicological implications of this heterogeneity in ·OH formation by PM, as can be easily determined by EPR, need further investigation.

# INTRODUCTION

Increased exposure to ambient particulate matter (PM) has been associated with respiratory, cardiovascular and malignant lung disease (Dockery *et al*., 1993; Cohen and Pope, 1995). *In vitro* studies indicate that the effects of PM may be due to its chemical composition or the size fraction of the particulates. In lung epithelial cells, the cytotoxicity of residual oil fly ashes and Utah-Valley dust has been linked to their transition metal content (Carter *et al*., 1997; Frampton *et al*., 1999). In macrophages, the toxicity of $PM_{10}$ was also shown to involve transition metals, but interestingly this effect was merely observed in its coarse (2.5-10μm) fraction, and not in its fine (<2.5μm) fraction (Monn and Becker, 1999). Despite this observation, higher metal concentrations are usually found in the fine mode, which is largely composed of particles of anthropogenic origin (Harrison and Yin, 2000). Metals have also been implicated in the inflammatory effects of PM (Carter *et al*., 1997; Frampton *et al*., 1999; Ghio and Devlin, 2001), as well as in the ability of PM to induce oxidative DNA damage (Van Maanen *et al*., 1999; Prahalad *et al*., 2001). Transition metals that are present in PM are considered to exert their effects predominantly via formation of hydroxyl radicals (·OH), generated by available iron via the Fenton-reaction (Donaldson *et al*., 1997). In addition to iron, several other 'Fenton-active' transition metals that usually occur in PM, such as chromium, vanadium and copper are also known to induce the ·OH-specific DNA adduct 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Kasai, 1997), albeit with considerably varying efficiency (Van Maanen *et al*., 1999; Lloyd *et al*., 1998). As such, these metal-specific findings are difficult to extrapolate to the ·OH generating properties of PM as an entirety, since this will depend on the concentration, bioavailability, and chemical speciation and oxidation state of each individual metal. Furthermore, ·OH generation may also be modified by other agents of this complex mixture. Therefore, we have recently developed a method using electron paramagnetic resonance (EPR) to measure the generation of ·OH by PM in the presence of $H_2O_2$ and 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO) as a specific spin trap (Knaapen *et al*., 2000), as an integrate of the Fenton-reactivity of a given PM sample.

Since the chemical composition of PM (e.g. metal concentrations) is well known to vary with time, sampling location, and size fraction (Harrison and Yin, 2000), we have anticipated that this would also be reflected in variability of ·OH generating capacities, as well as ·OH-associated effects. Therefore, the aim of our present study was to determine

the variation in ·OH generation and formation of 8-OHdG by coarse and fine PM, sampled over time at one sampling location, in relation to sampled mass and transition metal content, as well as to meteorological data that could affect its chemical composition.

# METHODS

## Collection and sample processing of particulate matter

Coarse (PM10-2.5µm) and fine (PM<2.5µm) fractions of $PM_{10}$ were sampled weekly in Düsseldorf, Germany in the period of July to December 1999. Coarse and fine PM were collected on Teflon filters using Graseby-Anderson dichotomous low volume samplers at a flow of 16.7 L/min. Filters were stored in the dark in a dry atmosphere until further analysis. The PM was removed from the filters by agitation (5 minutes) in 1ml of ultrapure water, and the suspensions were sonicated for 5 minutes. Resulting PM concentrations were estimated using comparative turbidometry against a standard dilution curve using a carbon-black suspension. Comparative analysis of gravimetric and turbidometric analysis of a total of 86 samples revealed the factor that was used to calculate mass from turbidometry (i.e. 3.13 for fine and 3.56 for coarse PM), and also showed that there was a small but non-significant difference in mass recovered from the filters containg coarse or fine PM on extraction (that is 88% for fine and 91% for coarse PM). The difference in the factors can be explained by the different blackness of coarse versus fine PM. Although the turbidometry method only represents an indirect estimate of the extracted amount of PM. It allowed us analyse the effects of freshly prepared (that is, non-frozen) PM suspension. This approach avoids prolonged storage, and freezing and thawing of PM suspensions, which can lead to altered leaching of metals or organic compounds and which could also affect particle agglomeration.

Particle suspensions were diluted in ultrapure water for further analysis at the concentrations as shown, and immediately used for analysis of ·OH generation and 8-OHdG formations. To determine temporal effects samples from 6 weeks (6 fine, 6 coarse) in the months July to September 1999 and 6 weeks in November/December 1999 (6 fine, 6 coarse) were tested (Table 3.1). The extracted mass ranged from 0.57 to 2.49 mg (fine PM), and 0.66 to 1.89 mg (coarse PM). Samples were all adjusted to equal concentrations, i.e. 0.57 mg/ml. From each sample a small liquid was used for determination of transition metals using inductively coupled plasma mass spectrometry (ICP-MS). For determination of the seasonal variability of ·OH formation and 8-OHdG formation, week-pairs of fine and coarse PM were randomised and analysed in two independent experiments, i.e. A and B (Table 1).

**Table 1. Sample characteristics of ambient PM sampled in Düsseldorf**

| | **Sampling** | | **Fine mode** | | **Coarse mode** | | **Meteorological parameters** | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Period | 7-days, start on | Exp [1] | conc.[2] (mg/ml) | EPR [3] (A.U.) | conc. [2] (mg/ml) | EPR [3] (A.U.) | *Predominant air mass origin* | Rain [4] (mm) | MLH [5] (km) | Source Influence Ruhr area[6] |
| I | 22.07.99 | B | 1.34 | 30.1 | 1.50 | 16.8 | NA, GB | 8.2 | 1.47 | 4 |
| | 29.07.99 | A | 0.93 | 10.1 | 0.75 | 27.8 | EE, CE | 0.0 | 2.49 | 0 |
| | 05.08.99 | B | 1.19 | 24.9 | 1.48 | 44.6 | NA, NF | 52.3 | 1.15 | 1 |
| | 12.08.99 | A | 1.14 | 22.4 | 0.66 | 29.7 | NA, GB | 35.4 | 1.45 | 0 |
| | 02.09.99 | B | 2.26 | 13.9 | 1.43 | 24.5 | EE, CE | 0.0 | 1.18 | 4 |
| | 09.09.99 | A | 2.49 | 19.4 | 1.10 | 31.6 | SG | 12.0 | 1.23 | 3 |
| II | 04.11.99 | A | 1.46 | 24.9 | 1.63 | 80.8 | NA, GB | 9.6 | 0.64 | 3 |
| | 11.11.99 | B | 1.16 | 35.7 | 1.89 | 80.4 | SC, EE | 6.3 | 0.49 | 3 |
| | 18.11.99 | B | 1.17 | 16.0 | 1.13 | 61.4 | NA, GB, EE ) | 14.6 | 0.25 | 3 |
| | 25.11.99 | A | 1.18 | 13.8 | 0.98 | 26.8 | NA, NF | 6.8 | 0.46 | 0 |
| | 02.12.99 | B | 1.68 | 13.1 | 1.34 | 44.8 | NA, GB | 11.9 | 0.64 | 0 |
| | 09.12.99 | A | 0.57 | 23.1 | 0.71 | 33.6 | NA, BG | 51.5 | 0.47 | 0 |

[1] Experiment (see method section for details);

[2] Mass of PM extracted from filters;

[3] ·OH generation determined by EPR;

[4] Weekly sum at station Brüggen; NF: Northern France

[5] Mixing layer height;

[6] No. of days when the local winds pointed predominantly from the core of the Ruhr area.

NA: North Atlantic; GB: Great Britain; EE: Eastern Europe; SC: Scandinavia; CA: Central Europe; SG: Southern Germany

## Electron paramagnetic resonance measurement

Hydroxyl radical formation by the coarse and fine PM was evaluated by Electron Paramagnetic Resonance (EPR) as described previously (Shi *et al.*, 2001). Briefly, 50µl of the particle suspension was mixed with 100µl of the spin-trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, 0.05M in distilled deionized water) and 50µl $H_2O_2$ (0.5M in PBS). The suspension was incubated for 10 min at 37ºC in a shaking water bath, and filtered through

a 0.1µm filter (Acrodisc 25 mm syringe filter, Pall Gelman Laboratory, Ann Arbor, USA). The filtrate was immediately transferred to a capillary and measured with a Miniscope EPR spectrometer (Magnettech). The EPR-spectra were recorded at room temperature using the following instrumental conditions: Magnetic field: 3360 G, sweep width: 100 G, scan time: 30 sec, number of scans: 3, modulation amplitude: 1.975 G, receiver gain: 1000. Quantification was done by accumulation of 3 different spectra, each averaging 3 different scans. All 4 peaks were quantified by measuring the amplitudes, and outcomes are expressed as the total amplitude in arbitrary units (A.U.).

**8-Hydroxydeoxyguanosine induction by PM in calf thymus DNA**

Induction of the ·OH specific DNA lesion 8-hydroxydeoxyguanosine (8-OHdG) by PM in isolated calf thymus DNA was estimated via a dot-blot assay that we developed, based on a method as described by Musarrat *et al*. (Musarrat and Wani, 1994). Freshly prepared suspensions of PM were incubated with 50µg of calf thymus DNA dissolved in Tris-HCl (10mM, pH=8.0) and $H_2O_2$ (1mM). In each experiment, DNA incubated without PM and $H_2O_2$, as well as DNA incubated with 0.1mM $FeSO_4$ and 1mM $H_2O_2$ were included respectively as negative and positive controls. Samples were incubated in the dark for 90 minutes at 37ºC in a shaking water bath, and then immediately centrifugated (6000 rpm, 5 min). 400µl of the supernatant was transferred to a fresh tube, and DNA was precipitated by the addition of 1/10 vol. NaAc (1.5M, pH=6.0) and 2 times vol. 100% ice-cold ethanol. The DNA was then washed twice using 70% ethanol (13000rpm, 5min), dried in the dark, dissolved in 30µl of Tris-HCl buffer and stored overnight at 4ºC. DNA concentrations were determined spectrophotometrically and the samples were diluted to a final concentration of 2.56µg/ml in 20xSSC. Of each sample, replicate 2-fold dilutions were blotted on a nitrocellulose-membrane using a dot-blot apparatus. To each blot, both negative and positive controls were added. The DNA was cross-linked by baking of the membrane for 90 min in a pre-warmed oven at 80°C. Blocking of the membrane was performed overnight using casein. Immunolocalisation of 8-OHdG was performed using the N45.1 monoclonal antibody (Toyokuni *et al*., 1997), and using the Vectorstain-ABC kit with diaminobenzidine-staining according to the recommended protocol (Vector Laboratories). The blots were analysed by computer-assisted densitometry scanning (BioRad), and expressed relatively to the density of the negative controls.

**Measurement of 8-Hydroxydeoxyguanosine in A549 epithelial cells**

A549 cells (American Type Culture Collection), were grown in Dulbecco's Modified Eagle's Medium (DMEM; Life Sciences), supplemented with 10% heat inactivated foetal calf serum (FCS; Life Sciences), L-glutamine (Life Sciences), and 30 IU/ml penicillin-streptomycin (Life Sciences) at 37°C and 5% $CO_2$. The induction of 8-OHdG in the epithelial cells was measured by immunocytochemistry as follows. A549 cells were seeded in 4-Chamber Slides (Falcon) at a concentration of 120,000 cells/chamber. After two days, cells were exposed to PM suspended in Hanks Balanced Salt Solution (HBSS) for 2 hours. Immunocytochemistry was performed using the Vectorstain-ABC kit (Vector Laboratories), and the same antibody (Toyokuni *et al*., 1997) was used for the dotblot assay.

**Analysis of metals by ICP-MS**

ICP-MS was used to determine the concentrations of V, Cr, Fe, Ni, and Cu in the aqueous suspensions of PM. Therefore, freshly prepared suspensions of PM were filtered through a 0.2µm Millipore filter (Minisart RC15 syringe filter, Sartorius AG, Göttingen, Germany). The filtrate was diluted with de-ionised water (1:5), and filtered again. The transition metals were analysed by sector field ICP-MS (ELEMENT by Finnigan MAT, Bremen, Germany) (Begerow *et al*., 2000) in the medium resolution mode ($m/\Delta m \cong 4000$) using the standard addition procedure for calibration. 50µl of the filtrate was diluted with 500 µl 0.08 N $HNO_3$ and 2000 µl ultrapure water and spiked with 50 µl of standard solutions containing 5–20 µg/L Ni, Cu, V, and Cr and 50–200 µg/L Fe, respectively.

**Meteorological analysis**

To identify eventual influences of meteorological parameters on properties of the sampled PM, the synoptic scale weather situation, atmospheric stability, local winds, and precipitation in the Düsseldorf area were assessed using three types of data: (a) wind direction, wind speed, temperature, and rainfall measured at the meteorological stations Brüggen (51.20 N; 6.13 E), and Gütersloh (51.93 N; 8.32 E); (b) daily radiosonde ascents at Essen (51.40 N; 6.97 E) at 1200 h UTC; (c) four-day backtrajectories (850 hPa level) based on model data from the European Centre for Medium-Range Weather Forecasts. The

radiosoundings were used to estimate the mixed layer height of the planetary boundary layer.

**Statistical analysis**

Comparison between fine and coarse PM or the PM samples of different sampling periods were made by t-test, or the non-parametric Mann-Whitney test (for 8-OHdG only). Spearman rank correlation was used to determine the relations between EPR activity, transition metals, and the formation of 8-OHdG. Therefore, the relative staining intensities as determined for 8-OHdG were ranked for each separate experiment (i.e. A or B, see table 1). As such rankings were made per experiment, respectively for coarse and fine PM together (i.e. ranking from 1 to 12) or for coarse and fine separately (i.e. ranking from 1 to 6).

## RESULTS

EPR measurements demonstrated that suspensions of both fine and coarse PM caused formation of ·OH in the presence of $H_2O_2$ (see fig 1). Dose-response curves were performed with both coarse and fine PM sampled in three randomly chosen weeks, to determine ·OH generating capacities of the PM suspensions at different concentrations (fig2). For all curves a highly significant fit ($r^2 > 0.97$, $p<0.005$) was observed when the concentration was expressed on a logarithmic scale. Interestingly, a large variation was observed in the ·OH generating capacities of fine as well as of coarse PM, sampled in different weeks. However, coarse PM had higher ability to generate ·OH than fine PM when compared at equal mass.

