Toxicity Screening of Wood Combustion Fine Dust Using a Microbial Test Battery

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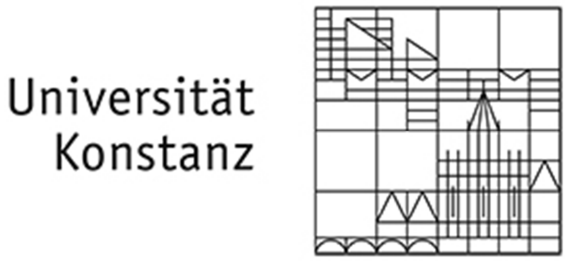

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Darin besteht das Wesen der Wissenschaft. Zuerst denkt man an etwas, das wahr sein könnte. Dann sieht man nach, ob es der Fall ist und im Allgemeinen ist es nicht der Fall.

Bertrand Russell (1872-1970)

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Summary

Claimed as renewable energy source, wood is increasingly used in stoves and boilers for heating purposes in western countries due to economic reasons and environmental consciousness. Consequently, domestic wood combustion contributes significantly to atmospheric loads of particulate matter (PM) nowadays. Epidemiological and controlled human, animal, and *in vitro* studies have proven that wood smoke exposure is injurious to human health. In this context, three standardized microbial assays, the bacterial contact assay BCT (*Arthrobacter globiformis*), the Umu-Test (*Salmonella typhimurium* TA1535/pSK1002) and the Yes-Test (*Saccharomyces cerevisiae* BJ3505) were adapted in this thesis in order to achieve a toxicological characterization of PM bound compounds in direct contact with the test organisms. Modifications included the assessment of bacterial and yeast viability via resazurin reduction, thereby replacing the conventional optical density measurement (OD600). This new endpoint indicated a higher sensitivity to compounds that affect organism's activity. Seven bulk fine dust samples and 13 filters loaded with PM with varying soot fractions, i.e. representative for complete and incomplete combustion, were tested as aqueous suspension or small filter cutouts. Regardless of the combustion type, all assumed types of toxicity were detected among the samples. Compared to other mode-of-action bioassays with nematodes and human cell lines in the overall project, the BCT turned out to be most sensitive for detection of baseline toxicity. Water content and type of wood of used pellets most likely influenced the baseline toxicity. The assumption that aqueous and thus bioavailable Zn may dominate PM baseline toxicity was not proven by X-ray diffraction or mixture experiments with Zn and PM samples. Expected over-additive toxicity of Zn when exposed simultaneously with the PAH fluoranthene was not observed. Furthermore, emitted fine dust that was subsequently oxidized in an atmospheric transport simulation chamber was assessed. Oxidized samples were only genotoxic when tested on the nitroarene sensitive NM3009 *Salmonella* strain not on the conventional *Salmonella* strain of the Umu-Test. Non-oxidized PM was not genotoxic, proving the formation of nitro-PAHs during atmospheric oxidation. The microbial test battery presented provides a screening technique for unknown fine particles that is rapid, sensitive, easy to handle and low-priced, while offering high-throughput testing. Therefore, it constitutes an excellent tool for evaluation of different stoves and boilers and can contribute to possible mitigation actions. For a cost-benefit-analysis of small-scale wood combustion devices, obtained toxicity results should be related to their total emission loads and heating values.

Zusammenfassung

Holz wird als erneuerbare Energiequelle angesehen und aus ökonomischen Gründen sowie ökologischem Bewusstsein in Öfen und Kesseln vermehrt zu Heizungszwecken eingesetzt. Daher trägt heutzutage Holzverbrennung in Privathaushalten deutlich zur Feinstaublast in der Luft bei. Epidemiologische Studien, kontrollierte Studien mit Menschen und Tieren, sowie *in vitro* Studien bewiesen, dass die Exposition mit Holzrauch schädlich für die menschliche Gesundheit ist. Vor diesem Hintergrund wurden in dieser Arbeit drei standardisierte mikrobielle Testsysteme für die toxikologische Charakterisierung von Feinstäuben angepasst: Der Bakterienkontakttest BKT (*Arthrobacter globiformis*), der Umu-Test (*Salmonella typhimurium* TA1535/pSK1002) und der Yes-Test (*Saccharomyces cerevisiae* BJ3505). Anpassungen umfassten die Messung von Bakterien- und Hefenvitalität über die Reduktion von Resazurin und ermöglichen den direkten Kontakt der Testorganismen mit den Feinstäuben. Dadurch wurde die konventionelle Messmethode über die optische Dichte (OD600) ersetzt. Der neue Endpunkt zeigte eine höhere Empfindlichkeit gegenüber Stoffen, die die Aktivität der Organismen beeinflussen. Sieben lose Stäube und 13 mit Feinstaub beladene Filter mit unterschiedlichen Rußgehalten, die von vollständiger und unvollständiger Verbrennung herrühren, wurden in Suspension oder als kleine Filterstücke getestet. Unabhängig von der Art der Verbrennung wurden alle vermuteten Toxizitäten innerhalb der Proben detektiert. Im Vergleich zu anderen mode-of-action bioassays mit Nematoden und humanen Zelllinien im Gesamtprojekt, zeigte sich, dass der BKT am empfindlichsten für die Erfassung von Basistoxizität ist. Wassergehalt und Holzart der verwendeten Pellets beeinflussten dabei höchstwahrscheinlich die Wirkung. Die Annahme, dass wasserlösliches und daher bioverfügbares Zn hauptverantwortlich für die Basistoxizität der Feinstäube ist, wurde nicht anhand von X-ray diffraction oder Kombinationsexperimente mit Zn und Feinstaubproben bestätigt. Eine erwartete über-additive Toxizität von Zn bei gleichzeitiger Exposition mit dem PAK Fluoranthene wurde nicht beobachtet. Darüber hinaus wurde Feinstaub, der in einer Atmosphärensimulationskammer oxidiert wurde, bewertet. Oxidierte Proben waren nur als genotoxisch zu erkennen, wenn sie mit dem NM3009 *Salmonella* Stamm, der empfindlich für Nitroarene ist, getestet wurden. Die Testung mit dem konventionellen *Salmonella* Stamm im Umu-Test ergab keine Genotoxizität. Nicht-oxidierte Proben waren nicht genotoxisch. Dies bewies die Bildung von Nitro-PAKs während der atmosphärischen Oxidation. Die hier vorgestellte mikrobielle Testbatterie bietet ein schnelles und kostengünstiges Screening von unbekannten Feinstaubproben mit einfacher Handhabung

und hohem Probendurchsatz. Daher stellt sie ein ausgezeichnetes Werkzeug für die Bewertung von Öfen und Heizungskesseln dar und kann zu möglichen Toxizitätsminderungsmaßnahmen beitragen. Für eine Kosten-Nutzen-Analyse von Kleinanlagen für die Holzverbrennung sollten Toxizitätsergebnisse auf deren Gesamtemissionen und Heizwerte bezogen werden.

1 Introduction

As a result of increasing costs for fossil fuels renewable energy resources such as wood have become highly popular in Europe and the U.S. within the last decades. For instance, in Germany and Finland approx. 25% of private households use wood logs, pellets, briquettes or woodchips for heating either as the exclusive energy source or in addition to the conventional fossil fuels (Fachagentur Nachwachsende Rohstoffe e.V., 2010, Ohlström et al., 2000).

Fine particles from such small-scale combustions represent a new source of air pollution that needs careful evaluation with regard to human and environmental risk assessment. Since stoves and boilers for residential heating are considerably less regulated by national governments than larger power plants, the increasing number of small wood incinerators may locally contribute significantly to the total environmental fine particle load. Indeed, high particle concentrations resulting from wood combustion used for heating purposes were observed especially in rural areas (Hellén et al., 2008). However, particle concentration is too general a descriptor for human and environmental risk assessment as size, total mass and the chemical composition of these fine particles vary due to factors like the type of burner and fuel, as well as the operating conditions employed (Kocbach Bølling et al., 2009, Tissari et al., 2009). Among the toxicologically interesting components of these fine particles are polyclyclic aromatic hydrocarbons (PAHs) and heavy metals (Larson and Koenig, 1994). There is increasing attention to possible toxicological effects on humans: In case-control studies the exposure with such particles were related to adverse health effects (Chen et al., 2006, Orozco-Levi et al., 2006). In both animal and controlled human studies exposure to wood smoke indicated an increase of disease related oxidative stress and neutrophils or cardiovascular risk factors in tissue and blood samples (Ghio et al., 2011, Park et al., 2004). These studies focus on the correlation between specific biomarkers and diseases associated with wood smoke. They do not provide a suitable tool for a rapid risk assessment of fine particles from different wood combustions since they face ethical issues, are time-consuming and high in cost.

However, in order to compare different small-scale wood combustion appliances, a standardized procedure in high-throughput manner is needed for toxicity testing of emitted fine dust. Toxicity assessment of varied combustion and stoves or boiler types would be highly beneficial for the technical advancement of improved devices and combustion conditions and thus also lower the impact of wood combustion fine particles on humans and environment. Representing principal cell functions, microorganisms may serve as eligible test subjects for primary toxicity testing of complex fine particles originating from the combustion

of wood. In contrast to human cell lines, internationally standardized toxicity tests with bacteria and yeast cells for soil, sediment or aqueous samples already exist. Against this background, this study presents a microbial test battery for a first and rapid toxicity screening of fine dust from wood combustion.

2 Main Goals and Outline of this Study

Main goal of this study was the examination of coherences between type of appliance, type of combustion, type of used wooden fuel and toxicity of emitted PM on the basis of a suitable test battery. Dominating chemical causatives for observed toxicity patterns of different fine dust samples (FPs) were looked for. The implementation of a suitable microbial test battery that can be applied for bulk fine dust and filters loaded with PM from wood combustion was prerequisite for a successful toxicity screening of particulate matter (PM). One integrative and two selective microbial tests were chosen of the numerous existing microbial test systems. The bacterial contact test (BCT), an integrative test, was explicitly developed for detecting baseline toxicity of solid samples (Rönnpagel et al., 1995). The Umu-Test (selective test with bacteria) was designed to detect genotoxic substances, whereas the Yes-Test (selective test with yeast) responds to estrogenic chemicals. The three test organisms have an affinity to particle surfaces, where contaminants are bound (Fretwurst and Ahlf, 1996, Rönnpagel et al., 1995, Weber et al., 2006). They were chosen in order to address the potential bioavailability of toxic compounds that have been mainly analyzed in wood combustion PM: polycyclic aromatic hydrocarbons (PAHs) and heavy metals. PAHs are known to be mutagenic and showed weak estrogenic activity in short-term bioassays (International Agency for Research on Cancer, 1983, Santodonato, 1997). However, heavy metals can also be genotoxic (Asakura et al., 2009). Since it has been demonstrated, that particle bound contaminants contribute to toxicity, which could not be extrapolated by testing chemical extracts of particles, this study focused on aqueous suspensions to assure the direct contact of test organisms and fine particle samples. Chosen microbial assays were adapted in such way that PM samples could be tested effectively. Besides the evaluation of the developed test battery, differences in toxicity of bulk fine dust samples and filter samples were studied using these modified bioassays. Relationships to particle size, carbon content or other physico-chemical characteristics of different combustions were examined. A comparison with other bioassays with nematodes and human cell lines in accompanying studies was conducted with regard to suitability and toxicity outcomes under consideration of their MoAs. Further research questions were developed on the basis of obtained results.

3 Scientific Background of Wood Combustion

3.1 Popularity Trend of Small-Scale Wood Combustion Devices

In the past, wood furnaces were commonly used for cooking and space heating. Simple devices, with open furnaces, for instance, have been mainly used by the poorer part of the population in developing countries (Faaij, 2004). Naeher et al. (2007) estimated that around 10% of the global direct energy consumption is provided by biomass (wood and agricultural wastes). Nowadays in industrialized countries this ancient energy source is undergoing a revival on a high technical level. Private wood combustion for primary or supplementary heating purposes has become very popular especially in residential rural areas as it has been reported for Denmark (Glasius et al., 2008), Finland (Hellén et al., 2008), Portugal (Gonçalves et al., 2011) and Germany (Bari et al., 2011). For example, Hellén et al. (2008) mentioned 2.2 million fireplaces in Finland, although Finland has only around five million inhabitants. In 2010 25% of German households were heated with wood (Fachagentur Nachwachsende Rohstoffe e.V., 2010) and such appliances typically range from a few kWth to 25 kWth (Faaij, 2004).

There are three main reasons for increased sales of domestic small-scale wood combustion devices: economical, ecological and life-style. In terms of money, heating with wood is much cheaper than with gas or oil. The German association for energy wood and pellets reported in March 2012 that heating with pellets (5.23 ct/kWh) costs 46% less than with oil (8.24 ct/kWh) (Deutscher Energieholz- und Pellet-Verband e.V., 2012). Similar scenarios are expected for other countries that depend on the import of fossil energy sources. Gas and oil prices are increasing worldwide (Deutsche Rohstoffagentur, 2012, Organisation of the Petroleum Exporting Countries, 2012). Financial support for the purchase of individual heating systems and/or the production of renewable energy that is exempted from tax are common policies in Germany, Austria, Finland, France, Netherlands, Spain and Sweden (Faaij, 2004). For instance, the purchase of domestic combustion applications is subsidized by the German government in order to promote the transition from fossil to renewable energy sources (Bundesamt für Wirtschaft und Ausfuhrkontrolle, 2013). From the environmental aspect wood is a renewable energy source. Thus, distributors of wood stoves advertise domestic wood combustion appliances with “heating with a clear conscience” (Frankfurter Allgemeine Zeitung, 2001). There is a third, less concrete reason why owners of family houses and apartments decide to install a wood combustion appliance: Heat from a wood stove is

perceived as more comfortable than heat from a radiator that is usually run by oil or gas boilers. Moreover, relaxing in front of the furnace of a wood stove is synonymous with a cozy home. Having your own wood stove is considered as “homey and cozy” (Stiftung Warentest, 2011, Umweltbundesamt, 2007).

3.2 Impact of Residential Wood Combustion on Air Quality

As a consequence, emissions from increasing numbers of private wood combustion appliances contribute significantly to air pollution levels nowadays. Compared to large-scale combustion plants which are regulated by law and contain gas-cleaning devices, small-scale combustion devices mostly lack dust collectors and are operated by private, untrained people (Wiinikka, 2008, BMU: Thrän and Pfeiffer, 2012). Stoves and boilers in private households can release high gaseous and particle emissions due to wrong handling or old, less sophisticated devices (Bari et al., 2011, Johansson et al., 2004). The German Environmental Agency (UBA) estimated 2007 that the amount of fine dust emitted from domestic wood combustion and small production companies is equal to fine dust emitted from automotive traffic - a rising tendency (Umweltbundesamt, 2007). Similar observations were made in Denmark and Finland (Glasius et al., 2006, Hellén et al., 2008). One third of total PM_{10} (particulate matter < 10 µm) emissions was associated with domestic wood combustion in a rural area in Italy (Caserini et al., 2010). Similarly, 18% was reported for Portugal (Borrego et al., 2010). In addition, inversion conditions enable high loads of ambient PM due to lowered atmospheric transport. Especially in winter, the main heating period, the influence of PM on air quality is highly noticeable visually and in measurements of specific biomass combustion products such as levoglucosan (Bari et al., 2011, Puxbaum et al., 2007). Levoglucosan is a combustion product of cellulose and is used as general molecular tracer for biomass burning. By means of levoglucosan level in ambient air samples the contribution of biomass burning to total air pollution is assessed (Puxbaum et al., 2007).

3.3 Completeness of Wood Combustion and its Influence on Emissions

Type and total masses of emissions from wood combustion can differ strongly. In general, the main components of wood are cellulose and lignin with small amounts of resinous materials and inorganic salts. Combustion converts these structures to smaller molecules, mainly CO_2 and water. Besides, inorganic gases (CO, NO and SO_2), volatile organic hydrocarbons (VOC), polycyclic aromatic hydrocarbons (PAHs) and PM are produced. The latter consists of salts with, for example, trace elements like potassium, soot and adsorbed organic material (Boman

et al., 2003, Larson and Koenig, 1994). Operational conditions are crucial for quantity and quality of emissions. Loading of the chamber influences furnace temperature which determines besides oxygen supply whether emitted particles contain mainly soot or alkali salts. Continuous oxygen supply and high initial temperatures (> 800 °C) result in grey fine particles as inorganic salts dominate the particle fraction (complete combustion). Air deficiency and low temperatures (300-500 °C) cause black fine particles consisting mainly of soot (incomplete combustion). Pellet stoves emit less fine particles than other stoves since pellets consist of homogenous material, combustion is operated automatically and thus is close to complete (Umweltbundesamt, 2007). With regard to size categorization, particles are typically classified as coarse (2.5 – 10 µm) or fine (≤ 2.5 µm) (Naeher et al., 2007), whereas wood smoke particles are on average ≤ 1 µm (Larson and Koenig, 1994, Naeher et al., 2007). The sizes of particles are strongly affected by the completeness of the combustion. Complete combustions generate less and smaller particles (< 125 nm) than incomplete combustions (< 600 nm) (Kocbach Bølling et al., 2009, Tissari et al., 2008). Ultrafine particles with ≤ 0.1 µm can reach the alveolar region of the lungs and trespass into bloodstream (Oberdörster et al., 2005). Due to their large surface area per mass, these ultrafine particles are considered to pose higher adverse health risks as studies on cells and animals revealed (Kocbach Bølling et al., 2009). Nevertheless, the influence of size may be outweighed by chemical or physical characteristics of applied fine dust samples. For example, in the study by Jalava et al. (2007) coarse particles from ambient air of six European cities elicited much higher inflammatory responses in a mouse cell line than fine particles. Besides the completeness of combustion, type and quality of wooden fuel affects emitted PM regarding mass, size and chemical composition (Johansson et al., 2003). The use of coniferous wood (softwood) leads to fewer emissions than deciduous wood (hardwood) as the latter contains more components that enable the building of aerosols. Rising water or bark content in wood fuels results in higher emissions (BMU: Thrän and Pfeiffer, 2012). Poor knowledge about handling wood stoves and the usage of old appliances heavily increase mass of gaseous and particle emissions (Bari et al., 2011, Johansson et al., 2004). How users' behavior, type of wood and combustion device influence emissions was recently observed in Greece. As a reaction to the financial crisis, the Greek government increased prices for heating oil by 40%. Not being able to afford oil, the Greek population started to collect any waste wood they could find on the street and burned it for heating purposes in old stoves. As a result of these uncontrolled wood burnings, severe deterioration in air quality has been reported by the environmental agency in Athens (Thomas Bormann, 2013, Schlötzer, 2012).

3.4 Regulations on Emissions from Small-Scale Wood Combustion Devices

In reaction to the increasing influence of wood combustion on air quality, political actions were made in western countries. For instance, German government enacted in 2010 a federal immission control ordinance that restricts emission values for new small-scale combustion devices (1. BImSchV, Bundesimissionschutzverordnung). Installation, constitution and operation of small scale wood combustion devices are also regulated. Modern (pellet) stoves are conforming to emission values requested from law. Legislation for old stoves and boilers is planned for the future. In order to reduce emissions originating from private wood combustion, the U.S. EPA has since 2005 supported campaigns where the replacement of old ovens by modern EPA-certified devices is sponsored. They produce about 70% less emission than old appliances. Besides education, some U.S. communities introduced "burn bans" during air inversions in winter when air quality deteriorates particularly as a result of emissions from wood combustions (U.S. Environmental Protection Agency, 2009). Current research focuses on the reduction of emissions from small-scale wood combustion appliances as primary action. Gas treatments of such devices as secondary action are less investigated (Wiinikka, 2008, BMU: Thrän and Pfeiffer, 2012). Nevertheless, the amount of emissions does not necessarily correlate with their overall toxicity. Thus, a toxicological characterization of emissions of different wood combustion devices and their relationship to the occurrence of diseases is needed in addition to measurements of emission levels.

3.5 Toxicity of Wood Combustion Emissions

3.5.1 Epidemiological Studies

In order to elucidate the development of research about the relationship of exposure to wood combustion emissions and adverse health effects, an overview of epidemiological findings published in the last ten years is given in the following (Table 1). Relevant sources of high emissions from wood combustion are forest fires and residential biomass burning. As cooking with biomass is common in developing countries indoor exposure to wood smoke is much higher than in developed countries where emissions of (modern) residential heating devices disperse into the outdoor atmosphere (Fullerton et al., 2008). In a review by Naeher et al. (2007) wood smoke concentration of 750 µg PM/ m^3 were defined as nonhazardous. Compared tor developed countries, much higher levels than 1 mg PM/ m^3 were reported for indoor concentrations in developing countries. Hence, it is not surprising that wood smoke-related diseases are predominantly reported in developing countries. Children are often considered in particular in such studies as their respiratory defense mechanisms are not fully

developed and thus are more susceptible to adverse effects of air pollution. In this line, Smith et al. (2000) conducted a critical review on the basis of epidemiological studies in developing countries and highlights acute respiratory infections as the major cause of death of small children. The authors reviewed 13 recent studies with 50-1000 children, mostly case-control studies, and detected a significant increased risk for ALRI for children living in households with biomass burning compared to the control group. However, they could not quantify the impact of wood smoke due to the inconsistency of data. Boman et al. (2003) evaluated nine studies with adults and children conducted in the U.S., Canada and New Zealand and found a significant relationship between the incidence of acute asthma and environmental PM. Relative risks increased when wood smoke was a major source of PM. Most listed reviews in Table 1 report significant relationships between exposure to emissions from wood or biomass combustion and ALRI. Although there was also evidence of cardiopulmonary disease and mortality with ambient air pollution with high contribution of wood smoke a significant proof was impossible due to a lack of data (Boman et al., 2003). This possible relationship was strengthened five years later in the review by Lewtas (1988) and Naeher et al. (2007). The authors concluded from cohort studies that fine dust from combustion (traffic and biomass burning) was an important risk factor for cardiopulmonary and lung cancer mortality. Similarly, Fullerton et al. (2008) concluded in their review that it was “highly likely” that cardiovascular diseases were related to exposure of biomass burning. According to Naeher et al. (2007) COPD, acute lower respiratory infections, blindness by cataracts and tuberculosis were associated with cooking with biomass in developing countries. Though, number and character of reviewed studies did not allow proof of a significant relationship. The same conclusion was also drawn by Fullerton et al. (2008). The authors reported that non-respiratory diseases such as tuberculosis, interstitial lung disease or blindness due to cataract occurred after exposure to emissions from biomass/wood burning predominantly in developing countries. Regarding tuberculosis, Laumbach and Kipen (2012) emphasized that epidemiological evidence for the association with wood smoke was not sufficient. As the number of studies about interstitial lung disease or cataract and biomass burning was also very limited, this may also apply to those illnesses. Zelikoff et al. (2011) and Laumbach and Kipen (2012) partially confirmed in their reviews evidences between both respiratory and non-respiratory diseases. The authors assumed that individual chemicals that occur during wood combustion most likely weaken the immune system and lead consequently to observed diseases like tuberculosis. Zelikoff et al. (2011) mentioned an *in vitro* study where metabolite-induced opacification and lens hyperplasia, hypertrophy, and epithelial cell multilayering

were detected after exposure to wood smoke. Consequently, adverse ocular effects may likely be associated with exposure to wood combustion emissions. According to the reviews of Fullerton et al. (2008), Naeher et al. (2007), Zelikoff et al. (2011) adverse birth outcomes like low birth weight are very likely due to maternal wood smoke exposure. More studies exist about the evidence of biomass/wood smoke exposure and (lung) cancer. The International Agency for Research on Cancer classified biomass smoke in their 2010 report as “probable carcinogenic” (Group 2a) (International Agency for Research on Cancer, 2010).

Besides case-control studies, stove exchange programs offer investigation possibilities about adverse health effects of wood smoke. In the review by Laumbach and Kipen (2012) such studies were reported. Open furnaces for cooking were replaced by improved appliances in 669 households in Mexico. Significant reduction of respiratory symptoms and reduced harm to lung function were detected one year later. In a similar study in Guatemala, incidences of severe pneumonia significantly reduced when improved cooking stoves were installed. The replacement of old wood stoves with high emissions by new certified devices led to fewer reports of asthma-related symptoms in children in the U.S. (Epidemiology, 2010). Such intervention studies underline the crucial role of quantity and quality of emissions of used wood stoves for toxicity outcomes.

In general, wood smoke-related diseases seem to challenge both developing and western countries, albeit different wood combustion appliances are used. Although many cohort and case-control studies about the relationship between wood smoke exposure and occurrence of illnesses exist, an evaluation or drawing critical values for indoor emissions from wood combustion is not possible for following reasons: The number of test subjects vary widely among studies. Confounding factors like cigarette smoking are sometimes not clarified nor considered. A differentiation of specific emitted compounds, both gaseous and particulate, is not made. Wood smoke exposure is rarely quantified and sometimes is limited to vague reporting of studied patients, about their cooking habits, for instance. The later drawback was also raised as an objection in the reviews by Lewtas (2007) and Naeher et al. (2007). To tackle this problem a recent review has suggested modeling exposures (Laumbach and Kipen, 2012).

Table 1: Epidemiological reviews about diseases related to biomass/wood combustion exposure in chronological order. n.i.: not indicated, x: evidence of relationship to biomass/wood smoke exposure, (A)RLI: (acute) lower respiratory infections, (C)OPD: (chronic) obstructive pulmonary disease.

Epidemiological reviews							
	Smith et al., 2000	Boman et al., 2003	Lewtas, 2007	Naeher et al., 2007	Fullerton et al., 2008	Zelikoff et al., 2011	Laumbach and Kipen, 2012
Number of reviewed studies	13	9	3	> 30	> 30	n. i.	> 30
Respiratory symptoms/diseases							
Asthma symptoms/ asthma		x		x		x	x
(A)LRI	x	x		x	x	x	x
Cardiopulmonary mortality			x				
(C)OPD				x	x	x	x
Lung cancer (mortality)			x	x	x	x	
Decreased lung function		x		x	x		
Interstitial lung disease					x		
Non-respiratory symptoms/diseases							
Adverse birth outcomes				x	x	x	
Cardiovascular diseases					x	x	x
Cataracts				x	x		
Mortality	x	x			x		
Premature death						x	x
Tuberculosis				x	x		x

Besides investigating direct links between wood smoke exposure and illnesses, human biomonitoring featured more prominently in research of toxicity of wood combustion emissions. In these kinds of molecular epidemiological studies biomarkers for metabolic genotype, DNA adducts, DNA repair, metabolites of polycyclic aromatic carbons (PAHs), and urinary mutagenic activity are monitored in humans who live in areas with combustion emissions, often not exclusively from wood combustion (Collins and Azqueta, 2012, Lewtas, 2007). Measurements are done in body fluids (e.g. blood, urine). As biomarkers monitor individual exposure concentrations and are likely to be precursors of diseases, such studies can provide more mechanistic insights into the genesis of diseases. Lewtas, 2007 concluded

by reviewing human biomonitoring studies that there was a significant correlation between exposure to $PM_{2.5}$ (particulate matter < 2.5 µm) of combustion emissions and metabolic genotype, DNA adducts, PAH metabolites, and urinary mutagenic activity. A very recent study analyzed urine samples of 79 pregnant Peruvian women for 1-hydroxypyrene, a metabolite of pyrene (Adetona et al., 2013). Higher hydroxypyrene concentrations were detected in women who cooked with wood or kerosene compared to those who used gas or coal briquette. Another study conducted in 2013 suggested levoglucosan, a tracer for wood combustion emissions in ambient air, as biomarker in human urine samples (Wallner et al., 2013). The authors measured levoglucosan and 1-hydroxypyrene in urine samples of mothers and children in different communities in Austria. There was evidence of a correlation between those biomarkers and wood smoke exposure. The last two studies show that the field of human biomonitoring with regard to wood combustion emissions may advance rapidly in the near future.

Nevertheless, classical and molecular epidemiological studies often face societal, ethical, legal and financial issues and thus are complemented by *in vivo/in vitro* studies (Zelikoff et al., 2011). Furthermore, compared to epidemiological studies, *in vivo/in vitro* studies can provide reproducible experimental set-ups.

3.5.2 *In vivo* Studies

3.5.2.1 Studies with Humans

Besides epidemiology studies, controlled human and animal studies are conducted in order to investigate the direct influence of wood smoke on lung function and on biomarkers related to disease factors in bodies. Similar to molecular epidemiological studies, exposure biomarkers comprise measurements of body fluids (e.g. blood, urine), tissue samples or exhaled breath. Particle concentrations below ~400 µg PM/ m^3 are used in human studies and are considered as nonhazardous, but environmentally relevant in residential areas (Riddervold et al., 2012, Zelikoff et al., 2011). Although they are conducted under controlled conditions, studies with humans are rare due to ethical and financial issues. A few are described in the following.

In the study by Barregard et al. (2006) 13 healthy human subjects inhaled wood smoke (240-280 µg PM/ m^3) and clean air for four hours each with an interval of one week in between. Blood and urine samples were taken before and after exposure. An increase of a cardiovascular risk factor and a slight effect on the balance of coagulation factors were detected; there was the indication of a possible increase in radical-mediated lipid peroxidation, which indicates inflammation. In another study ten volunteers were exposed to

both clean air and wood smoke particles (485±84 µg PM/ m³) at intervals of three weeks apart (Ghio et al., 2011). During exposure, test subjects exercised for the duration of two hours resting every 15 minutes. Pulmonary function, heart variability and repolarization were not affected by wood smoke exposure. In contrast, blood samples and bronchial and bronchoalveolar lavage exhibited an increase of neutrophils indicating systemic and pulmonary inflammation. In a recent study 20 atopic volunteers (allergic reactions observable on the skin) were exposed to clean air, and 200 and 400 µg PM/ m³ for three hours (Riddervold et al., 2012). Expiratory characteristics, nasal patency, and markers of airway inflammation in exhaled breath and nasal lavage were measured. Whereas there was weak evidence of inflammation in exhaled breath condensate, other endpoints were not affected significantly. With regard to the human studies presented, inflammation seems to be the prevalent effect in humans after short-term exposure to wood combustion emissions.

