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Mutagenicity and induction of CYP1A-enzymes from organic extracts of soil samples from nursery schools, evaluated by *in vitro* studies

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Marte Høye Thorsen

Picture at front page: Photographed and given admission to use by Yvonne Minde, 2011.

Abstract

Polluted soil can be a problem in urban areas, and can have a direct impact on human health upon exposure. The toxic potential of mixtures can be mediated by constituents and information about toxicity of mixtures is regarded as important, as it represents the real exposure situation.

The main purpose of this project was to measure the mutagenic and CYP1A inducing potential *in vitro* from organic extracts of soil, sampled in nursery schools in Oslo. Selection of soil was mainly based on the content of $\sum PAH_{16}$ and $\sum PCB_7$, chemical groups known to include CYP1A inducing and/or genotoxic and mutagenic compounds. Generally were Soil 1 considered as a "clean" sample, Soil 2 to Soil 4 contained increasing level of PAHs and Soil 5 contained mainly elevated levels of PCBs. Assessing varying samples in relation to chemical content was valuable due to potential differences in biological responses.

The soil samples were of top soil (0- 2 cm depth) and had been collected by NGU in connection to a geological survey of soil at playgrounds in nursery schools and schools in Norway. The soil was sampled in 2005- 2007, dried at 40 °C for one to two weeks, sieved in a 2 mm nylon sieve and stored in the dark at room temperature. Chemical analyses of both inorganic and organic compounds were performed before storage. In relation to this master project the organic pollutants in the selected soil samples were extracted by ultrasonic agitation in dichloromethane (DCM). Before experimental use the solvent was changed into dichloromethane (DMSO) by evaporating off the DCM using a water bath at 25 °C and a flow of nitrogen above. The dried extracts were redissolved in DMSO.

The Ames *Salmonella typhimurium* assay was used for measuring mutagenicity. The presence of primary and secondary mutagens was assessed by conducting the assay both with and without addition of a metabolic S9- mix. Induction of different point mutations was revealed by utilising two bacterial strains, TA98 and TA100, detecting frameshift and base- pair substitutions, respectively. Induction of CYP1A enzymes was assessed in the rat H4IIE hepatoma cell line, and measured immunologically by Western blotting. The exposure concentrations used in the CYP1A assay were based on results of cell viability, assessed by utilising MTT- assay for finding the highest non- cytotoxic exposure concentrations. Concentration ranges of the extracts were tested in both assays.

The mutagenic potential of extracts showed presence of secondary mutagenic compounds, and indicated absence or very low levels of primary mutagens. It was a general incidence of higher mutagenic activity with TA98 than TA100, reflecting highest induction of frameshift mutations. The inducing potential of extracts was in accordance with chemical analysis, showing a general increase in the potential of extract from Soil 1 to Soil 4, suggested to partly reflect differences in level of PAHs. The relative low potency of extract from Soil 5 was considered to be a reflection of a low content of PAHs and an expected dominance of PCBs, which have shown not to induce mutagens in the Ames assay.

Induction of CYP1A in H4IIE was measured after exposure to extracts of Soil 3, Soil 4 and Soil 5. Results clearly indicated presence of CYP1A inducers in the extracts. A positive

concentration- effect relationship was detected from exposure to extract of Soil 5. Extract of Soil 3 and Soil 4 did clearly induce CYP1A, but in a negative concentration dependent manner. These negative responses were suggested to indicate inhibition of CYP1A induction at the higher concentration, which may be linked to antagonism at the Ah- receptor.

The biological endpoints measured in the current project reflected the integrated effect from extract exposure, potentially affected by additivity, synergism and/or antagonism. Differences in toxicity between *in vitro* and *in vivo* conditions, along with several biological and environmental parameters can affect the biological responses. The results obtained in the current project indicated presence of potential hazards in the soil, but no further conclusion could be drawn about the actual hazard from humans exposure to polluted soil.

Sammendrag

Forurenset jord kan være et problem i urbane områder, og kan ved eksponering ha en direkte effekt på menneskers helse. Det toksiske potensialet av blandinger kan medieres av bestanddeler tilstede, og informasjon om blandingers toksisitet er ansett som viktig ettersom blandinger utgjør den reelle eksponeringssituasjonen.

Hovedmålet med dette prosjektet var å måle det mutagene og CYP1A induserende potensialet *in vitro* ved eksponering for organisk ekstrakt av jord, prøvetatt i barnehager i Oslo. Valg av prøver var hovedsakelig basert på innholdet av $\sum PAH_{16}$ og $\sum PCB_7$, som er kjemiske grupper kjent for å inkludere CYP1A induserende og/eller genotoksiske og mutagene forbindelser. Generelt var Jord 1 ansett som en ren prøve, Jord 2 til Jord 4 inneholdt økende innhold av PAHer, mens Jord 5 inneholdt forhøyede nivå av PCBer. Evaluering av ulike prøver i forhold til kjemisk innhold var ansett som verdifullt med hensyn på muligheten for varierende biologiske responser.

Jordprøvene var av overflatejord (0- 2 cm dybde) og var samlet inn av NGU. Dette i sammenheng med en geologisk undersøkelse av jord på lekeplasser i barnehager og på skoler i Norge. Jorden var samlet inn i 2005- 2007, tørket ved 40 °C i en til to uker, siktet i en 2 mm nylon sikt, og lagret i mørket ved romtemperatur. Kjemiske analyser av både uorganisk og organisk innhold var utført før lagring. I forhold til denne masteroppgaven ble organiske miljøgifter ekstrahert ved ultralyd behandling i diklormetan (DCM). Før eksperimentell bruk var løsemiddelet endret til dimetylsulfoksid (DMSO) ved å dampe av DCM ved bruk av et 25 °C vannbad under en strøm av nitrogen. De tørkede ekstraktene var løst i DMSO.

Ames *Salmonella typhimurium* assay ble benyttet for å måle det mutagene potensialet. Tilstedeværelse av primære og sekundære mutagener var undersøkt ved å utføre testen både med og uten tilsetning av en metabolsk S9- blanding. Induksjon av ulike punktmutasjoner var avdekket ved bruk av to bakteriestammer, TA98 og TA100. TA98 detekterte leserammeforskyvninger, mens TA100 detekterte baseparsubstitusjoner. Induksjon av CYP1A enzymer var undersøkt i cellelinjen H4IIE fra rottelever, og målt immunologisk ved bruk av Western blotting. Eksponeringskonsentrasjonene benyttet ved måling av CYP1A induksjon var basert på resultat av celleoverlevelse, undersøkt ved bruk av MTT metoden for å finne den høyeste ikke- cytotoksiske konsentrasjonen av ekstraktene. Konsentrasjonsserier av ekstraktene var undersøkt i alle analysene.