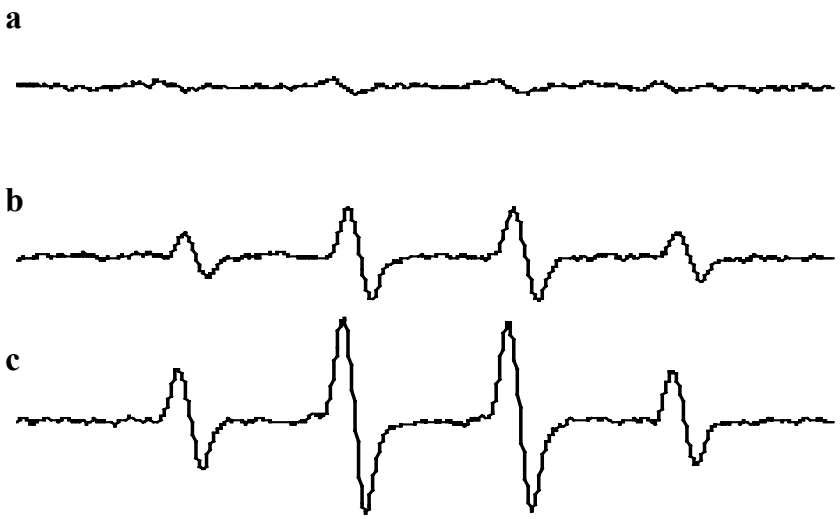



**Figure 1 DMPO-OH signal as measured by EPR of coarse and fine PM.** Following incubation with $H_2O_2$ and DMPO, EPR analysis showed the ·OH specific 1:2:2:1 quartet pattern for fine PM as well as coarse PM. (a) blank Teflon filter, (b) fine PM (2.2 mg/ml), (c), coarse PM (2.2 mg/ml)

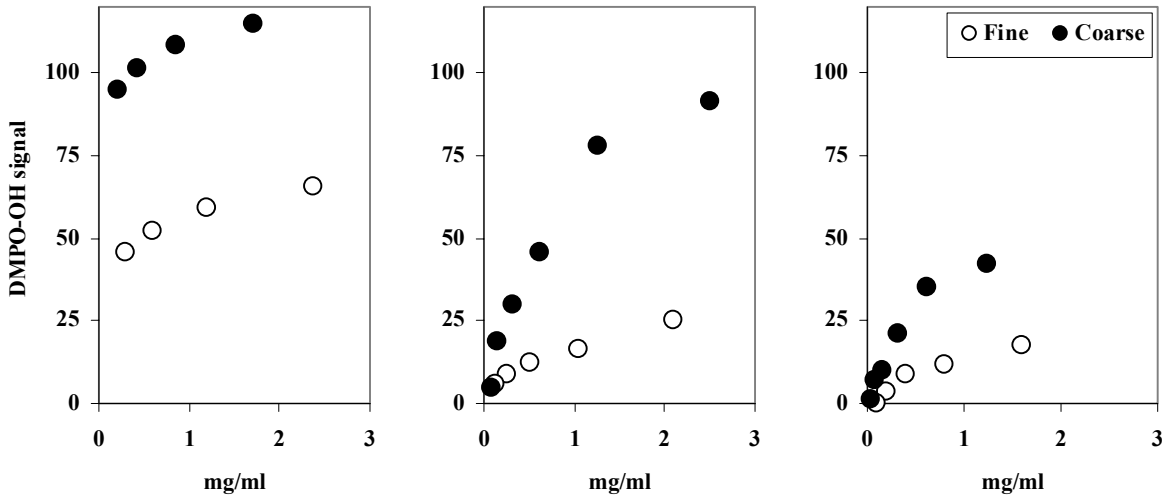



**Figure 2 Hydroxyl radical generation by serial dilutions of suspensions of coarse and fine PM, sampled in three different weeks.** Each graph shows ·OH generation for serial dilutions of a single weekly sample of respectively coarse or fine PM.Data are expressed as the intensity of the resulting DMPO-OH signal (see also Figure 1) in arbitrary units.

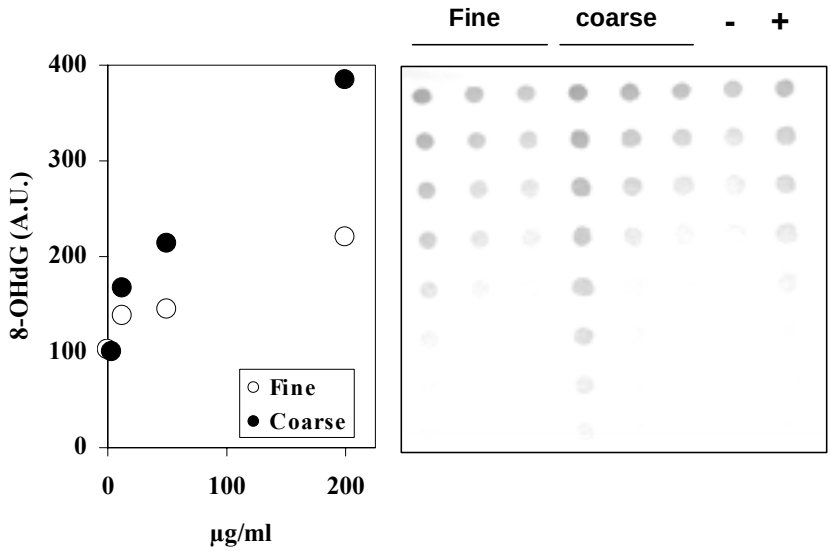
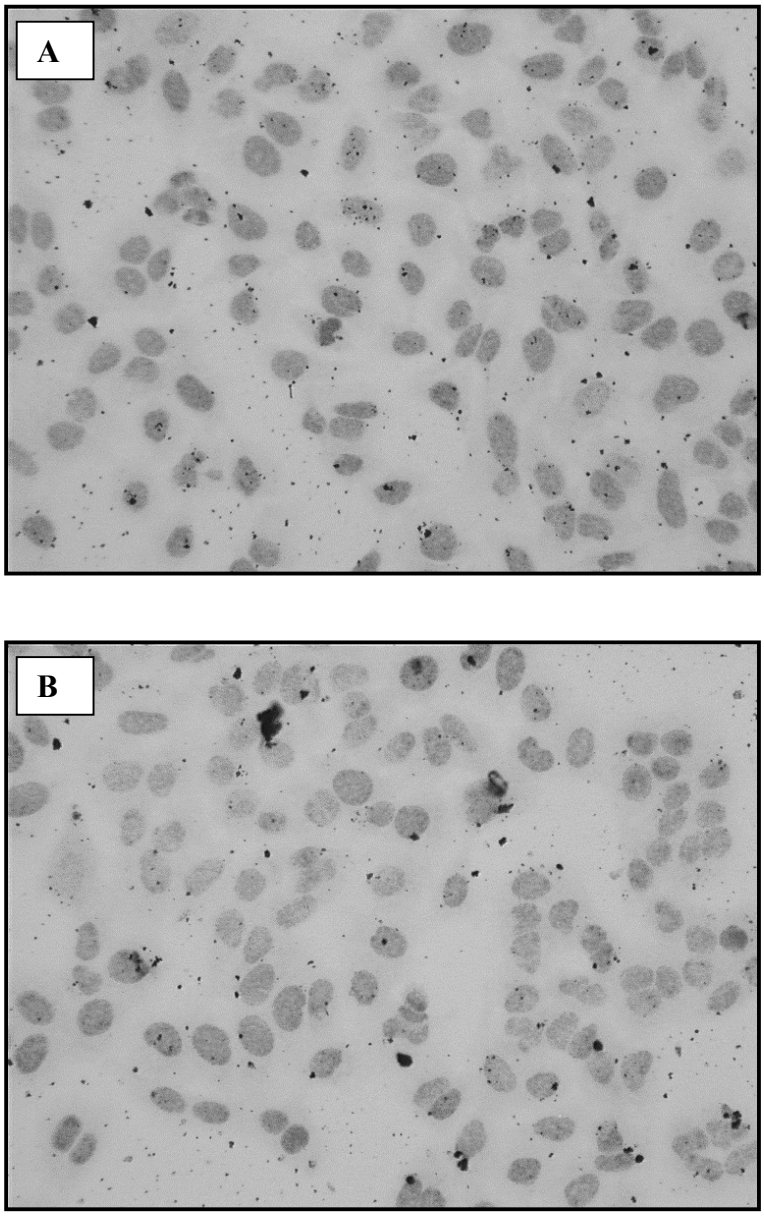



**Figure 3 Induction of 8-OHdG by fine and coarse fractions of PM in calf thymus DNA.** The right panel shows a representative dotblot of calf thymus DNA treated with serial dilutions of a suspension of respectively fine PM and coarse PM sampled from the same week, as well as the negative (-, DNA incubated with $H_2O_2$ but without PM) and positive controls (+, DNA incubated with $FeSO_4$ and $H_2O_2$). The graph on the left represents the same data for the PM per unit mass, as determined by densitometry analysis and expressed in arbitrary units (A.U.).

To see whether the observed ·OH generation relates to the induction of 8-OHdG coarse and fine PM were incubated with calf thymus DNA and analysed using an immunodotblot assay (Figure 3).



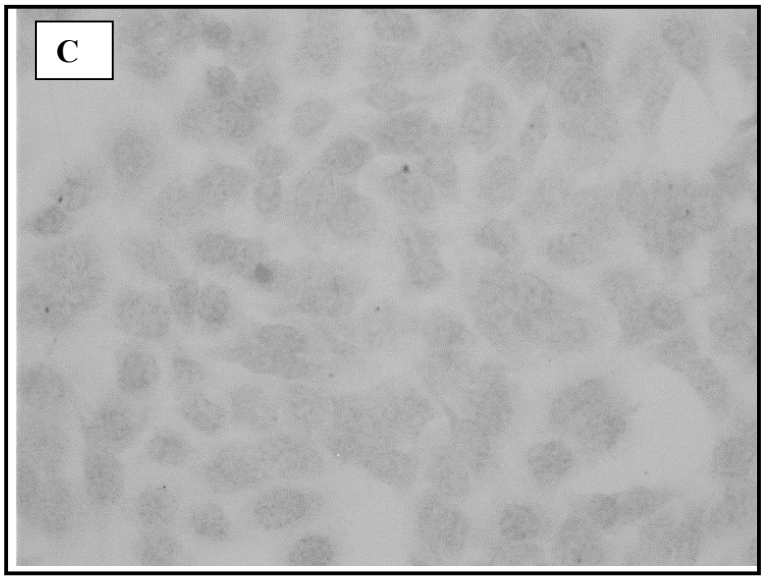



**Figure 4 Induction of 8-OHdG by fine and coarse fractions of PM in A549 human lung epithelial cells.** Cells were treated with PM suspensions for 2 hours, and 8-OHdG was determined by immunohistochemical staining. (a) fine PM; (b) coarse PM; (c) control. Pictures were taken at 400x magnification.

Both fine and coarse PM, as well as $FeSO_4$, used as positive control, caused formation of 8-OHdG in the presence of $H_2O_2$. In agreement with the observations using EPR, per unit mass the effects of coarse PM were stronger than the effects of fine PM. In order to determine the relevance of these acellular assays in a biological system, A549 human alveolar epithelial cells were treated with coarse and fine PM. Representative pictures are shown in figure 4. Both fractions of PM were able to induce 8-OHdG in the A549 cells upon 2 hours exposure. However, unlike the dotblot assay, no clear differences in the induction of 8-OHdG could be observed between coarse and fine PM.

To determine the possible influence of temporal variation, samples from two different periods (2 x 6 weeks) were analysed for ·OH generation using EPR as well as formation of 8-OHdG in calf thymus DNA (Table 2). As can be seen in the table, the ·OH generating capacities of the coarse particles sampled during the second period were significantly

higher than those sampled the first period. However, no differences in ·OH generation were found between both periods for the fine PM. A similar trend was observed for the formation of 8-OHdG although the differences did not reach significance. The observed temporal effects were not due to differences in sample storage time, since repeated measurements of samples collected with three parallel PM-samplers during the same week, did not show changes in ·OH generation due to sampling storage.

**Table 2 Hydroxyl radical generation, 8-OHdG formation in calf thymus DNA, and transition metal concentrations of fine and coarse PM sampled during 6 weeks in summer (period I) and 6 weeks in autumn/winter (period II).**

| | *Fine PM* | | *Coarse PM* | |
|---|---|---|---|---|
| | *Period I* [1] | *Period II* [1] | *Period I* [1] | *Period II* [1] |
| Mass (mg) [1] | 1.56 ± 0.56 | 1.20 ± 0.37 | 1.15 ± 0.38 | 1.28 ± 0.43 |
| EPR (A.U.) [2] | 20.1 ± 7.3 | 21.1 ± 8.5 | 29.2 ± 9.2 | 54.6 ± 23.3 * |
| 8-OHdG (rank) [3] | 3.3 [2.1 – 6.5] | 3.5 [1.8 – 5.9] | 7.5 [5.9 – 10.3] | 9.5 [8.8 – 12.0] |
| V (µg/g) | 214 ± 91 | 200 ± 100 | 81 ± 60 | 48 ± 57 |
| Cr (µg/g) | 143 ± 47 | 166 ± 56 | 70 ± 71 | 30 ± 12 |
| Fe (µg/g) | 2724 ± 1197 | 1752 ± 985 | 1161 ± 1703 | 239 ± 155 |
| Ni (µg/g) | 130 ± 63 | 192 ± 65 | 105 ± 71 | 92 ± 23 |
| Cu (µg/g) | 429 ± 96 | 287 ± 112 * | 570 ± 456 | 582 ± 240 |

[1] Mass of PM extracted from filters; ·OH generation determined by EPR; [3] relative inductions of 8-OHdG; all data are expressed as mean ± standard deviation, with the exception of 8-OHdG which is shown as median [25$^{th}$ percentile – 75$^{th}$ percentile] rank.
* Significantly different from period I ($p<0.05$, t-test)

When the samples of both period were considered together, coarse PM was found to have significant higher ·OH formation than fine PM ($n=12$, $p<0.01$, t-test), and also caused a

significantly higher 8-OHdG formation (n=12, p<0.001, Mann-Whitney). Figure 5 shows the correlation between ·OH generation and 8-OHdG formations for all samples. A significant correlation between ·OH formation and 8-OHdG was observed (n=24, Spearman's r=0.743, p<0.001), indicating that the ·OH generating properties of PM determine its ability to elicit oxidative DNA damage. Interestingly, this association was found both with the coarse mode and the fine mode, although the latter did not reach significance (i.e. coarse PM: n=12, r=0.580, p<0.05; fine PM: n=12, r=0.557, p=0.060). Neither ·OH generation nor 8-OHdG formations were correlated with the extracted mass.