3.5.2.2 Animal Studies

As in human studies, animals are exposed to emissions from wood combustion under controlled conditions; usually only acute effects are monitored. Test animals comprise mice, rats, rabbits, sheep, dogs and guinea pigs. Three example studies are described in the following after which an overview is derived by examining the findings of a review paper.

Rats as test objects were exposed to 1000, 10.000 µg PM/ m³ or clean air in the study by Tesfaigzi et al. (2002). Exposure times were 3 hours a day, 5 days a week for four or twelve weeks. Highest particle concentration caused reduced pulmonary functions, and particularly carbon monoxide-diffusing capacity. Furthermore, mild chronic inflammation and morphology changes of epithelia cells of the larynx and mucous cell lining in airways were observed. Number and pigmentation of lung macrophages (immune cells) increased. All in all, the immune system of the rats was weakened. Barrett et al. (2006) exposed ovalbumin-sensitized mice with preexisting lung inflammation to wood smoke (30, 100, 300, or 100 µg PM/ m³) and partially to ovalbumin for 6 hours a day or three days in a row. Ovalbumin-sensitized mice were used in order to differentiate between ovalbumin and wood smoke-induced lung inflammation reactions. After 18 hours of exposure bronchoalveolar lavage and blood sampling were conducted, showing that indices of allergic airway inflammation increased minimally, but only when ovalbumin had been given beforehand. In another study anesthetized adult male sheep were exposed to different concentrations of wood smoke by tubing the tracheostomy, four or five sheep for each concentration (Park et al., 2004). Concentrations were defined as 0, 5, 10 and 16 units which consisted of five breaths each unit. Blood plasma and exhaled breath samples were taken every six hours. After a total exposure

time of 48 hours sheep were euthanized and histologically examined. A dose-effect response was observed for a few oxidative stress indices in samples taken. Similarly, injury of tracheobronchial epithelium and lung parenchyma increased when doses increased. In order to ensure reproducible and defined dosing anesthetized animals are mechanically ventilated throughout exposure. This kind of exposure is questionable as it does not mimic normal breathing situations.

A detailed overview of outcomes in animal studies with wood smoke was presented in the review by Naeher et al. (2007). The authors focused on studies with most natural exposure routes (i.e. nose-only/whole body inhalation in conscious animals) and differentiated between acute and subchronic exposures. Among acute exposures three early studies conducted in the 1980s with rabbits described changes in lung cell morphology, including epithelial cell loss after high wood smoke emission exposure ($\leq$ 1707 ppm, $\leq$ 120 min). Further, effects on inflammation occurred as it was observed all throughout the studies reviewed by Naeher et al. (2007). In short-term experiments with guinea pigs and dogs a reduction in pulmonary compliance was observed as a consequence of inhalation of moderate levels of wood smoke. Animals recovered within several days. In a study of 2001 high-dose exposure to wood smoke led to altered pulmonary histology, induced an inflammatory response, increased static lung compliance, and increased lavageable cytokine levels (important for immune response) and cell counts in mice. As a sign of an upregulated immune response the number of alveolar macrophages increased while these cells were flatter and less active. Acute low-dose exposure to wood smoke caused less phagocytosis and intracellular killing of pathogen bacteria by macrophages in a study with rabbits conducted in 1984. The authors suggested that such weakened intrinsic immune defense may make the body more susceptible to infections. This relationship between weakened immune defense and occurring diseases like tuberculosis was also assumed almost 30 years later as described before (Laumbach and Kipen, 2012, Zelikoff et al., 2011). Acute high exposure of wood smoke caused epithelial cell loss, lipid peroxidation and changes in lung antioxidant enzymes due to oxidative stress in rats as described in a study of 2002. In the same vein, studies conducted in the 1990s with rats measured an increase of hydroxyl radical burdens as a sign of oxidative stress after exposure to wood smoke.

While there are numerous short-term animal studies, long-term studies are rare. Around the year 2000 three studies with rats revealed after repeated wood smoke exposure (e.g. 750 µg/m³, 1 hour per day for 4 days) suppressed bacterial clearance of pathogen bacteria. Moreover, production levels of superoxide anions for destroying such pathogens were

reduced. Another study exposed rats and mice with 30-1000 µg/ m³ for one week up to six months. Among other outcomes that were detected before in other studies, slight effects on hematology were observed. Long-term studies with rats or mice (6-15 months of wood smoke exposure) classified wood smoke as weak carcinogen inducer. Similarly, the review by Lewtas (2007) reported positive results in a mouse skin tumor initiation assay conducted on whole animals in three studies after exposure of particulate organic matter from wood combustion.

All effects considered, morphological alterations of lung tissue, inflammation, oxidative stress and reduced respiratory immune defense are the prevalent effects upon wood smoke exposure in animal studies. Furthermore, wood smoke may be potentially carcinogenic. Both human and animal studies presented here all reported inflammation as a reaction to wood smoke. Among many other relationships occurring in bodies, inflammation can be linked to oxidative stress, weakened respiratory immune defense, alterations of lung tissue and other adverse outcomes which in turn can be precursors of diseases. Thus, both molecular epidemiologic studies, and human and animal studies can provide more mechanistic insights into the development of illnesses. Still, the transferability of results from animals to humans may be difficult since the respiration tract of rodents that are commonly used in such studies is different, they breathe, for instance, via the nose, and breathing rates are distinct (Naeher et al., 2007). Consequently, deposition and clearance of fine dust or gaseous components may be distinct to those in humans. More comparative studies with humans and animal models are required in order to specify extrapolation values. However, as animal and human studies encounter ethical and legal issues as well as high expenses, toxicity of wood combustion derived fine particles is also investigated in *in vitro* studies.

3.5.3 *In vitro* Studies

3.5.3.1 General Approaches of in vitro Techniques

Compared to *in vivo* tests, in *in vitro* studies research is narrowed down to very specific mechanistic questions and endpoints. In general, obtained toxicity can be divided into specific and nonspecific mode of action (MoA) (Escher and Hermens, 2002). The latter describes the disturbance of membrane structures and functioning and is considered as the minimal toxicity of any chemical. In contrast, specific modes of actions are receptor-mediated toxicities like estrogenicity. Due to the universality of basal cellular structures and functions in different organisms and target tissues it is assumed that MoAs or target sites might be the same despite different organismic levels (Escher and Hermens, 2002). Consequently, it is assumed that

toxicity assessment of fine dust on the basis of cells or microorganisms may complement each other and can give hints on possible adverse effects on both humans and organisms. This was shown for the bacterial Ames-test for mutagenicity which predicts rodent carcinogens with a high degree of certainty (Reifferscheid and Heil, 1996). Environmental samples with endocrine disruption potential such as estrogenicity can impair the endocrine system and thus can cause developmental abnormalities in wildlife organisms and lead to cancer (Metzler, 2001). Hence, detecting estrogenicity in environmental samples in *in vitro* studies may alert possible effects on living organisms including humans.

The MoA of individual compounds that are formed during wood combustions have been studied widely (Table 2). Such health-damaging chemicals are either emitted as gases or associated with PM. Therefore, such MoAs were also expected when testing fine dust from wood combustion. In the majority of *in vitro* studies, only PM is tested, not gaseous emissions due to experimental feasibility reasons. However, novel approaches try to consider both particulate and gaseous phases of wood combustion by means of air/liquid exposures. It consists in the cultivation of test cells on a porous membrane in transwell inserts. Before emission exposure, medium is removed above the cells, whereas below the membrane the medium remains for a sufficient nutrient supply (Aufderheide et al., 2003). Examining the occurrence of single compounds by chemical analyses enables the possible MoAs of whole fine dust samples to be estimated. Based on such expected MoAs suitable biological test systems can be chosen. Depending on sample preparation and chosen test system, such toxicity testing of fine dust samples can provide more information about:

1. Mixture toxicities of complex fine dust.
2. Bioavailability of previously analyzed compounds.
3. Existence of compounds that were not chemically analyzed.

Table 2: Toxicologically relevant components that can form during wood combustion. Table adapted from Lewtas (2007), Naeher et al. (2007) and Zelikoff et al. (2011).

Chemical class	Example compound	Mode of action
Toxic gases	Carbon monoxide	Irritant, acute toxicity
VOCs (C2–C7)	Methyl chloride	Irritant, possibly carcinogenic
Saturated hydrocarbons	Hexane	Irritant, neurotoxicity
Unsaturated hydrocarbons	1,3-butadiene	Irritant, carcinogenic, mutagenic
Monoaromatics	Benzene	Irritant, carcinogenic, mutagenic
Polycyclic aromatic hydrocarbons (PAHs)	Benzo(163)pyrene	Carcinogenic, mutagenic, immunotoxic
Substituted PAHs	1,3 Dinitropyrene	Carcinogenic, mutagenic
Organic alcohols and acids	Methanol Acetic acid	Irritant, acute toxicity, teratogenic
Aldehydes	Formaldehyde	Irritant, carcinogenic, mutagenic
Phenols	Catechol	Irritant, carcinogenic, mutagenic, teratogenic
Quinones	Hydroquinone	Irritant, allergenic, redox active, oxidative stress and inflammation, possibly carcinogenic
Free radicals	Semi-quinone-type Radicals	Redox active, oxidative stress and inflammation, possibly carcinogenic
Metals	Arsenic	Carcinogenic, acute toxicity
Chlorinated dioxins	1,4-Dioxin	Irritant, may be carcinogenic or teratogenic
Particulate acidity	Sulfuric acid	Irritant

Besides known toxicants that are formed during wood combustion (Table 2), emitted PM can undergo atmospheric transformation processes in the environment and form new toxicants. Such chemical transformations occur due to reactions with gaseous and particulate molecules and UV radiation (Kocbach Bølling et al., 2009). Although wood smoke particles can change in size, morphology and chemical composition, research mainly focuses on chemical modification of substances groups during transportation. It is known that the photo-oxidation of PAHs leads to the formation of oxy- and nitro-PAHs (Arey, 1998, Lundstedt et al., 2007). These newly built molecules might have a higher toxicological relevance than their precursors (Arey, 1998, Yan et al., 2004). Comparing ambient air samples from six European cities the highest inflammatory activity was measured in summer samples from two Mediterranean cities (Barcelona and Athens) (Jalava et al., 2007). The authors suspected the high

photochemical activity and ozone concentration during summer to have transformed particles and their associated molecules into potent inflammatory inducers. However, to the author's knowledge basic research about atmospheric transformation processes and their influence on particle's toxicity is scarce and needs to be stepped up.

3.5.3.2 Toxicity Tests with Cell lines

Cell lines are cultures of eukaryotic cells and thus are assumed to give principle insights into the emergence of adverse health outcomes in whole organisms. Toxicity of fine particles from wood combustion has been studied on human and animal cell lines, such as, for instance from mice or hamsters as has been documented in certain studies (Danielsen et al., 2008, Tapanainen et al., 2011) and the review by Kocbach Bølling et al. (2009). Immortalized cell lines originate from (cancer) epithelia, fibroblast, macrophage or monocytic cells and are cultivated alone or in co-culture as lung epithelia cells, for example, which coexist with macrophages in the lung. In some cell studies the PM concentrations applied are indicated in µg PM/ m² instead of µg PM/ mL as cells grow in layers. Common tests with cell lines comprise the MTT test for cytotoxicity, the comet-assay and the micronucleus test for genotoxicity, the measurement of cell-signaling cytokines for inflammatory reactions and the depletion of glutathione as a signal of oxidative stress. In the MTT test the turnover of 3-(4,5-dimethylthiazol-2-yl)-2,5-10 diphenyltetrazolium bromide (MTT) by dehydrogenases in the cell is related to cytotoxicity. In the comet-assay DNA strand breaks are quantified by means of electrophoresis. In the micronucleus test the formation of a micronucleus during cell cycle is monitored. This kind of formation is a sign of chromosome aberration, thus, the test detects genotoxicity. As glutathione is an antioxidant, its depletion indicates oxidative stress. Besides, genetically modified cell lines are used in order to detect the presence of bioavailable chemical groups or certain receptor mediated endpoints. For this purpose, specific receptors such as the Ah receptor are inserted into the cell's DNA and coupled to detectable reporters such as the enzyme luciferase. Estrogenicity or the presence of PAHs were detected in ambient air samples and fine dust from wood combustion by the CALUX® assay that was constructed in this way (Walker, 1997, Wenger et al., 2009).

Tapanainen et al. (2011) exposed mouse macrophages for 24 hours to different concentrations (15 – 300 µg/ mL) of fine dust suspended in water. Fine dust samples were gathered from a pellet boiler, a conventional masonry heater and a sauna stove. Measured endpoints comprised cytotoxicity by means of the MTT test, production of a pro-inflammatory cytokine and chemokine, programmed cell death (apoptosis) and DNA damage by means of the comet

assay. Cytotoxicity, inflammation and genotoxicity were detected in all PM samples, whereas emissions from the conventional heater caused higher apoptosis rates and DNA damage than the pellet boiler. Particles from the sauna stove showed extensive cytotoxicity. Inflammatory responses were similar in all samples. Chemical analyses of PAH contents, for instance, did partially reflect toxicity results. The authors concluded that PM from incomplete combustion (conventional masonry heater and sauna stove) might be toxicologically more potent. In the study by Danielsen et al. (2008) both organic extracts of PM from combustion of wood and their respective native samples (2.5 – 100 µg/ mL) caused DNA damage (tested in the micronucleus test) in a human lung epithelial and a leukemia cell line. Similarly, the review by Kocbach Bølling et al. (2009) reported chromosome breakage detected in a micronucleus test after PM exposure of lung fibroblast cell line from Chinese hamsters. Exposure was conducted by suspending fine dust particles from different wood stoves in cell media. In the same review five studies with human macrophages, a human lung and epithelial cell line or co-cultures of monocytes and pneumocytes were compared. In all studies increased levels of cytokines were measured after exposure to PM of wood combustion. Besides, biological responses comprised cytotoxicity (MTT test), oxidative stress (glutathione depletion) and apoptosis.

Regardless of differences in sample preparation (organic extracts, aqueous extracts or native samples), cell types, PM samples or applied concentrations in cell studies, examined *in vitro* studies present a similar picture of toxicological outcomes after exposure to wood combustion-derived PM as *in vivo* studies: Inflammation, cytotoxicity, oxidative stress, and genotoxicity. Thus, *in vitro* cell studies accompany *in vivo* studies.

3.5.3.3 Microorganisms as Test Objects

Being unicellular as cells but less challenging in laboratory maintenance, bacteria are another promising tool for primary toxicity testing of complex fine particles originating from the combustion of wood. Representing principal cell functions, microorganisms have been used to assess common toxicities of environmental samples (Rönnpagel et al., 1995). On the basis of microbial bioassays environmental pollution was assessed by testing aqueous samples as well as solid matrices like soils and sediments. The best known bacterial test for mutagenic substances is the Ames-test. It was developed in the 1970s and has been used intensively for studying mutagenicity of both single substances and environmental samples since then (Ames et al., 1972, Reifferscheid and Heil, 1996). Organic extracts of wood smoke were tested as mutagenic in the Ames-Test in studies from the 1980s (reviewed by Naeher et al. (2007)) as well as in recent studies (Vu et al., 2012). Another commonly used bacterial assay is the

Vibrio fischeri bioluminescence inhibition test. The decrease in luminescence which indicates respiratory activity reflects baseline toxicity of exposed samples. With this bioassay Kováts et al. (2012) tested untreated outdoor aerosol samples collected in Budapest and discovered them to be ecotoxic. In the study by Barbosa et al. (2012) the bioluminescence test was part of an ecotoxicological test battery with five indicators applied on eluates of fly ashes from wood combustion. These fly ashes were classified as ecotoxic whereby the bioluminescence assay was most sensitive. For estrogenicity testing the human estrogen receptor (hER) gene was implemented into yeast (*Saccharomyces cerevisiae*) and coupled with the production of the detectable enzyme galactosidase. Soot particles that derived from wood combustion in Chinese villages were tested positively with such yeast bioassay (Wang et al., 2003). Additionally, as with the CALUX® assay with cell lines bacterial sensor-reporters are constructed in order to target specific chemical classes or compounds. Such reporter assays were used for environmental risk assessment of polluted sites with BTEX (benzene, toluene, ethylbenzene and xylenes), for instance (Tecon and van der Meer, 2008). Similar approaches were conducted by Funaska et al. (2003) with ambient air samples. In search of strongly mutagenic nitroarenes the authors tested urban atmospheric particles with a nitroarene sensitive *Salmonella* strain and obtained positive results. To the author's knowledge such bacterial sensor-reporters have not yet been applied to PM exclusively from wood combustion, but this may attract more attention in this research field in future.

In general, microbial bioassays offer a promising *in vitro* technique for toxicity screening of PM from wood combustion. They can be applied to solid samples like fine dust, are simple in laboratory handling and are efficient in terms of time and cost. Besides addressing solely human toxicity, ecotoxicity testing of fine dust may in future become more important for risk assessment since wood combustion emissions reach different environmental compartments as a result of atmospheric dispersion. Testing solid and aqueous environmental samples on microorganisms has a long tradition in ecotoxicology studies. Standardization of several tests (e.g. for Umu-Test or the *Vibrio fischeri* inhibition test) exist and are thus obtained results accepted from authorities (Bilitewski, 2007). Therefore, microbial bioassays may provide an excellent tool for testing both human and ecotoxicity of fine dust from wood combustion.

3.5.3.4 Main Drawbacks of in vitro Studies

No matter how they are exposed, *in vitro* models with cells or microorganisms have certain significant drawbacks: Due to their lower organization level they cannot mimic interactions between different cell types, tissues or organs that occur in higher levels. Nevertheless, they can give answers to specific mechanistic research questions such as binding processes of

xenobiotics on sensitive molecules like the DNA. Furthermore, i*n vitro* models exclude the complex inhalation process in the whole respiratory tract and its dependent processes like retention, clearance and alteration of particles (Kocbach Bølling et al., 2009). Therefore, *in vitro* studies should be complemented by geometric models that calculate deposition in the respiratory tract. The international commission of radiological protection considered in its model, for instance, breathing rate and percentage of air that finally reaches lung cells and blood stream (Bailey, 1994). Nowadays transport and deposition of aerosols are simulated in computational models. Such *in silico* models are generally in good concordance with *in vivo* studies (Longest and Holbrook, 2012). Advances in dosimetry models for pharmaceutical aerosols overlap with those for cigarette smoke and ambient air. Synergistic outcomes can be expected. In general, *in vitro* and *in vivo* studies cannot replace each other and should be considered as reciprocally complementary instead. *In silico* models might become more important in future for simulating deposition processes in lungs. Both *in vitro* and i*n vivo* studies may benefit from *in silico* models.

Altogether the given overview about the increasing use of (private) wood combustion, its impact on air quality and on human and environmental health underlines the necessity of toxicological evaluation of different small-scale wood combustion appliances. This is a prerequisite for further technical advancement and mitigation actions. The microbial test battery which is presented in this study may offer an excellent tool to meet this goal.

4 Material and Methods

4.1 Reagents and Chemicals

Medium and solutions in the BCT and the Umu-Test were prepared according to the German Standard Protocol (DIN 38412-48) and the Guideline ISO 13829 respectively. The solutions of the Yes-Test were prepared according to the descriptions provided by McDonnell et al. (1991a, b). Growth medium ingredients in the BCT, the Umu- and the Yes-Test were obtained from Carl Roth, Germany. Resazurine and resorufin (Fluka Chemie, Germany) were buffered with 3-(N-Morpholino) propanesulfonic acid (MOPS) (Carl Roth, Germany). For the Umu-Test, B-buffer and phosphate buffer were composed of disodium hydrogenphosphate dihydrate (Na2HPO4×2H20), sodium dihydrogenphosphate monohydrate (NaH2PO4×H2O), sodium dodecyl sulphate and magnesium sulphate heptahydrate (MgSO4×7H2O) from Carl Roth, Germany, and potassium chloride from Fluka Chemie, Germany. D(+)-Glucose (99.5%, Sigma-Aldrich, Germany), L-histidine-HCl, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) from Merck, Germany, were used in both the Umu- and Yes-Test. The reaction medium in the Yes-Test contained copper (II) sulfate 5-hydrate (CuSO4 * 5 H2O) from Merck, Germany. Antibiotics in the Umu- and the Yes-Test were ampicillin (Merck, Germany) and streptomycin (Carl Roth, Germany). Sodium carbonate (Na2CO3) from Carl Roth, Germany, served as stop reagent. O-Nitrophenol-ß-D-galactopyranoside (≥ 98%) (ONPG), lyticase and ß-mercaptoethanol (≥ 99%) was obtained from Sigma-Aldrich, Germany. Metabolic activation was carried out using β-Nicotinamide adenine dinucleotide phosphate sodium salt hydrate (3'-NADP) (≥ 98%) (Sigma-Aldrich, Germany) and S9 rat liver extract, induced by Arochlor (Trinova Biochem, Germany). Benzyldimethyl-hexadecyl-ammoniumchloride (BAC), mitomycin C (MMC), 17ß-ethinylestradiol (≥ 98%) (EE2), 2-aminoanthracene (≥ 96%) (AA) and 1,3 Dinitropyrene (99%) from Sigma-Aldrich, Germany served as positive controls. DMSO from Sigma-Aldrich Co. was used to dissolve lipid soluble chemicals. For combination experiments zinc chloride ($ZnCl_2$) (≥ 97%, p.a., Carl Roth, Karlsruhe), Fluoranthene (FLA) (p.a., Carl Roth, Karlsruhe) and a mixed cellulose ester membrane (Schleicher & Schüll, 0.2 µm) was used.

4.2 Tested Samples

4.2.1 Types of Samples

Fine particle samples (FPs) comprised bulk material from different stoves and boilers (9-15 kW) and PM loaded on Ø 47 or 150 mm quartz filters (MK 360, Munktell, Sweden) (Figure 1). Some samples were led through an atmospheric simulation chamber before deposition on filters (described in '4.2.2.3 Simulated Atmospheric Transformation of Emissions'). As model crystalline and amorphous quartz particles (d 50% = 0.012, 2, 4, 125 µm) were non-toxic to bacteria or yeast, size-segregated toxicity was not considered in this study.

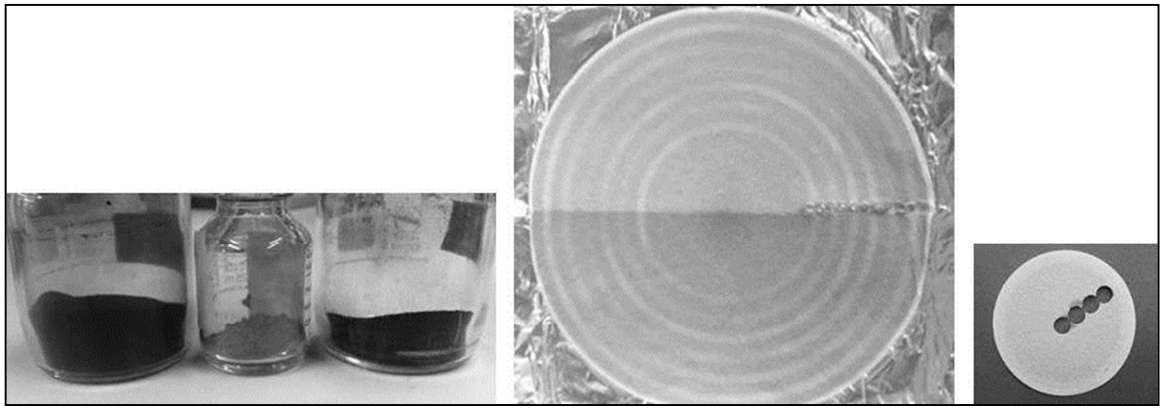


Figure 1: Bulk fine dust and fine dust precipitated on a Ø 150 mm quartz filter and Ø 47 mm.

4.2.2 Combustion Experiments

4.2.2.1 Bulk Fine Dust Samples

All fine dust samples that were collected by electrostatic precipitation were delivered as dust samples from the German Biomass Research Center (DBFZ), Leipzig and the Technology and Support Center, Straubing. In a field experiment about 800 kg non-pretreated wood briquettes (hard and soft wood) and 1 m³ split logs were burned for heating purposes in winter 2008/2009. The fireplace was a manually operated tiled stove oven (9 kW nominal heat output), installed in a private household. Fine particle sample A (FP A) is a mixed sample gained from the flue gas canal, the electrostatic precipitator and the chimney. FP C and D was produced by firing beech split logs in a wood stove in batch mode (for 270 and 245 min every 0.5 h logs were put into the stove). FP B and FP C were collected by an electrostatic precipitator, installed in the flue gas duct directly behind the furnace. For FP B, E, F and G an automatic pellet boiler was operated under experimental conditions with standardized wood

pellets. The furnace was carried out in the combustion laboratory and the fine dust precipitated electrically 3-4 m away from the furnace. The colors black and grey reflected the amount of soot that is generated depending on whether the combustion was close to complete (grey) or incomplete (black). Besides the soot amount gas emissions (e.g. CO values) characterized a complete or incomplete combustion. Table 3 gives an overview of fine particle origin.

Table 3: Origin of bulk fine dust samples.

Labeling of PM samples	Wooden fuel	Fire place	Type of Combustion
FP A	briquettes/ split logs	low quality stove, 9 kW	incomplete
FP B	pellets	pellet boiler, 15 kW	complete
FP C	split logs	high quality stove, 9	incomplete
FP D	split logs	high quality stove, 9	incomplete
FP E	pellets	pellet boiler, 15 kW	complete
FP F	pellets	pellet boiler, 15 kW	incomplete
FP G	pellets	pellet boiler, 15 kW	complete

4.2.2.2 Filter Samples

Precipitated particulate matter from a high quality pellet boiler (Pelletti II from Paradigma, 20 kW) was gained electrostatically on multiple quartz filters at the DBFZ. Electrical precipitation took place 3-4 m away from the furnace. One or more filters (Ø 45 mm) were used for chemical analysis, one for toxicity tests (Ø 150 mm). The combustions were close to complete as CO exhausts and produced carbon contents were very low. Used pellets varied in water content, location of cutting, type of wood and ash content (Table 4). If not indicated otherwise, pellets consisted of spruce without bark with a water content of 7.05 ± 0.95 %. Dried loaded filters were weighed and stored at -20°C until testing.

Table 4: Overview of variations of the pellets. The location indicated two different sites of cutting.

water content	location	type of wood	bark content
2.9 wt%	II	pine	10 wt%
6.5 wt%	III	cottonwood I	20 wt%
7.3 wt%		cottonwood II	30 wt%
12.1 wt%			40 wt%

4.2.2.3 Simulated Atmospheric Transformation of Emissions

DIN-Norm pellets of spruce were incinerated in an automatically loaded 7 kW pellet boiler (entry 1, Table 5) and beech logs were burned in a modern 5 kW wood stove (entry 2, Table 5). All combustions were close to complete as the CO emissions were ca. 4 ppm (pellet boiler) and 940 ppm (wood stove). Emitted fine dust were led through a 19 m^3 atmospheric simulation chamber and subsequently collected on quartz filters. A detailed description of the aerosol chamber LEAK at the Leibniz Institute for Tropospheric Research (TROPOS) is given elsewhere (Iinuma et al., 2009). Afterwards the flow through the chamber was stopped and the chamber was closed. Detected NO and NO_2-concentrations derived from the emissions. For aged samples O_3 (nighttime) or O_3+sunlight (daytime) was fed into the chamber. After ~ 2 h of chemical processing particles were sampled again (aged samples) (Q = 8 L/min, V= 0.48 m^3) for one hour on two pre-heated micro quartz fibre filters (Ø 47 mm, MK 360, Munktell, Sweden) per trial. One filter was used for elemental/organic carbon- and PAH-analysis, the second filter for toxicity testing. The FP weight was estimated on the basis of volume size distribution. Loaded filters were stored at -20°C until testing.

Table 5: Sampling set up and occurrence of gaseous components. rh: Relative humidity.

Sample name	min	rh [%]	O_3 [ppb]	NO [ppb]	NO_2 [ppb]	Sampling description
	0	28	0	90	14	Start sampling Daytime 1
Daytime 1	60	29	0	85	8	End sampling Daytime 1
	65	29	0	87	8	Feeding of O_3
	86	29	0	23	64	Stop Feeding O_3, close chamber
Daytime 1_aged	90	29	0	21	66	UV-lamps on
	210	32	8	31	48	Start Sampling Daytime 1_aged
	270	32	9	30	44	End Sampling Daytime 1_aged
Nighttime 1	0	23	0	115	6	Start sampling Nighttime 1
	60	27	0	116	7	End sampling Nighttime 1
	61	27	0	115	6	Feeding of O_3
	80	27	33	1	111	Stop Feeding O_3, close chamber
Nighttime 1_ aged	200	28	11	0	91	Start Sampling Nighttime 1_aged
	260	28	7	1	87	End Sampling Nighttime 1_aged
Daytime 2	0	18	0	34	1	Start Sampling Daytime 2
	60	24	0	57	8	End Sampling Daytime 3
	61	24	0	57	6	Feeding of O_3
	69	24	17	7	38	Stop Feeding O_3, close chamber
	76	24	23	1	53	UV-lamps on
Daytime 2_aged	196	25	35	7	39	Start Sampling Daytime 2_aged
	256	25	36	7	40	End Sampling Daytime 2_aged
Nighttime 2	0	25	0	23	0	Start sampling Nighttime 2
	60	28	0	39	8	End sampling Nighttime 2
	61	28	0	37	2	Feeding of O_3
	71	28	46	1	36	Stop Feeding O_3, close chamber
Nighttime 2_aged	191	27	41	1	23	Start Sampling Nighttime 2_aged
	251	26	36	1	13	End Sampling Nighttime 2_aged

4.2.3 Physico-Chemical Characterization

For bulk FPs and filter samples loaded with non-aged fine dust PAHs were analyzed according to the German Standard Protocol DIN EN 15549. Samples were extracted by accelerated triple solvent extraction with dichlormethane in Dionex ASE 150 Extraktor and

Dionex SE 500 Concentrator. The subsequent detection was carried out in an Agilent gaschromatograph 6890 and quantified with a mass selective detector Agilent 5975C. Elemental and organic carbon was determined by extraction and thermodesorption on the basis of the German protocol VDI 2465/1. The VDI 2465/1 method includes removal of organic carbon (OC) by solvent extraction and thermodesorption in nitrogen to determine the elemental carbon (EC) fraction by subsequent combustion. OC is determined by difference between total organic carbon (TOC, combustion of the whole sample) and EC. For metal detection FPs were treated in a microwave and suspended with HNO3 p.a., diluted with deionized water and analyzed with a ContrAA 700 atomic absorption spectrophotometer. Anions were analyzed after a 30 min water extraction (permanent shaking) by ion chromatography (DIONEX ICS 90) and photometry (nitrite). Particle sizes and zeta potential were measured in aqueous suspensions of bulk fine dust (not filter samples) with a Zetasizer Nano ZS (Malvern Instruments GmbH, Herrenberg, Germany). For technical reasons the zeta potential of the grey colored FPs (complete combustion) was determined 1 mg/mL suspensions, whereas for the black colored FPs (incomplete combustion) 0.1 mg/mL suspensions were used. Particle sizes and zeta potential measurements were kindly made by S. Gauggel at the University of Konstanz.