Det mutagene potensialet til ekstraktene viste tilstedeværelse av sekundære mutagene forbindelser, og indikerte fravær eller veldig lave nivåer av primære mutagener. Det var en generell forekomst av en høyere mutagen aktivitet i TA98 enn TA100, noe som reflekterte en høyere induksjon av leseramme- mutasjoner. Ekstraktenes induserende potensial var i samsvar med de kjemiske analysene, demonstrert ved en generell økning i potensialet fra Jord 1 til Jord 4. Disse resultatene var foreslått delvis å reflektere nivået av PAHer i jorden. Det relativt lave potensialet til Jord 5 var antatt å være en refleksjon av et lavt innhold av PAHer og en forventet dominans av PCBer, som har vist ikke å indusere mutagener i Ames test.

Induksjon av CYP1A i H4IIE var målt etter eksponering for ekstrakt av Jord 3, jord 4 og Jord 5. Resultatene indikerte klart tilstedeværelse av CYP1A indusere i ekstraktene. Positivt konsentrasjon effektforhold var detektert fra eksponering for ekstrakt av Jord 5. Ekstrakt av Jord 3 og jord 4 induserte tydelig CYP1A, men i en negativ konsentrasjonsavhengig måte. Disse negative responsene tydet på inhibering av CYP1A induksjon ved høyere konsentrasjon, noe som kan linkes til antagonisme ved Ah reseptoren.

De biologiske endepunktene som ble målt i dette prosjektet reflekterte den integrerte effekten fra ekstrakt eksponering, potensielt påvirket av additivitet, synergisme og/eller antagonisme. Den biologiske responsen kan medieres av biologiske parametere og miljøfaktorer, og det vil kunne være store forskjeller mellom toksisitet i *in vitro* og *in vivo* kontekst. Resultatene som var oppnådd i den gjeldende oppgaven indikerte tilstedeværelse av forbindelser med en potensiell risiko. En videre konklusjon om den reelle faren for mennesker ved eksponering for forurenset jord kunne ikke trekkes på basis av dette studiet.

Abbreviations

Ah	Aryl hydrocarbon
AhR	Aryl hydrocarbon receptor
APS	Ammoniumpersulphate
ARNT	Aryl hydrocarbon nuclear translocator
bHLH-PAS	basic helix-loop-helix-Per-Arnt-Sim
B[a]P	Benzo[a]pyrene
BSA	Bovine serum albumine
CYP	Cytochrome P450
DCM	Dichloromethane
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Oestrogen receptor
EROD	Ethoxyresourfin O- deetylase
eq.	Equivalents
FBS	Foetal bovine serum
GI	Gastrointestinal tract
HRP	Horseradish peroxidise
HSP	Heat- shock proteins
MTT	3-(4,5- dimethylthiazol-2-yl)- 2,5- diphenyl tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NGU	Geological Survey of Norway
NIPH	The Norwegian Institute of Public Health
NPD	4-nitro-o- phenylendiamine
P	Probability
PAH	Polycyclic aromatic hydrocarbons
Pb	Lead
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyls
Pen Strep	Penicillin Streptomycin
r^2	Correlation coefficient
ROI	Region of Interest Measurment
ROS	Reactive oxygen species
S.D.	Standard deviation
SDS	Sodium dodecyl sulphate
SDS- PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCDD	2,3,7,8,- tetrachlorodibenzo- p- dioxin
TEMED	N, N, N, N`- tetramethylene diamine
U.S. EPA	The United State Environmental Protection Agency
XRE	Xenobiotic responsive elements

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1 Introduction

Large quantities of pollutants are released to the atmosphere, hydrosphere and lithosphere from industrial and anthropogenic activity, leading to polluted soil that may constitute a genotoxic hazard to plants and biota (White & Claxton 2004). Urban soil has in the last years got an increased attention and a geochemical mapping of soil in Norwegian cities was started around ten years ago. Historically the urban ground has been a major sink for waste and pollution from anthropogenic activity. The soil has been used and reused several times, leading to partly high levels of pollutants in some areas. Urban soil is in general characterized to consist of building materials, fire remnants, domestic waste, industrial waste, carriage of soil and the local, natural soil. This has in turn lead to soil containing a mixture of chemical compounds, such as polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB) and heavy metals among others. The urban soil is dynamic and construction activity has lead to uncontrolled spread of pollution (Ottesen & Langedal 2008).

1.1 Environmental polluted soil at playgrounds and nursery schools

Ground pollution can constitute a threat to human health. In urban areas of Norway the highest health risk connected to polluted soil is often children's exposure to moderately polluted soil at playgrounds. The ground here can consist of a mixture of soil with different origin, such as soil present from before establishment, transported soil used for artificial landscaping and transported sand used in sandboxes (NGU 2005). The construction activity in cities that include digging and transport of soil can result in polluted soil being reused in sensitive areas where people play and live. The Geological Survey of Norway (NGU) has proven that organic material, such as sewage sludge and bog soil, has been mixed into polluted soil and later been sold to nursery schools (Ottesen & Langedal 2008).

A survey of surface soil in over 700 nursery schools in Oslo showed that 38% had pollution levels over recommendations, while there in Bergen were 39 out of 87 nursery schools with levels that were too high (Ottesen et al. 2008). An assessment of surface soil in ten nursery schools in Trondheim, showed that half of them had pollution levels higher than recommended (NGU 2008). The level of pollutants was compared with quality criterias for soil in nursery schools, playgrounds and schools, stated by The Norwegian Institute of Public Health (NIPH) (Alexander 2006).

Polluted soil may have a direct impact on human health by exposure through ingestion, inhalation or dermal absorption (Abrahams 2002). The ingestion can occur either deliberately or unintentionally. Young children are especially vulnerable to soil ingestion in their playing activity, due to their frequent hand- to- mouth activity and tendency to eat nonfood items (U.S. EPA 2008). Stanek and Calabrese (1995) estimated that the median soil ingestion for children between the age of 1- 4 years was 13 mg/day or less for 50% of the children and 138 mg/day or less for 95% of the children. Relative high ingestion levels of soil among children

makes it important that the soil is as clean as possible to diminish the health hazard (NGU 2005).

The United State Environmental Protection Agency (U.S. EPA) has sat recommendation values for soil ingestion to 30 mg soil/day for infants from 6 to 12 months and 50 mg soil/day for 1 to 6 year olds. These values include outdoor and indoor soil ingestion, but not dust which is another source of exposure. The values are limited by estimated quantities of soil ingested, and are not taking important factors as bioavailability and gastrointestinal absorption into consideration (U.S. EPA 2008). In risk assessment is the routinely employed soil ingestion rate 200 mg/day for children in the United State of America (White & Claxton 2004). The mentioned quality criteria's for soil pollution at playgrounds in Norway were sat on the basis of this rate and children's weight and toxicity of the compound (Alexander 2006).