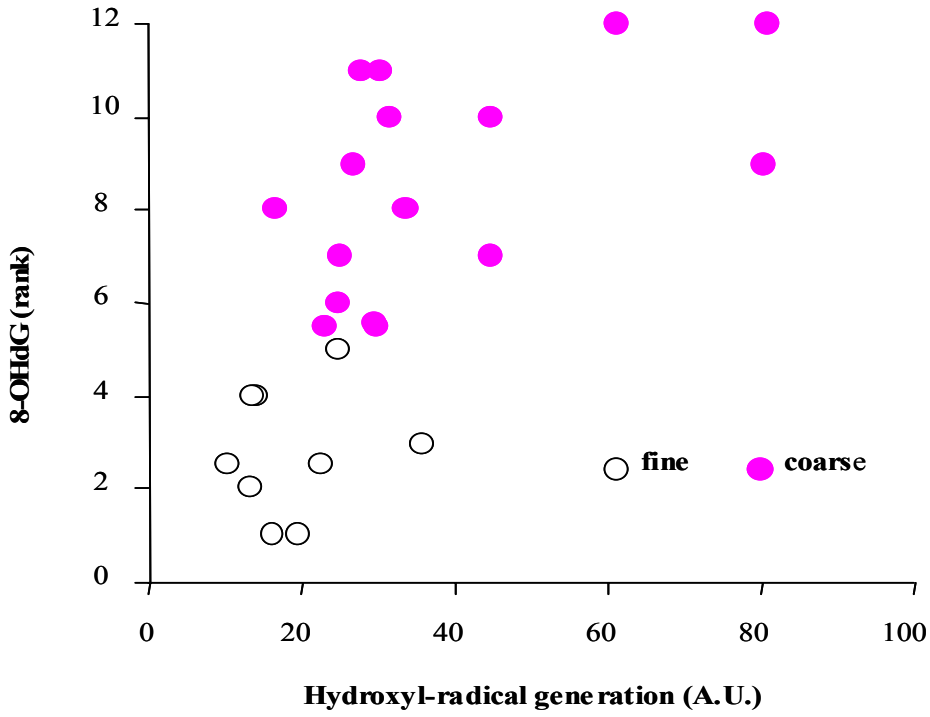



**Figure 5 Correlation between hydroxyl radical generating properties and 8-OHdG formation in calf thymus DNA for fine and coarse PM.** Hydroxyl radical generation as determined by EPR is expressed in arbitrary units, and for 8-OHdG data are expressed according to ranking of the samples as determined by densitometry analysis (see methods section for details).

To determine the role of transition metals in the oxidative properties of the PM in relation to the observed differences for both sampling periods, the suspensions of the PM were analysed for leachable Ni, Fe, Cu, V and Cr by ICP-MS. As shown in table 2, no differences in metal concentrations were observed for the PM sampled for both periods, with the exception of Cu which was significantly lower in the fine PM of the second

period. Furthermore, whereas both ·OH generation and formation of 8-OHdG were higher for the coarse PM, the concentrations of V, Cr, Fe (all $p<0.01$) and Ni ($p<0.05$) were found to be significantly lower for the coarse PM in comparison to the fine PM. Thus, the observed differences in hydroxyl radical generating capacities for both sampling periods as observed for the coarse PM were unlikely explained by different metal contents. Interestingly however, Cu was significantly correlated with ·OH generation ($n=24$, $r=0.644$, $p<0.001$), and to a lesser extent with the formation of 8-OHdG ($n=24$, $r=0.510$, $p<0.05$). This association was also observed for the coarse PM (i.e. hydroxyl radicals: $n=12$, $r=0.657$, $p<0.05$; 8-OHdG: $n=12$, $r=0.608$, $p<0.05$), but not for the fine PM. None of the other metals was found to correlate with ·OH or 8-OHdG, with the exception of Cr which showed a correlation with ·OH formation by the fine PM ($n=12$, $r=0.706$, $p<0.05$). Finally, the particle mass extracted from the filters was not different between both periods (Table 3. 2) and did not correlate with any of the metal concentrations.

Effects of meteorological parameters could be seen in several ways. The highest mass concentrations in the fine particle mode (occurred in period I, in the weeks starting on 02.09.99 and 09.09.99 (Table 1). Trajectory analysis showed that the air masses sampled during these weeks originated over South Russia, and South Germany, respectively. Such an accumulation of fine mode particles has been reported previously, and been ascribed to accumulation of primary and secondary aerosols in slow and non-precipitating air masses (Birmili *et al.*, 2001). Similar continental trajectory influence prevailed during the week starting on 29.07.99 (Table 1) but this time the fine particle mode mass concentration was low. During this week, the mixed layer height of the boundary layer was extremely high on average, eventually allowing for regeneration of the surface based aerosols with clean air from aloft. However, the continental nature of the backtrajectories had no visible effect on ·OH generation (Table 1) allowing a tentative suggestion that aged primary and secondary aerosols are not the predominant factors of toxicity in the fine particle mode.

As shown above, the highest rates of ·OH generation occurred in the coarse particle mode in period II, and especially during the first 3 weeks of sampling period II. All sampling weeks of period II featured weak vertical exchange and inversions, in contrast to period I, where atmospheric mixing was intense. It is well established that conditions such as in period II lead to an accumulation of atmospheric pollutants near the surface (Stull, 1988). The particular feature of the three weeks showing the highest ·OH generation was the predominant inflow of winds with a Northern component, often combined with a passage

of the air across the Ruhr area, featuring numerous industrial and other anthropogenic sources of PM. While a source contribution of the Ruhr area was evident from a meteorological point of view, there were no signs for increased metal concentrations during the period of concern (data not shown). This leads to the conclusion that another component in the coarse mode, which was not measured, might have been responsible for the observed effects in ·OH generation. The influence of recent precipitation was apparent, e.g., in the week starting at 09.12.99. The mass concentrations of the fine and the coarse mode were low (0.57 and 0.71 mg/ml, respectively) during this week, which was characterised by heavy rain (51.5 mm). For the entire campaign, the coarse mode mass concentrations seemed to be more susceptible to the recent occurrence of precipitation, whereas the fine mode mass concentrations seemed rather influenced by backtrajectories. However, neither precipitation nor backtrajectories correlated significantly with ·OH generation.

# DISCUSSION

Although associations between PM exposure and adverse health outcomes have been established in epidemiological studies (Dockery *et al*., 1993; Cohen and Pope, 1995) , there is still a debate on the actual constituents or characteristics of PM that play a role in these effects (Harrison and Yin, 2000). Transition metal-dependent ·OH formation has been considered as an important feature of the inflammatory effects of PM (Donaldson *et al*., 1997; Li *et al*., 1996). More recently, we and others (Van Maanen *et al*., 1999; Prahalad *et al*., 2000; 2001; Donaldson *et al*., 1997; Knaapen *et al*., 2000) showed that PM can induce oxidative DNA damage, including formation of the ·OH-specific and premutagenic DNA lesion 8-OHdG (Kasai, 1997; Kuchino *et al*., 1987; Marnett, 2000), and that this damage could be prevented with iron chelator desferoxamine and hydroxyl radical scavenger DMSO. These observations suggest a key role for Fenton-reaction driven ·OH formation in the induction of DNA damage. This is further supported by our current data, showing a strong association between ·OH generation by respectively coarse and fine PM samples from different sampling periods, and their abilities to induce 8-OHdG in calf thymus DNA.

Since 8-OHdG represents a premutagenic DNA adduct which has been implicated in carcinogenesis (Kasai 1997; Kuchino *et al*., 1987), the above data should also be viewed in relation to the recently established associations between $PM_{10}$ and lung cancer (Pope *et al*., 2002).

Obviously, 8-OHdG formation in cell free test systems, that is, using naked DNA, differs considerably from the induction of this DNA lesion in cell culture. However, in the present study we also demonstrated that both coarse PM and fine PM could induce 8-OHdG in A549 human epithelial cells. Notably, unlike the acellular assays (EPR, dotblot), where $H_2O_2$ was added to elicit Fenton-like reactions, DNA damage in the A549 cells occurred in the absence of the extracellularly added $H_2O_2$. This indicates the contribution of endogenous $H_2O_2$ to 8-OHdG formation in the epithelial cells. Indeed, it has been shown that DNA damage by PM can bge inhibited by catalase, and physiological levels of $H_2O_2$ have been shown to enhance ·OH generation by PM in an $H_2O_2$ concentration dependent manner. Organic constitutes within the PM have recently been proposed as another source of transition metal derived ·OH formation via formation of $H_2O_2$ from redox cycling of semiquinone radicals (Dellinger *et al*., 2001). Our findings are also in agreement with

observations by Prahalad *et al* (Prahalad *et al*., 2001). Using various (model) PM with highly different metal availability, such as coal and oil fly ashes, they showed a clear association between 8-OHdG induction in calf thymus DNA and in the DNA of BEAS-2B human bronchial epithelial cells. In our current study, the semi-quantitative nature of immunohistochemical staining, did not allow us to determine clear differences in 8-OHdG formation in the A549 cells for PM samples differing in size fraction or sampling period. Quantitative measurement of 8-OHdG using HPLC/ECD (Van Maanen *et al*., 1999) could not be performed, as the isolation of sufficient amounts of cellular DNA, would require at least 50-times the amount of PM as used in thepresent study. Our current method, however, allowed us to test low mass PM samples as typically collected on conventional low volume samplers. Another major advantage of the immunohistochemical detection of 8-OHdG is that it excludes possible artifactual oxidation of DNA during extraction from the cells and subsequent digestion for HPLC/ECD analysis (Toyokuni *et al*., 1997; Xu *et al*., 1999). As such, our data also demonstrate that ambient particulate matter, including its respirable fraction, is able to elicit oxidative stress----that is, in the form of intracellular ·OH generation, in epithelial lung cells (Kasai, 1997).

Importantly, in contrast to the highly contrasting (model) particles that were used by Prahalad and colleagues (Prahalad *et al*., 2001), we tested PM samples with rather similar characteristics. In fact, within respectively the fine and the coarse mode, only temporal (seasonal) variation existed for the different samples. Between the different modes as collected with the dichotomous samplers, the actual size distributions may overlap considerably as a consequence of the flow splitting ratio, which can result in significant amounts of fine particles in the coarse mode. Despite this low variability, clear differences were found in ·OH generation as determined by EPR and associated 8-OHdG formations in calf thymus DNA. For the coarse mode we found a significant difference between the ·OH generations of PM from two sampling periods---- that is, in summer versus autumn/winter season. Although differences did not reach significance with regard to 8-OHdG, our data might indicate the existence of seasonal variability in the ability of PM to elicit oxidative effects independent of its ambient concentration.

In the present study we used ICP-MS to determine the role of transition metals in the observed effects. Since previous studies indicate that availability rather than the concentrations of the metals are important for the induction of oxidative effects (Van

Maanen *et al*., 1999; Prahalad *et al*., 2000), readily leachable metal contents were determined, i.e. upon filtration of particle suspensions. Among the metals commonly present in PM, we chose V, Cr, Fe, Ni, and Cu because of their established role in the induction of 8-OHdG. For instance, Lloyd *et al*. (Lloyd *et al*., 1998) showed that Cr (III) and Fe (II), and to some lesser extent V (III), Cu (II) and Cr (IV) caused induction of 8-OHdG in salmon sperm DNA whereas Ni (II) and some other transition metals had no significant effect. Another study investigating the effectiveness of 8-OHdG formations by metals showed a ranking decreasing from V (IV), Fe (II), V (V), Fe (III), to Ni (II) (Prahalad *et al*., 2000). Recent EPR experiments in our laboratory with soluble metals or with particles that were coated with single metal salts, showed high ability for Cu (II), V (II), V (V), and Fe (II), and less ability for Fe (III) and Ni (II) to generate ·OH.

In our hands however, with the exception of Cu, no clear correlations between metal concentrations and 8-OHdG were observed. Furthermore, the concentrations of the V, Cr, Fe, and Ni, all appeared to be significantly higher in the fine PM, whereas the highest oxidative effects in terms of both ·OH-formation and induction of 8-OHdG were found for coarse PM. Although copper was found to correlate with the oxidative properties of the (coarse) PM and also tended to be higher in the coarse PM than in the fine PM, this does not fully explain why coarse PM shows higher ·OH generation for a number of reasons. Firstly, for fine PM no clear association was found between oxidative DNA damage and copper. Secondly, due to the multitude of transition metals usually present in the PM, and due to the intrinsic differences in the Fenton reactivity of each individual metal species (Lloyd *et al*., 1998), it would be unlikely that a single metal would explain for the observed oxidative effects. Most importantly however, although ICP-MS allows detection of low concentrations of metals as typically occurring in low mass environmental samples, this method do not allow determination of the chemical speciation of the metal. For instance, Prahalad and co-workers showed that the residual oil fly ash (ROFA) was 40-fold potent in causing 8-OHdG than oil fly ash (OFA), despite similar metal content and availability (Prahalad *et al*., 2001). Finally, the body of meteorological observations in this study also points to specific weather conditions under which the increased ·OH generation in the coarse mode was observed, in sampling period II. All sampling weeks of this period featured weak vertical exchange and inversions, in contrast to period I, where atmospheric mixing was intense. It is well established that conditions such as in period II lead to an accumulation of atmospheric pollutants near the surface. The particulate feature of the three weeks showing the highest ·OH generation were trajectories that are notably

influenced by regional anthropogenic sources, such as from the Ruhr area. The observed lack of correlation of this meteorological situation with increased metal concentrations (V, Cr, Fe, Ni, Cu) eventually points to other, yet unknown, anthropogenic components in the particle coarse mode that can affects its overall ·OH generating capacity. The high mass accumulations as observed for some weeks for the fine particle mode, which were ascribed to accumulation of primary and secondary aerosols in slow and non-precipitating air masses, and the continental nature of the backtrajectories had no visible effect on ·OH generation.