For filter samples originating from simulated atmospheric experiments PAHs and nitro-PAHs were analyzed. PAHs were detected by Curie Point Pyrolysis-GC/MS (Aufderheide et al., 2003). For nitro-PAH analysis 1.2 m³ were sampled on the filters, quantification was based on the method described by BMU: Thrän and Pfeiffer, 2012. The method was optimized for 7 nitro-PAHs (1-nitropyrene, 1-nitroperylene, 2-nitrofluorenone, 3-nitrofluoranthene, 7-nitrobenz[a]anthracene, 6-nitrochrysene, 6-nitrobenzo[a]pyrene). Quantification of 9-nitroanthracene and the dinitro-PAHs was not possible as no corresponding signal (mono- or di-derivatised amino-PAH) was found from the GC/MS analysis. Metals were not analyzed for aged FP since they were not relevant for chosen bioassay (Umu-Test with tester strain sensitive to nitro-PAHs).

4.2.4 Sample Preparation and Controls

A stock suspension (1000 mg/L) of each bulk fine dust samples was prepared with deionized water in falcon tubes, vortexted for 1 min, ultrasonicated (35 kHz) for 15 min and shaken horizontally for 24 h at room temperature before use. Deionized water without particles was employed as control for all three assays. In the bacterial contact test bulk fine dust samples were tested without prior overnight shaking additionally. Final test concentrations are specified in Table 6 and Table 7. For filter samples small cutouts (Ø 5 mm) of loaded filters

(Ø 150 mm and Ø 47 mm) were taken along the filter radius and placed in each well of a 96 well plate. Each Ø 150 mm quartz filter sample was tested 3 times with 6 internal replicates. Filter evaluation of aged samples (Ø 47 mm) consisted of 3 independent test repetitions with 4 small cutouts (internal replicates). The bioassays were performed as with the bulk fine dust replacing the FP suspension with deionized water. For the bacterial contact assay benzalkonium chloride (64 mg/L) served as positive control, for the Yes-Test 17ß-ethinylestradiol (1 ng/L), for the Umu-Test mitomycin C (0.25 mg/L), 2-aminoanthracene (2.0 mg/L) and 1,3 Dinitropyrene (0.1 ng/mL) served as positive controls.

4.3 Test Organisms

The bacterium *Arthrobacter globiformis* was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ – Braunschweig, Germany). Two *Salmonella* strains were used in this study: The conventional tester strain *Salmonella typhimurium* TA1535/pSK1002 and the nitro-PAH sensitive *Salmonella typhimurium* NM3009 strain. Both *Salmonella* strains and the recombinant yeast *Saccharomyces cerevisiae* BJ3505 (Protease deficient; MATα, PEP4::HIS3, prb-1-delta1.6R, HIS3-delta200, lys2-801, trp1-delta101, ura3-52gal2can1) were kindly provided by the German Federal Institute of Hydrology (BfG – Koblenz, Germany).

4.4 Applied Bioassays

4.4.1 Microbial Assays and Modifications

In the present study three standard microbial bioassays, common for testing soil, water and sediment samples, were adapted for the investigation of wood combustion fine particles. One integrative test for baseline toxicity and two selective assays for genotoxicity and estrogenicity were chosen according to expected MoAs.

The bacterial contact test (BCT) with *Arthrobacter globiformis*, an aerobe soil bacterium as target organism, is used to test baseline toxicity of solid environmental samples like soils or sediments (Rönnpagel et al., 1995). The test was performed according to the German Standard Protocol (DIN 38412-48) and miniaturized on the basis of Heise and Ahlf (2005). The initial inactivation step by heat denaturation of the samples was left out due to the absence of endogenous dehydrogenase activity in FPs. The fluorescence was measured every 15 minutes for 1 hour (Fluorimeter FLx800 TBIE, Bio-Tek). Its increase was directly proportional to the dehydrogenase activity and therefore a degree of the toxicity of the samples. In order to account for a potential quenching effect on the fluorescence signal of

dark stained FPs (Ahlf, 2007), a calibration for each FP was carried out as follows: 80 µL of ratios of isomolar (0.179 mM) resazurin and resorufin (4/0, 3/4, 1/2, 1/4) were added to all used concentrations of FP. By means of this calibration the produced mol% of resorufin was calculated after test performance and related back to the control samples as follows:

$$\textit{Relative Inhibition}\,[\%] = [1 - (\textit{Ss sample})/(\textit{Ss control})] \times 100 \qquad (1)$$

Ss sample: Slope of mol% - resorufin over 1 h of the control

Ss control: Slope of mol% - resorufin over 1 h of the sample

Among the selective tests, the Umu-Test was chosen to assess genotoxicity. *Salmonella typhimurium* TA1535/pSK1002 is used as genetically modified tester strain in the conventional Umu-Test. The coupling of the umuC-gene and the lacZ-gene, which encodes ß-galactosidase, enabels the detection of DNA damage. By inducing the transcription of the umuC-gene, DNA damage becomes visible. A second *Salmonella* strain was used in order to detect the possible formation of nitro-PAHs during simulated atmospheric transport. This NM3009 *Salmonella* strain overexpresses O-acetyltransferase Overexpression of O-acetyltransferase and nitroreductase genes was achieved via an insertion of a specific promoter thereby increasing the sensitivity of NM3009 exposed to nitro-PAHs. Both *Salmonella* strains were created by Yoshimitsu Oda (Oda et al., 1985, Oda et al., 1993).
Conventional growth measurement on the basis of optical density (OD) was replaced in both the Umu- and the Yes-Test. Instead, viability assessment of test organisms was performed via reduction of resazurin. This method is based on the hydrolytic activity of the test organisms using the blue redox-substrate resazurin and the subsequent kinetic measurement of the fluorescent product resorufin (Figure 2).

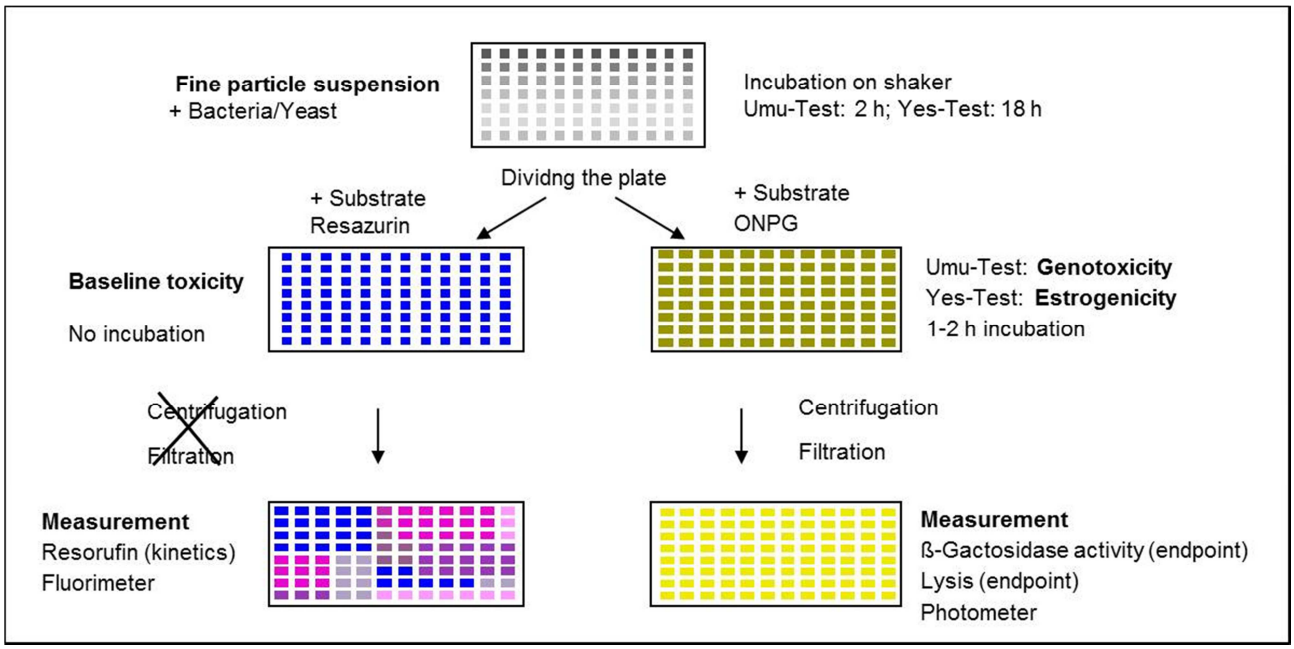


Figure 2: Methodological scheme of modified Umu- and Yes-Test.

The detection of the induction of the umuC-gene was performed on the basis of the standard protocol (Guideline ISO 13829) including viability testing and a filtration and centrifugation step for FP suspensions as follows: For better handling, the test volume in one well was reduced from 270 µL (conventional test) to 210 µL (modified test). For umuC gene detections 150 µL of the sample mixture were filtered through 0.2 µm filter plates by centrifuged (766 g for 10 min). The clear filtrate was measured at 420 nm photometrically for ß-galactosidase activity as in the conventional test (endpoint-measurement). After the incubation and before filtration absorbance of the controls were measured at 600 nm to check if the lysis of the cells was successful. Bacterial viability was determined by the addition of 90 µL resazurin (0.179 mmol, 1 mM Mops-buffer) to 180 µL of the original plate and a subsequent fluorimetric measurement (545/590 nm) of resorufin every 5 minutes (kinetics-measurement) until the fluorescence signal decreased (after 35±5 min). This bacteriotoxicity was only measured in the plates without metabolic activation (S9-mix) as the enzymes of the S9-mix metabolized the resazurin. But as both plates (with and without S9-mix) were prepared simultaneously growth data of the plate without S9-mix were used for calculation of both plates. A calibration plate was measured in parallel to the BCT.

The induction ratio (IR) of the modified Umu-Test was calculated as followed:

$$IR = 1/G \times (A420\ sample - A420\ blank)/(A420\ control - A420\ blank) \quad (2)$$

A420 sample: Extinction of the sample well at (420 ± 20) nm

A420 control: Extinction (mean value) of the control at (420 ± 20) nm

A420 blank: Extinction (mean value) of the blank at (420 ± 20) nm

G: Growth factor

$$G = (Ss\ sample)/(Ss\ control) \quad (3)$$

Ss sample: Slope of mol% - resorufin of the sample

Ss control: Slope of mol% - resorufin of the control

The receptor-mediated yeast estrogen screen (Yes-Test) using *Saccharomyces cerevisiae* as test organism was the second selective test in this study. The detection of estrogenicity is achieved by implementing a human estrogen receptor coupled to the lacZ gene. Although several optimizations of the test were proposed (De Boever et al., 2001, Kase et al., 2008), there has been no internationally standardized method so far. The loading of the plate was performed according to Kase et al. (2008), whereas the ß-galactosidase assay was started by the transfer of 60 µL of the suspension to 100 µL of lacZ mixture containing the substrate ONPG and subsequent incubation for 1 h at 37°C, 400 rpm. Having added the stop reagent and shaken at 1400 rpm, filtration, centrifugation and subsequent measurement at 600 nm and 420 nm was carried out as described above for the Umu-Test. Photometric viability measurement was also replaced by resorufin kinetics: 60 µL of resazurin (0.179 mmol, 20 mM Mops-buffer) were added to the original plate and mixed properly at 1400 rpm. Thereby the elevation of the pH from acidic (pH=3) to neutral – slightly alkaline conditions (pH=8) by the addition of buffered resazurin is notable. Resorufin was measured (545/590 nm) every 5 minutes while shaking the plate at 400 rpm until fluorescence descended (after 110±30 min). Every 10 min the plate was shaken for 30 s at 1400 rpm to prevent agglomeration and precipitation of the yeast. A calibration plate for resazurin and resorufin was done as in the previous tests. Growth and induction ratio was calculated according to equation (2) and (3). In order to relate the ß-galactosidase activity to the potency of estradiol, a dilution series of 50 and 100 ng/L of 17α-ethinylestradiol was tested in parallel to each assay.

4.4.2 Bioassays with Nematodes and Cell Lines

The main goal of the toxicological part of the overall project was to find an eligible test battery for a first toxicity screening of PM from wood combustion. The evaluation of all applied biotests in this project was done in this study. Bioassays which were not performed from the author of this study are described in the following.

Among unspecific MoA tests were one the MTT assay and the Nematode Reproduction Test. In the MTT assay dehydrogenases transform the yellow 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the purple formazan via NAD(P)H-dependent cellular oxidoreductase enzymes which is then measured spectrophotometrically. Thus a reduction of MTT transforming capability of human lung epithelial A-549 cell line as described in Gauggel et al. (2012), can be used to determine cytotoxicity (loss of viable cells) . A-549 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ – Braunschweig, Germany). The Nematode Reproduction Test with *Caenorhabditis elegans* determined the number of offspring per exposed test organism, as described in Guideline ISO 10872. *Caenorhabditis elegans* were obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota. Among specific MoA tests were an estrogen- and androgen receptor assay. The MELN- (MCF-7-ERE-bGlo-Luc-SVNeo) and the PALM- (PC3-AR-Luc-MMTV) cell lines, developed by the group of Patrick Balaguer at INSERM, Montpellier, France, detect estrogen and androgen agonists, respectively, due to combination of the estrogen- and the androgen receptor with the luciferase reporter gene. Measured luminescence is proportional to estrogen- or androgenicity, respectively. The MELN-assay was performed according to Witters et al. (2010), the PALM according to Freyberger et al. (2010) with the exception of cytotoxicity assessment, which was assessed by the MTT test. The commercially available PAH CALUX® bioassay (Biodetection Systems, Amsterdam, the Netherlands) links the Ah-receptor with a luciferase gene and thus detects Ah-receptor agonists like PAHs or dioxins, and is capable of detecting picograms (10-12 grams) of benzo[a]pyrene equivalents (EQs), largely depending on the sample matrix. Culture of PAH CALUX® cells as well as the bioassay were conducted as previously described by Gauggel et al. (2012). An overview of applied bioassays and tested concentrations is given in Table 6 and Table 7.

Table 6: Overview of applied tests of unspecific Mode of Action. BCT: Bacterial Contact Test.

Unspecific-Mode-of-Action-Tests			
	Baseline toxicity		
Name of the Test	BCT	MTT Test	Nematode Reproduction Test
Organism	*Arthrobacter globiformis*	Human lung adenocarcinoma epithelial cell line (A 549)	*Caenorhabditis elegans*
Duration of the Test	24 h	4 d	7 d
Exposure Time	2 h	48 h	96 h
Endpoint	Metabolic activity	Metabolic activity	Reproduction
Applied Concentration	0.03 - 400 mg/L	0.01 - 25 µg/cm² ≈ 0.018 - 44 mg/L	1 - 1000 mg/L

Table 7: Overview of applied tests of specific Mode of Action.

Specific-Mode-of-Action-Tests					
	Genotoxicity	**Endocrine Disruption Potential**			**Presence of PAHs**
Name of the Test	Umu-Test	Yes-Test	Estrogen receptor assay	Androgen receptor assay	PAH CALUX® Assay
Organism	*Salmonella typhimurium TA1535/pSK1002*	recombinant *Saccharomyces cerevisiae*	human breast cancer cells (MELN cells)	human prostate cancer cells (PALM cells)	rat hepatoma cell line (PAH CALUX® cells)
Duration of the Test	24 h	3 d	10 d	10 d	2 d
Exposure Time	2 h	18 h	19-20 h	24 h	4 h
Endpoint	SOS-Induction	Estrogenicity	Estrogenicity	Androgenicity	AHR binding
Applied Concentration	5-667 mg/L	0.05-667 mg/L	0.016 - 10 µg/cm² ≈ 0.03 - 18 mg/L	0.016 – 10 µg/cm² ≈ 0.03 - 18 mg/L	0.05 – 45 µg/cm ≈ 0.09 - 79 mg/L

4.5 Combined Exposure of Zn and FPs or Fluoranthene

Besides testing previously described PM samples, for combination experiments different $ZnCl_2$ concentrations (0.5, 4, 8, 16 µM) with bacteriotoxicities ranging from 0 to 40 % were tested together with all bulk FP and three least toxic filter samples (30 - 70% bacteriotoxicity) loaded with non-aged fine dust. Chosen Zn concentrations were below EC_{50}. Furthermore three non-toxic concentrations of the PAH fluoranthene (FLA) (1.2, 2.5, 4.9 µM) and seven $ZnCl_2$-concentrations (0.5, 4, 8, 16, 31, 63, 125 µM) were tested together. A stock solution of

$ZnCl_2$ (0.01 M) was prepared in ultrapure water and sterilized by membrane filtration. All subsequent dilutions were prepared in ultrapure water. A stock solution of FLA (250 mg/L) was done in 100% DMSO which was reduced to 1% in final FLA dilutions. Due to possible evaporation and attachment to glass walls, FLA-dilutions were prepared and immediately applied before test began.

4.6 XRD Measurements

Placed on a rotating holder, the crystalline structure of fine particle samples were measured by a Siemens D500 X-ray diffractometer that produces X-rays by a CU ceramic tube, type K FL Cu2K. 2Θ-Scanning ranged from 7 to 67° with increments of 0.05° and 3 sec per step. Pattern analysis was done with the software DIFFRAC^{plus} (Version 11, Bruker AXS) and DIFFRAC^{plus}EVA (Version 2005) that uses the JCPDS powder diffraction file of the International Centre for Diffraction Date (ICDD). Higher quantities of mineral phases result in higher peak heights though no exact quantification is possible. As only mineral structures are diffracted by X-rays, amorphous samples cannot be characterized by this method.

4.7 Statistical Analysis

In order to validate the Umu- and the Yes-Test the coefficient of variation (CV) was calculated for induction ratios. Significant differences to the control (no particles) were tested with the unpaired t-test (p=95%) with SPSS (bacteria, yeast, nematodes) or with a One-Way Anova with Dunnett's Multiple comparison Test followed by a Bonferroni post test using Graph Pad Prism ® (cell lines). EC_{50}-values were calculated when possible. LOEC refers to the lowest concentration applied that showed a significant effect. Bivariate correlations (Pearson) between chemical species and toxicity results were calculated with SPSS.

4.8 Overview of Applied Methods

This study consisted in three main research stages that are shown in Figure 3. First, toxicity screening of bulk FPs and filter samples was conducted on the basis of the modified microbial test battery. Obtained toxicity results were compared to those of nematode and cell line testing. A relationship to physico-chemical characteristics of test samples was looked for. Regarding possible relationships, Zn was suspected to play a major role for baseline toxicity. To clarify this assumption, Zn was exposed simultaneously with PM samples and FLA. Additionally, XRD measurements were used to examine existing Zn species. The third part consisted in genotoxicity testing of FPs that were artificially oxidized in an atmospheric

simulation chamber. Since it was expected that genotoxic nitroarenes predominantly form during atmospheric transport, a nitroarene sensitive *Salmonella* strain served as test organism.

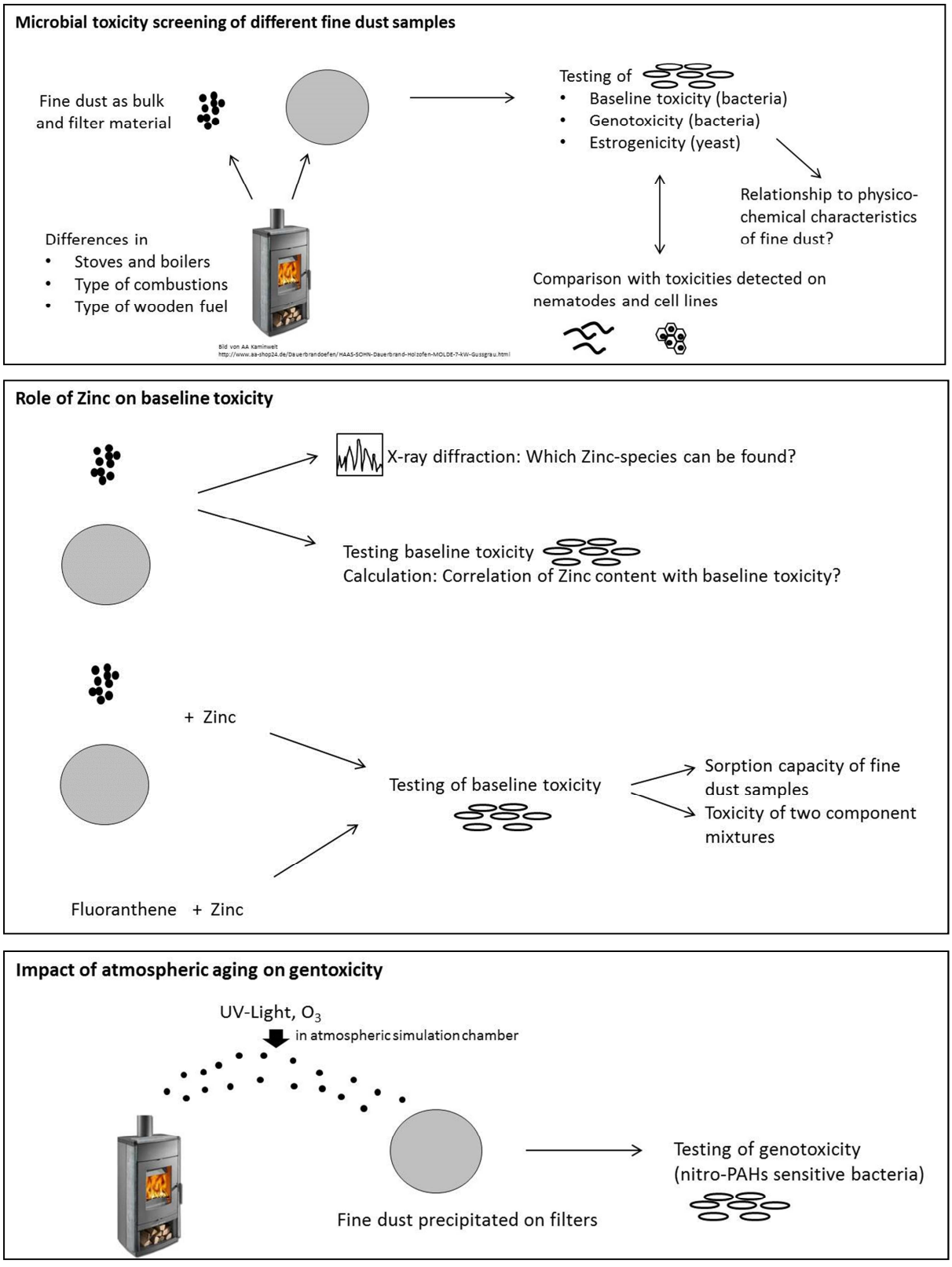


Figure 3: Methodological scheme of this study that consists in three main research parts.

5 Results

5.1 Parameters of Adapted Test Systems

In order to establish a biotest battery for the detection of PM bound chemicals, the test procedures had to be modified for the specific conditions. Suspensions of fine dust do not allow photometric measurements for the analysis of microorganisms' growth. Therefore, at first the standardized test procedures had to be checked for validation criteria and, in case of need, were modified.

5.1.1 Activity Measurement by Resazurin Turnover

In order to account for particle bound contaminants, test organisms were exposed to aqueous suspensions to assure the direct contact of test organisms and fine particle samples. Thereby, one crucial modification of selective bioassays was needed: In the original Umu- and Yes-Test procedures, the growth factor G is determined using optical density measurements at 600 nm (OD600). Since such turbidity measurement is disturbed by the presence of particles, determination of G needed to be changed. Therefore, conventional growth measurement was replaced by viability assessment via reduction of resazurin. This way, the adapted versions of the Umu- and the Yes-Test presented here employed resorufin kinetics (fluorimeter) in order to exclude fine particle induced turbidity and to provide for a more accurate count of viable bacterial cells than established with mere turbidity measurements. For calculation of the enzyme kinetic factor only the linear resorufin portion of the production curve (= equivalent to maximal resazurin turnover) was considered. Thus, monitoring of resorufin was stopped when the exponential phase was characterized with at least five data points. Speed of resorufin production and maximal resorufin amount after 40 min was Umu-Test > BCT > Yes-Test (Figure 4).

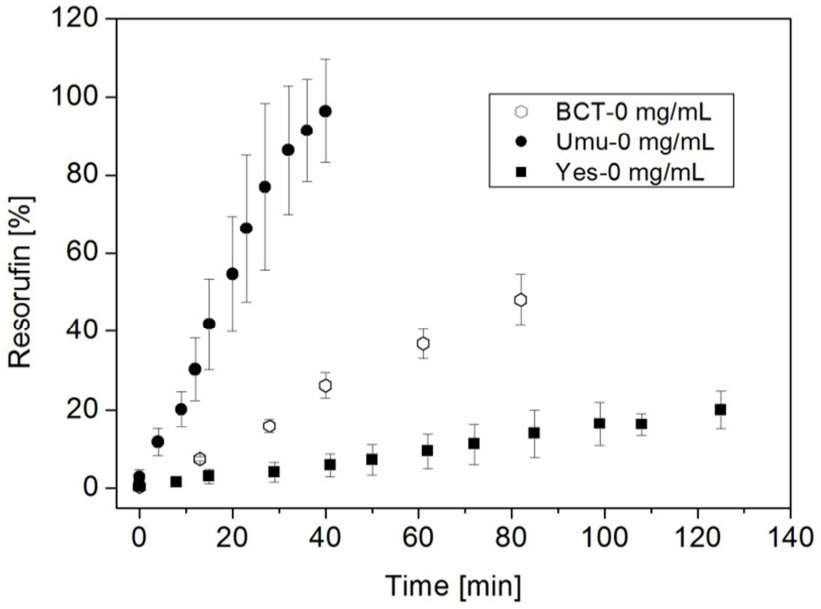


Figure 4: Production of resorufin in the Bacterial Contact Test (BCT), the Umu-Test and the Yes-Test under control conditions (no particles added).

The growth factor G calculation within the controls in the adapted method was compared to the conventional method. In the Umu-Test G was calculated for the positive control mitomycin C (MMC) (n=14) which inhibits DNA replication. Using OD600 G was on average 16% significantly higher than that determined using resazurin reduction (Table 8). In the Yes-Test G calculations for the solvent control, 1% ethanol, did not differ significantly from each other (n=8).

Table 8: Comparison of growth factor G calculations by optical density (OD600) and resazurin turnover and their statistical difference. MMC – Mitomycin C. EtOH – Ethanol.

G calculated		
	Umu-Test	Yes-Test
Measurements N	14	8
Chemical	MMC	1% EtOH
OD600	0.89 ± 0.07	1.14 ± 0.30
Resazurin turnover	0.75 ± 0.10	0.88 ± 0.19
Difference in G	15.9 ± 9.3 %	21.7 ± 11.1%
Statistically different by t-test	yes	no

5.1.2 Variability of Modified Test Systems

In the modified Umu-Test the coefficient of variation (CV) for the induction ratio IR was 20% (-S9) and 18% (+S9) respectively, in the adapted Yes-Test 16%. Dose-response curves of 17ß-ethinylestradiol (EE2) (1.56 – 100.00 ng/L) were determined in the Yes-Test in order to calculate ethinylestradiol equivalents (EEEQ). The mean EC_{50} of EE2 in the adapted test version was 44.0 ± 16.6 ng/L.

5.2 Bulk Fine Dust Samples

After modification of the toxicity tests, they were applied to bulk fine dust samples. For this validation phase larger quantities of fine dust were required collected by electrostatic precipitation and chemically analyzed. Results were examined for repeating toxicity patterns and relationships to, for instance, different combustion types and chemical analysis.

5.2.1 Chemical Analysis

The chemical analysis of the bulk FPs presented in Table 9 shows that incomplete combustion FPs resulted in high contents of carbon (black color), complete combustion FPs in low contents of carbon (grey color). Except this, no chemical substance class was correlated to the type of combustion.

Table 9: Overview of chemical categorization of bulk fine particle.

Labeling of PM samples	Combustion	Particle Size [nm]	Color	Carbon content [wt%]	PAH content [mg/kg FP]	Heavy Metal content [mg/kg FP]	Anion content [mg/kg FP]
FP A	incomplete	161.5 ± 54.3	black	25	816	121329	196
FP B	complete	208.6 ± 166.1	grey	5	11	30320	374
FP C	incomplete	200.9 ± 60.4	black	73	78	6966	35
FP D	incomplete	178.5 ± 25.7	black	66	23	27365	398
FP E	complete	123.4 ± 73.5	grey	5	31	20568	377
FP F	incomplete	157.4 ± 19.7	black	23	25	19372	456
FP G	complete	57.2 ± 18.8	grey	4	35	26648	378

The mean zeta potential ζ of the aqueous suspensions was -33.7 ± 4.5 mV for all PM suspensions. Based on the negative Zeta potential recorded, FPs did not demonstrate an increased likelihood of aggregation. Detailed chemical characterization of all FPs is provided in Table 10, Table 11, Table 12 and Table 13.

Table 10: 16 US EPA PAHs measured in bulk FPs.