1.2 Organic pollutants

Organic pollutants have been detected in a wide range of compartments in media and biota all over the world. The behaviour, fate and toxicity are dependent on chemical and physical characteristics of the compound and the nature of the environment (Safe 2000). Many of these organic compounds are known as persistent (El-Shahawi et al. 2010) and are often halogenated, mostly by chlorine. Stable carbon-chlorine bounds make them resistant to photolytic, chemical and biological degradation. Moreover the greater the number of chlorine substitutes and/or other functional groups, the more persistent the chemical is against breakdown (Chu et al. 2006). Characteristics as semi volatile enable chemicals to be transported over longer distances in the atmosphere, either as vapour or bound to solid particles of soil, sediment or ash, before deposition to the ground (El-Shahawi et al. 2010). These pollutants can be taken up by the biota, and the toxic effect can be highly complex and compound specific. Individual compounds can elicit several toxic responses in a tissue/organ-, species-, and sex- dependent manner (Safe 2000). Examples of highly studied organic pollutants found in the environment are illustrated in figure 1.2.

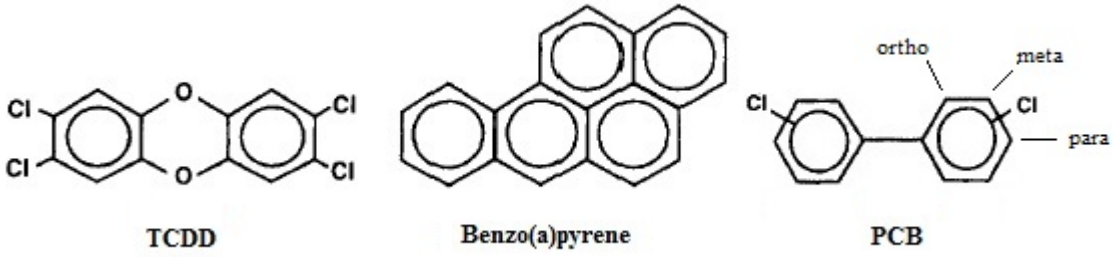

Figure 1.2 Chemical structures of three classes of organic pollutants; dioxin, polycyclic aromatic hydrocarbon (PAH) and polychlorinated biphenyl (PCB). Figure modified from Rowlands and Gustafsson (1997). The dioxin 2,3,7,8,- tetrachlorodibenzo- p- dioxin (TCDD) is known as the most potent organic pollutant, and toxic potency of similar compounds is often presented relative to TCDD (Safe 1994). The illustrated PAH is benzo[a]pyrene (B[a]P) which is also known as highly potent (Whitlock 1999). The polychlorinated biphenyl (PCB) illustrate the general structure and the positions where the substituted chlorine atoms are seated in the PCB congeners (Walker et al. 2006).

1.2.1 Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a collective term for organic pollutants composed of two or more fused aromatic hydrocarbon rings, normally occurring in mixtures. These pollutants have been found in high concentrations in urban soil (Ottesen et al. 2008; Ottesen & Langedal 2008), but also on remote places with no nearby sources (Baek et al. 1991). Especially older parts of cities in Norway have shown to be polluted by PAHs, derived mainly from incomplete combustion of fossil fuel in traffic, firing (wood, coal, oil) and city fires, as well as from tar- or creosote treated material (Ottesen & Langedal 2008). The finding of PAHs on remote areas is suggested to be caused by long- range transport in the atmosphere, and was one of the first pollutants in the atmosphere identified as carcinogenic (Baek et al. 1991). The largest contribution of PAHs in Norway originates from the aluminium industry and household wood firing (Alexander 2006).

Many PAHs are resistant to biochemical degradation in the solid material of the terrestrial environment, and possess characteristics as low aqueous solubility and high octanol- water distribution. The water solubility and the following availability for PAHs to the biota decreases with increasing molecular mass, while the stability of PAHs in soil increases at the same time as they tend to adsorb to soil particles. Bacteria normally degrade water dissolved chemicals, but the high molecular PAHs composed of five to seven rings are very little soluble in water. Compounds that are unavailable for microbial degradation can accumulate up to concerning levels in the environment (Johnsen et al. 2005). Accumulation in the biota is rare despite a hydrophobic character of most PAHs, caused by a high biotransformation rate into more excretible compounds (vanSchooten et al. 1997). Several PAHs can exert toxic effects upon organisms by being genotoxic, mutagenic and/or carcinogenic either in their native form, or after biotransformation to more toxic compounds (Meador 2008). Among the PAHs benzo[a]pyrene (B[a]P) is one of the most highly studied and potent compounds (fig. 1.2), known to be transformed into carcinogenic derivates (Klaunig & Kamendulis 2008). In a ground survey in Oslo it was found that B[a]P constituted about 10% of the sum PAH_{16} in most of the 1200 soil samples analysed (Alexander 2006).

1.2.2 Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are a group of synthetic, chlorine containing compounds known to be toxic, chemically stable, inflammable and bioaccumulative (Safe 1994). Theoretically there exist 209 different PCB congeners, which differ in the level of chlorination (1- 10) and the position of these atoms around the two aromatic rings that constitute the carbon skeleton (Thomas 2008). Stereochemistry of the congeners is dependent on the chlorine position, making the compound either coplanar, which is the most toxic form, or more globular. The coplanar form occurs with no substitution in the ortho position (fig. 1.2) (Nebert & Dalton 2006; Walker et al. 2006).

These chemicals have been highly used as organic diluents, plasticizers, flame retardants and as dielectric fluids for transformers and condensers (Safe 1994). The manufacture and use of PCBs has been banned since 1980, but leaching from waste products and withering of buildings and concrete constructions has led to spreading to the environment (Ottesen & Langedal 2008). Once introduced into the environment they degrade relative slowly and undergo cycling and accumulation in various environmental compartments and biota (Safe 1994). The water solubility decreases with increasing chlorination (Urbaniak 2007), reducing the microbial degradation by the same principle as explained for PAHs (part 1.2.1).

Negative health effects from exposure have been observed, but the acute toxicity is relatively low. Chronic exposure to PCBs in laboratory animals has elicited effects as hepatoxicity, dermal toxicity, reproductive and developmental toxicity, carcinogenicity and neurotoxicity, among other responses (Safe 1994). Short term *in vitro* exposure has shown to elicit induction of biotransformation enzymes (Safe et al. 1985; Nebert & Dalton 2006) and genotoxicity (Schilderman et al. 2000). The effect from exposure is dependent on factors such as; chemical properties, species, strain, age, sex, and route, duration and frequency of exposure. Impact on the environment and biota is additionally dependent on the individual congeners and their additive and/or non- additive (synergistic and antagonistic) effect, with themselves and other classes of chemical pollutants (Safe 1994).