Taken together these data support the use of a measurement that integrates the intrinsic redox activity of all different constituents within the PM. The EPR method described here is relatively simple and can be applied to low-mass samples of (different size fractions of) PM. The observed high correlation between ·OH-formation and the induction of 8-OHdG indicates that this overall measurement of ·OH generating activity of a PM sample, is a better predictor for the induction of oxidative DNA damage *in vitro* as shown here and previously (Van Maanen *et al*., 1999; Prahalad *et al*., 2000; 2001; Knaapen *et al*., 2000) and in the current study, than determination of the concentrations of individual transition metals. Since reactive oxygen species including ·OH are also implicated in transcriptional activation of Nuclear Factor (NF)-κB and associated up-regulation of inflammatory genes (Schins and Donaldson, 2000), the observed variation in terms of sampling season and the size fraction, may also be reflected in the inflammatory effects of PM. For instance, temporal differences have been described for the induction of inflammatory mediators in murine macrophage cell line (Salonen *et al*., 2000), and more recently we showed considerable regional variability in inflammatory mediator release from A549 cells (Schins *et al*., 2002). Whether these effects are related to the intrinsic ·OH generating properties which can be determined using the EPR method as described, is currently under investigation.

**ACKNOWLEDGEMENTS**

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# Chapter IV

# INVOLVEMENT OF HYDROXYL RADICAL GENERATION IN PARTICULATE MATTER INDUCED DNA DAMAGE

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**ABSTRACT**

Exposure to ambient particulate matter (PM) has been reported to be associated with increased respiratory, cardiovascular and malignant lung disease. Previously we showed that PM can induce oxidative DNA damage in A549 human lung epithelial cells. The aim of the present study was to investigate the variability of the oxidative DNA damaging properties of PM sampled at different locations as well as time, and to relate the observed effects to the hydroxyl radical (·OH) generating activities and transition metal content of these samples. Weekly samples of coarse (10-2.5µm) and fine (<2.5µm) PM from 4 different places (NRW, Germany) were analysed for H2O2-dependent ·OH-formation using Electron Paramagnetic Resonance (EPR) and formation of 8-OHdG in calf thymus DNA using an immuno-dotblot assay. DNA strand breakage by fine PM in A549 human lung epithelial cells was quantified using the alkaline comet assay. The soluble metal content of the PM was determined using inductively coupled plasma mass spectrometry. Both PM size distribution fractions elicited ·OH generation and 8-OHdG formations in calf thymus DNA. A significantly higher ·OH generation was observed for PM sampled at urban/industrial locations as well as for coarse PM, whereas higher soluble metal concentrations were found in fine fractions. Samples of fine PM also caused DNA strand breakage in A549 cells and this damage could be prevented using the hydroxyl radical scavenger mannitol. The observed DNA strand breakage appeared to correlate with the soluble metal content as well as the hydroxyl radical generating capacities of the PM samples but with different profiles for rural versus urban/industrial samples. In conclusion, when considered at equal mass, ·OH formation of PM shows considerable variability with regard to the sampling location and time, which is most likely driven by transition metal composition and related to its ability to cause DNA damage.

KEY-WORDS: Ambient particulate matter, Electron Paramagnetic Resonance, DNA damage

ABBREVIATIONS: 8-OHdG = 8-hydroxy-2'-deoxyguanosine; PM = particulate matter; EPR = Electron Paramagnetic Resonance, inductively-coupled plasma mass spectrometry (ICP-MS)

# INTRODUCTION

Epidemiological studies have demonstrated that increased exposure to ambient particulate matter (PM) is associated with increased respiratory, cardiovascular and malignant lung disease, thereby increasing morbidity and mortality (Daniels *et al*., 2000; Pope *et al*., 2002). However, it is not clear how exposure to PM, typically as low as 30 $\mu g/m^3$, can produce the health effects observed in epidemiological studies and which components of PM mediate these effects. Although epidemiological and toxicological evidence suggest that it is the fine ($PM_{2.5}$) and even the ultrafine ($PM_{0.1}$) fraction that is responsible for the observed effects, there is no general agreement (Monn and Becker, 1999; Zhang *et al*., 2002). The wide range of endpoints suggest that more than one component may be driving the health effects (Donaldson *et al*., 1998; Dreher, 2000). Some leading hypotheses include transition metal content (Molineli *et al*., 2002), particle size and surface (Donaldson *et al*., 1998), endotoxin contamination (Monn and Becker, 1999) or organic compounds, such as polycyclic aromatic hydrocarbons (PAHs) (Sakai *et al*., 2002).

A remarkable consistency with regard to the adverse health effects of PM has been observed throughout many epidemiological studies irrespective of the locations where these studies have been carried out, i.e. with different PM composition. However, recent research has demonstrated that the adverse health outcomes vary from city to city and therefore indicate that specific components or properties of PM may be involved (Katsouyanni *et al*., 2001). Both *in vivo* and *in vitro* studies with residual oil fly ashes (ROFA) (Kodavanti *et al*. 2001) and Utah-Valley PM (Frampton *et al*., 1999; Ghio and Devlin, 2001) indicate that PM-induced health effects may be due to its chemical composition and more specifically to its transition metal content. Transition metals are considered to exert their effects predominantly through the formation of hydroxyl radicals (·OH) via the Fenton-reaction (Donaldson *et al*., 1997), and have been implicated in the inflammatory effects (Carter *et al*., 1997; Frampton *et al*., 1999; Ghio and Devlin, 2001) as well as in the DNA damaging properties (Van Maanen *et al*., 1999; Prahalad *et al*., 2001; Knaapen *et al*., 2002) of PM.

We, along with others have previously demonstrated that PM induces DNA strand breakage and formation of the hydroxyl radical (·OH) specific lesion 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Halliwell, 1999; Marnett, 2000) in lung epithelial cells (Knaapen *et al*., 2000; Prahalad *et al*., 2001; Knaapen *et al*., 2002; Dellinger *et al*., 2002).

Using electron paramagnetic resonance (EPR), we demonstrated that PM generates ·OH in suspension and showed that this ·OH generation, as well as the induction of DNA damage, is transition metal dependent (Knaapen *et al*., 2000; 2002). More recently, we have shown that the ·OH generating properties of PM as well as its ability to induce oxidative damage in naked DNA varies considerably with sampling time (Shi *et al*., 2003). The aim of the present study was to determine the ·OH generating properties of coarse and fine PM collected over time at several contrasting locations within Germany in relation to their chemical composition and furthermore, to establish whether these characteristics would relate to different capacities to induce DNA damage.

# METHODS

## Reagents

5,5-dimethyl-1pyrroline-N-oxide (DMPO), Hanks' balanced salt solution (HBSS), ethidium bromide, Dulbecco's modified eagle's medium (DMEM), phosphate-buffered saline (PBS), diaminobenzidine-tetrahydrochloride (DAB), mannitol, desferoxamine and DMSO (dimethyl sulfoxide) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Hydrogen peroxide ($H_2O_2$) was purchased from Fluka (Seelze, Germany). Calf thymus DNA was obtained from Life Technologies Inc. (Gaithersburg, MD, USA). Hydrogen peroxide ($H_2O_2$) was purchased from Fluka (Germany). Vectastain-ABC kit was obtained from Vector Laboratories, Burlingame, CA. For EPR experiments, double-distilled de-ionised water was used.

## Collection and sample processing of particulate matter

Coarse (PM10-2.5µm) and fine (PM<2.5µm) fractions of $PM_{10}$ were sampled in weekly intervals at four different locations in Nordrhein Westfalen (NRW), Germany in the period of February to May 2000. The locations were Borken (Bo) representing a rural site, and three urbanised/industrialised sites in Dortmund (Do) and Duisburg (Du-M and Du-B). Coarse and fine PM were collected on pre-weighed Teflon filters using Graseby-Anderson dichotomous low volume samplers at a flow of 16.7 L/min.

Filters were stored in the dark in a dry atmosphere until further analysis. The PM was removed from the filters by agitation (5 minutes) in 1ml of ultra pure water, followed by sonicationfor 5 minutes. Resulting PM concentrations were estimated using comparative turbidometry against a standard dilution curve using a carbon-black suspension. Although this method only represents an indirect estimate of the extracted amount of PM, it allowed for the analysis of freshly prepared (i.e. non-frozen) PM suspensions. This approach avoids prolonged storage and freeze - thawing of PM suspensions, which can lead to altered leaching of metals or organic constituents and which also could affect particle agglomeration (Donaldson *et al*., 1997). Upon reconstitution of the filters from which the PM was extracted, the actual extracted mass was also determined gravimetrically, to allow a comparison with the turbidometry determinations. Particle suspensions were diluted in ultra pure water for further analysis at the concentrations shown, and immediately used for analysis of ·OH generation, 8-OHdG formation, and DNA strand breakage experiments.

Samples were all adjusted to equal concentrations, i.e. to 320µg/ml for coarse PM, and 380 µg/ml for fine PM respectively. From each sample a small aliquot was used for determination of transition metals using inductively coupled plasma mass spectrometry (ICP-MS). For determination of the regional variability of ·OH formation and 8-OHdG formation, pairs of fine and coarse PM were analysed randomly with regard to sampling period and sampling location. A random selection of fine PM was also used for determination of DNA strand breaks in A549 human lung epithelial cells.

**Analysis of metals by ICP-MS**

Inductively coupled plasma mass spectrometry (ICP-MS) was used to determine the concentrations of V, Cr, Fe, Ni, and Cu in the aqueous suspensions of PM. Therefore, freshly prepared suspensions of PM were filtered through a 0.2µm Millipore filter (Minisart RC15 syringe filter, Sartorius AG, Göttingen, Germany). The filtrate was diluted with de-ionised water (1:5), and filtered again. The transition metals were analysed by sector field ICP-MS (ELEMENT by Finnigan MAT, Bremen, Germany) (Begerow *et al*., 2000) in the medium resolution mode ($m/\Delta m \cong 4000$) using the standard addition procedure for calibration. 50µl of the filtrate was diluted with 500 µl 0.08 N $HNO_3$ and 2000 µl ultra pure water and spiked with 50 µl of standard solutions containing 5–20 µg/L Ni, Cu, V, and Cr and 50–200 µg/L Fe, respectively. Platinum was measured in the low-resolution mode ($m/\Delta m = 3000$), with a detection limit of 0.01 ng/l.

**Electron paramagnetic resonance measurement**

Hydroxyl radical formation by the coarse and fine PM was evaluated by Electron Paramagnetic Resonance (EPR) as described previously (Shi *et al*., 2003). Briefly, 50µl of the freshly prepared particle suspension was mixed with 100µl of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, 0.05M in distilled deionised water) and 50µl $H_2O_2$ (0.5M in PBS). The suspension was incubated for 10 min at 37ºC in a shaking water bath, and filtered through a 0.1µm filter (Acrodisc 25 mm syringe filter, Pall Gelman Laboratory, Ann Arbor, USA). The filtrate was immediately transferred to a capillary and measured with a Miniscope EPR spectrometer (Magnettech). The EPR-spectra were recorded at room temperature using the following instrumental conditions: Magnetic field: 3360 G, sweep width: 100 G, scan time: 30 sec, number of scans: 3, modulation amplitude: 1.975 G, receiver gain: 1000. Quantification was done by accumulation of 3

different spectra, each averaging 3 different scans. All 4 peaks were quantified by measuring the amplitudes, and outcomes are expressed as the total amplitude in arbitrary units (A.U.). Comparative evaluation of this quantification protocol with the method of double integration as usually applied in EPR studies (Schins *et al*., 2002), demonstrated nearly identical results for DMPO-OH spectra as observed with PM samples (n=30, Pearson's, r=0.99, P<0.001).

**8-Hydroxydeoxyguanosine induction by PM in calf thymus Dann**

Induction of the ·OH specific DNA lesion 8-hydroxydeoxyguanosine (8-OHdG) by PM in isolated calf thymus DNA was estimated via a dot-blot assay that we have developed recently (Knaapen *et al*., 2002), based on a method as described by Musarrat *et al* (Musarrat and Wani, 1994). Freshly prepared suspensions of PM were incubated with 50µg of calf thymus DNA dissolved in Tris-HCl (10mM, pH=8.0) and $H_2O_2$ (1mM). In each experiment, DNA incubated without PM and $H_2O_2$, as well as DNA incubated with 0.1mM $FeSO_4$ and 1mM $H_2O_2$ were included respectively as negative and positive controls. Samples were incubated in the dark for 90 minutes at 37ºC in a shaking water bath, and then immediately centrifuged (6000 rpm, 5 min). 200µl of the supernatant was transferred to a fresh tube, and DNA was precipitated by the addition of 1/10 vol. NaAc (1.5M, pH=6.0) and 2 times vol. 100% ice-cold ethanol at –20°C for 1 hour. The DNA was then washed twice using 70% ethanol (13000rpm, 5min), dried in the dark, dissolved in 30µl of Tris-HCl buffer and stored overnight at 4ºC. DNA concentrations were determined spectrophotometrically and the samples were diluted to a final concentration of 2.56µg/ml in 20xSSC. Of each sample, replicate 2-fold dilutions were blotted on a nitrocellulose-membrane using a dot-blot apparatus. To each blot, both negative and positive controls were added. The DNA was cross-linked by baking of the membrane for 90 min in a pre-warmed oven at 80°C. Blocking of the membrane was performed overnight using casein. Immuno-localisation of 8-OHdG was performed using the N45.1 monoclonal antibody (Toyokuni *et al*., 1997), and using the Vectorstain-ABC kit with diaminobenzidine-staining according to the recommended protocol (Vector Laboratories). The blots were analysed by computer assisted densitometry scanning (BioRad), and expressed relatively to the density of the negative controls. The relative staining intensities as ranked for each separate dot blot experiment, were used for statistical evaluation.

**Treatment of A549 cells with fine PM.**

A549 cells (American Type Culture Collection) were grown in DMEM supplemented with 10% heat inactivated fetal calf serum, L-glutamine, and 30 IU/ml penicillin-streptomycin at 37°C and 5 % $CO_2$. For experiments, cells were trypsinized at confluence, seeded into 60 mm culture dishes, and grown until confluence. Cells were washed 2 times with HBSS and then treated with freshly prepared samples of fine PM as described before upon dilution in HBSS at a final concentration of 20 µg/cm$^2$. Cells were incubated for 3h at 37ºC (100 % relative humidity, 5 % $CO_2$). A total of 15 fine PM samples were randomly selected in a total of three independent experiments measuring each sample in duplicate. In additional experiments, mannitol (final concentration 25 mM) was used as a hydroxyl radical scavenger.