PAHs (mg/ kg fine dust)	FP A	FP B	FP C	FP D	FP E	FP F	FP G
Naphthalin	< 4	< 0.4	12.4	3.7	< 0.4	0.6	1.0
Acenaphthylene	7.0	< 0.4	< 7	< 0.4	< 0.4	0.9	0.9
Acenaphthene	< 4	< 0.4	< 7	< 0.4	< 0.4	0.0	0.0
Fluorene	< 4	< 0.4	< 7	< 0.4	< 0.4	0.8	0.4
Phenanthrene	88.5	0.8	44.2	2.3	2.5	5.9	3.0
Anthracene	20.5	< 0.4	< 7	0.5	1.1	2.2	1.3
Fluoranthene	188.0	1.3	11.1	1.6	3.0	5.5	3.3
Pyrene	187.5	1.2	10.5	1.7	3.1	4.9	3.3
Benzo[a]anthracene	54.5	0.7	< 7	1.5	2.9	1.4	3.3
Chrysene	63.0	1.0	< 7	1.1	2.1	1.2	2.5
Benzo[b]fluoranthene	72.5	1.8	< 7	1.8	3.7	0.9	4.0
Benzo[k]fluoranthene	21.5	0.5	< 7	0.8	1.7	< 0.4	1.9
Benzo[a]pyrene	34.0	0.7	< 7	1.3	3.1	< 0.4	3.3
Indeno[1,2,3-c,d]pyrene	41.0	1.4	< 7	2.8	3.5	< 0.4	3.5
Dibenzo[a,h]anthracene	5.0	< 0.4	< 7	< 0.4	0.6	< 0.4	0.5
Benzo[g,h,i]perylene	32.5	1.2	< 7	2.6	3.1	< 0.4	3.0
Sum	815.5	10.6	78.2	22.8	31.5	25.5	35.4

Table 11: Detected metal contents of bulk FPs.

Metals (mg/ kg fine dust)	FP A	FP B	FP C	FP D	FP E	FP F	FP G
K	69363.5	879.2	18999.3	205762.1	137773.6	106704.5	206703.3
As	0.0037	0.0029	4.0	16.1	< 2.7	< 3.3	< 3.7
Cd	87.2	31.8	130.3	120.3	175.1	386.9	226.4
Cr	3.4	11.1	2.9	9.3	113.9	82.2	116.6
Cu	4.1	3.8	38.5	377.3	323.0	193.5	461.5
Ni	0.5	< 2.4	11.5	117.1	8.9	3.2	7.9
Pb	1462.6	127.6	1705.7	1585.5	not analyzed	not analyzed	not analyzed
Mn	2138.0	3692.9	2055.6	8011.2	8035.2	2501.7	11575.1
Zn	4178.8	11963.3	4023.8	14869.9	8574.5	15050.0	11106.2
Al	not analyzed	not analyzed	353.0	1215.8	1056.9	576.4	1499.6
Fe	114917.4	14616.5	699.8	3843.9	3336.9	1154.1	3153.8
Sum without K, Al, Pb	121329.3	30319.5	6966.5	27364.9	20567.5	19371.6	26647.6

Table 12: Detected anion contents of bulk FPs.

Salts (mg/ kg fine dust)	FP A	FP B	FP C	FP D	FP E	FP F	FP G
Fluoride	1.0	< 0.004	< 0.004	0.3	0.3	0.5	< 0.004
Chloride	42.4	55.2	< 0.004	41.6	46.3	79.2	39.7
Nitrite	< 0.004	27.2	< 0.004	38.5	15.5	< 0.004	34.6
Nitrate	1.1	< 0.004	< 0.004	8.5	12.9	9.9	8.3
Phosphate	< 0.004	4.8	< 0.004	< 0.15	< 0.004	< 0.004	< 0.004
Sulfate	151.6	287.2	35.2	309.1	302.3	366.0	295.8
Sum	196.0	374.4	35.2	397.9	377.2	455.6	378.4

Table 13: Detected carbon contents of bulk FPs.

Carbon content (% w/w)	FP A	FP B	FP C	FP D	FP E	FP F	FP G
EC	18.0	2.0	60.0	60.0	2.4	17.0	3.2
OC	6.7	2.5	13.0	6.0	2.8	6.0	0.4
Sum	24.7	4.5	73.0	66.0	5.2	23.0	3.6

5.2.2 Biotest Results

5.2.2.1 Unspecific-Mode-of-Action-Tests

Baseline toxicity of FPs was assessed employing the BCT (bacteria), the MTT Test (human cell line) and the nematode reproduction test. Except of FP C all samples from incomplete combustion showed baseline toxicities in at least one assay (Table 14), whereas all samples from complete combustion were toxic. In the BCT FP A and FP C were not toxic. The LOEC of toxic FP F and G was below the minimal applied concentration in the BCT. FP F was most toxic according to the EC_{50} value. The maximal applied concentration in the MTT test (25 µg/cm² ≈ 44 mg/L) did induce cytotoxicity only in FP D with a LOEC corresponding to this concentration. In the nematode reproduction test all FPs except FP C and D had a significant effect on the offspring production (Figure 5). No clear dose-response-relationship was observable in neither of the FPs. The amendment of 1 mg/L of FP E, F and G caused less offspring than higher concentrations. Indeed, FP E showed tendencies of an inverse dose-response-relationship. The LOEC or EC_{50} values of neither of the baseline toxicity tests did correlate with type of combustion, total or single chemical species. Overall, the results show different sensitivities towards applied FPs.

Table 14: Effect concentrations of the FPs in the baseline toxicity tests. Applied concentrations are indicated behind test name. – no significant effect. ▭ FP from "complete" combustion.

Unspecific-Mode-of-Action-Tests				
Baseline toxicity				
	BCT (0.03 - 400 mg/L)		MTT Test (0.01 - 25 µg/cm² ≈ 0.018 - 44 mg/L)	Nematode Reproduction Test (1 - 1000 mg/L)
	LOEC [mg/L]	EC_{50} [mg/L]	LOEC [µg/cm²]	LOEC [mg/L]
FP A	-	-	-	10
FP B	25	153	-	10
FP C	-	-	-	-
FP D	0.06	239	25	-
FP E	0.06	149	-	1
FP F	< 0.03	97	-	1
FP G	< 0.03	134	-	1

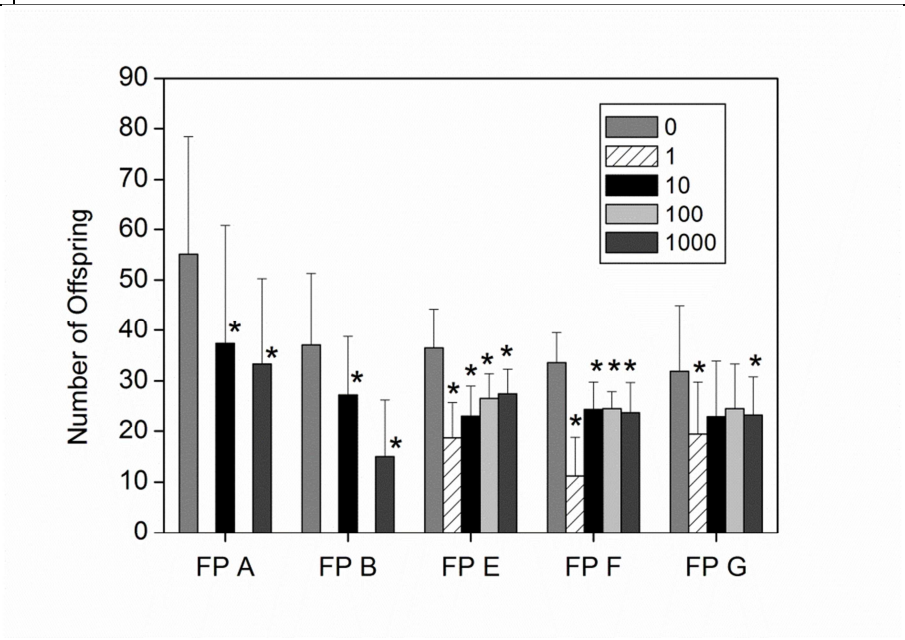


Figure 5: Average number of offspring in the nematode reproduction test (± SD) when treated with 0, 1, 10, 100, 1000 mg/L of FP A, B, E, F, G. 1 and 100 mg/L were not tested in FP A and B. FP C and D was not significantly different from the control (0 mg/L) and thus not plotted. * Significantly different from the control in t-test.

5.2.2.2 Specific-Mode-of-Action-Tests

Although the Umu- and the Yes-Test were especially developed to detect specific toxicities, viability (baseline toxicity) was also assessed via resazurin reduction in these assays. This is of great importance since neglected baseline toxicity can cause false positive target toxicities, namely genotoxicity and estrogenicity (Toolaram et al., 2012). FP A and G showed nonspecific toxicity (baseline toxicity) in the Umu-Test and the Yes-Test. FP D and E were baseline toxic only in the Umu-Test (Table 15). Genotoxic activity was detected for FP A, B and C with LOEC corresponding to the highest (FP B and FP C) or second highest (FP A) applied concentration. Compared to FP A and FP C, FP B was positively tested also without metabolic activation by S9 mix in the Umu-Test. FP A induced estrogenicity in the Yes-Test with a full dose-response curve allowing the calculation of a LOEC and an EC_{50} value. All other FPs were tested negatively in the Yes-Test. Full dose-response curves were obtained in the PAH-CALUX® bioassay for FP A, B, E and G with different LOEC and EC_{50} values. In remaining FP C, D and F the highest applied concentration caused minimal positive effects and thus high LOECs. The MELN and PALM assay did not show any positive results (data not shown). This means that among the tests for endocrine disruption potential, estrogenicity was only detected by the Yes-Test, though only in one sample. Altogether, the PAH-CALUX® bioassay seemed to offer highest sensitivity among the specific MoA assays.

Table 15: Effect concentrations of the FPs in the receptor-mediated tests. No significant effect was detected with the MELN and PALM cells. Applied concentrations are indicated behind test name. – no significant effect. / calculation not possible. ▭ FP from "complete" combustion.

Specific-Mode-of-Action-Tests								
	Genotoxicity Umu-Test (5 – 667 mg/L)			Estrogenicity Yes-Test (0.05 – 667 mg/L)			Presence of PAHs PAH-CALUX® Assay (0.05 - 45 µg/cm² ≈ 0.09 - 79 mg/L)	
	Nonspecific Toxicity LOEC [mg/L]	Genotoxicity LOEC [mg/L] without S9	Genotoxicity LOEC [mg/L] with S9	Nonspecific Toxicity LOEC [mg/L]	Estrogenicity LOEC [mg/L]	Estrogenicity EC_{50} [mg/L]	LOEC [µg/cm²]	EC_{50} [µg/cm²]
FP A	667	-	333	667	42	432	0.5	0.7
FP B	-	667	667	-	-	-	4.6	6.2
FP C	-	-	667	-	-	-	45.0	/
FP D	83	-	-	-	-	-	45.0	/
FP E	333	-	-	-	-	-	4.6	9.7
FP F	-	-	-	-	-	-	45.0	/
FP G	10	-	-	42	-	-	14.4	16.2

5.2.3 Bacterial Baseline Toxicity in Relation to Zn Content

As Zn, a known toxic compound, was highly abundant within all fine dust samples (bulk and filter samples) (Table 11, Figure 8) a relationship between toxicity and Zn was assumed. In consequence, pure Zn-toxicity was determined in the BCT. Furthermore, Zn contents were calculated for each tested FP concentration and plotted against measured baseline toxicity of FP concentrations (Figure 6). With the exception of FP A and C, measured Zn-toxicity (EC_{50} = 16 µM) was in good concordance with FP-toxicity as with rising Zn content detected baseline toxicity increased as well. As a possible explanation for the lack of toxicity in FP A and C, it was taken into account that non-bioavailable Zn-species were present in non-toxic FP A and C in comparison to all other toxic FPs. Therefore, all FPs were examined for specific Zn-species by XRD. The identification of bioavailable and non-bioavailable Zn-

species would allow statements about the influence of chemical specification of Zn on observed toxicity patterns. If non-bioavailable Zn species were only found in non-toxic FP A and C, but bioavailable Zn species in all other FPs, the assumption that Zn is mainly responsible for measured baseline toxicity of FPs would be intensified.

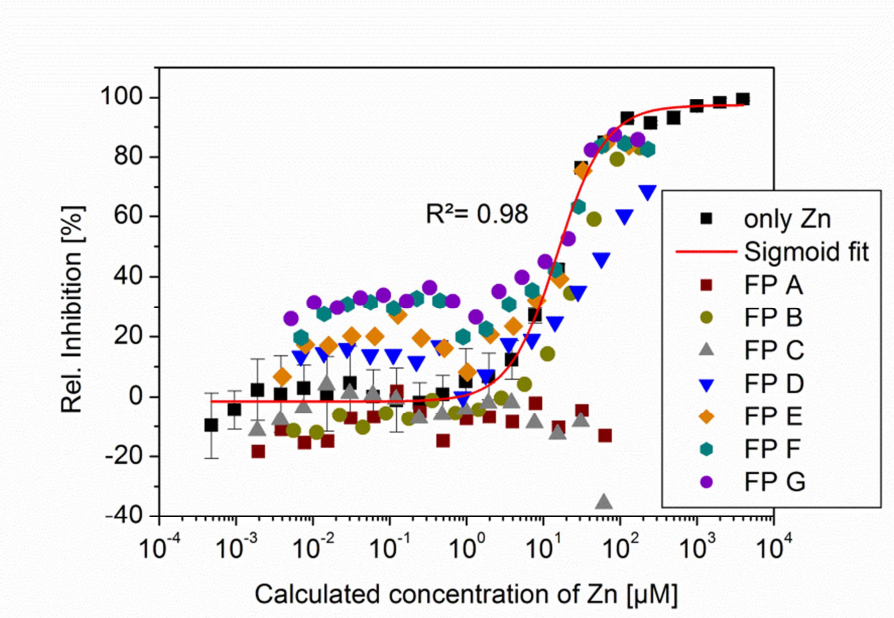
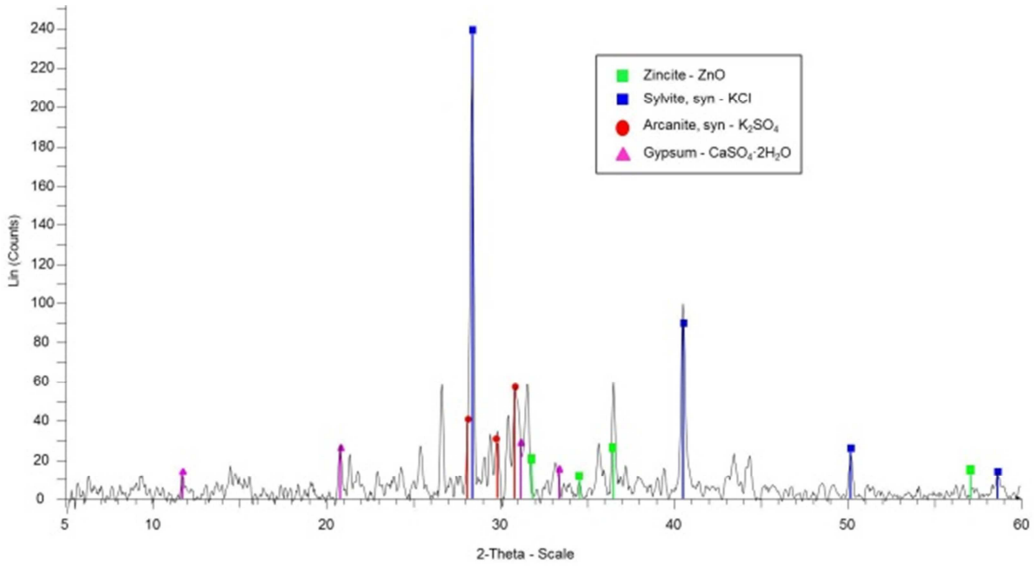


Figure 6: Zn concentration calculated on basis of applied FP concentration versus measured relative inhibition in the BCT compared to pure Zn-toxicity (± SD). For clarity purposes standard deviations for FPs were omitted.

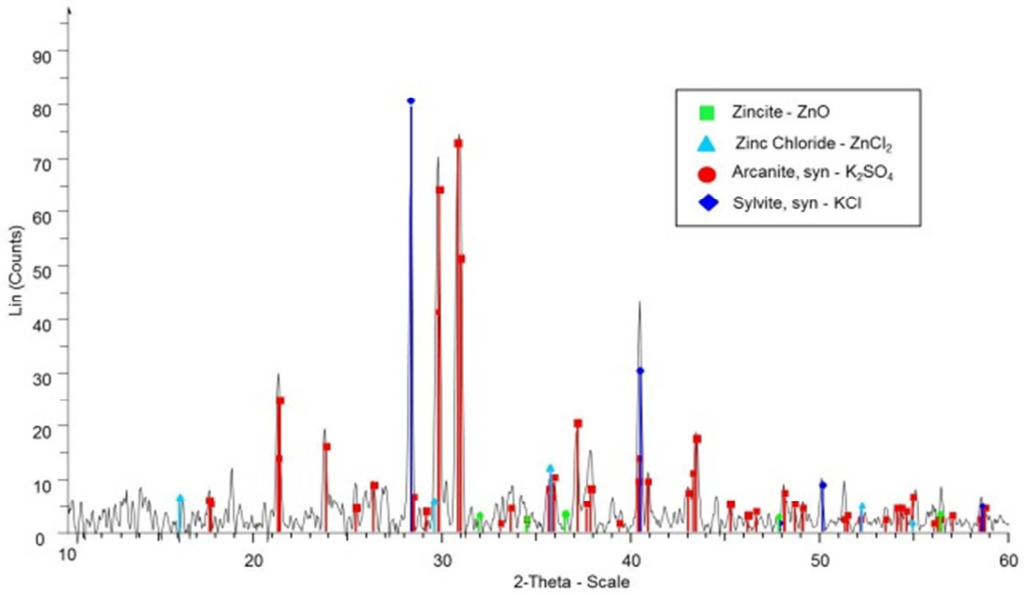
5.2.4 XRD Results

XRD patterns were examined for most abundant chemical structures and Zn species. Heights of diffraction patterns differed strongly among the samples (Figure 7). This indicated that differences in quantities of chemical compounds were pronounced. Diffractable chemical composition was dominated by KCl and K_2SO_4. This agrees well with high amounts of these ions measured in the chemical analysis (Table 11, Table 12). FP C was not crystalline at all as no peaks occurred. Besides K_2SO_4, FP D showed not identifiable crystalline carbonaceous components (not sketched in the graph). FP C and D were mainly composed of carbon (Table 13). According to XRD results such can be considered as totally or mostly amorphous structures for FP C and D respectively (Figure 7). While the diffraction pattern of FP A indicated the presence of ZnO, FP B exhibited clearly $ZnCl_2$ and possibly little amounts of ZnO. FP E showed little contents of both ZnO and $ZnCl_2$. $ZnSO_4$ was not identified reliable in any of the samples as the main peaks of e.g. KCl overlapped the much smaller peaks of Zn-sulfates. No crystalline Zn-species was found in FP F and G.

FP A



FP B



FP C

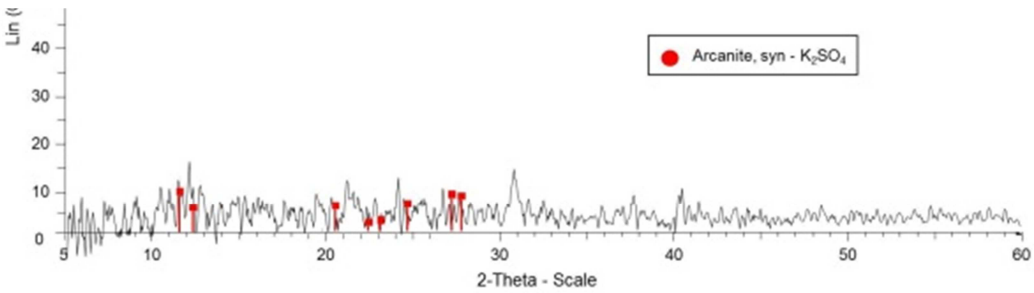
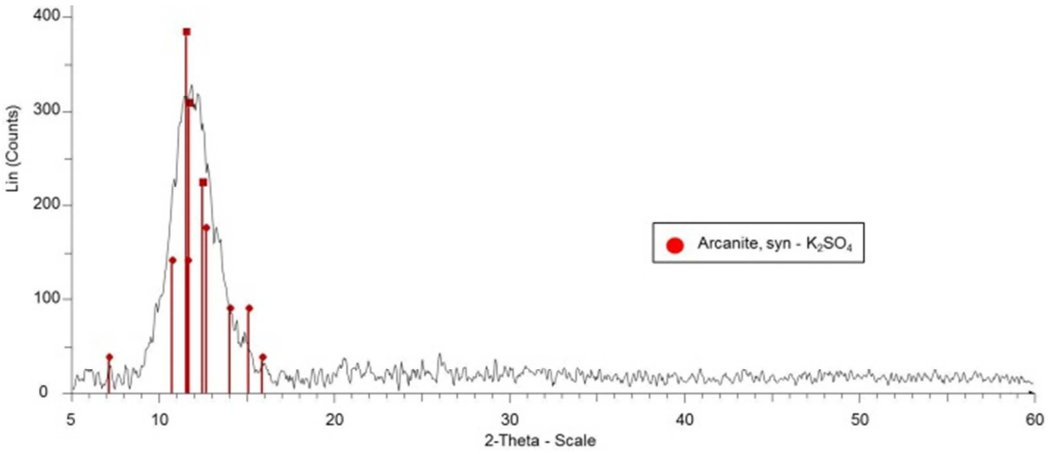
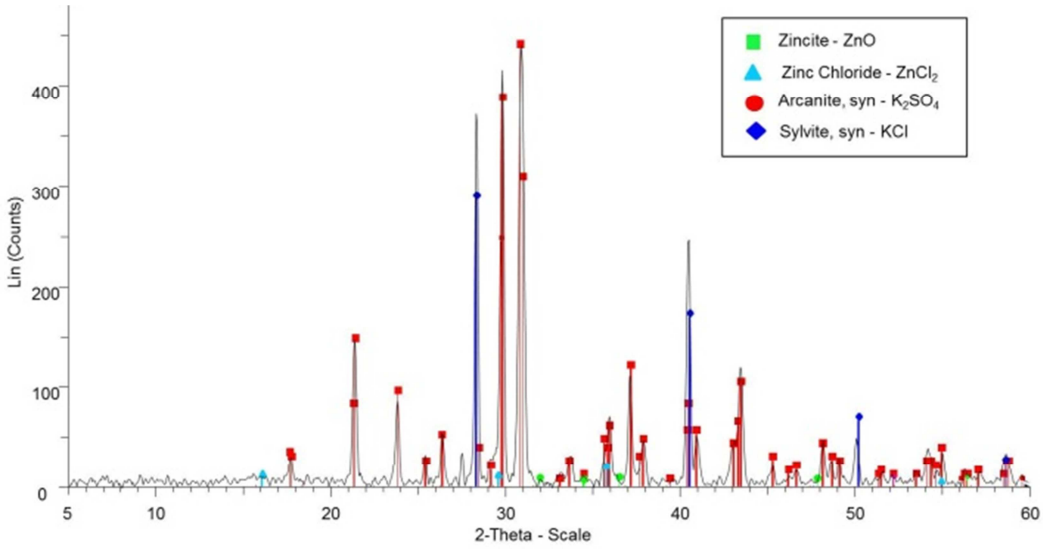


Figure 7: Diffraction spectrum and identified main chemical and Zn species in FPs. In order to provide a qualitative not quantitative overview of detected species y-axis' scales are different among FPs.

FP D



FP E



FP F

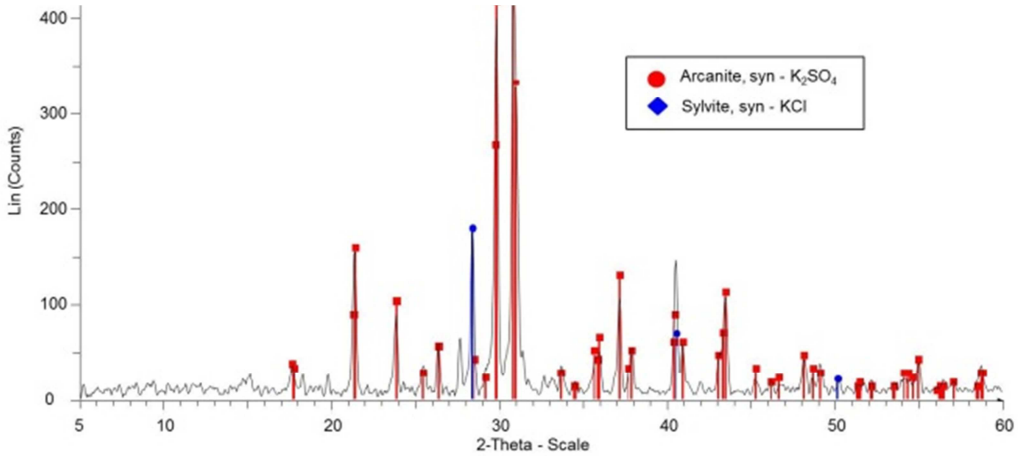


Figure 7 (continued): Diffraction spectrum and identified main chemical and Zn species in FPs. In order to provide a qualitative not quantitative overview of detected species y-axis' scales are different among FPs.

FP G

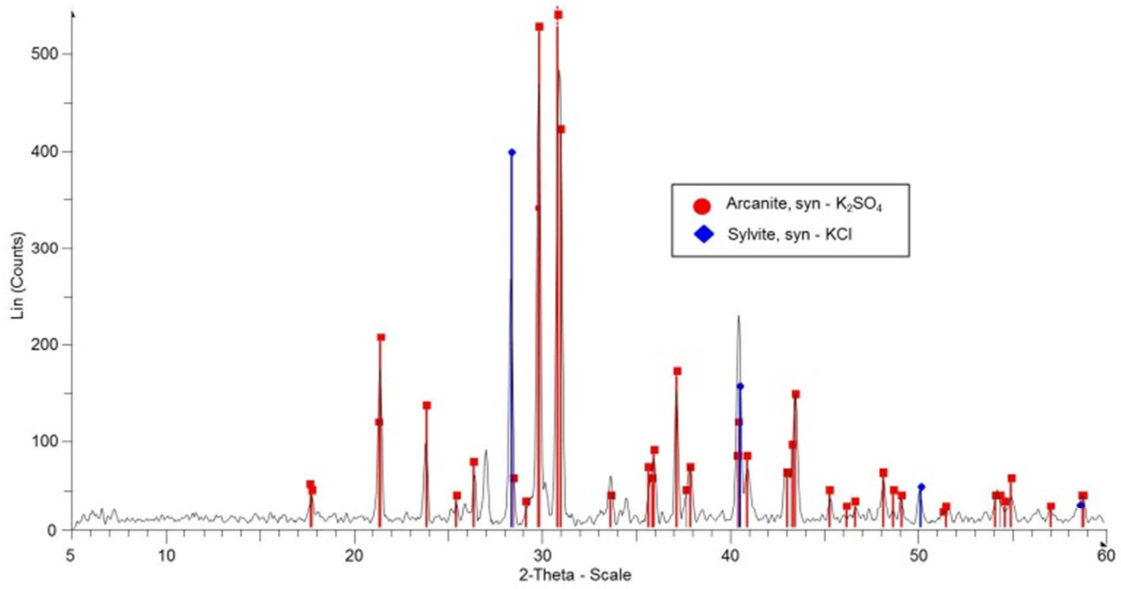


Figure 7 (continued): Diffraction spectrum and identified main chemical and Zn species in FPs. In order to provide a qualitative not quantitative overview of detected species y-axis' scales are different among FPs.

5.3 Filter Fine Dust Samples

For quartz filters loaded with PM a suitable test application that circumvent an extraction of PM from filters was looked for. Furthermore, filter material should not disturb bacterial activity. The latter was proven by testing blank filters in the BCT. Regarding exposure, small cutouts of Ø 5 mm covered with 1.13±0.03 mg PM incl. blank filter were placed in each well of the 96 well plate and test bacteria were added to ensure direct contact between PM and bacteria.

5.3.1 Chemical Analysis

Filter samples loaded with fine dust had negligible carbon contents as it is typical for complete combustions. PAH concentrations were not detectable or low, ranging from 0.5 - 12 µg total PAHs per g of loaded Ø150 mm filters (Table 16). With increasing bark content metal contents, which were dominated by Zn, increased as well (Table 16, Figure 8). Detailed chemical characterization can be found in "10.1 Supporting Information".

Table 16: Total metal and anion content of filter samples loaded with non-aged fine dust.