1.3 Bioavailability and toxicity of pollutants in soil

The environmental fate and toxicity of pollutants in soil can be highly affected by the soil type, due to differences in sorption and bioavailability. For example, can fine clay particles possess a negative charge and therefore retain high levels of positively charged ions of toxic metals (e.g. copper, chromium, arsenic, nickel and cadmium), while organic pollutants with limited water solubility usually get sorbed to soil organic matter and clay particles (White & Claxton 2004). Organic compounds can occur in different forms and phases in soil (Ruby et al. 1999), and the following bioavailability can be affected by the chemical compound (Ellickson et al. 2001; Cave et al. 2010), total PAH to soil organic carbon content (Cave et al. 2010), physical and chemical weathering, biological processes, infiltration of water and disturbance from anthropogenic activity (Ruby et al. 1999). Increased soil- pollution contact time or so called "aging", with the inherent processes of surface sorption, intra- particle diffusion, biodegradation and entrapment within humic complexes, has also been claimed to decrease compounds bioavailability (Kelsey et al. 1997; White et al. 1997; Reid et al. 2000).

Soil bound pollutants can upon ingestion become released in the gastrointestinal (GI) environment and is consequently ready for absorption into the systemic circulation of organisms, termed the bioaccessible fraction (Ruby et al. 1999). From this fraction only some of the absorbable parts will be taken up into the blood, and this is the actual bioavaliable portion (Ellickson et al. 2001). The remaining fraction fixed to indigestible particles will leave the body without exerting any effect (Oomen et al. 2002). Bioaccessibility of organic pollutants has shown to be affected by the presence of food in the gastrointestinal tract, increasing the accessibility of compounds, due to the presence of fat and bile salt that mobilize hydrophobic organic compounds (Oomen et al. 2000; Tang et al. 2006).

Both *in vivo* (Ellickson et al. 2001) and *in vitro* (Hack & Selenka 1996; Oomen et al. 2000; Ellickson et al. 2001; Cave et al. 2010) digestive models have been used to assess mobilization of chemicals during ingestion. Oomen et al. (2000) and Cave et al. (2010) showed, by using several digestive models that simulated the human GI tract that the bioaccessible fraction was less than 50- 60%, for both metals and PAHs, respectively. In a study by Ellickson et al. (2001) the bioavaliable fraction of metals was determined to be only a small fraction of the bioaccessible parts. Cautions should be taken when considering bioaccessibility, as the results may be affected by the utilised method, resulting in over- or under- estimation of the accessible fraction (Johnsen & Karlson 2007). Furthermore can the bioavailability also be organism and species specific (Reid et al. 2000). In terms of toxicity related to soil ingestion, lead (Pb) is the element that has attained highest concern (Abrahams 2002). In a cooperation project between NGU and scientists in New Orleans (Mielke et al. 2007) the relationship between Pb pollution in soil and children's health was examined. This study revealed a positive relationship between levels of Pb in soil and in the blood of children in the same neighbourhood. Pb is a known neurotoxin that can affect children's concentration and learning abilities.

1.4 Bioactivation and Cytochrome P450 enzymes

When exposed to organic, lipophilic chemicals an important defence mechanism in several organisms is biotransformation of compounds into more hydrophilic, excretible compounds. Biotransformation consists of serial enzymatically catalyzed reactions, altering the physiochemical characteristics of chemicals from those favouring absorption across the membrane, to those favouring elimination through urine or bile. The enzymatic biotransformation reactions are separated into two main groups. The first group include hydrolysis, reduction and oxidation reactions, which earlier were termed phase I, and the second group include conjugation reactions, earlier termed phase II. Biotransformation of chemicals is mainly a detoxification process, but sometimes compounds can be transformed into more toxic electrophilic metabolites, a term called bioactivation. These electrophilc metabolites can bind critical proteins and DNA bases, potentially causing harmful effects. The balance between biotransformation and bioactivation is often a key determinant of toxicity (Nebert & Dalton 2006; Parkinson & Ogilvie 2008).

The Cytochrome P450 (CYP) enzymes are among the phase I enzymes that play a key role in biotransformation of both endogenous and exogenous compounds, and are found in all eukaryotes and some prokaryotes. The basic reaction is monooxygenation, in which one atom of oxygen is incorporated into the substrate during a catalytic cycle. The first electron added in the cycle is derived from NADPH- Cytochrome P450 reductase, while the second electron and a proton (H^+) are provided from NADPH- Cytochrome P450 reductase or Cytochrome b_5. The oxidation reactions catalyzed by CYP include hydroxylation, epoxidation and hetroatome (S-, N- and I-) oxygenation among others, in addition are some reduction and peroxidation reactions also catalyzed by the CYP enzymes (Parkinson & Ogilvie 2008). The enzymes have evolved and greatly diversified during the evolution from aqueous to terrestrial life forms, and are classified according to their genetic relationship. For example do CYP1A1 refer to gene family 1, genetic subfamily A and the last number refer to the individual gene (Boelsterli 2007).

1.4.1 CYP enzymes involved in biotransformation of organic pollutants

The enzymes of CYP family 1- 3 are mostly active in the metabolism of organic pollutants. These enzymes are predominantly localized on the smooth endoplasmic reticulum membrane in liver cells, but also in the intestine, nose epithelial, lung and skin, among other organs. An increased activity reflects increased transcription of cognate genes, affecting the inactivation or activation rate of potentially toxic compounds, as these enzymes tend to bioactivate some of the organic compounds to genotoxic metabolites. These metabolites might have a mutagenic or carcinogenic effect (Whitlock 1999). Depending on the nature of the exposure compound, bioactivation and the following toxicity can be organ and species specific, due to the induction of different isoforms of CYP enzymes.

The cytochrome P450 enzymes; CYP1A1, CYP1A2 and CYP1B1 are all enzymes important in toxicology as they mediate transformation of organic pollutants and their toxicity. They are all induced via the aryl hydrocarbon receptor (AhR) by dioxin like chemicals, such as some

PAHs and coplanar PCBs (Mandal 2005; Nebert 2006). The proposed mechanism of AhR induction and following gene transcription is illustrated in figure 1.4.1. Recent studies have also demonstrated AhR induction from globular PCBs, raising the question on several possible mechanisms of AhR activation (Alonso et al. 2008).