**DNA strand break analysis (comet assay).**

DNA strand break formation in A549 cells was determined by the comet assay (Singh *et al*., 1988) according to the guidelines recently proposed by an expert panel (Tice *et al*., 2000). Fully frosted slides were covered with a layer of 0.65% agarose using a cover slid and stored overnight at 4ºC. Following treatment, A549 cells were harvested from the dishes using trypsin and were then suspended in HBSS. Cytotoxicity in A549 cells caused by exposure procedures and cell processing was evaluated using Trypan Blue dye exclusion. Subsequently, 25 µl of the cell suspension (approximately 2x10$^6$ cells/ml) was mixed with 75 µl 0.5% low melting point agarose. This mixture was then added to the slides, on top of the first agarose layer using a cover glass. Slides were stored 45 minutes at 4ºC to allow solidification, and covered with another layer of low melting point agarose (100 µl). Following solidification for at least 45 minutes at 4ºC, slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% Sodium Lauryl sarcosinate, pH 10, 10 % DMSO and 1 % Triton X-100 added just before use) and stored overnight at 4ºC. The following day, slides were rinsed with distilled water and placed in an electrophoresis tank filled with ice-cold buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 30 minutes. Electrophoresis was conducted at 300 mA and 25 V for 15 minutes. Slides were then neutralized 3 x 10 min using neutralization buffer (0.4 M Tris, pH 7.5). All steps described were performed in the dark or under dimmed red light to prevent additional DNA damage. Slides were stained with ethidium bromide (20 µg/ml in $H_2O$) and comet appearances were analysed using an Olympus BX60 fluorescence microscope at 1000x magnification. On every single slide 50 cells were analysed randomly, and classified into

one out of five categories according to tail length (0,1,2,3,4, in which 0 = no tail). For final analysis a 'comet-score' of each individual slide was calculated, according to the method described by Collins *et al* (Collins *et al*., 1993): *Comet Score = sum (class 1 cells + 2x class 2 cells + 3x class 3 cells + 4x class 4 cells)*. Using this formula a minimally damaged sample will have a score of 0, whereas a maximally damaged sample obtains a comet score of 200.

**Statistical analysis**

Comparison between fine and coarse PM or the PM samples of different sampling sites (rural area (Bo) versus other sites) were carried out using t-test, or the non-parametric Mann-Whitney test (for 8-OHdG only). All correlation was determined using simple linear regression analysis. Since transition metal data lacked normal distribution, log transformed values were used in these comparisons.

## RESULTS

The mean mass of the PM as sampled at the different locations and as extracted from the Teflon filters using our protocol are summarised in Table 1. The sampled masses as well as the extracted masses were remarkably similar for the four different locations, with the exception of coarse PM from Dortmund (DO), which was significantly higher than coarse PM from Borken (BO).

**Table 1 Characterisation of fine (1a) and coarse (1b) PM sampled at four different locations**.

1a, Fine PM

| | Borken | Dortmund | Duisburg-Meid | Duisburg-Bus |
|---|---|---|---|---|
| Number of samples | 12 | 7 | 14 | 7 |
| Sampled mass (mg) | 2.2±0.6 | 2.7±0.7 | 2.3±0.8 | 2.1±0.7 |
| Extracted mass (mg) | 2.0±0.6 | 2.5±0.6 | 2.1±0.8 | 1.8±0.7 |
| Recovery (%) | 88.9±11.6 | 92.3±4.8 | 89.4±24.5 | 86.2±10.3 |
| Estimation of extracted PM (mg/ml)(turbidometry) | 0.5±0.1 | 1.0±0.4 | 0.6±0.2 | 0.6±0.2 |
| Al (µg/l) a | 1480±1085 | 817±555 | 1657±1795 | 1123±772 |
| Fe (µg/l) a | 1912±863 | 3153±1635 b | 3150±941 b | 3028±1684 |
| Cu (µg/l) | 235±90 | 216±110 | 354±166 | 397±250 |
| V (µg/l) a | 66±39 | 64±49 | 78±34 | 86±45 |
| Cr (µg/l) a | 41±27 | 105±38 b | 76±45 b | 90±54 b |
| Ni (µg/l) | 64±25 | 39±23 | 90±47 | 58±34 |
| Cd (µg/l) | 20±8 | 92±60 | 38±21 | 577±904 b |
| Pb (µg/l) a | 691±512 | 480±159 | 1894±2138 | 1968±2177 |
| Pt (ng/l) a | 76±43 | 47±22 | 70±33 | 79±53 |

1b. Coarse PM

| | Borken | Dortmund | Duisburg-Meid | Duisburg-Bus |
|---|---|---|---|---|
| Number of samples | 14 | 7 | 14 | 6 |
| Sampled mass (mg) | 1.8±0.5 | 2.7±1.0 b | 2.3±1.1 | 2.3±1.0 |
| Extracted mass (mg) | 1.6±0.5 | 2.7±0.9 b | 2.0±0.6 | 1.8±0.7 |
| Recovery (%) | 93.5±14.3 | 98.3±7.7 | 105.1±46 | 84.1±29.3 |
| Estimation of extracted PM (mg/ml)(turbidometry) | 0.5±0.2 | 0.6±0.1 | 0.6±0.3 | 0.6±0.2 |
| Al (µg/l) | 876±687 | 376±545 | 852±774 | 700±637 |
| Fe (µg/l) | 532±665 | 212±255 | 599±1218 | 923±1824 |
| Cu (µg/l) | 221±118 | 157±147 | 279±200 | 255±174 |
| V (µg/l) | 22±9 | 14±10 | 22±25 | 38±47 |
| Cr (µg/l) | 8±5 | 7±2 | 11±11 | 45±80 b |
| Ni (µg/l) | 27±25 | 15±15 | 57±29 | 97±187 b c |
| Cd (µg/l) | 6±6 | 6±6 | 99±339 | 72±92 |
| Pb (µg/l) | 39±71 | 6±8 | 100±167 | 281±502 b |
| Pt (ng/l) | 36±22 | 26±13 | 31±20 | 31±6 |

All data are expressed as mean ± standard deviation

a. Significantly different from coarse fraction ($p<0.05$, t-test)

b. Significantly different from rural samples ($p<0.05$, t-test)

c. One outlier (6873 µg/l) was excluded.

Using our extraction protocol (vortexing and sonication) the mass recovered from the Teflon filters appeared to be quite high for the coarse fraction (mean: 97 %, range: 84-105 %) as well as the fine fraction (Mean: 89 %; range: 86—92 %). For both PM size fractions, the recovery appeared to be a constant proportion of the filter loading as indicated by a linear correlation between the sampled and recovered mass (Fine: $r=0.92$, $P<0.001$; Coarse: $r= 0.69$, $P<0.001$). Since turbidometry is the only method that can be used to estimate mass for immediate use with small amounts of PM, we analysed the relationship between gravimetric and turbidometric data and found a highly significant correlation between mass and density for both the fine and coarse fractions (Figure 1).

Similar correlation coefficients were found for the fine (r= 0.58, n= 40, P<0.01) and the coarse (R=0.41, n= 41, P<0.01) fractions, but the slope of the line shows that in our hands the concentrations expressed by turbidometry lead to an underestimation (30 %) of extracted mass.

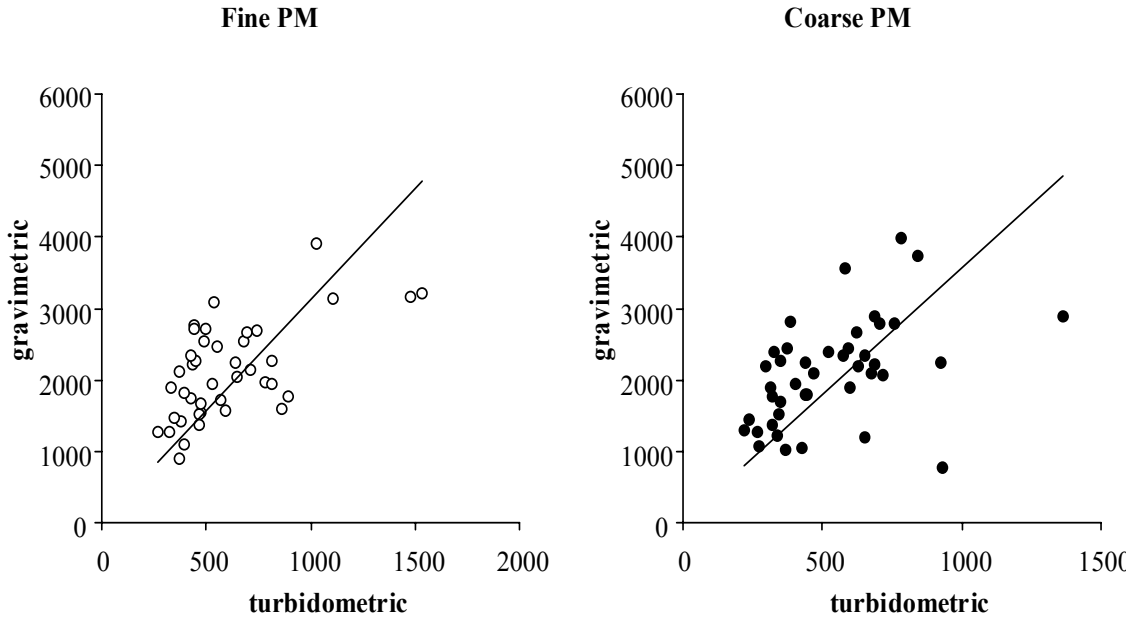


**Figure 1 Comparison of gravimetric determination and turbidometry estimation of extracted mass from the Teflon filters (37 mm).** Significant correlation between mass and density for both the fine (R= 0.58, n= 40, P<0.01) and the coarse (R=0.41, n= 41, P<0.01) fractions were found, but the slope of the line shows that the concentrations expressed by turbidometry leads to an underestimation (30 %) of extracted mass.

To determine the role of water-soluble metals in the oxidative properties of the PM in relation to the observed differences for sampling locations, the suspensions of PM were analysed for leachable Al, Fe, Cu, V, Ni, Cd, Cr, Pb, and Pt by ICP-MS. The results of these metal determinations are shown in Table 1. A considerable variation was observed in the soluble metal content of the PM samples among the different sampling locations, as well as with regard to the size fraction of the PM (see Table 1). As can be seen in the table, concentrations of soluble metals tended to be higher in samples from the urban/industrialised locations, especially within the fine PM. In general, and irrespective of the sampling location, higher metal concentrations were found in the fine fractions compared to the coarse fractions (significant for Al, Fe, V, Cr, Pb and Pt, P<0.05).

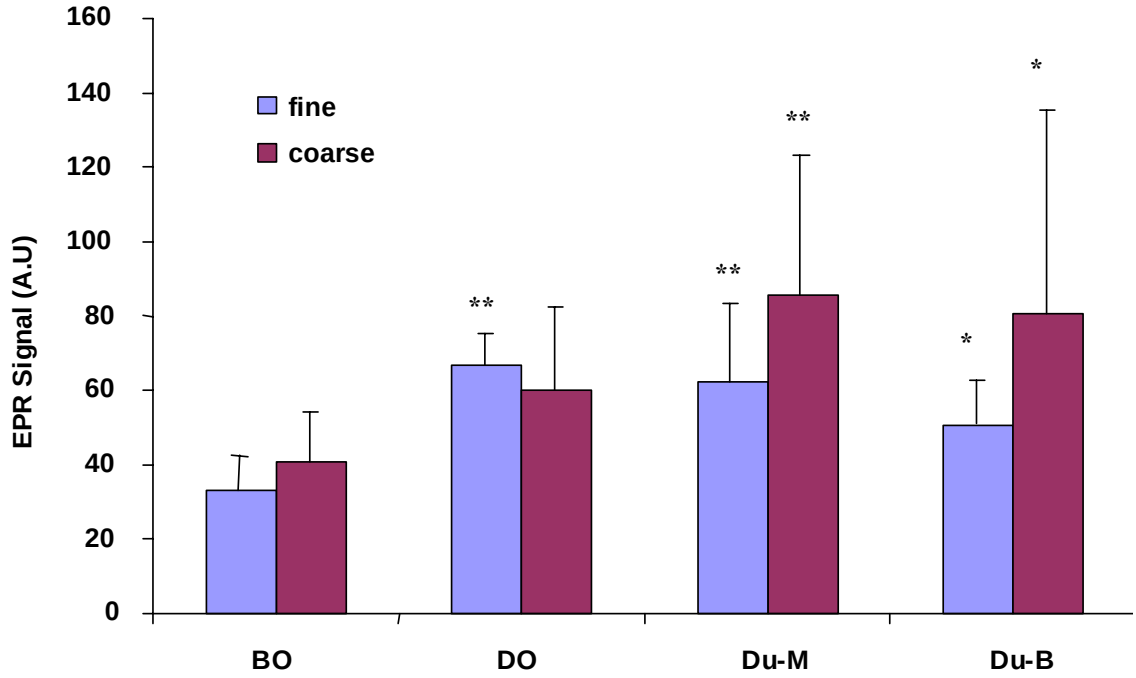


**Figure 2 Hydroxyl radical generation of coarse and fine PM of suspensions sampled at four locations.** Data are expressed as mean and SD of the intensity of the resulting DMPO-OH signal in arbitrary units from the weekly samples of coarse and fine PM. PM samples from the urban/industrial areas (DO, Du-M, Du-B) showed higher ability to generate hydroxyl radical than the PM samples from the rural location (BO). The asterisks indicate a statistically significant difference with the rural location (BO), at *$p<0.05$ and ** $p<0.01$ respectively.