Labeling of filter samples	total US EPA PAHs (µg/g loaded filter)	total metals without kali and alkali metals (mg metal ion per g loaded filter)	total anions (mg anions per g loaded filter)
water content_2.9%	12.04	0.23	3.55
water content_6.5%	0.70	0.18	3.78
water content_7.3%	0.48	0.33	4.49
water content_12.1%	-	0.17	3.09
location II	4.02	0.17	2.51
location III	-	0.15	2.43
type of wood_pine	4.30	0.16	1.69
type of wood_cottonw. I	1.71	0.25	6.44
type of wood_cottonw. II	1.70	0.31	9.21
bark_10 wt%	-	0.34	4.81
bark_20 wt%	0.94	0.4	5.54
bark_30 wt%	1.30	0.62	6.79
bark_40 wt%	-	0.66	3.18

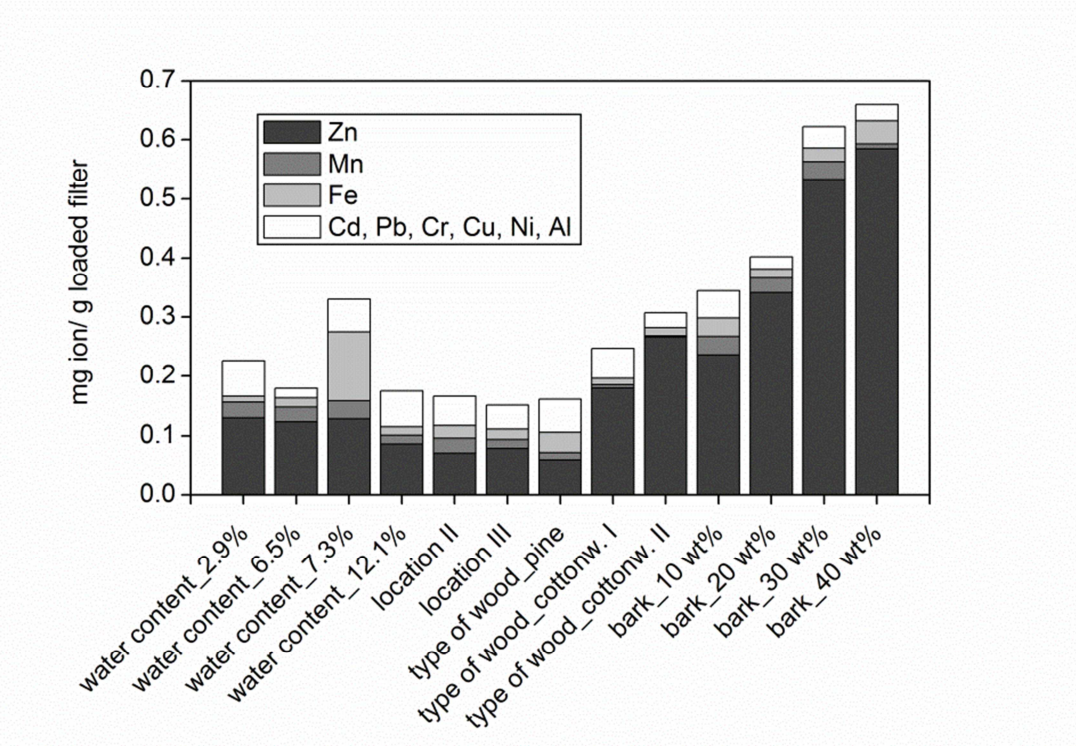


Figure 8: Contents of most abundant metals excluding kali and alkali metals of PM collected on filters from complete combustions with different types of pellets.

5.3.2 BCT and Umu-Test Results

Direct testing of filter samples was successful and thereby this exposure way constitutes a novel and rapid method avoiding the extraction of PM from filter material. This way, PM sample is less disturbed in structure and chemistry. All filter samples caused baseline toxicity in the BCT (Figure 9). Pellets consisting of pine caused less toxicity than cottonwood or spruce. Spruce was used for all pellets if not indicated otherwise. Higher water contents resulted in lower toxicities. Such causal relationship was not observed for location or bark content. The Umu-Test showed elevated induction ratios for the sample “water content_2.9%”, “type of wood_pine” and “bark_30 wt%”, predominantly after metabolization with S9 (data not shown). But since decreased bacterial growth occurred simultaneously, observed genotoxic effects could not be separated from bacteriotoxicity and were probably false positive. No estrogenicity was detected in the filter samples in the Yes-Test.

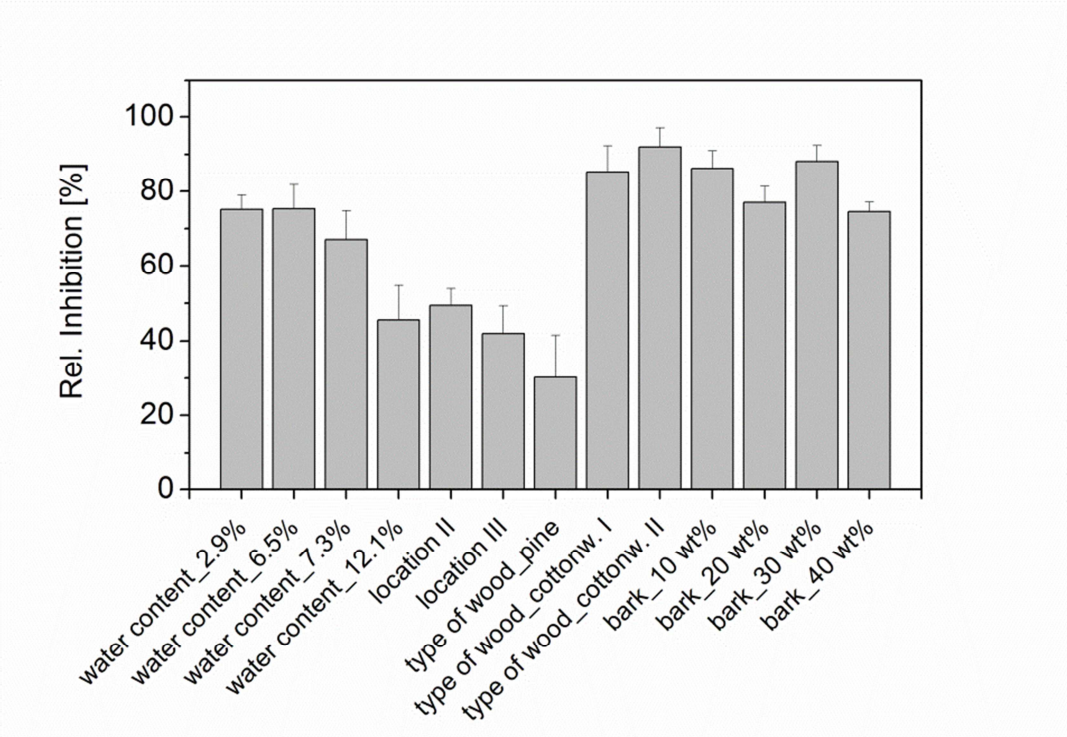


Figure 9: Baseline toxicity (± SD) expressed as relative inhibition to blank filter (control) of fine particles collected on filters from complete combustions with different types of pellets.

5.4 Experiments with Zn

On the basis of toxicity results of bulk fine dust and filter samples, Zn was expected to play a crucial role for baseline toxicity of test samples. In order to investigate this assumption more profoundly, experiments with Zn were conducted.

5.4.1 Zn Addition to Bulk FPs

As pure Zn-toxicity was in good concordance with Zn-content and toxicity of bulk FPs except for FP A and C (see “5.2.3 Bacterial Baseline Toxicity in Relation to Zn Content”), Zn possibly dominated baseline toxicity. From the mechanistic point of view this would make sense since Zn inhibits the bacterial respiratory electron transport system (Choudhury and Srivastava, 2001). In order to study expected relationship, toxic Zn was added FPs. It was assumed that the admixture of toxic Zn to FPs would increase toxicity in the BCT. But only when negligible carbon content (complete combustion) characterized FPs. Otherwise Zn is adsorbed to carbonaceous compounds like soot (incomplete combustion) and thereby becomes less bioavailable. This would cause less Zn-toxicity than expected. Indeed, an over-additional effect was expected within FP from complete combustion (low carbon content) due to the mixture of Zn and PAHs present in FP. Such mixture effect of PAHs and metals was

observed before in bacteria extracted from soil (Gogolev and Wilke, 1997). To examine this possible combination mechanism, Zn and fluoranthene (FLA) were tested together.

For Zn-FP-combination experiments Zn concentrations (0.5 – 125 µM $ZnCl_2$ causing 0-90 % relative inhibition) plus highest non-toxic FP concentrations were chosen: 6 mg/L of all FPs and additionally 400 mg/L of non-toxic FP A and C. In Figure 10 and Figure 11 measured toxicity of combined exposures of Zn and FPs was compared to pure Zn-toxicity. Using the highest non-toxic concentration of FP A and C (400 mg/L), the addition of Zn to FP C reduced Zn-toxicity much more than with FP A (Figure 10).

Zn-toxicity was not (FP A) or less (FP C) reduced when FP concentration was lowered to 6 mg/L (Figure 11). The addition of FPs from both complete and incomplete combustion (in non-toxic 6 mg/L concentration) lowered Zn-toxicity in middle range Zn-concentrations and did not alter Zn-toxicity in high concentrations (Figure 11). Within FPs from complete combustion the amendment of FP E and G caused higher toxicities of 0.5 and 4 µM Zn than only Zn. All other FPs caused lower or unchanged Zn-toxicity at low Zn-concentrations (0.5, 4 µM). These results show that FPs from both complete and incomplete combustion show sorption capacities for Zn. Over-additional toxicity was only observed within two FP from complete combustion amended with low Zn concentrations. Thus, expected mechanism exerted by combined incubation of PAHs (present in FPs) plus Zn is questionable as it would have occurred within all FPs from complete combustion and higher Zn concentrations as well.

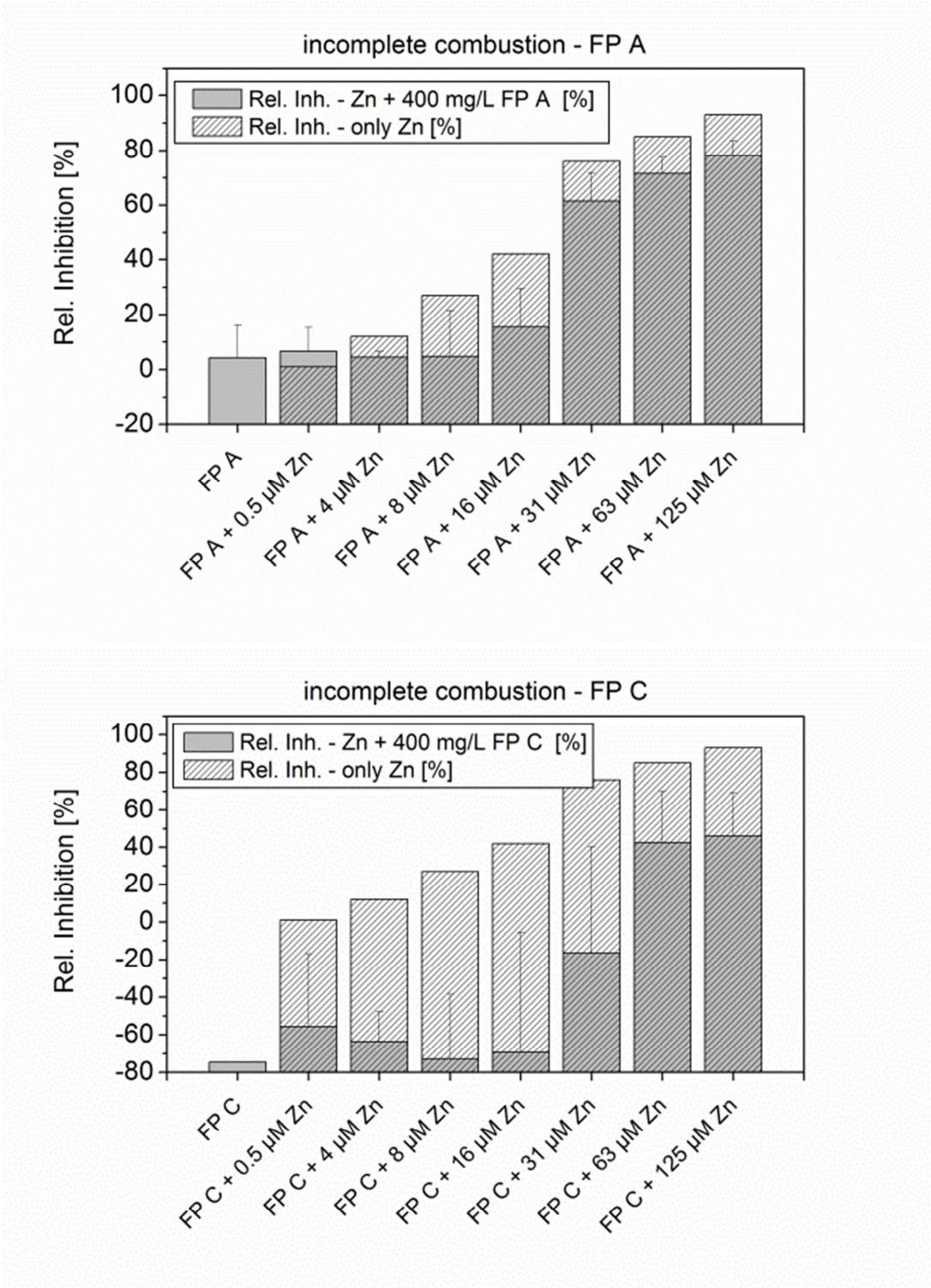


Figure 10: Pure Zn-toxicity (± SD) compared to combined toxicity of FP A and C in non-toxic concentration (400 mg/L) and Zn.

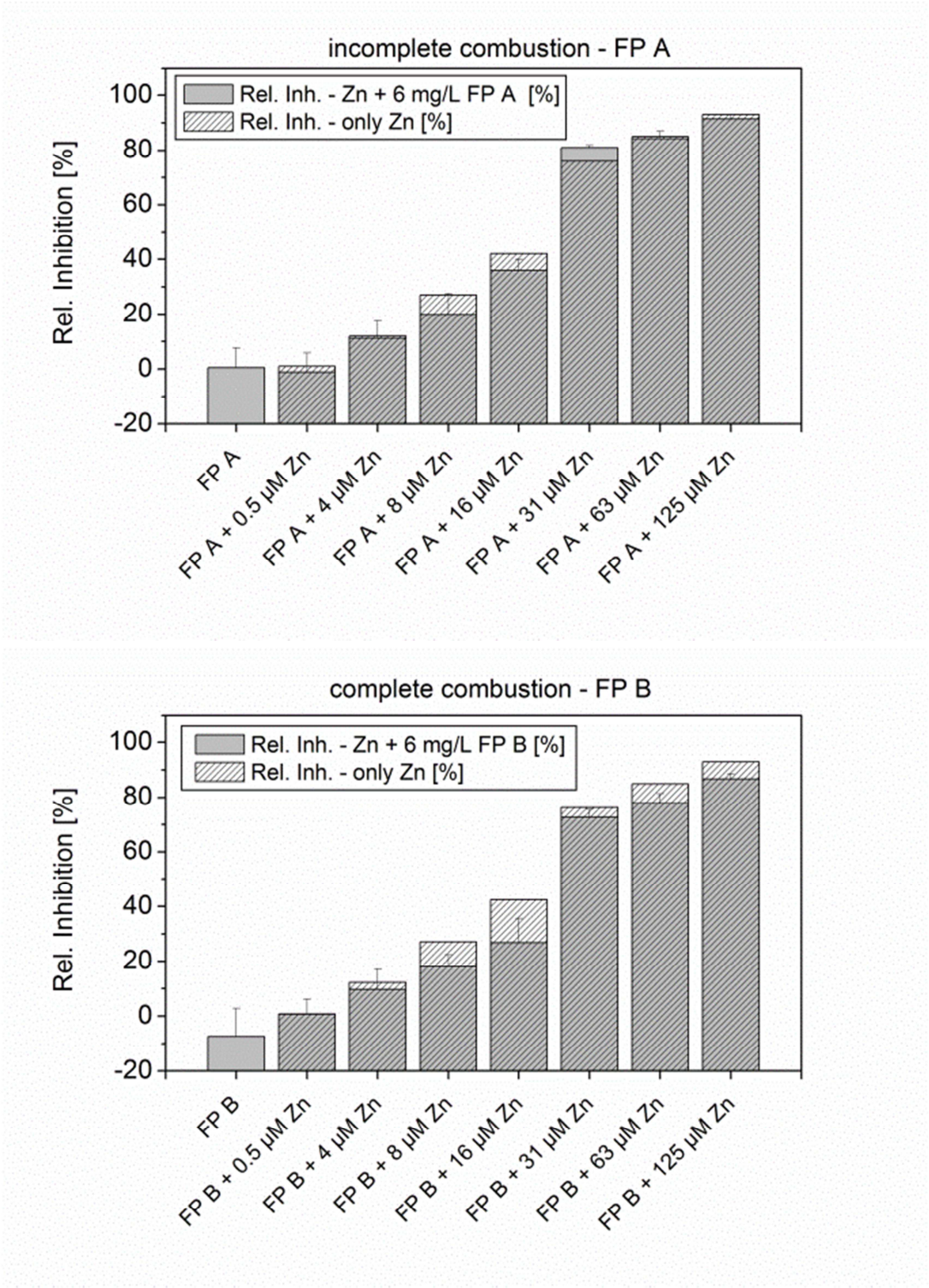


Figure 11: Pure Zn-toxicity (± SD) compared to combined toxicity of all FPs in non-toxic concentration (6 mg/L) and Zn.

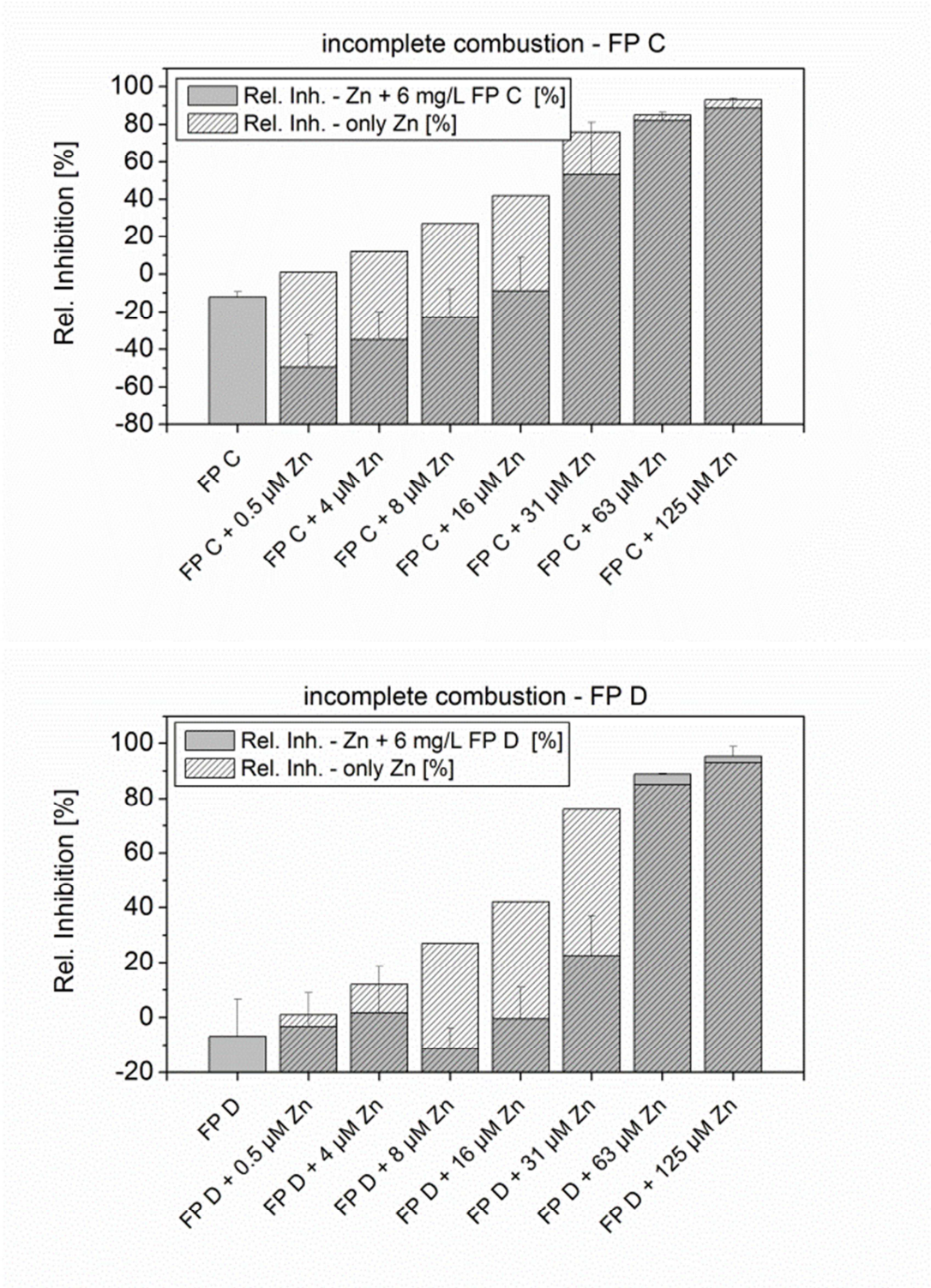


Figure 11 (continued): Pure Zn-toxicity (± SD) compared to combined toxicity of all FPs in non-toxic concentration (6 mg/L) and Zn.

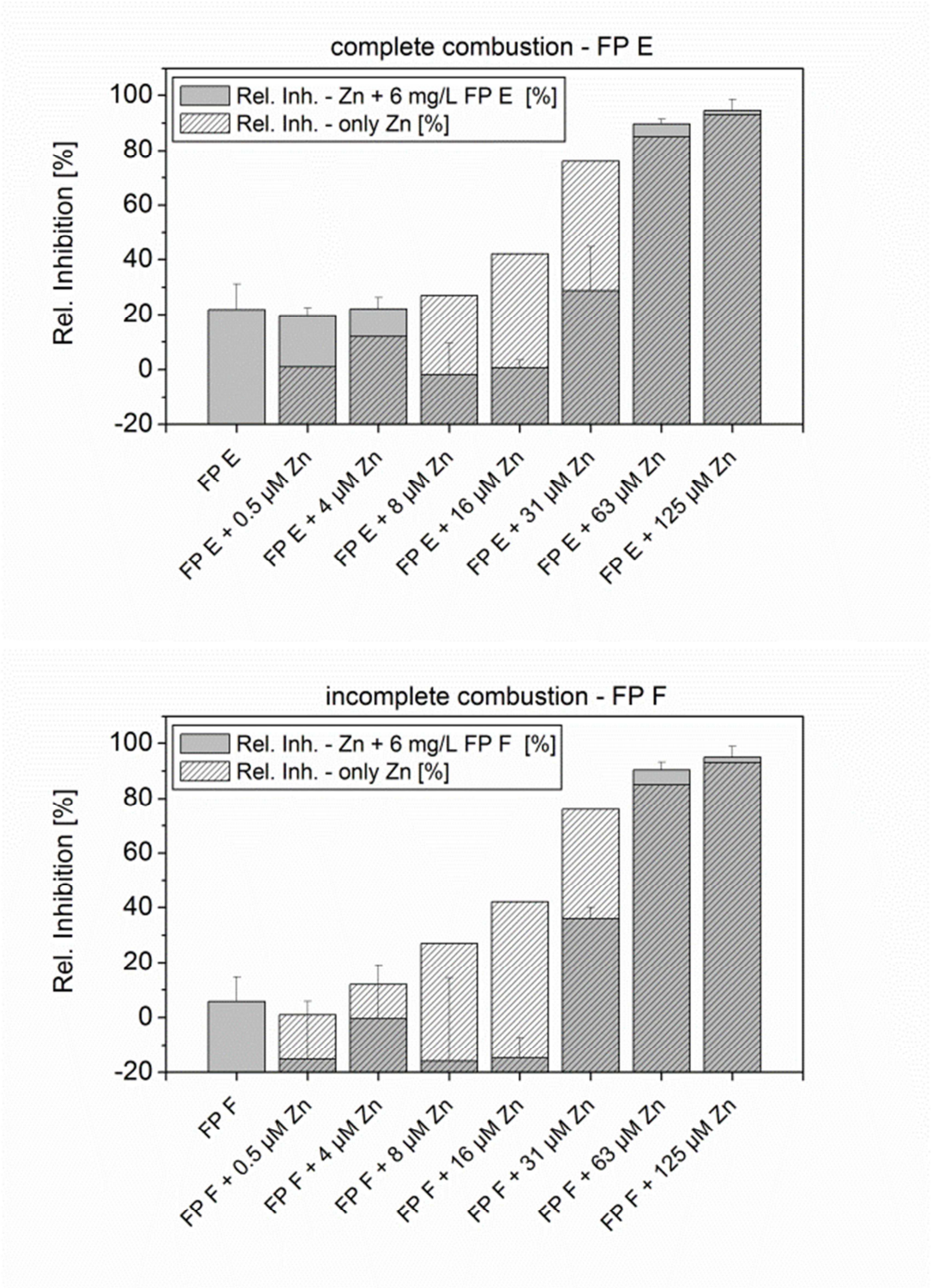


Figure 11 (continued): Pure Zn-toxicity (± SD) compared to combined toxicity of all FPs in non-toxic concentration (6 mg/L) and Zn.

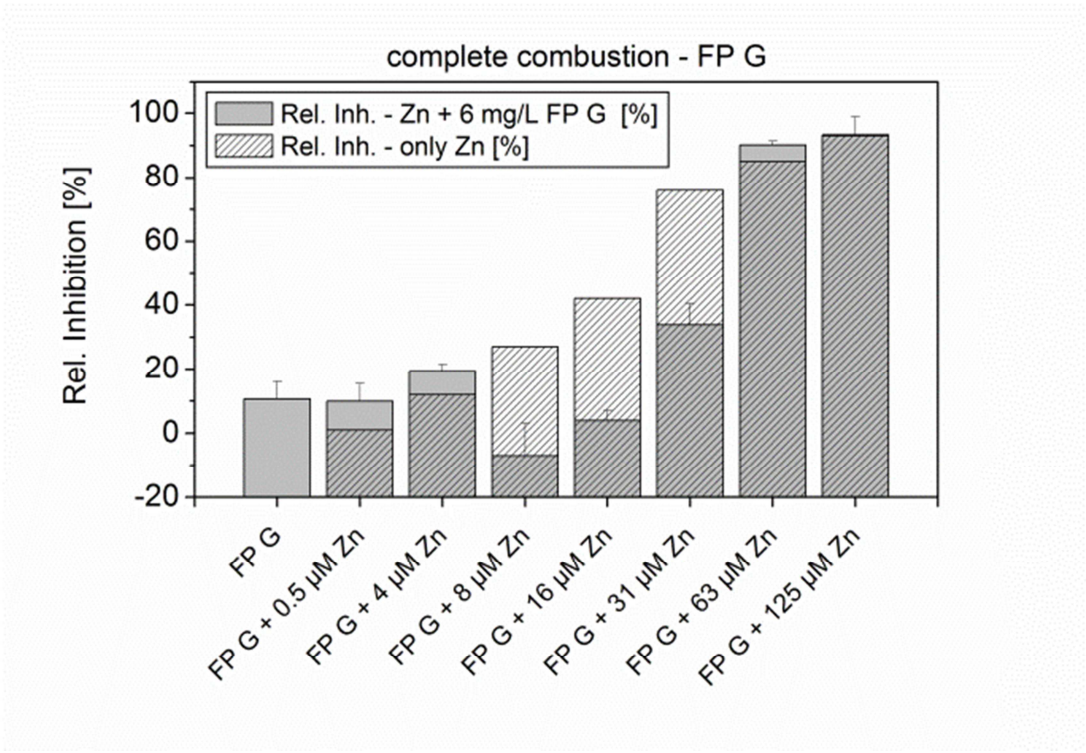


Figure 11 (continued): Pure Zn-toxicity (± SD) compared to combined toxicity of all FPs in non-toxic concentration (6 mg/L) and Zn.

5.4.2 Zn Addition to Filters Loaded with Fine Dust

In order to assess the possible enhancement of Zn-toxicity by fine dust from complete combustions (see explanation in "5.4.1 Zn Addition to Bulk FPs"), four concentrations of Zn were tested together with three filter samples that derived from complete combustions. Chosen Zn concentrations (0.5, 4, 8, 16 µM) alone caused relative inhibitions ranging from 0 to 40 %. Filters with least toxicity (30 - 70%) were used for combination experiments: "water content_7.3 %", "water content_ 12.1%" and "type of wood_pine". Pretrials showed that the presence of blank filter did not alter Zn-toxicity (data not shown). In Figure 12 measured and calculated additive toxicity was compared. Measured inhibition of filter samples plus 0.5 or 4 µM of Zn was higher than predicted by calculation. 16 µM of Zn caused less inhibition than the sum of both toxicities, whereas 8 µM Zn did not cause apparent differences between calculated and measured toxicity. This toxicity patterns indicated an over-additive toxicity only within lower Zn concentrations. As higher Zn concentrations caused less toxicity than expected by calculation, the assumed mechanism, the enhancement of Zn by PAHs that were present in PM, was questionable.

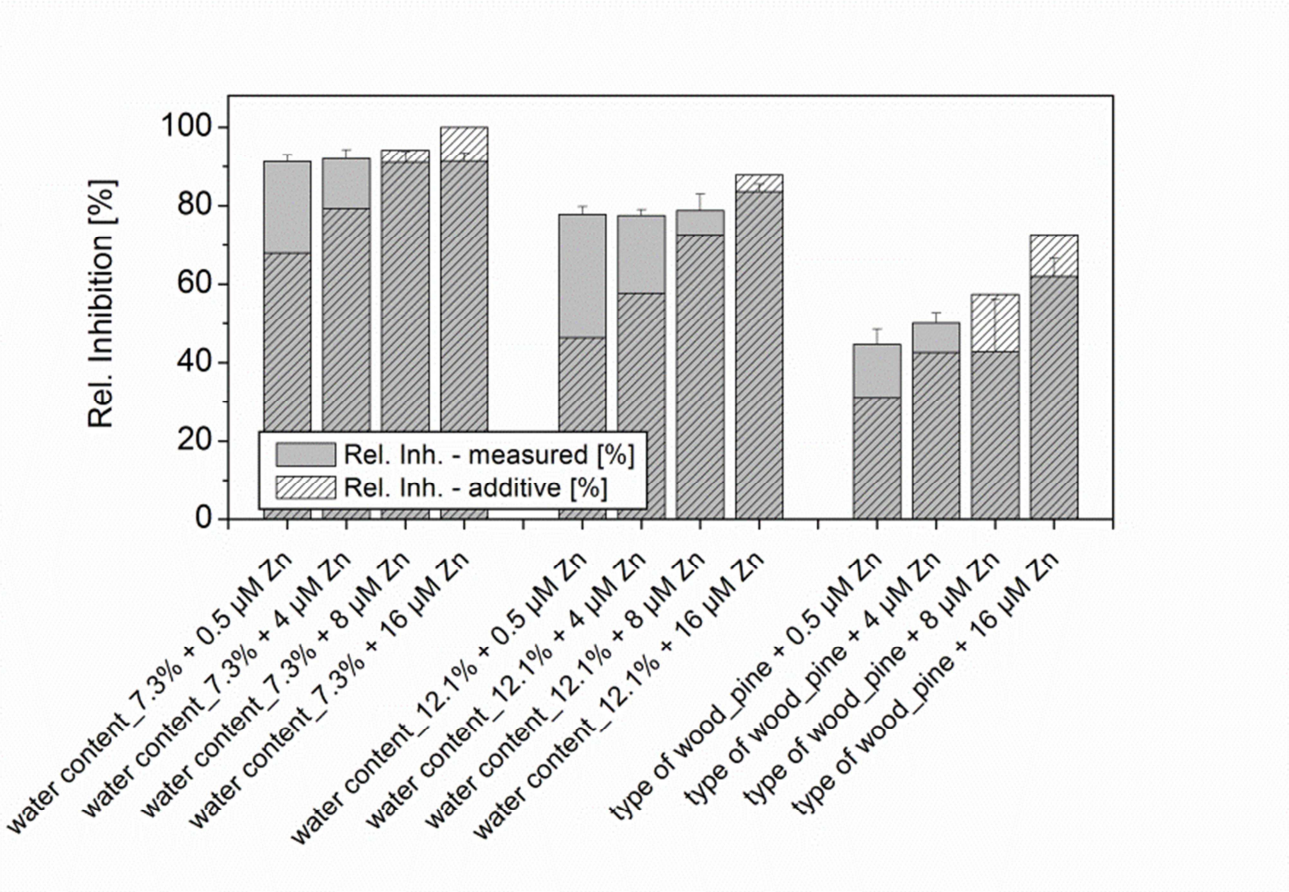


Figure 12: Baseline toxicity (± SD) of different concentrations of Zn plus three filter samples loaded with fine dust originating from complete combustions with pellets varying in water content and type of wood. “Rel. Inh. – measured” indicates relative inhibition measured in the BCT, “Rel. Inh. – additive” indicates relative inhibition when summing up pure Zn-toxicity and previously measured toxicity of filter samples.