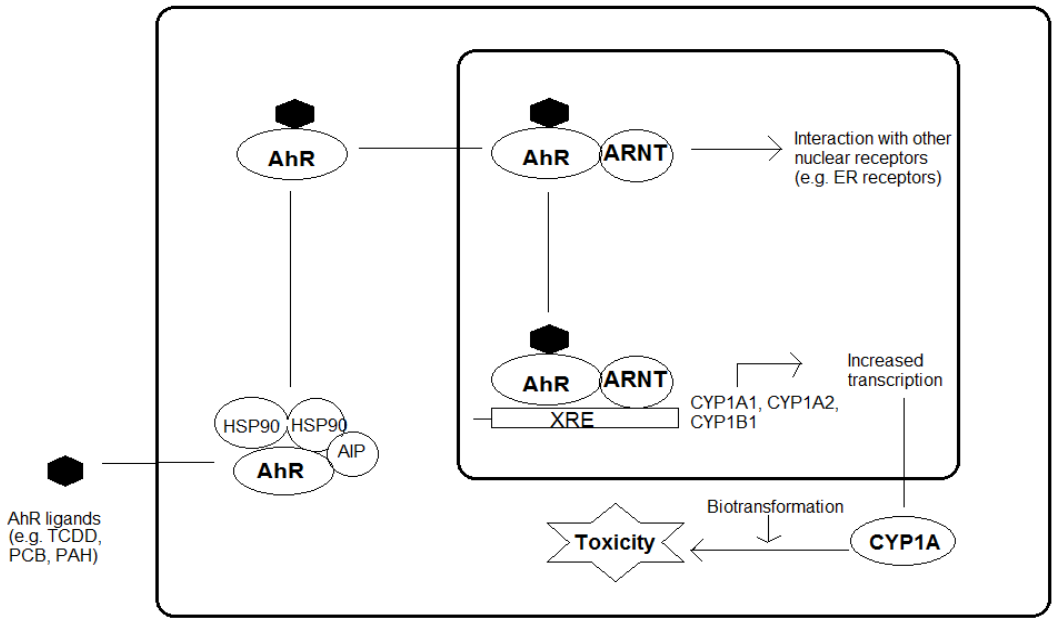

Figure 1.4.1. Proposed mechanism of induction of aryl hydrocarbon receptor (AhR) and the following gene transcription. Figure modified from Parkinson & Ogilvie (2008). Environmental pollutants as TCDD (Whitlock 1999), some PCBs (Safe 1994) and PAHs (Nebert et al. 2000) induce gene transcription of the following cytochrome P450 enzymes; CYP1A1, CYP1A2 and CYP1B1. This by binding to the Ah- receptor (Mandal 2005). The AhR is a ligand-activated, basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) transcription factor belonging to the PAS receptor superfamily. This is a family of nuclear- signaling molecules, found in the nucleus or cytosol (Hahn 2001). The Ah- receptor dissociates from molecules of heat- shock proteins (hsp90) upon ligand binding (Nebert et al. 1993) and is transported from cytosol to nucleus where it heterodimerize with aryl hydrocarbon nuclear translocator (ARNT). This AhR- ARNT complex binds xenobiotic responsive elements (XREs) in the regulatory region of connected genes, followed by a subsequent recruitment of transcription factors. A process resulting in gene transcription (Safe 2001) and a subsequent increase in CYP1A/1B enzymes, which can alter the toxicity of the parent AhR ligands (Whitlock 1999). The AhR regualte also transcription of at least four other genes placed in a gene battery together with the respective CYP enzymes (Nebert et al. 2000). These are identified as conjugating biotransformation enzymes (Nebert et al. 1993) involved in cell cycle regualtion (Nebert et al. 2000). Complex interactions between the AhR and other nuclear receptors have been identified, e.g the modulation of the oestrogen receptors (ERα and ERβ) by a mechanism called oestrogen receptor hijacking (Brosens & Parker 2003; Ohtake et al. 2003).

1.4.2 Effects from induction of CYP1A

The induction of enzymes via AhR can in some cases be deleterious due to the possibility of generating toxic and genotoxic metabolites which can be mutagenic and/or carcinogenic by bioactivation. These metabolites can be reactive with cellular proteins or DNA, and formation of reactive oxygen species can cause oxidative stress (Nebert et al. 2000; Mandal 2005).

Epoxides and oxides are among the metabolites that are formed in the CYP catalyzed biotransformation. These intermediates are highly reactive with proteins and DNA, but are normally converted to less reactive and more excretible products by the detoxifying enzyme epoxide hydrolase. Among the compounds that induce CYP1A1 is B[a]P, which is readily metabolized by the same enzyme to phenolic products and epoxides. These intermediates are by further conversion transformed into corresponding dihydrodiols by epoxide hydrolase. In some cases metabolites can be formed that are inaccessible for further hydroxylation by sterical hindrance (Parkinson & Ogilvie 2008). These derivates can be genotoxic by being DNA intercalating adducts and/or by causing DNA oxidative damages, potentially initiating a mutagenic chain of events that can lead to tumour formation (Nebert et al. 1993). The enzymatic transformation of B[a]P into both non- toxic and toxic derivates is illustrated in figure 1.4.2.

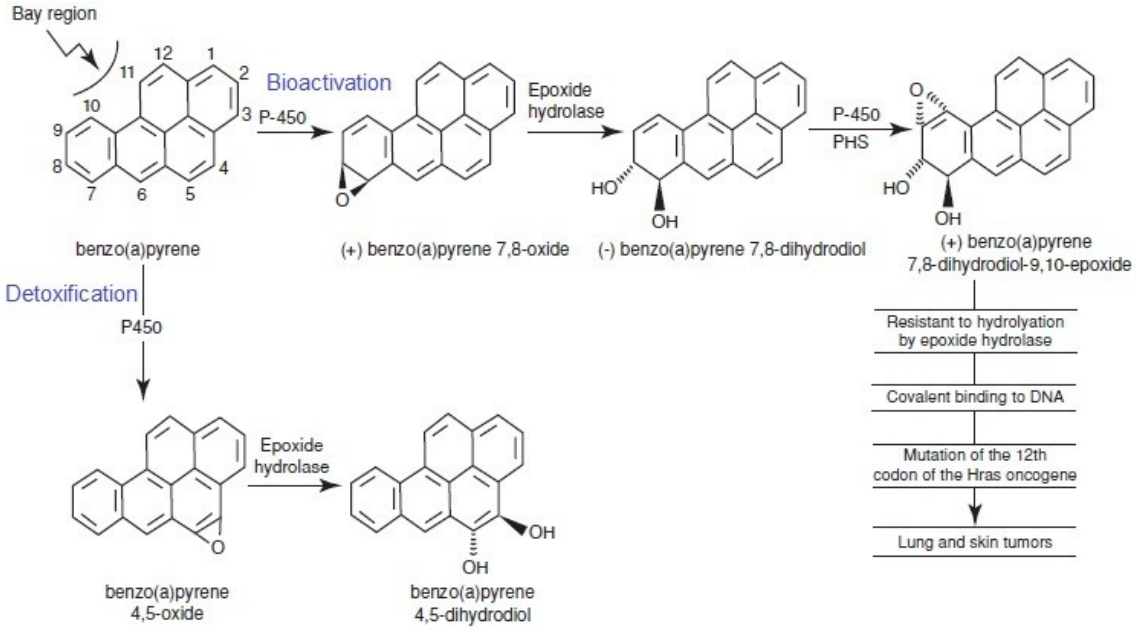

Figure 1.4.2. Biotransformation of benzo[a]pyrene (B[a]P). Figure modified from Klaunig and Kamendulis (2008). B[a]P can be detoxified or bioactivated to structurally different compounds in enzymatic reactions, involving CYP1A1 and epoxide hydrolase. The oxidizing of B[a]P by CYP1A1 to a 7, 8- oxide can by further reactions, involving epoxide hydrolase and CYP1A1, result in a 7,8- dihydrodiol- 9,10- epoxide. This epoxide is resistant to hydroxylation and might covalently bind DNA, causing genetic damages that possible result in mutations and carcinogenicity (Klaunig & Kamendulis 2008).