Since hydroxyl radicals (·OH) produced via Fenton-like reactions have been considered as a key feature of PM, we used electron paramagnetic resonance (EPR) to quantify ·OH formation by PM as an entirety. The measurements performed in the present study demonstrated that suspensions of all PM samples collected during this study showed the typical spectrum for DMPO-OH, differing only in signal amplitude and characteristics of ·OH formation. The results of the EPR measurements are shown in Figure 2. As can be seen in the figure, urban PM had significantly higher hydroxyl radical generation than rural PM. The coarse fractions generally showed a higher signal than the fine fractions, with the exception of samples from Dortmund (DO). In addition to differences in both PM size and sampling location, considerable temporal variability in ·OH formation was also observed. Figure 3 shows, as an example, the ·OH generation for consecutive sampling

weeks of both fine and coarse PM from the rural location (BO) and an urban location (Du-M). As can be seen in the figure, irrespective of the week-to-week variations of the ·OH signals, coarse PM consistently showed higher ability to generate ·OH than fine PM for these locations. Hydroxyl radical formation as determined by EPR for fine PM correlated significantly with the soluble metal content of Fe, Cu, Cr, Cd, and Pb ($P<0.01$). For coarse PM the EPR signals were correlated to the concentrations of Cu, Ni, Cd, Pb ($P<0.01$).

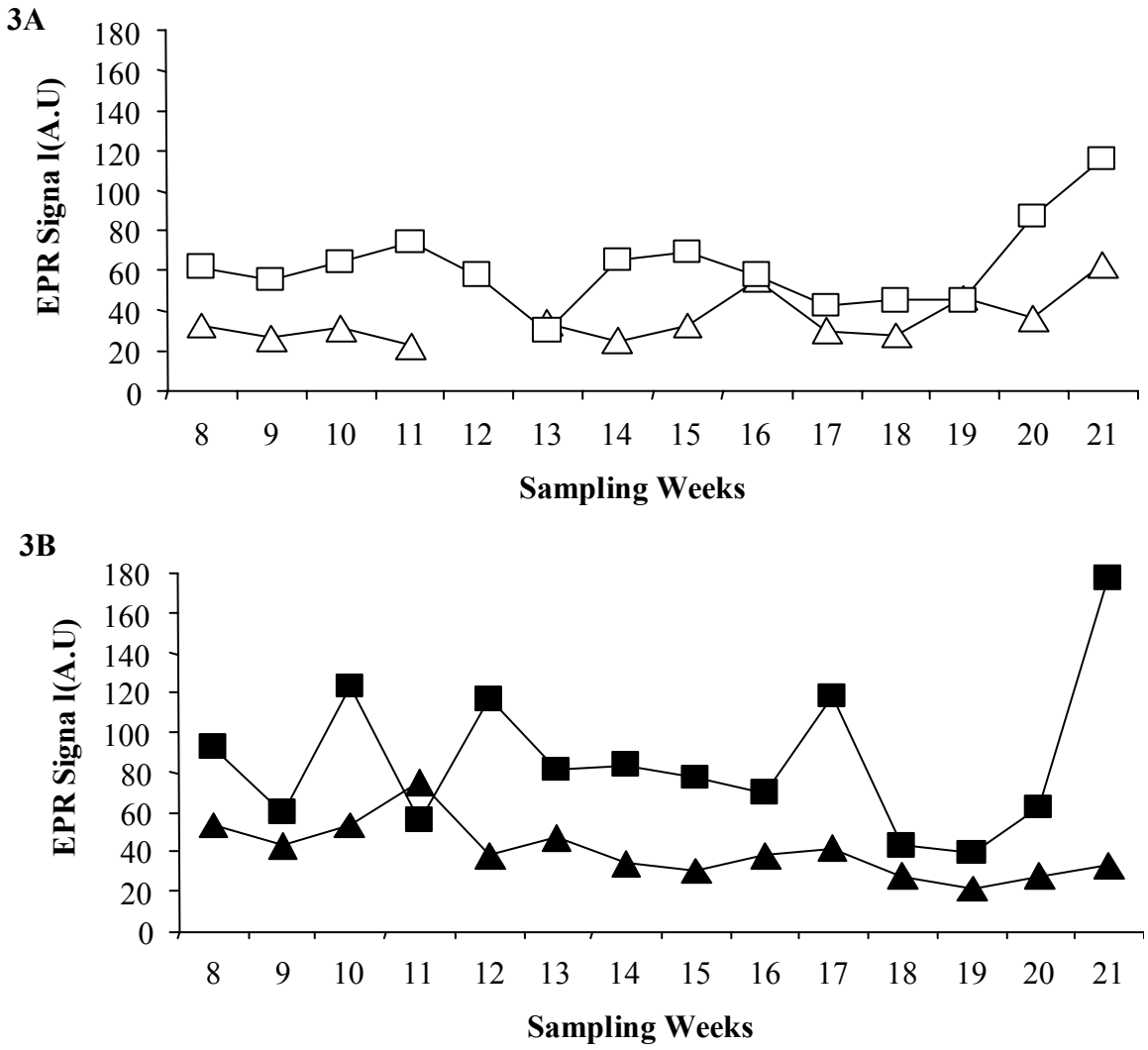


**Figure 3 Time course of week means of DMPO-OH formation of the fine (A) and the coarse (B) fraction of PM as sampled in the rural town (BO) (▲) and a location of the city of Duisburg (Du-M) (■).** Data are expressed as the intensity of the resulting DMPO-OH signal in arbitrary units. PM sampled at urban area cause higher ability to generate hydroxyl radical. Variations were observed for both fractions and locations, large variations were found among urban samples.

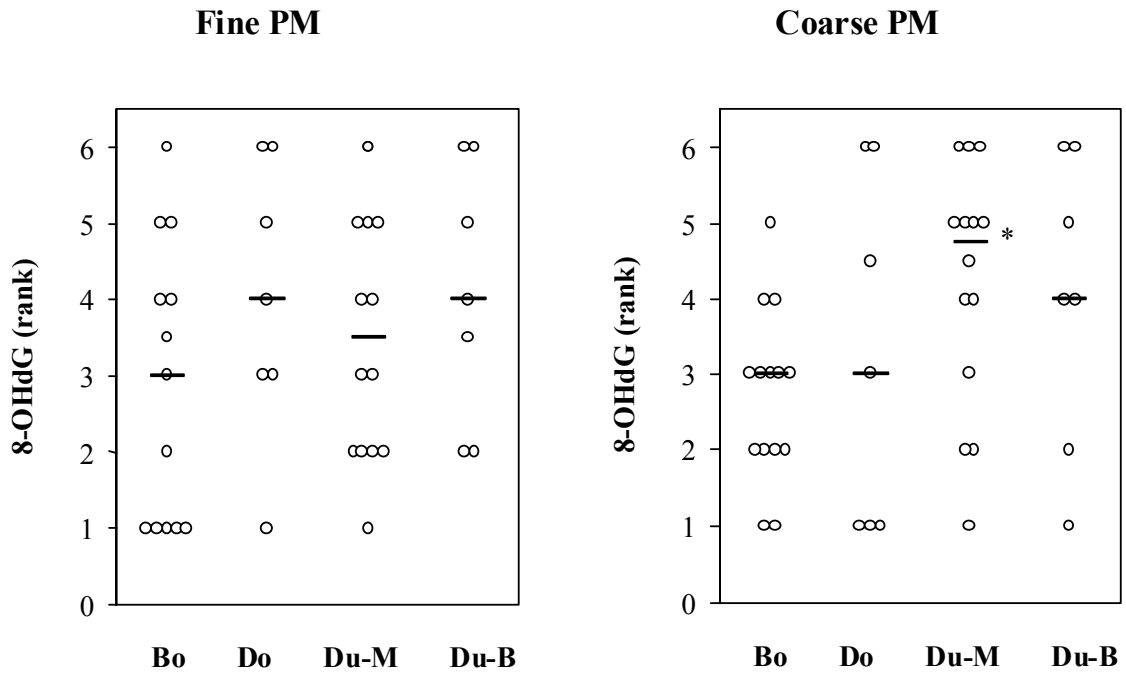


**Figure 4 Induction of 8-OHdG by fine and coarse fractions of PM in calf thymus DNA. The data showed are ranked 8-OHdG in accordance with the relative staining intensities for each separate experiment (rank from 1-6).** For each sampling location the horizontal line represents the median value. Urban area PM demonstrated a higher ability to induce 8-OHdG than rural PM. Significant differences were observed only in urban PM (Du-M) ($p<0.05$) when compared to rural particles (BO).

In order to evaluate whether the observed variability in ·OH generation relates to the induction of 8-OHdG, coarse and fine fractions of PM were incubated with calf thymus DNA in the presence of 1 mM $H_2O_2$ and analysed using an immunodotblot assay. Both fine and coarse PM caused formation of 8-OHdG and the effects tended to be stronger for the PM samples collected at the urban/industrialised locations (Figure 4). However, significantly higher 8-OHdG was only observed with coarse PM from Duisburg-M when compared to coarse PM from the rural location Borken. A significant correlation was found between ·OH generation and the induction of 8-OHdG (n=81, Spearman's r=0.48, $p<0.001$). The induction of 8-OHdG was also related to metal concentrations. For fine PM, the formation of 8-OHdG in calf thymus DNA was generally associated with the same metals as those that correlated with the EPR measurements, that is Fe, Cu, Cr, Cd, and Pb. However, for coarse PM, the induction of 8-OHdG was only related to some extent with the concentrations of Fe and Cu ($P<0.05$).

To determine the significance of these observations for cellular DNA damage, A549 cells were treated with fine PM samples, randomly selected from both urban and rural locations. DNA strand breakage results are shown in figure 5. As can be seen in panel A of this figure, no significant difference was found in DNA damage between the different sampling locations, but a considerable variability among the samples was observed, in line with the hydroxyl radical generating properties of the different samples. As can be seen in panel B of the figure the ·OH generating capacities of the PM samples tended to correlate with their ability to elicit DNA strand breakage in the A549 cells. When samples were subdivided into rural and urban samples significant correlations between ·OH generation and DNA strand breakage was observed albeit with different slopes, i.e. for rural samples (n=5, Spearman's r=0.9, $p<0.05$) and urban samples (n=10, Spearman's r=0.76, $p<0.05$).

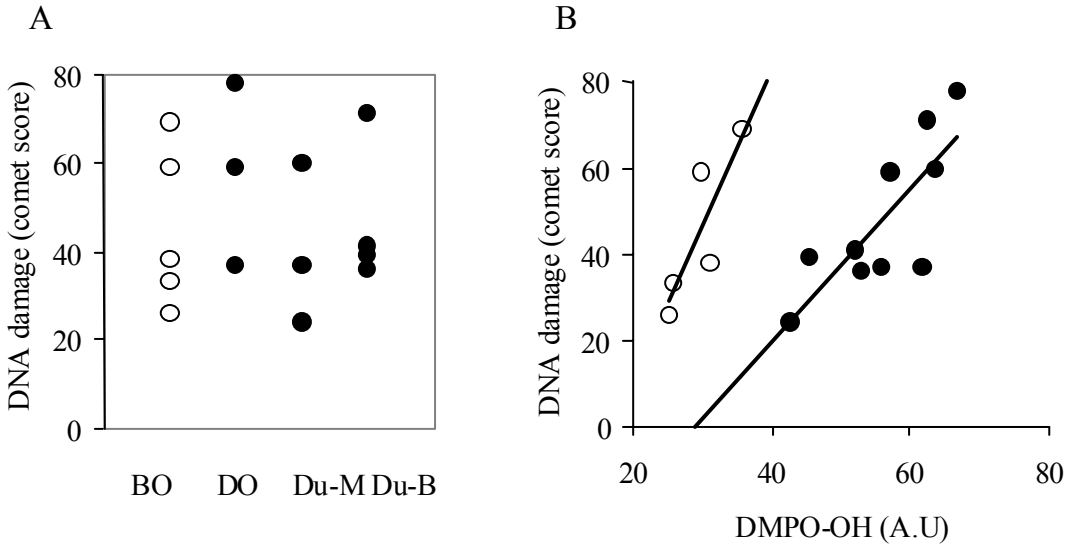


**Figure 5 Induction of DNA strand breakage by fine PM from different sampling locations in A549 cells (A), and its relation with hydroxyl generation (B).** A549 cells were treated for 3 hours with fine PM samples from different locations at equal dose (20µg/cm$^2$). No significant differences in DNA damage were found between the different sampling locations (panel A). When samples were subdivided into rural and urban/industrial locations (panel B), a significant correlation was observed between the hydroxyl radical generating ability of the samples as determined by EPR and the induction of DNA strand breakage in A549 cells, respectively for rural samples (○) (n=5, Spearman's r=0.9, $p<0.05$) versus urban samples (●) (n=10, Spearman's r=0.76, $p<0.05$).

The role of transition metals in the observed DNA damaging properties of these PM samples is shown in table 2. As can be seen in the table, DNA strand breakage by both

rural and urban/industrial samples tended to relate to the iron content. However, it appeared that the DNA damaging properties of the rural samples, but not the urban samples were in addition associated to several other metals such as lead and nickel. In order to further evaluate whether hydroxyl radical generation, as determined by EPR, may indeed be involved in the observed DNA damaging effects of PM, subsequent experiments were performed using the hydroxyl radical scavenger mannitol. The effect of mannitol on DNA strand breakage by fine PM is shown in Figure 6. As can be seen in the figure co-incubation with mannitol caused a significant reduction of DNA strand breakage in the A549 cells.