5.4.3 Zn Addition to Fluoranthene

In order to examine the possible mechanism for previously observed over-additional toxicity in combination experiments with Zn plus fine dust, Zn and FLA were tested together. Such enhancement of Zn by FLA was shown before on isolated soil bacteria (Gogolev and Wilke, 1997). A full dose-response curve of FLA could not be monitored since FLA was not soluble above 20 µM (Figure 13). An EC_{50} of 7.1 µM was calculated for obtained FLA dose-response curve. Combined exposure of non-toxic concentrations of FLA (1, 2 and 5 µM) and rising concentrations of $ZnCl_2$ caused the same or less toxicity than calculated by addition of both toxicities (Figure 14). The over-additional effect did not occur.

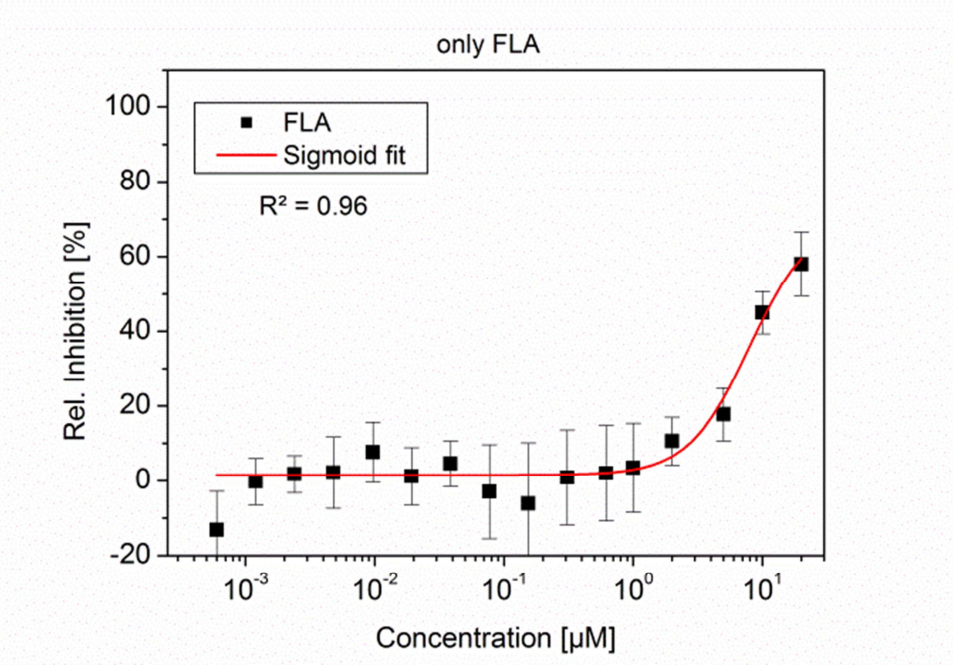


Figure 13: Baseline toxicity (± SD) of fluoranthene (FLA), higher concentrations were not applicable due to limited solubility in 1% DMSO.

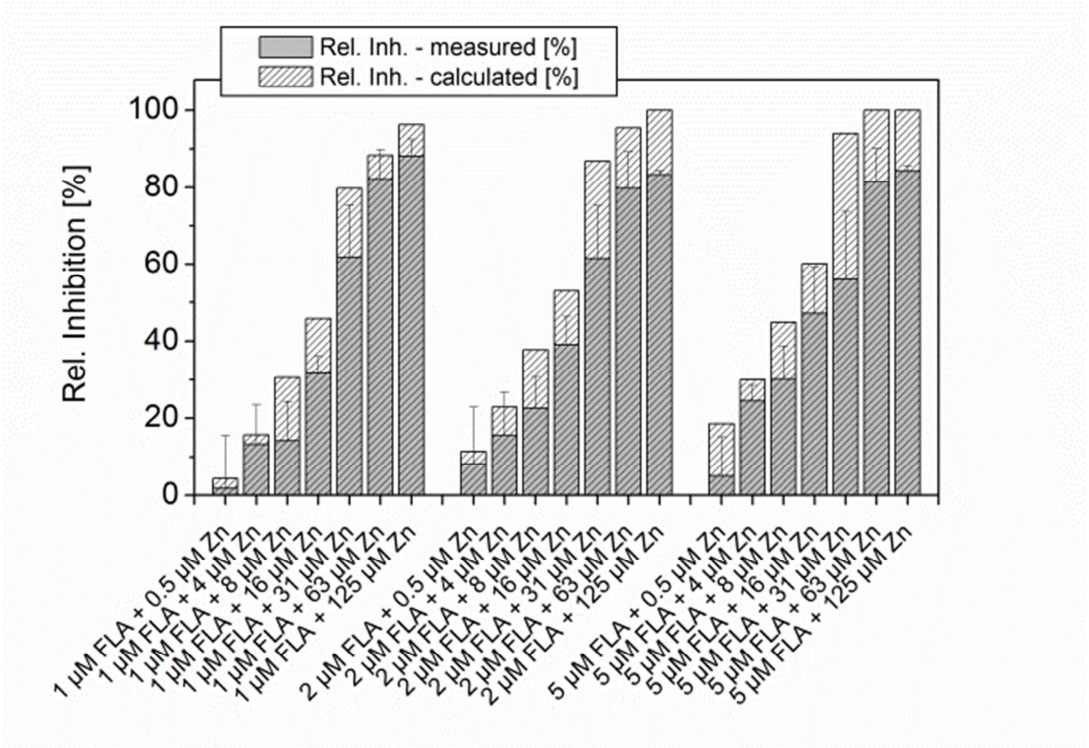


Figure 14: Combined baseline toxicity (± SD) of Zn and fluoranthene (FLA). “Rel. Inh. – measured” indicates relative inhibition measured in the BCT, “Rel. Inh. – calculated” indicates relative inhibition when summing up Zn- and FLA-toxicity.

5.5 Experiments in the Atmospheric Simulation Chamber

Once fine particles are emitted into the environment, they undergo atmospheric transformation processes due to reactions with gaseous and particulate molecules and UV radiation (Kocbach Bølling et al., 2009). For instance, oxy- and nitro-PAHs are built by photo-oxidation of PAHs (Arey, 1998, Lundstedt et al., 2007). Whereas oxy-PAHs might be less mutagenic than their parent original PAH (Lundstedt et al., 2007), it is known that nitro-PAHs are more mutagenic than their precursors when tested in the Ames-Test (Arey, 1998, Pitts, 1987). In order to detect expected formation of such toxicologically highly relevant nitroarenes during atmospheric oxidation, simulation experiments were conducted in an atmospheric simulation chamber. Oxidized PM precipitated on filters which were subsequently tested for genotoxicity in the Umu-Test using a nitroarene sensitive *Salmonella* strain.

5.5.1 Chemical Analysis

During transformation in the atmospheric simulation chamber O_3, NO and NO_2 were monitored (Table 17). Detected NO and NO_2-concentrations derived from the boiler and stove emissions. The soot amount of subsequently taken filter samples was low (< 15%) providing for grey fine particles as it was expected for complete combustions (data not shown). Aged samples contained less non-substituted PAHs than their equivalent non-aged samples (Table 18). Despite the reasonable detection limit (1.25 ng/µL equals 2.5 µg per filter) no nitro-PAH was detected in any of the fine dust samples.

Table 17: Sampling set up and occurrence of gaseous components.

Sample name	min	O_3 [ppb]	NO [ppb]	NO_2 [ppb]	Sampling description
	0	0	90	14	Start sampling Daytime 1
Daytime 1	60	0	85	8	End sampling Daytime 1
	65	0	87	8	Feeding of O_3
	86	0	23	64	Stop Feeding O_3, close chamber
Daytime 1_aged	90	0	21	66	UV-lamps on
	210	8	31	48	Start Sampling Daytime 1_aged
	270	9	30	44	End Sampling Daytime 1_aged

Table 17 (continued): Sampling set up and occurrence of gaseous components.

Sample name	min	O_3 [ppb]	NO [ppb]	NO_2 [ppb]	Sampling description
Nighttime 1	0	0	115	6	Start sampling Nighttime 1
	60	0	116	7	End sampling Nighttime 1
	61	0	115	6	Feeding of O_3
	80	33	1	111	Stop Feeding O_3, close chamber
Nighttime 1_ aged	200	11	0	91	Start Sampling Nighttime 1_aged
	260	7	1	87	End Sampling Nighttime 1_aged
Daytime 2	0	0	34	1	Start Sampling Daytime 2
	60	0	57	8	End Sampling Daytime 3
	61	0	57	6	Feeding of O_3
	69	17	7	38	Stop Feeding O_3, close chamber
	76	23	1	53	UV-lamps on
Daytime 2_aged	196	35	7	39	Start Sampling Daytime 2_aged
	256	36	7	40	End Sampling Daytime 2_aged
Nighttime 2	0	0	23	0	Start sampling Nighttime 2
	60	0	39	8	End sampling Nighttime 2
	61	0	37	2	Feeding of O_3
	71	46	1	36	Stop Feeding O_3, close chamber
Nighttime 2_aged	191	41	1	23	Start Sampling Nighttime 2_aged
	251	36	1	13	End Sampling Nighttime 2_aged

Table 18: Analyzed PAHs in exhaust, - below detection limit, * genotoxic in Umu-Test with NM3009 strain.

PAHs in ng/m³								
	Daytime 1	Daytime 1_aged	Nighttime 1	Nighttime 1_aged *	Daytime 2	Daytime 2_aged *	Nighttime 2	Nighttime 2_aged *
Fluorene	7.9	6.6	16.3	1.1	20.9	1.9	4.0	6.2
Phenanthrene	-	-	5.1	-	80.7	18.7	2.1	2.3
Anthracene	-	-	1.4	-	7.9	0.3	-	-
Fluoranthene	1.3	-	5.6	-	151.8	27.0	1.8	2.8
Pyrene	2.1	-	5.2	-	129.5	31.0	3.6	3.0
Retene (Phenanthrene, 1-methyl-7-[1methylethyl]-)	8.3	1.4	1.1	4.8	16.9	3.0	9.1	3.8
Benzo(b)naphtho(1,2-d)thiophene	-	-	-	-	-	-	-	-
Cyclopenta(cd)pyrene	-	-	-	0.2	20.3	5.9	0.5	0.5
Benz(a)anthracene	-	-	-	-	29.1	9.8	0.6	0.8
Chrysene+Triphenylene	-	-	-	-	24.4	11.4	0.7	0.8
2,2-Binaphthyl	-	-	-	-	2.0	0.9		
Benzo(b)fluoranthene	-	-	-	-	65.3	31.0	2.2	1.9
Benzo(k)fluoranthene	-	-	-	-	22.1	11.4	0.8	1.1
Benz(e)pyrene	-	-	-	-	42.0	21.7	2.4	2.5
Benz(a)pyrene	-	-	-	-	36.2	12.3	0.8	0.7
Indeno(1,2,3-cd)pyrene	-	-	-	-	11.9	3.6	-	-
Dibenzo(ah)anthracene	-	-	-	-	1.1	-	-	-
Benzo(ghi)perylene	-	-	-	-	20.1	6.2	-	-
Coronene	-	-	-	-		-	-	-
Sum	19.5	8.1	34.6	6.1	682.3	196.1	28.7	26.4

5.5.2 Umu-Test Results

NM3009 *Salmonella* showed enhanced sensitivity towards the reference compound 1,3 Dinitropyrene (LOEC = 0.08 ng/mL) compared to the conventional *TA1535/pSK1002 Salmonella* strain (LOEC = 0.41 ng/mL). No genotoxicity of the filter samples was detected with the conventional Umu-Test. All aged samples with the exception of one (sample Daytime 1_aged) were tested positive in the Umu-Test with the nitroarene sensitive strain (Figure 15). In comparison, none of their parent not-aged sample provided for a significant positive response. This indicated the formation of nitroarenes during prolonged presence in the atmospheric simulation chamber. Sample Daytime 2_aged provided for a higher IR than the corresponding nighttime aged sample. There was no linear correlation of IR and deposited FP masses, though, lowest masses were calculated for non-genotoxic Daytime 1 samples (aged and not-aged). Among genotoxic samples, 5 h incubation time caused significant higher induction rates than 2 h incubation.

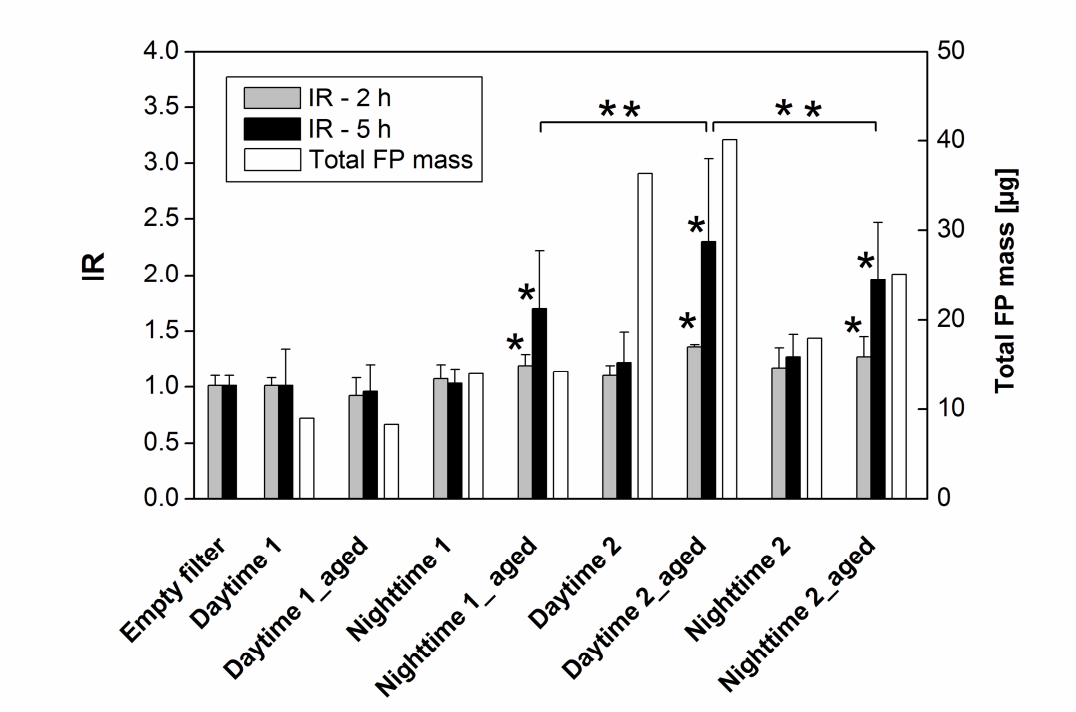


Figure 15: Total fine particle sample (FP) masses on whole filters (not cutouts) and induction ratio (IR) in the Umu-Test with the NM3009 *Salmonella* strain after 2 and 5 h of incubation, the Umu-Test with the conventional tester strain was negative (data not shown), * significantly different from control (empty filter), ** significantly different from each other.

6 Discussion

The increasing use of wood combustion appliances in private households makes risk assessment of related emissions necessary. For this purpose, epidemiological as well as *in vivo* and *in vitro* studies have been conducted in the past. *In vitro* studies mainly focus on the use of immortalized human cell lines. As described in "3.5.3.3 Microorganisms as Test Objects" and in the following discussion microorganisms might provide another promising tool in *in vitro* toxicity testing of PM in direct contact manner. Afterwards, toxicity screening of tested FPs is discussed in relation to possible causatives. A comparison with other bioassays regarding suitability and toxicity outcomes follows. Zn was suspected to influence baseline toxicity predominantly. This expectation was examined more precisely by testing Zn together with PM as well as one selected PAH. Finally, the importance of atmospheric transformation processes for PM's genotoxicity is shown by discussing Umu-Test results that were obtained for artificially oxidized fine dust.

6.1 Evaluation of Modified Test Systems

6.1.1 Reasons for Selected Microbial Biotests

In general, bioassays are used for toxicological characterization of single chemical compounds, their mixtures and environmental samples (Escher et al., 2005). The impact of xenobiotics on the integrity of structure or function of single biomolecules like proteins, DNA or other cellular components like the cell wall can be studied on microorganisms as well as on cell lines in microscale *in vitro* tests. Main idea is that certain MoAs are the same within different species regardless of the organismic level, therefore extrapolation of biotest results to higher organisms are aimed for. Biotests require small sample volumes and are thus eligible for screening a large number of sampless. In contrast to cell lines, microorganisms are easily and rapidly cultivated. Moreover, bioassays with microorganisms are simple in handling, less expensive and test results are obtained within short time. Microorganisms are unicellular and represent principle cell functions. These characteristics made them a popular tool for toxicity testing of environmental samples from water, soil or sediment (Fai and Grant, 2010) and thus can also serve for PM testing. Summary effects of environmental samples such as baseline toxicity are often assessed on the basis of standardized microbial bioassays like the freshwater algal test or the *Vibrio fischeri* bioluminescence inhibition test. Standardized microbial biotests targeting specific toxicities like mutagenicity or genotoxicity, for instance, comprise

the Ames-Test and the Umu-Test (Bilitewski, 2007). Microbial receptor based tests for other specific MoAs like neurotoxicity or endocrine disruption potential are not standardized yet.

In this study two bacterial bioassays and yeast test were chosen due to following reasons. The BCT was preferable applicable to assess baseline toxicity of PM since its test organism *Arthrobacter globiformis* features a high affinity to surfaces (Figure 16). *Salmonella typhimurium* and *Saccharomyces cerevisiae*, test organisms of the Umu- and Yes-Test, are also known to attach to particle surfaces (Fretwurst and Ahlf, 1996, Weber et al., 2006). This trait makes these test organisms highly beneficial for testing solid matrices like FPs. As particle-bound contaminants can add to toxicity (Ahlf et al., 2009) whole particle testing provide more realistic exposure routes than extract testing and thus was performed in this study.

The BCT was standardized for soil samples (DIN 38412-48) and miniaturized by Heise and Ahlf (2005). The Umu-Test was standardized for aqueous samples (Guideline ISO/FDIS 13829). Under the supervision of the German Federal Institute of Hydrology (BfG) standardization for the Yes-Test is currently launched. Standardization of chosen test systems makes them reproducible and accepted by authorities. Hence, the evaluation of different small scale wood combustion devices on the basis of presented microbial test battery would supposedly be approved by decision-makers and could, for instance, influence political actions that aim for mitigation of emissions from wood combustion.

Furthermore, applied bioassays were selected since certain unspecific and specific toxicities were expected from PM from wood combustion. Genotoxicity (Umu-Test) and estrogenicity (Yes-Test) as well as narcosis (BCT) were assumed to be the main MoAs caused by polycyclic aromatic hydrocarbon (PAH) and heavy metal contamination of PM from wood combustion found in this and other studies (Table 10, Table 11) (Larson and Koenig, 1994, Kocbach Bølling et al., 2009). The latter are known to induce baseline toxicity in microorganisms (Giller et al., 1998). Some heavy metals can cause mutagenicity and genotoxicity in bacterial bioassays (Codina et al., 1995). Single PAH species can induce both genotoxicity and estrogenicity in genetically modified bacteria and yeast (Nakamura et al., 1987, Santodonato, 1997). The Umu- and the Yes-Test for assessing genotoxicity and estrogenicity respectively were employed on fine particles earlier. Funaska et al. (2003) examined organic extracts of atmospheric suspended particles in the Umu-Test. Similarly, organic extracts of wood combustion particles from Chinese villages were positively tested in the Yes-Test (Wang et al., 2003).

The BCT was preferred over other bacterial viability tests because it might be more sensitive to toxicants like metals compared to other bacterial solid-phase tests like the *Photobacterium phosphoreum* test (Rönnpagel et al., 1995). Moreover, inherent resazurin reduction capacity measured in the BCT reflects bacterial viability better than mere growth measurement (Niles et al., 2009). The Umu-Test was picked instead of the widely used Ames-Test, as the former works with only one bacterial strain and test results are obtained within one day (Ames-Test: 48 h). Furthermore, small volumes and no sterile working conditions as in the Ames-Test are required (Hamer et al., 2000, Oda et al., 1985). For single substance testing there is a very good concordance (93%) between genotoxicity results in the Umu-Test and rodent carcinogenicity (Reifferscheid and Heil, 1996). This indicates a possible transferability of Umu-Test results to higher organisms, thereby supposedly allowing predictions about human and environmental toxicity.

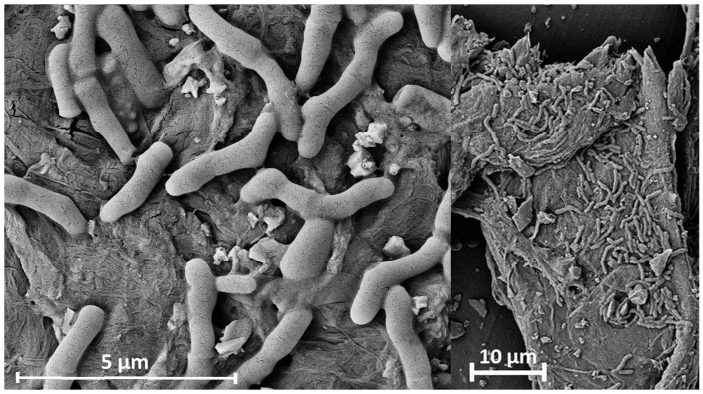


Figure 16: *Arthrobacter globiformis* on cellulose, small cutout and large cutout.

Besides microorganisms that were used in this study, the nematode *Caenorhabditis elegans* and different human cell lines were evaluated for suitability for toxicity testing of PM in the overall project. Compared to bacteria, yeast and cell lines, nematodes ingest particles and thus provide for both an additional exposure route and a multicellular organism. Deriving from human tissue, human (cancer) cell lines are commonly used in toxicological studies assessing fine dust.

6.1.2 Beneficial Modifications of the Bioassays

As used microbial test subjects have an affinity to solid phases as described in “6.1.1 Reasons for Selected Microbial Biotests”, it is assumed that contact between test organisms and PM occur when aqueous suspensions of PM are used. This way, applied bioassays account for both the aqueous and solid phase of PM which might be crucial for a realistic environmental

exposure route (Kováts et al., 2012). In contrast to suspension testing which is used in this study, in the majority of the *in vitro* studies PM from wood combustion deposited on filters were extracted with organic solvents like methanol, dichloromethane, acetone, hexane or benzene and trespassed to a 1% DMSO solution before exposure to cells or microorganisms. This procedure is done due to two reasons. First, whole fine particle material needs to be separated from the filters. Second, authors expected the highest toxicity from the organic phase as toxicologically relevant groups like PAHs are soluble in it. However, testing either the organic phase or the aqueous phase of whole particles can over- or underestimate the toxicity of whole particles themselves depending on type of toxicity and strength of adsorption. For example, in organic extracts organic components are desorbed from PM which under normal circumstances e.g in lung would remain in the particle matrix. In the study by Baulig et al. (2009) organic extracts of atmospheric PM caused higher biological responses in human bronchial epithelial cells than aqueous extracts of the same fine dust samples. Moreover, toxicity results of whole particle testing differed from those of their respective extracts. In the study by Danielsen et al. (2008) organic extracts of PM caused higher DNA damage in a human lung epithelial and leukemia cell line than their respective native samples, although lower concentrations were applied from organic extracts (2.5 or 25 µg/mL of organic extracts, 25 or 100 µg/mL from native PM). Such differences in magnitude of toxicities were also observed by Fretwurst and Ahlf (1996). The authors investigated the influence of a model sediment phase on the genotoxicity of three genotoxins in the Umu-Test. Prior to testing genotoxins were shaken together with the particle phase. Subsequently, porous water was obtained by centrifugation. Both porous and whole particle suspensions were tested in the Umu-Test. Results showed that genotoxicity was underestimated when testing only porous water because genotoxic compounds accumulated on the particulate matter and became less bioavailable. These studies show that toxicity findings obtained from extracts differ among type of used solvent and might not predict toxicity from whole particles. Consequently, the conclusion drawn by Kocbach Bølling et al. (2009) “particles from poor combustion induced more severe effects on both cytotoxicity and DNA damage than particles from more complete combustion conditions” might be deficient and need further investigation since the authors based this statement on reviewed toxicity studies that used various particles exposure ways, namely organic and aqueous extracts or particles suspended in cell media. With regard to type of combustion, the amount of soot which is typically higher in incomplete combustion than in complete combustion can heavily influence bioavailability of chemical compounds. Soot can adsorb toxicants and make them less bioavailable (Talley et al., 2002).

This could mean that PAHs that are present in PM of both types of combustions might be less bioavailable in PM from incomplete combustion (Gauggel et al., 2012). In this line, the influence of the particle matrix on Zn-toxicity was studied in this thesis and is discussed in "5.4 Experiments with Zn" later on. Testing fine dust suspensions allows direct contact between whole PM sample and test organism and thus accounts for bioavailability of adsorbed toxicants and therefore was used in this study. As PM undergoes physico-chemical changes when applied as suspension, their physical characterization (size, zeta potential) was an obligatory prerequisite prior toxicity assessment additionally to chemical characterization.
In order to enable prefered whole-particle testing in chosen bioassays, one important modification of the Umu- and the Yes-Test was needed: measurement of optical density for determination of conventional growth was replaced by viability assessment via reduction of resazurin. This modification was beneficial due to two reasons. First, solid samples as fine dust cannot be tested by OD measurement as turbidity disturbs the photometrical signal. In contrast, the fluorescence signal of resorufin, which is the resulting product of resazurin reduction, is purely influenced by turbidity. Consequently, resazurin reduction can be employed on PM (that causes turbidity). Secondly, growth assessment via OD is less sensitive than viability testing with resazurin turnover as non-active cells give also a positive signal in photometrical OD measurement. Further discussion about increased sensitivity of the resazurin method is given in "6.1.3 Growth Calculation via Resazurin Reduction". Resazurin, also known as AlamarBlue®, has been used since the 1950s in dairy production for the detection of bacterial and yeast contaminations. Nowadays it is applied on cell lines, yeast and bacteria in order to estimate cell proliferation and assess baseline toxicity (Fai and Grant, 2009, O'Brian et al., 2000, Rönnpagel et al., 1995). The turnover of resazurin is directly linked to bacterial viability (Niles et al., 2009), but cell location of the reduction and responsible enzymes such as certain dehydrogenases and oxidoreductases were identified only partially (O'Brian et al., 2000). Resazurin is nontoxic and cells do not need to be killed prior measurement as with tetrazolium substrates in the MTT-assay or dehydrogenase activity measurements in soil (O'Brian et al., 2000, Thalmann, 1968). For these reasons this relatively old viability test was considered as "simple, rapid, efficient, reliable, sensitive, safe and cost-effective" (O'Brian et al., 2000) and employed in this study.

6.1.3 Growth Calculation via Resazurin Reduction

The linear shape of all three resorufin production curves suggested a diffusive efflux from water soluble resorufin from cells (Figure 4). Variations of observed resorufin kinetics may have resulted from dissimilar membrane structures of *A. globiformis* (BCT), *S. typhimurium*

(Umu-Test) and yeast (Yes-Test) entailing differences in resazurin uptake and resorufin release. Eukaryotic yeast with a relatively thick cell wall, in comparison to the prokaryotic bacterial cell wall, is less permeable and thus provide for a lower mass transfer into the cell (Walker, 1997), leading to a smaller velocity of resorufin production and time-delayed maximal turnover of resazurin in the Yes-Test compared to the BCT and Umu-Test. Indeed, as a result of genetic modifications *S. typhimurium* produce less lipopolysaccharides (rfa-mutant) thereby increasing the permeability of the cell membrane to many chemicals (Oda et al., 1985), and therefore presumably increasing also the influx of resazurin as well as the efflux of the resorufin.