Reactive species such as $RH^{-}\bullet$, $O_2^{-}\bullet$, H_2O_2 and FeO^{3+} can be formed if the CYP catalysed oxidation cycle of compounds (explained part 1.4) is mistakenly terminated (Parkinson & Ogilvie 2008). These species can react with cellular proteins and nucleic acids, causing oxidative stress. Oxidative stress is defined as damages in organisms that are mediated by oxygen- or free- radicals. Especially CYP1A1 and CYP1A2 have shown to cause formation of oxidative stress through reactive intermediates that are produced in their biotransformation of compounds. The roll of oxidative stress as a major signal in initiating apoptosis of cells connect the CYP1A1/1A2 enzymes and their production of reactive intermediates to cell cycle control and apoptosis. These reactive intermediates can initiate a signal transduction cascade that lead to activation of transcription factors important in cells decision between division, apoptosis, growth arrest and differentiation (Nebert et al. 2000). This process can be important in tumour formation.

1.5 Genotoxicity

Damages in the genetic material of organisms occur naturally thousands of times per day in single cells, but these damages are normally repaired rapidly by the cells repair machinery. Additional damages termed genotoxic effects can be caused by exposure to chemical or physical agents intercalating with the DNA or genetic processes of living cells. Genotoxicity covers a broad spectrum of endpoints, including unscheduled DNA synthesis, DNA- adducts, DNA strand breaks and chromosomal aberrations, among other effects in the genetic material. These damages are not necessarily transmissible to other generations of cells or individuals, while genetic damages termed mutations are permanent damages confined to single genes, which by cell division are transferred to the next generation (Preston & Hoffmann 2008). Mutations can possible lead to cancer if occurring in critical genes as oncogenes or tumour suppressor genes (Baird et al. 2005; Gregus 2008). Metabolic activation of PAHs have shown to induce such critical mutations *in vivo* and it has been detected that PAHs might induce tumours by causing changes in cellular gap- junction communication, thereby promoting tumour formation (Baird et al. 2005).

Genotoxic damages are manifested in organisms when repair fails to restore the native state because of the machinery being; overwhelmed, exhausted, impaired or inefficient (Gregus 2008). PAH induced damages in the DNA are considered to be incorporated as mutations when error- prone DNA replication occurs across unrepaired DNA lesions (Lagerqvist et al. 2008). The degree of repair seems to be influenced by the specific DNA binding adduct, as some are recognized and repaired by the nucleotide excision repair machinery while others elude it (Buterin et al. 2000; Dreij et al. 2005). Exposure to genotoxic compounds can initiate processes of; repair, cell death or manifestation of damage (Gregus 2008), illustrated in figure 1.5.1. The genotoxic effect from a chemical is however dependent on its cellular target and often on metabolic activation (part 1.4) (Kirsch-Volders et al. 2003).

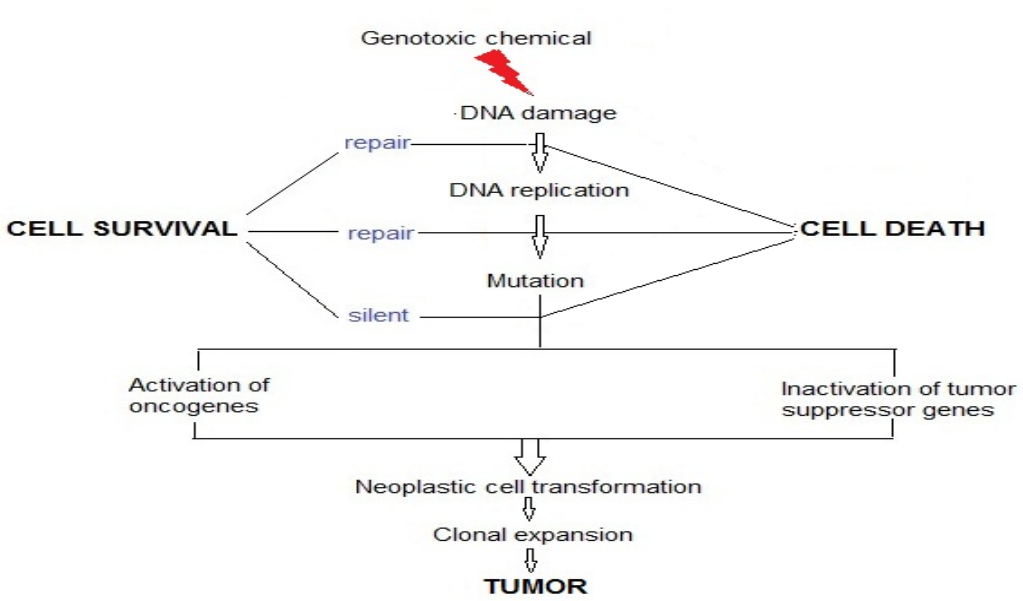

Figure 1.5.1 The process of; repair, cell death or manifestation of damage in cells exposed to genotoxic chemicals. Figure modified from Gregus (2008). Exposure to chemicals resulting in genotoxic effects can proceed to formation of tumours, if the induced DNA damages avoid the repair machinery and are manifested as mutations in genes important for cancer development. Damages occurring in the genetic material of cells are normally repaired and if not the cell can undergo growth arrest and remain in a non- dividing state. If the damages are incompatible with cell survival or constitute a neoplastic risk by having a potential to replicate, the cell can undergo controlled cell death (apoptosis) instead of other defence mechanisms. The response to exposure depends on cell type, location, environment and extent of damage (Evan & Littlewood 1998). If the damaged cell precedes with replication the DNA damage can be incorporated as a mutation in the genetic material (Buterin et al. 2000). If this mutation results in activation of oncogenes or inactivation of tumour suppressor genes this may lead to clonal expansion of the cell. This increases the likelihood of forming additional mutations, which can eventually result in formation of a tumour (Baird et al. 2005; Gregus 2008).

1.6 Pollutants occurring as complex mixtures in the environment and hazard assessment

Pollutants found in environmental compartments such as soil, air, water and biota, normally occur in complex mixtures (Fay & Mumtaz 1996). Complex mixtures is defined to consist of tens, hundreds or thousands of different compounds, where the composition is not qualitatively or quantitatively fully known (Fay & Feron 1996). Exposure and chemical toxicity data is mainly focused on individual compounds and not on complex mixtures, that actually represent the real exposure situation (Cassee et al. 1998). Measured toxicity of environmental samples can frequently differ markedly from the predicted toxicity. This can be due to incomplete chemical analysis, by missing the presence of some toxic molecules, and in the case of soil samples there is also a question of availability of compounds (Walker et al. 2006).

When assessing chemical mixtures and potential health effects from exposure is it important to understand the basic concepts of the combined action and interactions. It is also important to keep in mind that the outcome of effect may be dependent on the dose level of mixtures.