**Table 2 Correlations between soluble metal content and hydroxyl radical generation of fine PM and induction of DNA strand breakage in A549 human lung epithelial cells.**

| | DNA strand breakage | | |
|---|---|---|---|
| | All samples (n=15) | Rural (n=5) | Industrial (n=10) |
| Al | -0.074 | 0.563 | -0.316 |
| Fe | 0.656** | 0.785 | 0.726* |
| Cu | 0.382 | 0.732 | 0.322 |
| V | -0.315 | -0.416 | -0.335 |
| Cr | 0.459 | 0.789 | 0.356 |
| Ni | 0.440 | 0.899* | 0.286 |
| Cd | 0.436 | 0.696 | 0.512 |
| Pb | 0.565* | 0.919* | 0.431 |
| Pt | 0.261 | 0.874 | -0.084 |
| EPR-signal | 0.441 | 0.863 | 0.796** |

Data expressed are Pearson's linear correlation coefficients between log transformed metal content (log transformed), hydroxyl radical generating capacity (EPR) and DNA strand breakage in A549 cells. Significant correlations are indicated with * ($p<0.05$) and ** ($p<0.01$)

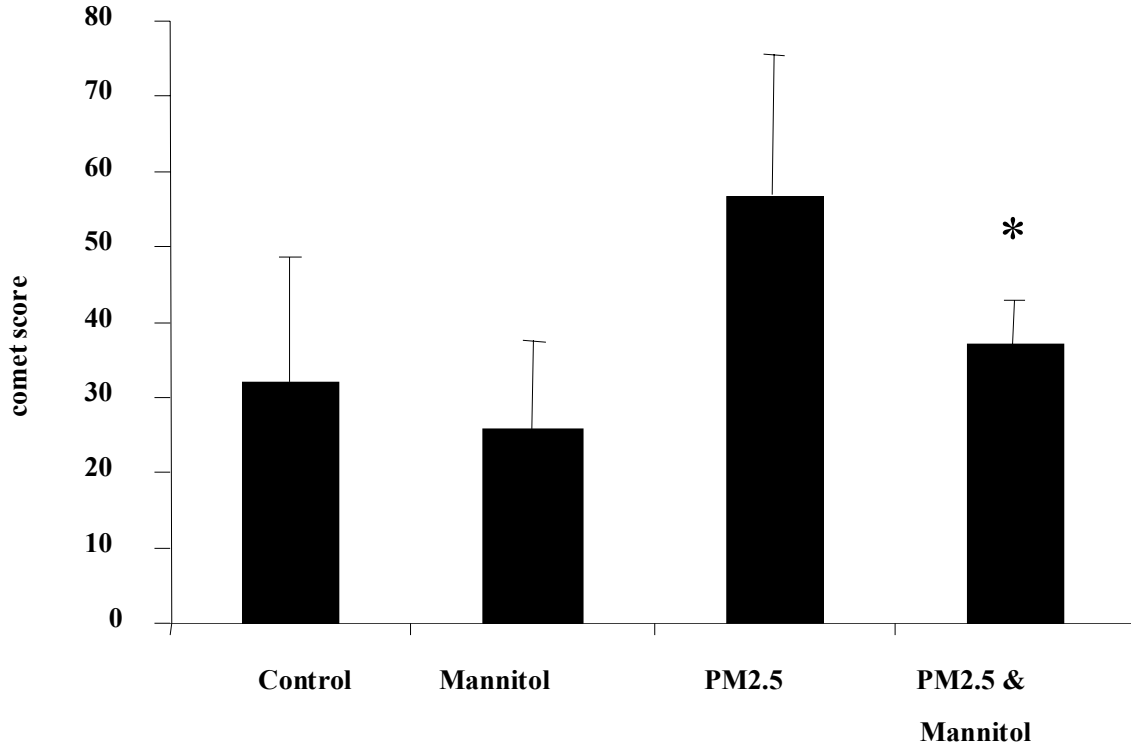


**Figure 6 DNA strand breakage by fine PM in the presence or absence of the hydroxyl radical scavenger mannitol.** A549 cells were treated for 3 hours with fine PM suspended in HBSS (125µg/ml) and in the presence or absence of mannitol (25mM). Mannitol caused a significant inhibition of PM induced DNA strand breakage ($P<0.05$).

## DISCUSSION

Although associations between PM exposure and adverse health effects have been established in epidemiological studies (Daniels *et al*., 2000; Pope *et al*., 2002), it is still unclear which constituents or characteristics are involved herein. Transition metal-dependent generation of hydroxyl radicals has been put forward as an important feature of inflammatory and toxic effects of PM (Gilmour *et al*., 1996; Ghio *et al*., 1996; Knaapen *et al*., 2000; Prahalad *et al*., 2001; Knaapen *et al*., 2002; Molinelli *et al*., 2002). In the present study, we have investigated the DNA damaging properties of PM sampled at different locations, in relation to its transition metal content and ability to generate hydroxyl radicals (·OH). All samples of coarse and fine PM collected during our sampling campaign showed clear ·OH generation and 8-OHdG formations in calf thymus DNA, albeit at considerable sample-to-sample variability. A significantly higher ·OH formation was observed with PM samples from urban/industrial locations, as well as for coarse PM, whereas generally higher soluble metal concentrations were found in fine fractions. Samples of fine PM caused DNA strand breakage in A549 cells, which could be prevented with the hydroxyl radical scavenger mannitol. DNA strand breakage appeared to correlate with the soluble metal content as well as the hydroxyl radical generating capacities of the PM samples, but with different profiles for rural versus urban/industrial samples.

The data in our present study is in line with earlier observations that PM can elicit DNA damage in lung epithelial cells via inherent oxidative properties. It is generally known that reactive oxygen species (ROS) are able to cause DNA strand breaks as well as DNA oxidation, processes that have been implicated in the initiation stage of carcinogenesis (Marnett, 2000). Among the major products of oxidative DNA damage is the premutagenic lesion 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Kasai *et al*. 1984; Kuchino *et al*, 1987; Floyd *et al*., 1990; Moriya and Grollman, 1993; Kasai, 1997). Studies using naked DNA (plasmid, calf thymus) indicate that the iron content of PM may be a key factor for DNA breakage (Donaldson *et al*., 1997; Knaapen *et al*., 2000), and 8-OHdG formation (Prahalad *et al*., 2001; Knaapen *et al*., 2002). However, various other metals within the PM which have the potential to participate in Fenton chemistry and/or have been reported to elicit DNA damage, such as copper, chromium, cadmium, lead and nickel, may also be involved (Lloyd *et al*., 1998; Yang *et al*., 1999; Blasiak and Kowalik, 2000; Liang and Dedon, 2001; Oikawa *et al*., 2002; Fracasso *et al*., 2002; Wozniak and

Blasiak, 2002). Keeping in line with these studies, several of these metals were found to correlate with 8-OHdG formations in our study. However, with the complex mixture that PM represents, individual metals largely differ in their concentration as shown here, and allow for various chemical interactions among the various metals and/or other constituents (Fenoglio *et al*., 2000; Dreher *et al*., 1997) For instance, it has been demonstrated that hydroxyl radical generation of Fe (II) can be greatly enhanced by Fe (III) which, as such, is less potent in hydroxyl radical formation (Urbanski and Beresewicz, 2000). Unfortunately, the ICP-MS analysis as applied in our study does not allow for identification of the valency state of these and other metals. Therefore, we proposed EPR as a tool to determine the over all Fenton reactivity of PM samples (Knaapen *et al*., 2002; Shi *et al*., 2003). Indeed, a clear correlation between hydroxyl radical generation by PM and the induction of 8-OHdG in naked DNA was observed in our study, which was also in line with our previous observations (Shi *et al*., 2003).

Obviously and in contrast to acellular methods such as EPR and naked DNA damage assays, the oxidative DNA damaging properties of PM in cellular systems involves more complex biochemical and physical processes, where various constituents of PM can interact with components on the surface as well as inside the cell. Our EPR method, as well as the 8-OHdG assay are both performed in the presence of exogenous hydrogen peroxide and therefore most likely reflect the Fenton-reactivity of the samples. Several other mechanisms have been considered, e.g. generation of ROS such as hydroxyl radical by PM as a result from its surface area (Brown *et al*., 2001), as well as organic constituents (Li *et al*., 2002). It has been found that ·OH generation may also be modified by other organic and inorganic components, such as sulphate (Ghio and Samet, 1999) and semiquinone (Dellinger *et al*., 2001). Previously, we and others have shown that PM induces both DNA strand breaks and 8-OHdG in lung epithelial cells (Knaapen *et al*., 2000; Prahalad *et al*., 2001; Don Porto Carero *et al*., 2001; Dellinger *et al*., 2001; Knaapen *et al*., 2002; Shi *et al*., 2003). Inhibition of PM-induced DNA strand breakage with hydroxyl radical scavengers such as tetramethylthiourea (TMTU) or dimethyl sulfoxide (DMSO), the antioxidant enzyme catalase and the iron chelator deferoxamine suggest that ·OH produced by the Fenton reaction may play a predominant role in these cellular systems (Dellinger *et al*., 2001; Knaapen *et al*., 2002). The data from our present study are in support of these observations, since for $PM_{2.5}$ a correlation was observed between DNA damage in A549 cells and the concentrations of various Fenton active metals as well as the

hydroxyl radical generation within this PM size fraction. Furthermore, it was found that the hydroxyl radical scavenger mannitol inhibits strand breakage by fine PM. With regard to the current effects as observed with fine PM, we have recently demonstrated, using immuno-cytochemistry, that this fraction as well as coarse PM elicits 8-OHdG formations in A549 cells (Shi *et al*., 2003). However, in this study we were not able to correlate ·OH generation or metal content of PM to this oxidative DNA lesion, due to the semi-quantitative nature of the method. Quantitative analysis of 8-OHdG e.g. using high performance liquid chromatography with electrochemical detection (HPLC-ECD) (Schins *et al*., 1995) necessitates incubations of large cell numbers to obtain sufficient DNA, and therefore high mass of PM. In past decades, a more useful approach for the quantitative detection of DNA damage has been developed, i.e. the alkaline comet assay (Singh *et al*., 1988; Tice *et al*., 2000). Importantly, several transition metals including Fe (III) and Cu (II), have been shown to correlate with strand breakage and 8-OHdG formation in DNA (Toyokuni and Sagripanti, 1996), and 8-OHdG formation is considered to represent a marker for cellular oxidative stress (Kasai, 1997). Taken together, our previous studies with 8-OHdG (Shi *et al*., 2003) and our current observations on DNA strand breakage in relation to EPR measurements and the effect of mannitol, indicate that the comet assay represents an indicator of oxidative stress by PM in A549 cells.

Importantly, we showed that PM elicits DNA strand breakage in cultured cells in the absence of exogenous hydrogen peroxide, whereas hydroxyl radical generation by PM using EPR is measured upon addition of a rather high concentration of $H_2O_2$. Previously, we showed that micromolar levels of $H_2O_2$, which is concentrations as occurring in physiological conditions, already enhance hydroxyl radical generation by PM (Knaapen *et al*., 2002). Importanly, and complementary to our EPR work (Knaapen *et al*., 2000; Shi *et al*., 2003), EPR measurements of dry PM samples also show spectra indicative of semiquinone radicals similar to those found in cigarette tar samples, suggesting that $H_2O_2$ formation via redox cycling of these compounds can contribute to ·OH generation (Dellinger *et al*., 2001).

Our data demonstrate that although PM samples from different areas have the ability to generate hydroxyl radicals and cause oxidative DNA damage, the patterns may differ per sampling location. On the one hand, PM samples from industrial/urban location showed higher ability for the formation of hydroxyl radical than rural PM when compared at equal

mass. On the other hand, no clear differences were found in the induction of 8-OHdG in naked DNA or DNA strand breakage in lung epithelial cells by PM samples from different locations. Despite these observations, for both rural and urban samples, DNA damage appeared to increase with increasing hydroxyl radical generating capacities. Different profiles of transition metal content as observed for these contrasting locations, and which are indicative of location-specific sources, are most likely responsible for this. For instance, whereas DNA strand breakage by the urban/industrial $PM_{2.5}$ was merely related to its soluble iron content, DNA damage by rural $PM_{2.5}$ was additionally characterised by relatively high amounts of the traffic-marker metals Pb and Pt (Kylander *et al*., 2003), as well as for Cr and Ni. Interestingly, Pb, Ni and Cr, as well as (traffic) combustion particles that typically contain low metal concentrations, have all been shown to elicit DNA strand breakage via oxidative mechanisms (Nagashima *et al*., 1995; Yang *et al*., 1999; Wozniak and Blasiak, 2000; Blasiak and Kowalik, 2000; Don Porto Carera *et al*., 2001). Obviously however it should be emphasized that our results are observed with a relatively low number of PM samples, and therefore further studies are needed to unravel the relative impact of the possible interactions between these and other metals as contained within PM on cellular DNA damage.

In conclusion, we have demonstrated that PM, both coarse and fine, sampled over time and at different locations have the ability to generate hydroxyl radicals, induce 8-OHdG formation in calf thymus DNA and elicit DNA strand breaks in human lung epithelial cells. Importantly, PM shows considerable variability in each of these endpoints with regard to the sampling location and time. DNA strand breakage appeared to correlate with the soluble metal content as well as the hydroxyl radical generating capacities of the PM samples, but with different profiles for rural versus urban/industrial samples. The observed location-specific characteristics of PM are in agreement with recent observations in epidemiological research (Katsouyanni *et al*., 2001), and forward a message that geographic or site related physical-chemical characteristics of PM impact on its ability to elicit cellular oxidative stress and DNA damage via mechanisms involving ·OH generation by Fenton reactive metals and possibly other, yet unidentified, constituents.

**ACKNOWLEDGEMENTS**

The PM samples used for this study have been collected within the framework of the so-called HOTSPOT study, a health surveillance program in Nordrhein-Westfalen (NRW), supported by the Landesumweltamt (LUA) in Essen, Germany. We acknowledge Ms Olschewski and Mr Schwarz from the LUA for their assistance in PM sampling. The ICP-MS determinations of the PM samples were carried out within the former Department of Analytical Chemistry at the Institute of Environmental Hygiene (MIU), Düsseldorf, Germany under the supervision of JB. We are also grateful to Martina Thurfeld for the metal analysis.