Lowered resazurin reduction by MMC (0.25 mg/L) in the Umu-Test indicated baseline toxicity, which would have remained undetected by the optical density method (Table 8). Earlier studies using 0.25 mg/L MMC in *S. typhimurium* did not report toxicity for growth determined by OD600 (Giuliani et al., 1996). Hence, viability assessment via resazurin turnover might be more sensitive than OD600 measurement. In comparison, in the Yes-Test the difference between growth of 1% ethanol calculated by resazurin turnover and OD600 was not significant. Viability impairment of yeast by 1% ethanol was not expected as it is commonly used as negative control in yeast assays (De Boever et al., 2001, Denier et al., 2009). In earlier studies higher sensitivity of the resazurin reduction method compared to mere turbidity measurement was shown for bacteria and yeast. For instance, in a preliminary work of our research group MMC and other known genotoxins were tested in the Umu-Test using resorufin fluorescence reading and turbidity measurement for cytotoxicity detection (Toolaram et al., 2012). The latter proofed to be less sensitive for bacterial viability assessment than resorufin fluorescence reading. Similarly, Fai and Grant (2009) tested fungicides on yeast comparing both viability assessment methods. The authors measured resorufin fluorescence at a fixed point of time not as kinetics as in this study. The resorufin fluorescence inhibition bioassay turned out to be both faster and more sensitive than the growth inhibition bioassay (OD600). In view of above data and discussed literature, resazurin reduction was considered as more sensitive for viability testing than the conventional turbidity measurement. Furthermore, resazurin is non-toxic to neither bacteria nor humans, water soluble and thus, easy in handling and disposing (Fai and Grant, 2009). Its use in the BCT makes this baseline toxicity test highly attractive for high-throughput screening of PM.

6.1.4 Reproducibility and Robustness of Adapted Umu- and Yes-Test

Results obtained with the modified Umu-Test proofed to be stable when repeated, as variations within measured IR values (IR -S9: 20%, +S9: 18%) were similar to CV (IR –S9:

18%, +S9: 25%) reported from an interlaboratory study in the ISO-norm (Guideline ISO/FDIS 13829). Regarding IR values of the adapted Yes-Test (CV: 16%), Dhogge et al. (2006) reported in an interlaboratory study a lower CV (< 9%) for the conventional Yes-test. This difference might be a result of the relatively high variations in growth factor G (22%) in the modified version. Pretrials showed that despite shaking the plate before splitting it for separate viability and estrogenicity measurement, agglomeration of yeast cells was not completely dissipated. Hence, in each test a slightly different amount of cells was pipetted into the subsequent plate resulting in varying G. Nevertheless, the modified Yes-Test was reliable for testing estrogens as the mean EC_{50} of EE2 (44.0 ± 16.6 ng/L) was similar to the mean EC_{50} of 51.6 ± 11.9 ng/L that was measured by van den Belt et al. (2004) using the conventional Yes-Test.

6.2 Combined Biotest Battery

6.2.1 Chemical Composition and Toxicity

An “incomplete” combustion typically results in FP with high contents of carbon, a “complete” combustion in turn is characterized by FP containing low concentrations of total carbon (Alves et al., 2011, Kocbach Bølling et al., 2009). This is in line with chemical analysis of FPs (Table 13). No significant relationship between LOEC`s or EC_{50}’s of the individual endpoints and the chemical composition of the FPs (single components and total contents) or type of combustion (incomplete or complete) was detected. Barbosa et al. (2012) obtained a similar result for eluates of fly ashes of wood combustion, tested in an ecotoxicity battery. The authors described their findings like this: “it was not found any relationship between the chemical and the ecotoxicological behavior”. This lack of correlation was not surprising since biological responses are a complex result of contaminant/particle interaction and can be superimposed by organisms' activity. In addition, toxicant’s bioavailability in fine dust is crucial for their detection in toxicity tests. For instance, carbonaceous components like soot typically reduce bioavailability of known toxicants like PAHs as it was shown for other solid matrices like sediments (Talley et al., 2002). Besides differences in bioavailability, a mixture of toxic compounds can cause toxicities distinct from the sum of toxicities of single compounds. Risk assessment of contaminated particles combines chemical composition and toxic effects because potential toxic compounds which were not included in chemical analysis may also influence the bioassay results (Vu et al., 2012). However, some tendencies between chemical characterization and toxicity results were observed and are described within the following discussion of biotest results. For instance, Zn was suspected to predominantly

influence BCT results as it was shown in “5.2.3 Bacterial Baseline Toxicity in Relation to Zn Content”. As carbon contents were lower in PM from complete combustion than from incomplete combustion, it was assumed that the latter FPs would exert higher sorption capacity than PM from complete combustion. Increased sorption and thus decreased bioavailability of toxicants would result in lower toxicity. This expected influence of carbon content on toxicity was examined in detail by addition of toxic Zn to PM samples from both complete and incomplete combustion (see “6.3.1.3 Sorption Abilities of FPs”). It was expected that simultaneous exposure of Zn and PM from incomplete combustion would result in lowered Zn-toxicity in contrast to PM from complete combustion. Regarding toxicity outcomes of mixtures of toxic compounds that are present in FPs from wood combustion, Zn and fluoranthene was tested together since an over-additive toxicity was assumed as explained in “6.3.3 Zn and FLA Combination Effects”.

Besides examining the role of Zn and sorption capacities of tested PM, a suitable biotest battery for toxicity testing of FPs from wood combustion was looked for. Therefore, a comparative discussion of results obtained from different bioassays targeting the same MoAs is given in the following. Although it is assumed that chemical compounds with the same MoAs cause the same effects in different organisms (Escher and Hermens, 2002), divergences in exposure routes and organism’s sensitivities need to be considered and thus discussed.

6.2.2 Unspecific Toxicity in Bacteria, Cells and Nematodes

In this study all FPs from complete combustion were baseline toxic in comparison to FPs from incomplete combustion (Table 14). This contrasts studies with cell lines that detected higher cytotoxicities (baseline toxicity in cell lines) of organic extracts from FPs from incomplete combustion compared to complete/normal combustion (Jalava et al., 2010, Kocbach Bølling et al., 2009). Conducting the MTT assay on mouse macrophages, Jalava et al. (2010) tested methanol extracts of particulate emissions from normal and incomplete wood combustion. Samples from normal combustion induced less cytotoxicity than those from incomplete combustion. However, as described before in “6.1.2 Beneficial Modifications of the Bioassays” toxicity results of organic extracts cannot be directly compared with test results of this study as organic extracts of FPs typically induce different toxicity patterns compared to aqueous suspensions. The extraction of PM by organic solvents can make toxicologically relevant organic compounds like PAHs more bioavailable than they would be when whole particles would be tested. This way, an overestimation of toxicity is possible by testing extracts.

Regarding differences between all baseline toxicity tests, the BCT was more sensitive than the MTT assay. FP doses applied in the BCT overlapped the ones in the MTT test. Entire dose response curves and thus LOEC and EC_{50} values were obtained for five FPs in the BCT, whereas in the MTT test only a LOEC was detectable for one FP (Table 14). This difference in sensitivity might be due to the cell culture medium used in the MTT assay. The MTT medium of this study contained 10% of fetal bovine serum (FBS). High loads of proteins in FBS can bind e.g. metals and presumably diminish their bioavailability. Borenfreund and Puerner, 1986 showed that the toxicity of metals decreased 3-4 times in the neutral red assay with mouse fibroblasts when FBS was 10% instead of 1%

Besides the influence of cultivation media on toxicant's bioavailability, interspecies and interassay differences in toxicity responses to the same toxicants are known. Rudzok et al. (2011) conducted cell viability assays with different underlying cellular mechanisms e.g. the MTT test, cell count and resazurin uptake. Using these bioassays 17 xenobiotics like insecticides or pharmaceuticals and four metals were tested on a human hepatoma cell line and a protozoa species. The protozoa which is a eukaryotic single cell organism was 20 times more sensitive to applied xenobiotics and metals than the cell line. Independently from target object, the resazurin assay, also commercially available as AlamarBlue® assay, was most sensitive according to obtained LOECs. As with the BCT, the AlamarBlue® test is based on resazurin turnover which is an indicator for metabolic activity of used cells. The results of Rudzok et al. (2011) are very conforming with this study despite different target cells and test samples. Similar results were obtained in the study by Barbosa et al. (2012). The authors tested eluates from fly ashes from wood combustion in growth or mobility inhibition tests with the bacterium *Vibrio fischeri*, a freshwater and a marine micro-crustacean and two microalgaes. The freshwater organisms were more sensitive than marine organisms, whereas the bacterial *Vibrio fischeri* inhibition test was most sensitive. Specific reasons for higher sensitivity of protozoa or bacteria were not given in neither of the studies of Barbosa et al. (2012) or Rudzok et al. (2011). As it was mentioned above, cell medium might have influenced bioavailability of toxicants. Furthermore, toxic effects might occur earlier in unicellular organisms like protozoa or bacteria due to rapid growth rates compared to micro-crustacean, for instance, which pertain to a higher organismic level. Due to longer generation cycles of multicellular organisms and also cell lines, test time would presumably need to be prolonged in order to increase toxicity response in these test subjects. Nevertheless, sensitivity differences in different species in above mentioned studies and in this study cannot be fully explained.

Regarding the nematode reproduction test, surprisingly, lowest FP concentration (1 mg/L) showed higher toxicities than higher ones (Figure 5). Possible explanations could be that promoting effects at higher concentrations presumably compensated suppressing effects on the offspring that occurred at lower concentrations. Such nematode reproduction stimulation was reported for estrogens (benzylphtalate, n-octylphenol) and antiestrogens (tamoxifen) (Höss and Weltje, 2007). FP E showed a tendency of an inverse dose-response. The estrogen 4-nonylphenol (65.6 – 106.5 µg/L) caused an inverse dose-response-effect on nematode reproduction (Höss et al., 2002). Höss et al. (2002) suggested an interference of endocrine controlled processes like molting as possible reason for observed effect. Compounds like PAHs, which are commonly found in fine dust from wood combustion as in used FPs, were tested positively in an estrogen-sensitive reporter gene assay (ER-CALUX) (Wenger et al., 2009). Thus, PAHs could have served as estrogenic stimulating agents in FP E and other FPs. Nevertheless, the number of offspring never exceeded the one of the control when exposed to FP A, B, E, F or G. Hence, toxicants suppressing reproduction still dominated the toxicity pattern in the nematode reproduction test especially in the lower concentrations. Such toxicants could have been e.g. chemically detected heavy metals which typically decrease nematode reproduction (Jonker et al., 2009). FP C and D did not affect reproduction of nematodes. Both FPs derived from incomplete combustion and exhibited highest soot amounts among all FPs (Table 13). Therefore, an adsorption of potentially relevant contaminants might have lowered their bioavailability to nematodes. Adsorption of toxic cationic heavy metals on soot was described by Groszek (1997). As FP D was toxic in the BCT and the MTT test but not in the nematode reproduction test, cell lines and bacteria might have desorbed contaminants from soot. FP C did not trigger baseline toxicity in none of the tests. Either an irreversible adsorption of contaminants to soot occurred or concentrations of toxicants in FP C were too low to induce positive responses in chosen test systems. The non-toxicity of FP C could indicate a possible transferability of the test systems, but with the precondition of broader concentration ranges in all tests. In order to achieve more precise LOEC values in the nematode reproduction test, the range of applied concentrations should be increased in future tests. But as the classic test is conducted in petri dishes and one test takes seven days, work hours and used material would be multiplied. Test results with bacteria can be obtained within 1-3 days. In comparison, bioassays with cell lines often take several days until first results are achieved, e.g. the MTT Test takes four days. Since rapid, cost-effective and easy to handle bioassays in microscale are demanded by industry, especially since the EU REACH regulation has entered in force (Fai and Grant, 2010), microbial biotests as chosen in

this study should be preferred for primary toxicity screening. Furthermore, the studies by Lundstedt et al. (2007), Barbosa et al. (2012) and this study showed that unicellular organisms with an independent metabolism like protozoa and bacteria are suitable for toxicity testing and may provide for higher sensitivities than cell lines or multicellular organisms like algae, nematodes or micro-crustacean. Therefore, with regard to time constraints and test sensitivity, the BCT with the proposed test protocol might be most applicable for short-term testing of baseline toxicity of FPs from wood combustion (incomplete and complete).

6.2.3 Specific Toxicity in Bacteria, Yeast and Cells

The detection of test organism's viability was of great importance in the Umu- and Yes-Test in order to avoid false positive target toxicities, namely genotoxicity and estrogenicity (Toolaram et al., 2012). FP A and D caused baseline toxicity in the Umu- and the Yes-Test (Table 15). FP D and E were baseline toxic only in the Umu-Test. It was assumed that identic internal effect concentrations would cause similar baseline toxicities because the target enzyme group (dehydrogenases) was the same in test organisms, bacteria and yeast (Reifferscheid and Heil, 1996). Physiological differences could lead to different influx of toxicants into the cells leading to deviating internal effect concentrations and thus different resulting toxicities as it was described in "6.1.3 Growth Calculation via Resazurin Reduction". Furthermore, the influence of different cultivating media on toxicant's bioavailability should be considered as a possible reason for observed distinct baseline toxicities in the Umu- and the Yes-Test.

Significant induction of the DNA repair mechanisms (Umu-Test) mainly occurred after metabolic activation with S9 at the highest applied concentration of FP A, B and C (Table 15). Mutagenicity was detected before on organic extracts of wood smoke as Naeher et al. (2007) reviewed. More recently, Cohn et al. (2011) tested organic extracts from PM samples that derived from biomass combustion. A significant correlation between PAH concentration and mutagenicity tested in the Ames-Test with S9 activation was found. As it is known that certain PAHs can be activated by S9 and positively tested in the Umu-Test (Nakamura et al., 1987), such substance group might have influenced to positive Umu-Test results. The PAH CALUX® assay was designed for detecting the presence of PAHs. It gave positive responses for all FPs. However, the results of the Umu- or the PAH CALUX® assay did not correlate with total analyzed PAHs or single PAHs. Except FP A, all FPs contained the same range of total PAH content (Table 10). FP C, D and F caused lowest responses (highest LOEC) in the PAH CALUX® assay (Table 15) and contained the highest carbon contents besides FP A. This indicated decreased bioavailable PAH contents in those samples, presumably due to

PAH adsorption to organic carbon. As it was described before for e.g. sediments (Talley et al., 2002), different carbon contents of the FPs could have reduced bioavailability of PAHs. FP A had the highest PAH content and gave the greatest response in both the Umu-Test, the Yes-Test and the PAH CALUX® (lowest LOECs). Consequently, it is assumed that either PAHs or substances that occur in parallel with PAHs during combustion had an influence on toxicity results in presented specific-mode-of-action tests, though, not a linear one. Regarding the Umu-Test, other activated genotoxic but not analyzed compounds might have contributed to positive effects as it was expected in other studies with fine dust from wood combustion. Vu et al. (2012) tested organic extracts from wood combustion in the Ames test. The authors did not find any correlation between emission factors of carcinogenic PAHs and mutagenicity. They suspected nitro-PAHs and Cl-PAHs to be the main genotoxic causatives. Other phenolic compounds that fit to the Ah receptor like dioxins could have contributed to observed positive results in PAH CALUX® assay.

Although PAHs can be detected in the Umu- and the PAH CALUX® assay, the distinct underlying mechanisms in both bioassays should be considered. The Umu-Test measures indirect genotoxicity: Single stranded DNA, trinucleotides and oligonucleotides caused by genotoxins lead to activation of the SOS response system which is artificially coupled with the production of ß-galactosidase. In contrast, the PAH CALUX® assay detects the activation of the intrinsic Ah-receptor upon binding of ligands, e.g. PAHs. This means that non-genotoxic PAHs or other compounds like dioxins with an affinity to the Ah-receptor will also trigger the production of subsequently measured luciferase. Hence, the Umu-Test and the PAH CALUX® assay have different aims and are thus not comparable despite the fact that both detect PAHs.

As PAHs exhibit a weak ER binding (Santodonato, 1997) and only FP A with the highest PAH content was estrogenic in the YES-Test (Table 15), PAHs presumably contributed to this result. It was surprising that FP A was tested positive in the Yes-Test, but not in the MELN test, although both estrogenicity reporter assays are based on the same underlying mechanism: the fusion of an estrogen response element (ERE) with reporter genes (luciferase for MELN, lacZ for Yes-Test). This was in contrast to the study by van den Belt et al. (2004). The authors detected as maximum a 15 times higher sensitivity of the MVLN-assay (transformed MCF-7 human breast cancer cell line) towards 17ß-estradiol, estrone, 17α-estradiol and nonylphenol compared to the Yes-Test. Applied concentrations in this study might give explanations: the LOEC of the Yes-test (42 mg/L) was still higher than the maximal applied concentration in the MELN test (10 µg/cm², which equals approximately

18 mg/L). Exposure times were similar in both tests (~20 h), but FPs were shaken overnight for the Yes-Test as pretrials revealed higher induction rates in the Yes-Test when particles were shaken for 24 h before exposure. Hence, estrogenic substances probably desorbed from FP A when shaken overnight and thus presumably became bioavailable. As the application of higher concentrations in the MELN assay was not feasible, a comparison regarding sensitivity of both assays was not possible.

6.3 Role of Zn for Baseline Toxicity in Bacteria

Besides kali and earth alkali metals, Zn is the most abundant metal in wood smoke as it was shown in this study in "5.2.1 Chemical Analysis" and in other studies (Jalava et al., 2007, Kocbach Bølling et al., 2009). Since toxicity of FPs increased with rising Zn contents among most of tested samples (see "5.2.3 Bacterial Baseline Toxicity in Relation to Zn Content"), Zn was assumed to play a major role in BCT outcomes. Additionally, Zn is known as inhibitor of the bacterial respiratory electron transport system (Choudhury and Srivastava, 2001) and thereby exerted clear toxicity in the BCT when tested alone (Figure 6). It was hypothesized that baseline toxicity only occurred when bioavailable Zn-species, which were identified by X-ray diffraction, were present. Furthermore, it was examined if the presence of FPs would diminish pure Zn-toxicity when Zn was incubated together with FPs with high carbon contents (incomplete combustion) due to adsorption of Zn to soot. Pretrials suggested an over-additive toxicity of Zn and FPs from complete combustion (negligible carbon content). In order to study such expected mechanism of mixture toxicity, Zn was tested together with FLA. Both chemical compounds dominated metals or PAHs, respectively. Combined exposure of metals and PAHs revealed over-additive toxicity before (Gogolev and Wilke, 1997), thus, this mechanism was assumed to occur in fine dust from wood combustion.

6.3.1 Bulk FPs

6.3.1.1 Zn-Toxicity

Detected pure Zn-toxicity in the BCT (EC_{50} = 16 µM) was higher than reported by Nweke et al. (2007). The authors determined an EC_{50} of 206 µM for isolated *Arthrobacter* species from a river sediment by dehydrogenase activity measurement via reduction of TTC. This increased tolerance towards Zn-ions might have evolved due to elevated background concentration of Zn in sampled sediments. In contrast, detected total inhibitory concentration (IC_{100}) of Zn at around 1000 µM for the isolated *Arthrobacter* bacteria in Nweke et al. (2007) agrees well with this study (Figure 6). Supposedly, Zn-detoxification systems like specialized Zn-efflux

pumps that develop explicitly in Zn resistant bacteria (Choudhury and Srivastava, 2001) might be exhausted at certain Zn levels causing similar IC_{100} in *Arthrobacter globiformis* (BCT) and isolated *Arthrobacter* species in Nweke et al. (2007).

6.3.1.2 Identified Zn-species in bulk FPs

The presence of particles can strongly influence bioavailability of metals (Liß and Ahlf, 1997). In particle-free solutions specification of metals predominantly determines their toxicity. The ionic form of trace metals like Zn is mainly transported via ionic channels into cells and subsequently exerts toxicity (Ahlf et al., 2009). It was assumed that the influence of PM from complete combustion on metal's bioavailability would be small due to low carbon contents. Therefore, FPs were examined for bioavailable Zn ions ($ZnCl_2$, $ZnSO_4$) and non-charged, non-aqueous and thus less bioavailable Zn crystals (ZnO) by XRD measurement. Depending on type of wood, temperature and oxygen supply, ZnO and $ZnCl_2$ are formed during wood combustion (Sippula et al., 2009, Jöller et al., 2007). ZnO is formed from gaseous Zn at T < 1100 ° C when oxygen is sufficiently supplied (Sippula et al., 2009). As FP A, C, D and F resulted from "incomplete" combustion the formation of ZnO under low temperatures was expected. But only FP A showed considerable ZnO amounts in XRD spectrum. Zn was highly abundant in all FPs in chemical analysis, but crystalline Zn-species was little or not detected by XRD. Consequently, Zn was mostly bound in a non-crystalline form which was not detectable by XRD. Similarly, Wiinikka (2008) expected the formation of ZnO in wood pellet combustions by model prediction that was based on combustion conditions. The authors did not identify ZnO by XRD measurements and hence assumed that "Zn was bound in a more complex solid phase than ZnO". Such binding could result from Zn-complexation and adsorption of Zn ions on scarce hydrophilic sites of carbon surfaces (Groszek, 1997). Crystalline Zn-complexes with anions like abundant sulfates could not be identified reliable in XRD spectrum as their small peaks were overlapped by e.g. KCl. Amorphous Zn-complexes cannot by detected by XRD. Hence, it remains unclear in which formation Zn was present in all FPs except of FP A and B.

The presence of little bioavailable ZnO would explain the lack of baseline toxicity in FP A in contrast to bioavailable and hence toxic $ZnCl_2$ in FP B (Figure 7). Furthermore, an irreversible adsorption of Zn or any other baseline toxicants to carbon presumably occurred in non-toxic FP C which exhibited highest carbon contents (Table 13). Bacteria as a competing ligand (Ahlf et al., 2009) were not able to make Zn, which was chemically detected, or any other particle-bound toxicants bioavailable in FP C. As toxic FP D contained similar carbon contents than FP C, bacteria possibly desorbed Zn and other toxicants from particles or

toxicants were desorbed by shaking FPs overnight causing finally bacteriotoxicity. Nevertheless, a relationship between (non-)bioavailable Zn-species and toxicity was not found in any other FPs than FP A and B. The influence of carbon content on baseline toxicity remained as unclear as the role of Zn for baseline toxicity of bulk FPs. Either other baseline toxicants than Zn or Zn in non-identifiable by XRD, but bioavailable form contributed to detected baseline toxicities of FP D, E, F, G in the BCT.

6.3.1.3 Sorption Abilities of FPs and Possible Over-Additive Zn-Toxicity in Presence of FP

It was assumed that the addition of toxic Zn to non-toxic FP would cause over-additive toxicity only when FPs had very low carbon contents due to complete combustion (FP B, E, G) (Table 13). Carbonaceous matrices like soot which is highly abundant in PM from incomplete combustion can reduce bioavailability and thus lower toxicity due to sorption (Talley et al., 2002). It was expected that the higher the inherent carbon content of FPs was the lower would be measured baseline toxicity in the BCT when toxic Zn was added. In this line, it was assumed that PM from complete combustion with low soot contents would have negligible sorption capacities and thus not influence Zn-toxicity. Regarding the over-additive toxicity, it was hypothesized that organic components as PAHs with a mode of action 1 (MoA1), namely narcosis, which are bioavailable in fine dust enhance baseline toxicity of Zn in an over-additive manner. Sikkema et al. (1994) reported increased fluidity of the membrane layer of liposomes prepared from *E. coli* bacteria by accumulation of hydrocarbons e.g. toluene or phenanthrene in the membrane. This increased fluidity resulted in an elevated influx of protons, a decreased proton motive force and thus a disturbance of energy production. It was assumed that PAHs can cross bacterial cell walls and embed in the subsequent cytoplasmatic membrane (Gogolev and Wilke, 1997). Hence, it was suggested that the influx of cations like baseline toxic Zn increase and result in enhanced baseline toxicity as it was shown for protons (Sikkema et al., 1994). Gogolev and Wilke (1997) extracted viable bacteria from soil and exposed them simultaneously to Zn and fluoranthene. The authors made the alteration of membrane's permeability responsible for observed over-additive toxicity.

Within non-toxic samples in the BCT (FP A and C) the addition of 400 mg/L of FP C to Zn reduced Zn-toxicity more than addition of FP A (Figure 10). This is presumably due to considerably higher carbon content of FP C (Table 13) and thereby higher sorption capacity for Zn. This sorption capacity of both FPs decreased when 6 mg/L of FP was tested (Figure 11). It was assumed that less binding sites for Zn were available when particle concentration decreased. Nevertheless, the carbon content of FPs did only partially predict resulting Zn-

toxicity. For instance, FP A and FP F had similar carbon contents (23-25 % w/w, see Table 13) but FP A did not alter Zn-toxicity when added in a concentration of 6 mg/L. In comparison, FP F (6 mg/L) lowered Zn-toxicity especially in middle Zn-concentrations (Figure 11). In general, both FP types from complete and incomplete combustion decreased Zn-toxicity and thereby most likely both types had inherent sorption capacity for Zn. Although, carbonaceous matrices like organic carbon are known to decrease bioavailability of metals (Ahlf et al., 2009), other mineral particle surfaces like terminal, negatively charged chemical groups like OH^- or SO_4^{2-} may adsorb Zn and build stable complexes. Consequently, bioavailability is decreased and thus also toxicity. This phenomenon is known for minerals and clays like kaolinite in soils which can adsorb Zn and other metals (García-Miragaya and Dávalos, 1986).

FP E and G showed tendencies of over-additive toxicity in lower concentrations (Figure 11). This may be explained by embedded PAHs in bacterial membrane resulting in an increased influx of Zn as mentioned before. Such apparent over-additive toxicity disappeared in subsequent Zn concentrations in this study, indeed, less toxicity than additive toxicity appeared. Detoxification by Zn-transporters could have taken place within higher Zn concentrations (Choudhury and Srivastava, 2001). Though, it remains unclear why possible detoxification (if occurring) was triggered only by high Zn concentration and not by lower ones.

All in all, reduced Zn-toxicity was detected within FPs from complete and incomplete combustion. Hence, all FPs showed sorption capacities. Partially observed over-additional toxicity of Zn tested together with fine dust from complete combustion could not be explained sufficiently.

6.3.2 Pure and Mixture Toxicity of Filter Samples

Excluding kali and alkali metals, Zn also dominated the metal-fraction in filter samples (Figure 8) which is typical for complete combustions (Jalava et al., 2007). Wiinikka (2008) reported that with rising bark content of pellets, Zn content also increased. This was very conforming with the findings of this study (Figure 8). Coherence between type of pellet and baseline toxicity was only observed for type of wood and water content. Though, a calculation of correlations lacked for enough data. PAH content was probably too low to cause observed baseline toxicity (Sikkema et al., 1994). Osmotic stress by ions could be excluded by pretrials. When comparing Figure 8 and Figure 9 a relationship of Zn and baseline toxicity was suggested. Nevertheless, low Zn-contents in the samples from pellets with different water contents did also result in high toxicity (Figure 9). Zn-concentrations were calculated for each

tested filter cut-out and compared with pure Zn-toxicity. Detected baseline toxicity exceeded pure Zn-toxicity. Besides other (not identified) baseline toxicants, mixture effects like those of metals and PAHs might have occurred within the filter samples and contributed to baseline toxicity. To assess such possible combined effects of Zn and fine dust, mixture experiments were conducted.

As discussed in "6.3.1.3 Sorption Abilities of FPs" over-additional toxicity of Zn and PM was expected due to PAHs present in PM. Filter samples were preferable to study expected over-additional toxicity since they derived from complete combustion and thus had little carbon contents resulting in supposedly negligible sorption capacities for Zn. Combination experiments consisted in addition of $ZnCl_2$ to selected filter samples (Figure 12). As within two bulk FPs from complete combustion only lower Zn concentrations caused over-additive toxicities within the filter samples (Figure 11). This could be result of embedded PAHs as it was described before in "6.3.1.3 Sorption Abilities of FPs". Sample "water content_7.3%" and "type of wood_pine" had detectable PAH amounts, "water content_12.1%" not (Table 16). Not analyzed PAH metabolites or substitutes may have added up to the total PAH load. Postulated detoxification mechanism at higher Zn concentrations remained questionable as explained before. Hence, an assumed over-additive toxicity by addition of Zn to filter samples (complete combustion) was not confirmed as it only appeared in low Zn concentrations.

6.3.3 Zn and FLA Combination Effects

In order to study expected mechanism of over-additional toxicity of Zn and a bioavailable organic components with a MoA1, fluoranthene was chosen. FLA was the most prevalent PAH in FPs besides pyrene and is considered as a narcotic PAH (MoA1) with a $\log K_{ow}$ of 5.16 (EG Wasserrahmenrichtlinie10/23/2000.). It was expected that FLA in non-toxic concentrations would enhance the potency of Zn as it was discussed in "6.3.1.3 Sorption Abilities of FPs and Possible Over-Additive Zn-Toxicity in Presence of FP".

Detected pure FLA toxicity (EC_{50} = 7.1 µM) was in the range of FLA toxicity to nematodes in the reproduction test (EC_{50} = 4.5 µM) (unpublished data of our own laboratory). Gogolev and Wilke (1997) observed a much lower toxicity on bacteria extracted from soil. Counting viable bacterial colonies the authors observed an inhibition of 27% for 494 µM FLA. This pronounced difference in toxicity may be due to adsorption of FLA to soil particles in the study by Gogolev and Wilke (1997). The authors spread soil dilutions on agar plates that were amended by FLA.

Expected over-additional toxicity did not occur when testing Zn and FLA together (Figure 14). Indeed, the opposite toxicity pattern was observed. Decreased bioavailability of Zn due to

complexation by FLA or DMSO was considered as a cause. Both molecules are able to build complexes with Zn (Oprunenko et al., 2002, Meek et al., 1960). In a Zn-DMSO combination trial no difference to Zn-toxicity was detected (data not shown). Hence, decreasing Zn bioavailability by complexation with DMSO was excluded as possible reason for observed lowered toxicity. The complexation of Zn by FLA may exist between the π-electrons of FLA and the cation Zn^{2+} and thus is probably weak as it is no ionic binding (Oprunenko et al., 2002). Therefore, a decrease of Zn-toxicity due to complexation of Zn-ions by FLA was less probable. The underlying mechanism of how FLA impaired the influx of Zn into bacterial cell remained unclear and would require a more mechanistic approach like assessing membrane integrity and labeling of Zn.

In summary, previously observed over-additive toxicity in fine dust samples (both bulk and filter samples) from complete combustions could not be explained by expected mechanism of combined exposure to Zn and a low molecular PAH as FLA.