Interactions between chemicals in mixtures can frequently occur, modifying the magnitude of effect and possible also the nature of effect. Modifying interactions can result in higher (synergistic, potentiating) or lower (antagonistic) effects, that deviate from the usual additive toxicity. Additive toxicity is used for non- interactive processes where the individual compounds do not interfere with each other's toxicity. The chemicals can act by the same mechanism and differ only in their potencies (dose additivity), or by different modes of action and possible also by different nature and site of effect (response additivity). Interactions can occur at the physicochemical and/or biological level. It can appear in the toxicokinetic processes of uptake, distribution, metabolism and excretion of compounds, and/or in the toxicodynamic phase, influencing chemical interactions with receptors, cellular targets or organs. A clear toxicodynamic effect influencing toxicity is a chemically induced change in enzyme induction and/or inhibition, for example resulting in a changed level of enzymes crucial for bioactivation or detoxification of chemicals (part 1.4) (Cassee et al. 1998).

Chemical analysis and toxicity information can be important parameters for understanding possible health hazards from exposure to environmental samples such as soil. But soil characterization do not tell anything about the actual toxicity or the potentially interacting effects (Courty et al. 2008). Exposure experiments with soil extracts (Ragnvaldsson et al. 2007) and mixtures at known concentrations (Cassee et al. 1998; Feron & Groten 2002) can therefore be valuable in understanding the actual toxic potential of polluted soil (Ragnvaldsson et al. 2007). The risk assessment approach for assessing simple and complex mixtures can be divided into whole- mixture analyses, also designated top- down approaches, or component interaction analysis also termed bottom- up approaches, studying fewer compounds at known concentrations (Feron & Groten 2002). There is a need for understanding interactions in mixtures and to establish approaches for evaluating carcinogenic potential of mixtures in soil. This was underscored in a study by Mattson and co-workers (2009), exposing HepG2 cells to soil extracts containing mixtures of PAHs. These exposures elicited responses in DNA damage signalling in an unpredictable manner, indicating presence of mediating interactions.

In vitro tests can be applied as first screening for identifying presence of potential hazards in environmental samples and indications on interactions (Maron & Ames 1983; Kopponen et al. 1994). These assays have a low cost, short duration and ethical advantages, compared with *in vivo* studies. Bacteria, yeast and isolated mammalian cells have been widely used test organisms for this purpose, and several endpoints have been assessed, genotoxicity being most widely applied (Kopponen et al. 1994). Numerous genotoxicity and mutagenicity assays for identifying compounds causing damages to the DNA have been developed. Assays employing prokaryotes enable detection of agents inducing gene mutations and primary DNA damage, while eukaryotes are used for a greater damage extent, varying from gene mutations to chromosome aberrations (Houk 1992). A widely used *in vitro* test on prokaryotes is the Ames *Salmonella thypimurim* assay (Ames et al. 1975; Maron & Ames 1983). This assay have given valuable information about mutagenic potentials of chemicals and environmental samples, but despite information about mutagenic hazards is the risk for human and terrestrial biota difficult to quantify (White & Claxton 2004).

The induction of CYP1A enzymes has been a widely used biomarker in the assessment of environmental samples and mixtures (Kopponen et al. 1994; Østby & Krøkje 2002; Østby et al. 2006; Jensen & Krøkje 2008). This enzyme is very relevant in combination with genotoxicity due to the role of CYP1A in bioactivating chemicals into genotoxic, mutagenic and carcinogenic compounds (explained in part 1.4). A biomarker has been defined as a biological response to exposure that deviate from the normal status, at the individual level or below (Spurgeon et al. 2005). The CYP1A enzymes; CYP1A1 and CYP1A2, are also interesting due to the fact that these enzymes are possessed by all mammalian species, and are functionally and regulationally highly conserved (Parkinson & Ogilvie 2008). The rat hepatoma cell line H4IIE has been extensively used for assessing the *in vitro* induction of CYP1A (Tillitt et al. 1991; Kopponen et al. 1992), and earlier results showing good agreement between *in vitro* and whole animal studies, have been considered to support the value of results (Safe 1989).

1.7 Aim of study

Pollutants in the environment can have negative health effects upon exposure, and it is important to understand the biological effects and interactions between compounds affecting the toxic potential. In this master project *in vitro* biological effect studies will be performed, exposing test organisms to organic extracts of soil sampled in nursery schools in Oslo. The soil samples have earlier been collected by NGU in connection to a geological survey of children's playing environment in Norway. Soil to be assessed will be a selection of samples that according to the chemical analysis have been detected to contain different amount of organic pollutants as PAHs and PCBs.

First aim of this study is to measure the mutagenic potential of the organic extracts of soil in the Ames *Salmonella thypimurium* assay (Ames et al. 1975; Maron & Ames 1983). The presence of both primary and secondary mutagens will be assessed by conducting the assay with and without addition of an exogenous metabolic system (S9- mix), and screening for induction of different type of point mutations by employing two different *S. typhimurium* strains. The strains to be included are TA98 and TA100, which can be used for detecting frameshift mutations and base- pair substitutions, respectively.

The second aim of this project is to assess the *in vitro* induction of CYP1A1/A2 biotransformation enzymes in the rat hepatoma cell line H4IIE, after exposure to extracts of soil. The enzymes will be measured quantitatively by immunological detection of CYP1A by Western blotting (Towbin et al. 1979).

In both the mutagenicity and CYP1A induction assays a concentration range of the extracts will be tested.

1.7.1 Hypothesis

- The mutagenic potential of the soil extracts will mainly be of a secondary nature, due to the requirement for metabolic activation of several pollutants in order to exert a genotoxic effect.

- The level of organic pollutants in the soil will reflect the biological responses induced by the extracts, showing a general higher inducing potential of extracts derived from the soil containing the highest level of organic pollutants.

- There will be a relation between the mutagenic and CYP1A inducing potential of the extracts.

2 Material and methods

2.1 Soil samples

Ground pollution can be a problem in urban areas, and elevated levels of especially lead, PAHs and arsenic, in addition to some PCBs and mercury, have been found in many nursery schools and playgrounds in Norway. With the aim of reducing children's exposure, a national mapping and remediation project of soil in playing areas of nursery schools, schools and playgrounds in Norway was officially initiated in 2007, starting with the nursery schools in the ten largest cities, and five larger industrial areas (Klif 2006; Ottesen et al. 2008). Cleanup by change or covering of polluted soil, and change of copper-, chrome- and arsenic-impregnated wood around sandboxes, were demanded if the chemical analysis revealed levels of pollutants exceeding the health based quality limits for surface soil, sat by NIPH. These limits gave estimated concentrations of relevant environmental pollutants that with safety margins were considered safe for children to get exposed for (Alexander 2006).