# Chapter V General Discussion

Epidemiological studies have demonstrated the association between ambient particulate matter exposure and adverse health effects (Daniels *et al*., 2000; Pope *et al*., 2002). From these studies it is estimated that per 10 μg/$m^3$ increase in the annual concentration of $PM_{2.5}$, mortality increases with 1.4 %, while respiratory disease such as bronchitis or asthma exacerbation increase by as much as 4 % (WHO, 1999). It is by no means clear how exposure to PM, typically as low as 30 μg/$m^3$ when compared to occupational particle exposure which can be as high as several hundreds of μg or even mg/$m^3$ level, can produce the health effects observed in epidemiological studies and which components of PM mediate these effects. Although epidemiological and toxicological evidence suggest that it is the fine ($PM_{2.5}$) and even the ultrafine ($PM_{0.1}$) fraction that is responsible there is no general agreement (Oberdorster *et al*., 1994; Wichmann *et al*., 2000). The wide number of endpoints suggests that more than one component may be driving the health effects (Donaldson *et al*., 1998; Dreher, 2000). The ability of PM to generate reactive oxygen species (ROS) has been suggested as a unifying factor in adverse health effects (Gilmour *et al*., 1996; Prahalad *et al*., 2001; Schins, 2002). Among these ROS, the hydroxyl radical is of great concern, since it is a highly electrophilic species, known for its ability to attack enodgenous molecules such as DNA (Halliwell 1999). It has been suggested that surface area (Brown *et al*., 2001), redox active metals (Prahalad *et al*., 2001) and organic components (Squadrito *et al*., 2001; Li *et al*., 2002) influence the direct as well as the indirect (through inflammation) capacity of particles to generate oxidative stress. Numerous methods have been developed to detect ROS *in vitro* or *in vivo*. Generally these methods are roughly divided into direct and indirect assays. The indirect assays include the measurement of changes in endogenous antioxidant levels or an increase in biochemical markers of oxidative damage. Other indirect methods include changes in endogenous antioxidants such as catalase, superoxide dismutase, peroxidases and non-specific antioxidant molecules such as ascorbic acid, glutathione and uric acid. The measurement of biomarkers related to oxidant stress concerns the products of lipid peroxidation (malondialdehyde), protein oxidation and oxidative DNA damage.

For direct ROS measurement, EPR is one of the most sensitive and definite methods, especially for the detection of very reactive oxygen species, such as ·OH measurement

(Halliwell and Gutteridge, 1985; Takeshita *et al*., 2002). In this thesis we applied EPR with spin trap to evaluate the capacity of ambient particulate matter to induce hydroxyl radical generation in different PM fractions, temporal as well as regional variations. In addition, we have related this activity to the effect of PM to cause DNA damage in target cells and nude DNA.

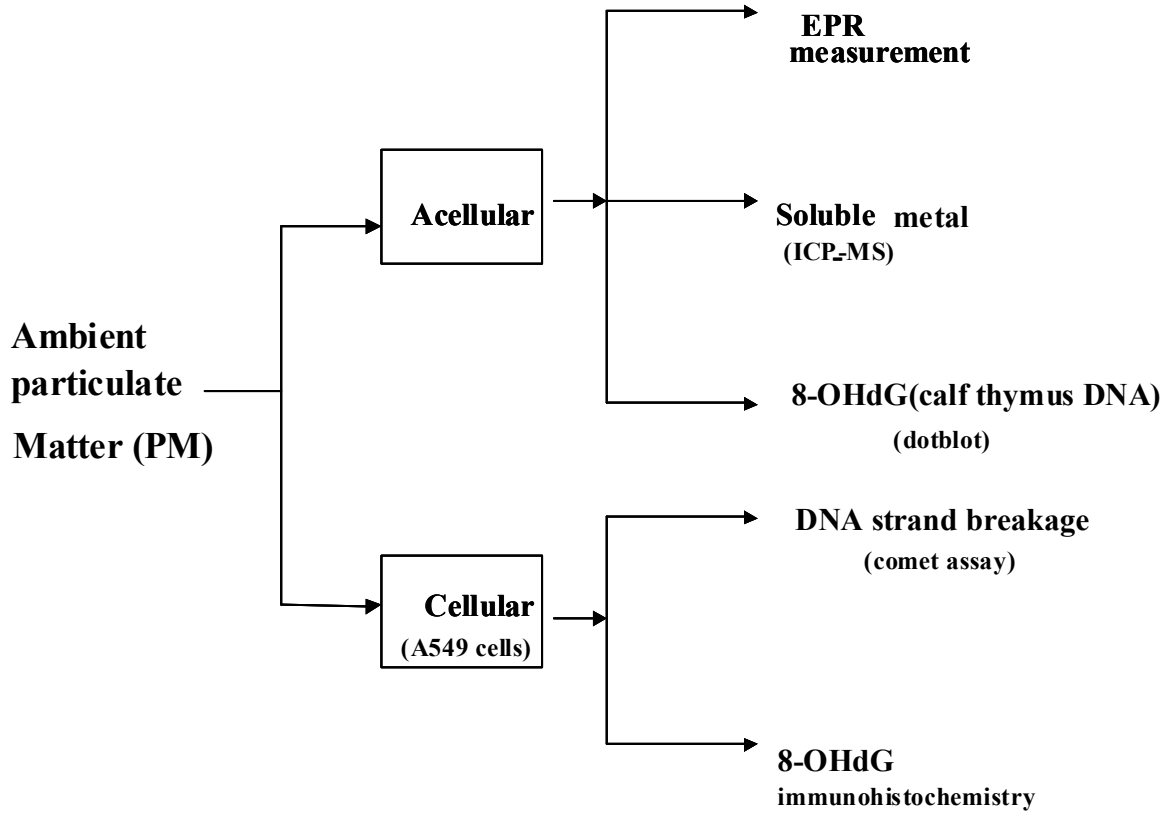


**Figure 1 Scheme of framework in the present thesis to investigate PM induced hydroxyl radical formation as well as chemical analysis.**

A method was developed for the measurement of ·OH generation by different particulate matter, including ROFA, TSP, coarse and fine PM fractions using EPR with the spin trap DMPO (Chapter II). All those particles were shown to generate hydroxyl radicasl in the presence of hydrogen peroxide and this was related to particle mass, size and the source of particles (such as ROFA particles). Hydroxyl radical generation was facilitated by exogenous hydrogen peroxide and was inhibited by the metal chelator desferoxamine. This implies the involvement of a Fenton reaction, i.e. the participation of hydrogen peroxide and transition metals in this particle caused ·OH formation (Fenton, 1894). On the other hand carbon black particles coated with different soluble metal salts also showed hydroxyl radical generation, that varied with different metals as well as with different oxidant states of the same metal. Higher ability of free radical generation was found in those particles coated with $Cu^{2+}$, $V^{2+}$, $V^{5+}$, $Fe^{2+}$ and lower with $Fe^{3+}$, $Ni^{2+}$ and $Zn^{2+}$. This is in agreement with Fenton active metals to cause oxidative DNA damage in naked DNA and the highest activity being found with $Cr^{3+}$, $Fe^{2+}$, $V^{3+}$ and $Cu^{2+}$ (Lloyd *et al*., 1998). Furthermore both soluble and insoluble fractions of particle contribute to particle induced ·OH formation and this varies with particle specifications, further confirming early observations from Ghio *et al* (Ghio *et al*., 1999), who showed that metals present in the insoluble particle fractions have catalytic activity. In general, EPR measured hydroxyl radical generation by particles was well correlated to particle induced 8-OHdG which has been demonstrated previously for soluble metals (Floyd *et al*., 1986). When comparing PM fractions, the concentrations of the V, Cr, Fe, and Ni, appeared all to be significantly higher in the fine PM, whereas the highest oxidative effects in terms of both ·OH formation and induction of 8-OHdG were found for coarse PM. Interestingly, with the exception of Cu, no consistent correlation between soluble metal content and hydroxyl radical generation was observed. This finding suggests that the oxidant activity of PM in this method is dependent on (i) the availability of Fenton active metals, and (ii) the valence of these metals, and (iii) the presence of specific combinations that can from redox couples. Studies with model ROFA showed that vanadium were very well able to catalyze ·OH formation from $H_2O_2$ while still being on the particle surface. In addition, the kinetics of ·OH formation was different between metals, with the highest formation rate for $Fe^{2+}$ (Chapter II). We therefore suggest that metal-ions such as $Fe^{2+}$ can initiate the Fenton reaction, generating ROS that can reduce other metals such as $Cu^{2+}$ to the redox-active $Cu^{+}$. These interactions might also explain why addition of desferoxamine usually blocks the whole ·OH formation in PM, although at low concentration this chelator is specific for iron and aluminum. It also explains why

correlations between soluble metal content and ·OH variation vary so much and are sometimes absent.

Since ·OH can react with DNA and produce many products (Marnett, 2000), the ·OH-specific DNA adduct 8-hydroxy-2'-deoxyguanosine (8-OHdG) was measured in biological systems using naked DNA and epithelial cells (A549 cells)(Chapter 3 and 4). Temporal variations of hydroxyl radical generation (EPR and 8-OHdG in naked DNA) were found in those samples which have been sampled at one sampling site (Chapter 3). EPR measured ·OH generation by particles was well correlated to particle induced 8-OHdG which has been demonstrated previously for soluble metals (Floyd *et al*., 1986). Similar studies have been carried out with PM sampled at different sites (Chapter IV). Weekly samples of coarse and fine PM from rural and industrial/urban areas (Duisburg/Borken, Hettstedt/Zerbst) elicited ·OH generation and DNA damage, caused DNA single strand breakage in A549 cells as well as induced 8-OHdG formations in calf thymus DNA. DNA damage was significantly related to ·OH generation and both DNA damage and ·OH generation were found correlated to several metals, such as Fe, Cu, Cr, Cd and Pb. When considered at equal mass, ·OH formation shows considerable variability with regard to the sampling places as well as the fractions of PM. A significantly higher ·OH generation was observed for PM sampled at urban/industrial area. Although higher soluble metals were found in fine fractions, coarse fractions showed higher oxidant ability, which is in agreement with the observation by Greenwell *et al*. (Greenwell *et al*., 2002), who has demonstrated that both urban $PM_{2.5}$ (fine fraction) and $PM_{2.5-10}$ (coarse fraction) caused significant plasmid DNA damage, the coarse fraction displaying higher oxidative capacity and the soluble components have been found responsible for most of the bioreactivity in both PM sizes.

A tendency is present among scientists and legislators to consider the fine and most likely the ultrafine fractions as the most harmful. This is based on (i) the traditional toxicological conception of harmlessness of components that make up the bulk of the coarse fraction and which dominate the mass of $PM_{10}$ e.g. salt, ammonium sulphate and ammonium nitrate plus crustal minerals such as clays, and (ii) toxicological and *in vitro* evidence that small combustion derived carbon centred, transition metal rich particles have considerable biological activity. PM is a heterogeneous mixture which contains a lot of organic compounds and materials (organic carbon, pollen fragment, ammonium, allergens and

endotoxins) and inorganic compounds (elemental carbon, sulphate, nitrate, chloride, metals) as described in the first chapter of this thesis. Our findings certainly suggest that the particle size and composition has a profound effect on its intrinsic oxidant activity, by affecting solubilisation, offering reducing elements and catalysing surface and other redox active metals. Particle induced ·OH generation has been suggested to be modified by other organic and inorganic components, such as sulphate (Ghio and Samet, 1999) and quinoid, semiquinone or nitroaromatic compounds (Kumagai *et al*., 1997; Dellinger *et al*., 2001). Metal-metal interactions certainly influence the oxidant activity of PM fractions with considerable variation of composition (Fenoglio *et al*., 2000; Dreher *et al*., 1997). It has been demonstrated that hydroxyl radical generation of Fe (II) was greatly enhanced by Fe (III) which is less active in hydroxyl radical formation (Urbanski and Beresewicz, 2000). The interactions between transition metals or transition metal with other components in particle certainly greatly influence metal induced hydroxyl radical formation. The result for this possible modification is that (organic) components in coarse fractions can favour the metal mediated hydroxyl radical formation. This supports the concept of using an assay that integrates bioavailability and redox activity of metals in the presence of particles that are able to catalyse radical formation.

Our results have demonstrated that EPR with spin trap can measure reproducibly the intrinsic oxidant capacity of particulate matter, which correlates to PM induced damage in naked DNA as well as cultured human epithelium cells. However, our direct mixing of PM with spin trap and $H_2O_2$, naked DNA or even to cultured cells is quite different from human exposure to PM. With the exception of dosimetric specification of PM, which involves deposition, translocation and clearance of PM in the human body, the anti-oxidant capacity also greatly influences potential adverse effects caused by PM. As we have described in the first chapter of this thesis the human body has an extensive anti-oxidant capacity, which includes enzyme systems and non-enzymatic components, mostly small molecule anti-oxidant substances, like ascorbic acid, GSH and uric acid. A lot of anti-oxidant substances have been found in human lung surface lining liquid and these substances will react with particle deposited on to the lung surface. It has been demonstrated that low molecular components of fresh lung lavage were found to offer most antioxidant protection (Zielinski *et al*., 1999; Sun *et al*., 2001; Greenwell *et al*., 2002). Recently, we tested how data on PM induced ·OH formation in an oxidant ($H_2O_2$) environment relate to particle induced anti-oxidant depletion in an environment without

$H_2O_2$. Different size fractions of ambient particles ($PM_1$, $PM_{2.5}$, $PM_{10}$) were shown to deplete GSH and ascorbate and the depletions were correlated well with their ability to generate hydroxyl radical measured by EPR (ascorbate ($r^2$=0.32), GSH ($r^2$=0.85)) (Shi *et al*., 2003). Ongoing studies in collaboration with larger epidemiological programs (ECRHSII, PAMCHAR, HEPMEAP) will now have to reveal how particle oxidant activity is related to different sources (traffic, industry, rural) and various health endpoints including lung function.

For an organism the deposited particle in the lung can introduce extra oxidants which can lead to an imbalance of prooxidant-antioxidant status and cause so called oxidative stress. On the other hand, this interaction will greatly change characteristics of PM and produce a new particle with a specification completely different from the original one (Kendall *et al*., 2002). However, these changes will vary among different particles, for example surrogate Epithelial Lining Fluid (sELF) has shown significant amelioration of DNA damage by the coarse fraction but less effect was found against the fine PM fractions (Greenwell *et al*., 2002). This may be helpful for the explanation why different size fractions dominate different biological and health effects, such as coarse PM size fractions have been thought to cause asthma or asthma-like syndromes (Zhang *et al*., 2002). EPR with spin trap has been used to measure free radical formation *in vivo* (Kadiiska *et al*., 1997; Leonard *et al*., 2002) and demonstrated that particles can greatly increase free radical formation in animals (Han *et al*., 2001). Recent work in our lab has shown that instillation of uF carbon black particles in rats can increase the oxidant stress as shown, by *ex-vivo* measurement in bronchoalveolar lavage (data not shown). These findings support the concept that particles can induce free radical formation and cause oxidant stress which has been associated with many human diseases.

For its sensitivity, relative easiness to be handled and requiring little mass the *in vitro* EPR measurement has been proven a valuable method in understanding PM induced effects as well as to assess its activity. Although some aspects need further research ongoing application in epidemiological studies will have to reveal how oxidant activity will perform as metric alternative to mass.

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