6.4 Influence of Atmospheric Transport on Formation of Nitroarenes

When fine dust from wood combustion is emitted into ambient air, atmospheric transformation processes include structural and chemical changes and thus can alter toxicity (Lundstedt et al., 2007). In order to study such processes, experiments in atmospheric simulation chambers were conducted. This study focused on the formation of nitroarenes during simulated transformation processes. Nitroarenes are highly mutagenic and their chemical detection is difficult and not yet standardized (Arey, 1998). By means of the Umu-Test using the nitroarene sensitive NM3009 *Salmonella* strain and the conventional *Salmonella* strain, the bioavailable fraction of nitro-PAHs was determined in non-aged and aged samples that were produced in an atmospheric chamber that simulated day and night chemistry. For nitro-PAHs chemical alteration by enzymes is necessary to exert toxicity (Oda et al., 1993). In the NM3009 Salmonella such metabolization is accelerated by overexpression of specific enzymes. Thus, as it was expected, the NM3009 tester strain was more sensitive to the positive control 1,3 Dinitropyrene (LOEC = 0.08 ng/mL) than the conventional Salmonella strain (LOEC = 0.41 ng/mL). Detected LOECs were comparable to values reported earlier by Oda et al. (1993). The authors defined LOEC values as the lowest concentration causing IR > 2 (in this study LOEC was calculated on the basis of the t-test) and obtained a LOEC of 0.1 ng/mL for the NM3009 strain and 0.4 ng/mL for the conventional tester strain. The presence of nitro-PAHs in the filter samples was only detected by the Umu-Test (NM3009 strain) (Figure 15), not within applied chemical analysis.

Consequently, sensitivity of the Umu-Test was higher than chemical detection limit (2.5 µg per filter). The formation of multiple-substituted nitro-PAHs during oxidation processes would possibly explain such higher sensitivity. In chemical analysis only mono-substituted nitroarenes could be detected. In contrast, multiple-substituted nitro-PAHs e.g. dinitro-PAHs can be detected in the Umu-Test as they are metabolized by the nitroarene sensitive tester strain to their genotoxic form. During combustion such substituted PAHs are generated simultaneously to PAHs. But in ambient air the gas-phase radical-initiated formation of nitroarenens is more important as source than direct emissions from nitroarenes (Arey, 1998). Secondary formed nitro-PAHs are transformation products of PAHs that can occur at day- and nighttime. During daytime, PAHs react with OH radicals that are formed via photolysis and subsequent NO_2-additon. During nighttime nitro-PAHs are formed by reaction of PAHs with nitrate radicals or N_2O_5 (Arey, 1998, Pitts, 1987, Kamens et al., 1990). As the amount of non-substituted PAHs diminished increased stay in the chamber in this study (Table 18), these reactions are expected to have taken place in the atmospheric simulation chamber, supposedly due to transformation to substituted PAHs like nitro-PAHs. The latter is also supported by the decreasing NO_2 content with increasing time (Table 17). FP masses differed among samples. Hence, it remained unclear, if chemical oxidation of PAHs produced significantly higher nitro-PAHs during day- or during nighttime processes. The formation of low molecular nitro-PAHs like nitrofluorene and nitropyrene was likely in sample Daytime 1_aged (Table 18). However, this sample did not show genotoxicity probably due to low masses of deposited fine dust and thus decreased nitro-PAH concentration that were presumably below Umu-Test sensitivity (Figure 15). Differences in induction ratios among the other aged samples might be influenced by total FP masses, though, no clear correlation was found. Prolongation of incubation time typically increases inducibility in the Umu-Test (Nakamura et al., 1987) leading to higher IR when incubated 5 h instead of 2 h as more nitroarenes are metabolized into their genotoxic compounds.

When calculating fine dust concentrations on the basis of deposited PM mass on filter cutouts, concentrations of samples positive for genotoxicity with the nitroarene sensitive *Salmonella* strain ranged from 1 – 3 µg FP/mL. These values are similar to the LOEC of 0.05 – 5 µg/mL determined by Funaska et al. (2003) albeit the latter authors tested methylene chloride extracts of urban atmospheric particles rather than whole particles as presented here. Funaska et al. (2003) used the same tester strain as in this study. The assumption that nitro-PAHs, primarily or secondarily formed, contribute significantly to the total direct-acting mutagenic potential of ambient air and wood combustion exhausts (Arey, 1998, Vu et al., 2012) is supported by the

findings of this study, especially as only the nitroarene sensitive *Salmonella* strain but not the conventional strain demonstrated genotoxicity in tested FP samples.

In general, nitroarens are toxicologically highly relevant. Whereas oxy-PAHs, which are also formed by atmospheric transformation processes, might be less mutagenic than their parent original PAH compound (Lundstedt et al., 2007), nitro-PAHs are often more mutagenic when tested in the Ames-Test (Arey, 1998, Pitts, 1987). In fact, some nitro-PAHs are classified as the most potent direct-acting bacterial mutagens (Rosenkranz and Mermelstein, 1983). Therefore, their consideration in toxicity testing of PM samples is highly important. As chemical analysis of nitroarenes, especially of multi-substituted nitroarenes, is difficult and not standardized, presented Umu-Test with the NM3009 tester strain provides for detection of bioavailable nitroarenes with very high sensitivity. Furthermore, the lack of S9 liver extract addition makes the test way easier in sample handling than the conventional Umu-Test. All in all, this study shows that atmospheric transformation might be crucial for (geno-)toxicity of emitted PM from wood combustion. Hence, adverse health effects might not only derive from toxicants adsorbed on PM, but also their atmospheric transformation products.

7 Conclusion

To the author's knowledge this is the first study that used microbial bioassays for toxicity testing of PM from wood combustion in a direct contact manner. The feasibility of chosen and modified test systems, the BCT, the Umu-Test, and the Yes-Test was demonstrated by testing bulk FPs and PM loaded on filters. Modifications were needed for the Umu- and Yes-Test as conventional test system can be applied only for aqueous samples. Hence, presented microbial test battery allows a rapid, easy to handle and low-priced screening of unknown fine particles. Whole-particle testing accounted for particle-bound contaminants and ensured realistic bioavailability. Direct particle contact between test organism and fine dust sample is highly recommendable in toxicity testing since extraction of organic or aqueous components alters particle matrices and neglects toxicant's bioavailability. Chemical composition typically does not predict toxicity findings of whole particles since the particle matrix can influence such bioavailability of chemical compounds as discussed in this study. In this line, it was expected that only FPs with high carbon contents (incomplete combustion) would exert sorption capacity for toxic Zn, not FPs from complete combustion with low carbon contents. Surprisingly, all FPs revealed sorption capacities for toxic Zn, regardless of their content of total carbon. This result emphasizes the crucial role of the particle matrix for bioavailability of pollutants independently from type of combustion. In general, Zn is highly abundant in wood combustion derived FPs. Expected importance of Zn for PM's baseline toxicity was not confirmed. Indeed, combination experiments with Zn, FLA and fine dust samples caused contradicting results. For instance, simultaneous testing of FLA and Zn did not show expected over-additive toxicity. XRD measurements did only partially provide further insights of bioavailable and non-bioavailable Zn crystals. To clarify the role of Zn for baseline toxicity in future attempts, mechanistic studies with e.g. Zn-resistant bacteria or labeled Zn would be meaningful. In view of experiments with Zn, Zn was not identified as leading chemical compound for baseline toxicity. The finding of a leading chemical compound for toxicity would have been highly beneficial as mitigation actions regarding toxicity could target on the removal of such compound. Such mitigation actions would result in significantly decreased toxicity of emitted PM.

With respect to evaluation of the chosen tests, the BCT turned out to be most sensitive in contrast to other baseline toxicity tests with cell lines and nematodes in the overall study. The Umu- and Yes-Test still need to be evaluated for sensitivity compared to other bioassays. Since other applied receptor-mediated toxicity tests in the overall study targeted other MoAs

or test concentrations did not overlap, a comparison with Umu- or Yes-Test was not possible. Nevertheless, expected toxicities, namely baseline toxicity, genotoxicity and estrogenicity were detected by means of selected biotests. Therefore, primary toxicity screening of unknown fine dust samples targeting three distinct MoAs is possible using the test battery. Since MoAs are assumed to be the same among different organismic levels (Escher et al., 2005), transferability of obtained toxicity results to higher organisms including humans is expected, but would need more profound investigation.

Regarding toxicity results among fine dust samples, PM from both complete and incomplete wood combustion caused positive results in all microbial test systems. Hence, a distinction of types of combustions by means of toxicity was not possible. This was surprising as inspected studies concluded that PM from incomplete combustion would show higher toxicity potentials than PM from complete or normal combustions (Jalava et al., 2010, Kocbach Bølling et al., 2009). Different exposure ways (organic extract testing versus whole-particle testing) and thus different bioavailability of PM's toxicants might be the main reason for this discrepancy with literature. However, it should be emphasized that, in general, complete combustion produces less particulate mass and noxious gases e.g. CO, NO in total than incomplete combustion. Therefore, relating obtained LOEC or EC_{50} values to emitted particulate mass/gases would facilitate a quantitative comparison of different combustion devices in terms of toxicity. Besides that, it would be beneficial for both regulators and consumers to relate toxicity results and emissions to the intrinsic heat value (kWh) of used boilers and stoves. This way, a cost-benefit analysis including toxicity outcomes of emissions can be developed for each wood combustion device. Risk assessment of PM on the basis of microbial bioassays may accelerate technical advancement of appliances and combustion conditions and thus also contribute to a reduction of environmental and human impact of wood combustion fine particles. Such technical improvements for toxicity mitigation include secondary actions as the installation of precipitators in the flue gas channel. The efficiency of such precipitators could be verified by presented microbial test battery in future projects.

This study concentrated mainly on distinguishing complete and incomplete combustion with regard to toxicity. But as results showed that both types of combustion induced positive toxicity results, characterization of combustions needs to be extended. A first attempt in this study included variations in quality of burned wooden fuel, namely water content or wood type of used pellets. The type of wood influenced baseline toxicity in the BCT. Other characteristics like water or bark content may alter toxicity as well. In literature, toxicity patterns among different types and quality of burned wood are studied insufficiently yet. As

existing toxicological studies rarely name the type of wood or form of wooden fuel (e.g. pellets or logs), reviewing and evaluating the influence of wooden fuel on toxicity outcomes is difficult (Naeher et al., 2007). In order to minimize PM's toxicity that is caused by certain properties of used wood fuels, future studies should investigate such relationships. Consequently, the optimal wooden fuel for each wood combustion device should be finally found with regard to toxicity potential of produced PM.

Exposure of humans and environment to fine dust from wood combustion takes place after emission and subsequent atmospheric transport. During atmospheric transport, transformation processes of fine dust take place. They are highly complex as they are driven by many factors such as intensity of UV-light, temperature or other present gaseous and particulate molecules (Naeher et al., 2007). Therefore, a prediction of how and if toxicity of emitted PM change after release into atmosphere is complicated. Atmospheric transformation processes make assessment of PM's impact on human and environmental health even more difficult as changes in toxicity of PM are highly uncertain and thus exposure to toxic PM can vary heavily. Hence, it is of tremendous importance to find ways to include atmospheric transformation processes into risk assessment. To the author's knowledge the impact of night and day chemistry as well as atmospheric oxidation on PM's toxicity was presented first in this study. The relevance of nitro-PAHs in atmospherically aged FPs for genotoxicity was shown by using a nitroarene sensitive bacterial strain in the Umu-Test. Results suggested that the formation of genotoxic nitroarenes took place when fine dust was transported in atmosphere. Chemical detection of nitroarenes is difficult and may not be as sensitive as used tester strain as shown in this study. Therefore, the modified Umu-Test that was introduced in this study may help to understand how atmospheric alteration of fine dust influences genotoxicity outcomes. Atmospheric transformation processes should be taken into account in comprehensive risk assessment of PM in future studies as they can change chemical and physical properties and thus toxicity findings in *in vitro* tests. But as atmospheric alterations comprise a variety of chemical and physical reactions, the embedment of such in toxicity studies would require close collaborations of atmospheric chemists, physicists, and toxicologists. This might remain a challenge.

8 Outlook

8.1 Trends in Small Scale Wood Combustion Devices

In the recent years Europe and the U.S. have expanded the construction and installation of sophisticated wood combustion appliances for domestic heating. Such automatically operated stoves and boilers abet complete combustions and thereby low emissions. Against the background of the end of fossil fuels, the use of CO_2-neutral biomass instead is wanted and thus supported from policy sides by subsidizing the purchase of pellet stoves, for instance (Faaij, 2004). Educational activities about the correct use of stoves and boilers were lanced (Umweltbundesamt, 2007). Campaigns for stove exchange programs and "burn bans" are further examples for policy driven actions (U.S. Environmental Protection Agency, 2009). Additionally, ordinances and directives regulate emission values of new small-scale wood combustion devices, e.g. an important regulation in Germany is called the German Federal Immission Control Ordinance (1. BImSchV). The probable link between adverse health effects and wood smoke exposure has been recognized in the recent years not only from scientists but also from politicians. Compared to western countries, developing countries may face much more severe impacts on health by wood smoke as cooking with biomass and thus direct exposure to its emissions is common in these countries (Fullerton et al., 2008). In contrast, most of the research about quantity, characterization and toxicity of wood smoke is done in the U.S. and Europe. Thereby, use and construction of improved wood combustion appliances take also mainly place in these countries. As the burning of biomass is cheap and traditionally anchored in developing countries, improvements regarding wood smoke exposure might be facilitated by political awareness and consequently politically driven actions in future. Scientific studies with specific research questions often precede such politically actions as can be exemplarily seen in the EU REACH regulation. This regulation forces chemical industry to provide chemical safety data about their chemical product. This data in turn can be obtained, for example, in microbial tests (Fai and Grant, 2010).

As presented microbial test battery makes a high-throughput screening of PM possible, many combustion devices could be assessed with regard to, for instance, the relationship between operation of the appliances or used wooden fuel on PM's toxicity. Therefore, presented bioassays might be highly valuable as toxicity test tool and thus should be used in such studies.

8.2 Advances in Toxicity Testing of Fine Dust

8.2.1 Improvements in Combustion Experiments

Wood smoke and associated health-damaging pollutants differ in composition from emissions from other combustion processes. Indeed, every emitted fine dust might be unique in toxicity pattern as wooden fuel and completeness of combustion vary among existing wood stoves and boilers. In order to narrow down the reasons for PM's toxicity from device side, combustion parameters as well as wooden fuel characteristics would need to be changed successively in one appliance. Collected PM should be tested in bioassays, obtained toxicity results should be related to gaseous/ particulate emissions and compared to other appliances. This procedure would entail a large-scale experimental setup in order to allow the identification of factors influencing toxicity and finally an evaluation of different stoves and boilers. The resulting high number of gained FPs would require a first toxicity screening in high-throughput manner. This can be fulfilled by the presented microbial test battery.

8.2.2 Challenges in PM Characterization

Testing whole particles via submersed exposure as performed in this study can cause morphological and chemical changes of samples. Therefore, a physico-chemical characterization of the suspensions is required in order to account for such particle matrices changes. Besides characterization, standardized test procedures for testing fine dust would enable comparative examinations of different studies. Microbial bioassays are partially standardized for other solid matrices like sediments and thus applicable to PM from wood combustion. Standardized tests with cell lines are scarce yet. Characterization and standardization are also demands of scientists working with artificially produced nanomaterials. In the course of the fast rising number of newly emerging nanomaterials, the OECD gave recommendations for sample preparation and dosing of nanoparticles that are tested in bioassays (OECD, 2010). Such approach would be highly recommendable for testing fine dust from wood combustion.

8.2.3 New Exposure Techniques

In order to minimize physical and chemical modifications of FPs during test application and to simulate realistic exposure routes, there exists an exposure technique at the air/liquid interface (Aufderheide et al., 2003, BMU: Thrän and Pfeiffer, 2012). Human lung cells are grown on a porous membrane in transwell inserts. Before exposure with the test atmosphere, the medium above the cells is removed whereas below the membrane enough medium remains for a sufficient nutrient supply. This way, cells are directly exposed to the test gas

which thereby does not undergo physical changes like in submerged testing. Different biological responses were detected in cell lines using this air/liquid *in vitro* method (Diabaté et al., 2008). It is an advantageous method for direct exposure in mechanistic studies and may gain importance in future. Though, the complexity in handling the gas dosing to the test cells and the high costs of the whole test system makes it less applicable for a high-throughput-screening of different wood combustion devices with varying emissions.

8.2.4 Genetically Modified Microorganisms as Test Objects

Exposure of PM suspension in microbial assays copes with sample alterations in water, but allows first toxicity screening of many samples. Besides, microbial bioassays may help to minimize the use of animal products such as S9 mix (bacteria) and fetal calf or fetal bovine serum (cell lines). In the case of PAHs, metabolic activation of PAHs takes place in higher organisms before causing damage to DNA (Neilson, 1998). Bacteria that are used in bioassays are not capable to exert such activation, therefore, an external enzyme mix, called S9 mix, is added in bacterial genotoxicity tests in order to allow the detection of PAHs that need to be activated first (Guideline ISO/FDIS 13829). Tests with genetically designed bacteria with inherent metabolic activation system, as the NM3009 *Salmonella* strain in this study, avoid the use of S9 which is gained from rats. Fetal calf or fetal bovine serum serves as growth medium ingredient in tests with cell lines (Masters and Palsson, 1999). The avoidance of fetal calf or fetal bovine serum in tests with cell lines is difficult as cell lines require complex media with specific nutrients and growth factors (Masters and Palsson, 1999). Therefore, microbial tests that do not require animal products as the Umu-Test with the NM3009 *Salmonella* strain might constitute alternatives to tests with cell lines. Furthermore, low costs of microbial assays may "play an important role in the acceptance of a specific test system" (Reifferscheid and Heil, 1996) and thus make them economically attractive for evaluation of wood combustion fine dust. Hence, the author of this thesis is convinced that the presented test battery will play a more important role in risk assessment of PM from wood combustion in future. Another promising approach beyond laboratory studies is the use of bacterial sensor-reporters in the field. They are based on genetically modified bacteria that detect target compounds like nitroarenes in this study. Polluted soil sites were already assessed this way (Tecon and van der Meer, 2008). Future scenarios could comprise microbial onsite measurements at selected locations. Predestinated sites would be e.g. rural valleys with high occurrences of domestic wood combustion appliances. Especially valleys suffer from high PM concentrations when air inversion conditions appear. Such onsite measurement by living

organisms is known from monitoring of drinking water quality. Thereby fish and mussels are used as living onsite sensors. Nevertheless, these are future scenarios.

8.2.5 Necessary Linkage between Acute and Chronic Effects

Besides bacteria and yeast, effects of PM on the molecular level are studied on cell lines and multicellular organisms of different organismic levels like nematodes, animals or humans. Such studies comprise among other endpoints the overexpression of certain genes that are involved in xenobiotic's metabolism in body fluids. Examining the underlying molecular processes after fine dust exposure is the key for understanding potential chronic adverse health outcomes in whole organisms. Therefore, mostly acute (partially also chronic) toxic effects measured in short-term bioassays with high concentrations need to be linked to chronic toxic effects observed in whole organisms or even populations which are typically exposed to low concentration. In order to extend the knowledge about the relationship between acute and chronic effects, PM samples that are tested in bioassays should be taken from ambient air at study sites where epidemiological studies in e.g. hospitals are conducted in parallel. Epidemiological studies can provide information about occurring illnesses and ambient PM air concentrations. Such classical epidemiological studies could be complemented by human biomonitoring studies. The latter offer more realistic exposure concentrations by measuring e.g. PAH metabolites or certain DNA adducts in body samples that are related to combustion processes. Such molecular biomarkers reflect personal exposure concentrations better than environmental concentrations. But as certain body defense mechanisms like metabolization of PAHs that are targeted by human biomonitoring are also triggered by emissions from other combustion processes, source identification is necessary. For atmospheric samples (not body samples) levoglucosan is an accepted marker for wood combustion. This tracer was also measured in urine samples from humans and thus might provide for a necessary molecular biomarker in human biomonitoring studies (Wallner et al., 2013). Internal exposure concentrations of wood smoke could be monitored on the basis of levoglucosan. This example shows that human biomonitoring approaches could be advantageous in long term studies.

All in all, advances in *in vitro*, *in vivo* and epidemiological studies are expected regarding study design, exposure techniques, comparability, transferability, and finally toxicological insights resulting from wood smoke exposure. A linkage of different studies is aimed for.

8.3 Ecotoxicity of FP

Today's fine dust research focuses on human health, whereas the impact of PM from wood combustion on ecological systems has been little investigated. As emissions from wood stoves may reach different environmental compartments via atmospheric transport, ecotoxicological characterization of PM should be considered as well. Barbosa et al. (2012) gave an example of a possible ecotoxicity test battery. The authors tested aqueous eluates from fly ashes from an industrial biomass boiler. Performed bioassays comprised the luminescence inhibition test with *Vibrio fischeri*, mobility inhibition tests of a freshwater and a marine micro-crustacean (*Daphnia magna* and *Artemia franciscana*), and growth inhibition of a freshwater and a marine microalgae (*Selenastrum capricornutum* and *Phaeodactylum tricornutum*). All samples showed toxicity in all test organisms. Freshwater organisms were more sensitive than marine organisms, whereas the bacterial *Vibrio fischeri* test was most sensitive. In another study of 2012 native outdoor aerosol samples were tested on *Vibrio fischeri* and showed positive results (Kováts et al., 2012). Besides unspecific endpoints like growth inhibition, ecotoxicological risk assessment should also target specific MoA. Specific MoA like endocrine disruption can have a great impact on developmental processes, sexual differentiation, and reproduction (Eggen et al., 2004) and thus can threaten both human and environmental health. As specific and unspecific toxicities can cause adverse health effects, microbial bioassays may help to direct both human and environmental mechanism-based assessment. In order to provide for a more comprehensive ecotoxicological risk assessment, both information about environmental exposure and toxicity are required. Selected organisms that represent different environmental compartments should be tested with environmentally relevant PM concentrations. Obtained results should be compared to those retrieved from bioassays, for instance, as used in this study. This way, information about extrapolation from effects on simple test organisms like bacteria to higher organisms is improved. Concepts for the transformation of acute data to chronic data, like the species sensitivity distribution, exist for ecological risk assessment of substances (Duboudin et al., 2004). These concepts should be applied to PM risk assessment in order to gain more information about long-term effects of PM. Anyhow, as ecosystems are characterized by highly complex interactions, bioassays and also tests with higher organisms may always only partially reveal potential threats to whole ecosystems. Nevertheless, the recent studies of Barbosa et al. (2012) and Kováts et al. (2012) and the results of this study underline that simple microbial bioassays can serve as a first (eco-)toxicity screening of fine dust in a risk assessment procedure.

9 References

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10 Appendices

10.1 Supporting Information

Table S1: Anion content in filter samples loaded with non-aged fine dust.

mg anions per g loaded filter					
	Chloride	Nitrite	Nitrate	Sulfate	total
water content_2.9%	0.26	0.37	0.05	2.87	3.55
water content_6.5%	0.25	0.36	0.07	3.11	3.78
water content_7.3%	0.35	0.34	0.08	3.72	4.49
water content_12.1%	0.20	0.30	0.05	2.54	3.09
location II	0.30	0.22	0.06	1.93	2.51
location III	0.35	0.19	0.06	1.82	2.43
type of wood_pine	0.14	0.00	0.07	1.47	1.69
type of wood_cottonw. I	0.16	0.36	0.05	5.87	6.44
type of wood_cottonw. II	0.24	0.40	0.05	8.52	9.21
bark_10 wt%	0.28	0.36	0.07	4.11	4.81
bark_20 wt%	0.48	0.28	0.06	4.72	5.54
bark_30 wt%	0.57	0.31	0.06	5.84	6.79
bark_40 wt%	0.93	0.00	0.03	2.22	3.18

Table S2: Metal content in filter samples loaded with non-aged fine dust.

mg metal ion per g filter									
	K	Ca	Na	Mg	Zn	Mn	Fe	Cd, Pb, Cr, Cu, Ni, Al	total metals (without K, Ca, Na, Mg)
water content_2.9%	6.05	0.06	0.02	0.02	0.13	0.03	0.01	0.06	0.23
water content_6.5%	6.72	0.05	0.04	0.02	0.12	0.02	0.01	0.02	0.18
water content_7.3%	6.65	0.03	0.05	0.04	0.13	0.03	0.12	0.06	0.33
water content_12.1%	5.17	0.07	0.03	0.02	0.09	0.01	0.01	0.06	0.17
location II	3.03	0.13	0.02	0.02	0.07	0.02	0.02	0.05	0.17
location III	1.58	0.05	0.02	0.02	0.08	0.01	0.02	0.04	0.15
type of wood_pine	0.95	0.07	0.02	0.03	0.06	0.01	0.03	0.05	0.16
type of wood_cottonw. I	13.91	0.02	0.13	0.01	0.18	0.00	0.01	0.05	0.25
type of wood_cottonw. II	20.57	0.04	0.17	0.03	0.27	0.00	0.01	0.03	0.31
bark_10 wt%	8.21	0.04	0.05	0.02	0.24	0.03	0.03	0.05	0.34
bark_20 wt%	5.55	0.04	0.04	0.02	0.34	0.02	0.02	0.02	0.40
bark_30 wt%	6.74	0.13	0.05	0.04	0.53	0.03	0.02	0.04	0.62
bark_40 wt%	1.99	0.03	0.03	0.02	0.59	0.01	0.04	0.03	0.66

Table S3: Analyzed PAHs in filter samples loaded with non-aged fine dust. – below detection limit (< 213 ng/ g filter).

ng/ g filter	Acenaphthylene, Acenaphthene, Fluorene	Phenanthrene	Anthracene	Fluoranthene	Pyrene	Benzo(a)anthracene	Chrysene	Benzo[b]fluoranthene	Benzo[k]fluoranthene	Benzo[a]pyrene	Indeno[1,2,3-c,d]pyrene	Dibenzo[a,h]anthracene	Benzo[g,h,i]perylene	total
water content_2.9%	-	277		3093	4843	395	331	480		464	741		1419	12043
water content_6.5%	-	-	-	304	400	-	-	-	-	-	-	-	-	704
water content_7.3%	-	-	-	213	267	-	-	-	-	-	-	-	-	480
water content_12.1%	-	-	-	-	-	-	-	-	-	-	-	-	-	-
location II	-	-	-	1312	2005	-	-	-	-	-	240	-	459	4016
location III	-	-	-			-	-	-	-	-		-	-	-
type of wood_pine	-	-	-	1083	1589	224	-	336	-	235	331	-	501	4299
type of wood_cottonw. I	-	-	-	725	987	-	-	-	-	-	-	-	-	1712
type of wood_cottonw. II	-	-	-	736	965	-	-	-	-	-	-	-	-	1701
bark_10 wt%	-	-	-			-	-	-	-	-	-	-	-	-
bark_20 wt%	-	-	-	400	544	-	-	-	-	-	-	-	-	944
bark_30 wt%	-	224		491	581	-	-	-	-	-	-	-	-	1296
bark_40 wt%	-	-	-	-	-	-	-	-	-	-	-	-	-	-

10.3 Abbreviations

ALRI	Acute lower respiratory tract infection
BCT	Bacterial Contact Test
BfG	Bundesanstalt für Gewässerkunde (German Federal Institute of Hydrology)
BImSchV	Bundesimmissionsschutzverordnung (German Federal Immission Control Ordinance)
BMU	Bundeministerium für Umwelt, Naturschutz und Reaktorsicherheit (German Federal Ministry of Environment, Nature Conservation and Nuclear Safety)
COPD	Chronic obstructive pulmonary disease
CV	Coefficient of variation
DBFZ	Deutsches Biomasseforschungszentrum (German Biomass Research Center)
DIN	Deutsches Institut für Normung (German Institute for Standardization)
EC_{50}	Half maximal effective concentration
ER	Estrogen receptor
FLA	Fluoranthene
FP	Fine particle sample
G	Growth factor
IARC	International Agency for Research on Cancer
IC_{100}	Total inhibitory concentration
IfT	Leibniz-Institute of Troposphere Research
IR	Induction ratio
LOEC	Lowest observed effect concentration
KIT	Karlsruhe Institute of Technology
MELN	MCF-7 human ERE-ßGlob-Luc-SV-Neo
MoA	Mode of Action
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-10 diphenyltetrazolium bromide
OECD	Organization for Economic Co-operation and Development
PAHs	Polycyclic aromatic hydrocarbons
PALM	PC3 human androgen receptor-Luciferase-MMTV
PM	Particulate matter
$PM_{2.5}$	Particulate matter < 2.5 µm
PM_{10}	Particulate matter < 10 µm
SD	Standard deviation
Ss	Slope of mol%-resorufin of the sample

TCC	Triphenyl tetrazolium chloride
TFZ	Technologie- und Förderzentrum (German Technology and Promotion Center)
TUHH	Technical University of Hamburg-Harburg
US EPA	United States Environmental Protection Agency
VOC	Volatile organic components

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Weitere Personen waren an der Abfassung der vorliegenden Arbeit nicht beteiligt. Die Hilfe eines Promotionsberaters habe ich nicht in Anspruch genommen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Eigenabgrenzung / Kooperationen

Die in Kapitel „Material and Methods“ beschriebene physikalische Charakterisierung der Feinstaubproben sowie alle Versuche mit Zelllinien wurden von Susanne Gauggel der Universität Konstanz durchgeführt. Die chemische Charakterisierung übernahm Irene Richardt-Brauer am Institut für Umwelttechnik und Energiewirtschaft (IUE) der Technischen Universität Hamburg-Harburg (TUHH).

Vorarbeiten zur Adaptation des Umu-Tests wurden von Anju Priya Toolaram im Rahmen einer von mir betreuten Diplomarbeit gemacht. Die Ergebnisse des Umu-Tests mit *Salmonella typhimurium* TA1535/pSK1002 wurden in einer weiteren Diplomarbeit unter meiner Betreuung von Paula Queiroz de Aquino erzielt. Der Nematoden-Reproduktionstest wurde von Nutcha Kootiratrakarn im Rahmen einer Diplomarbeit mit Birte Hegemann als Betreuerin durchgeführt (siehe Kapitel „Material and Methods“). Alle genannten Diplomarbeiten wurden am IUE der TUHH erstellt.

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