The soil samples chosen to be assessed in this project were from five nursery schools in Oslo. Sampling of surface soil (0-2 cm depth) and reporting were performed by NGU. Soil was sampled in 2005- 2007, dried at 40 °C for one- two weeks, sieved in a 2 mm nylon sieve, and stored in the dark at room temperature. Analysis of organic chemical content was performed by Analycen AS in Moss, and the inorganic analyses in NGU's laboratory (NGU 2005). The selected soil samples are given in table 2.1 (complete chemical data given in appendix A-1). The selection was based on; chemical data, quality limits for soil pollution, possibility to extract chemicals by the extraction procedure, compatibility between the chemical compounds and bioassays, and prioritization of compounds proven to be genotoxic and CYP1A inducing. The compounds of interest were PAHs and PCBs, resulting in a "clean" sample and samples containing levels below, at and above the quality limits for $\sum PAH_{16}$, and a sample containing elevated levels of $\sum PCB_7$.

Table 2.1 Soil samples from nursery schools in Oslo, used in this project. The chemical data of relevant environmental pollutants (mg/kg) in soil samples, and the health based quality criteria sat by NIPH, for soil in nursery schools, playgrounds and schools, categorized as "normal" playgrounds (Alexander 2006). Numbers written in bold illustrate values exceeding the quality limits.

Sample ID	B[a]P	[1]$\sum PAH_{16}$	[2]$\sum PCB_7$	[3]As	[3]Ni	[3]Cr	[3]Cd	[3]Pb	[3]Hg
1	<0.01	<0.20	<0.004	2.2	7.2	7.40	<0.1	2.5	<0.01
2	**0.8**	**8.1**	0.0054	4.9	30.1	32.4	0.36	90.7	0.23
3	**3.1**	**20**	<0.004	4.5	29.1	28.9	0.29	40.7	0.08
4	**6.4**	**87**	0.0084	6.2	21.2	19.3	0.45	80.8	0.28
5	0.11	1.5	**1.97**	6.1	32.7	27.6	0.14	42.3	0.08
[4]**Quality limits**	0.5	8	0.5	20	135	[5]5 (Cr^{6+})	10	100	1

[1] Sum PAH includes naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3,cd]pyrene. [2] Sum PCB include 2,4,4'-Trichlorobiphenyl (PCB 28), 2,2',5,5'-Tetrachlorobiphenyl (PCB 52), 2,2',4,5,5'-Pentachlorobiphenyl (PCB 101), 2,3',4,4',5-Pentachlorobiphenyl (PCB 118), 2,2`,3,4,4`,5,-hexachlorobiphenyl (PCB 138), 2,2`,4,4`,5,5`-hexacholorbiphenyl (PCB 153), 2,2',3',4,4',5,5'-heptachlorobiphenyl (PCB180). [3] As- arsenic, Ni- nickel, Cr- chrome, Cd- cadmium, Pb- lead, Hg- mercury. [4] Quality limits for normal playgrounds- taking

exposure routs as soil intake by ingestion, inhalation of gas/dust and dermal absorption into consideration (mg/kg). [5] Quality limit for chrome VI. Analysis for Cr VI are only performed if total chrome concentrations are over 40 mg/kg, as chrome normally occurs in the form of Cr III, which is much less bioavailable and toxic (Ottesen & Haugland 2007).

The soil samples will from here on be referred to by using their given sample ID, consequently using the names Soil 1, Soil 2, Soil 3, Soil 4 and Soil 5.

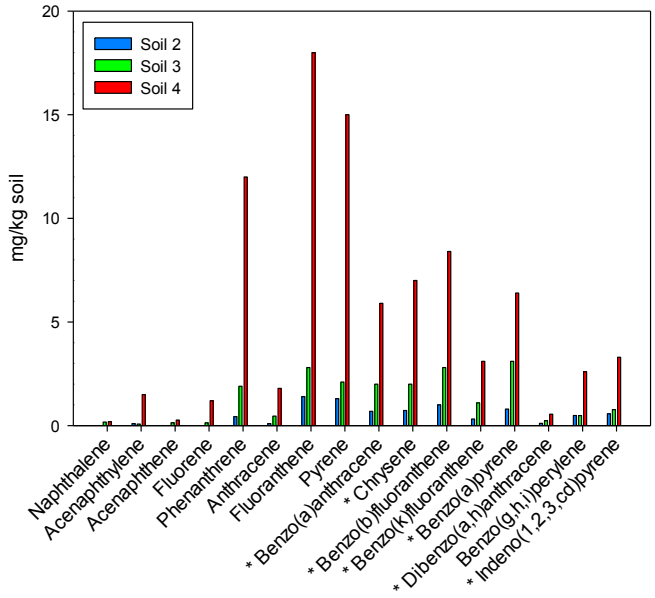

Figure 2.1 PAH- profile from chemical analysis of Soil 2, Soil 3 and Soil 4. Given as mg/kg dried soil. PAHs marked with asterisk are classified as probable human carcinogens according to U.S. EPA (NTP 2005).

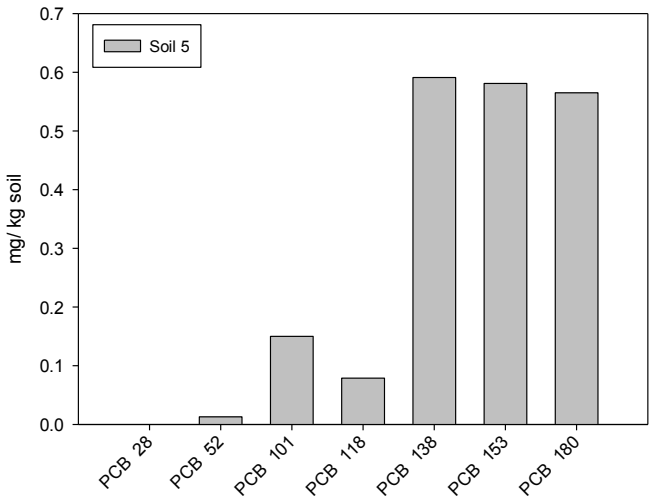

Figure 2.2 PCB profile from chemical analysis of Soil 5. Given as mg/kg dried soil.

The chemical PAH profile of Soil 2, Soil 3 and Soil 4 given in fig. 2.1 shows that Soil 4 has the overall highest content of the PAHs, followed by Soil 3 and then Soil 2. Soil 4 is clearly dominated by fluoranthene, pyrene, phenanthrene, and contains high levels of

benzo[b]fluoranthene, chrysene, benzo[a]pyrene and benzo[a]anthracene, in the following order. The chemical analysis of Soil 2 and Soil 3 do not designate any compounds as main constituents, but it is a general higher level of the high molecular weight compounds, especially those in the middle (PAHs listed from left to right in fig. 2.1 according to molecular size). The PCB profile of Soil 5, given in fig. 2.2 clearly shows a dominance of PCB 138, PCB 153 and PCB 180. Soil 1 and Soil 5 were not included in fig. 2.1 due to very low levels of PAHs, while only Soil 5 was included in fig. 2.1, since this is the only soil sample with high content of PCBs.

2.2 Organic extract of soil samples

Organic chemicals in the soil were extracted by ultrasound agitation in dichloromethane (DCM) (part 2.2.2), owing to the usefulness of ultrasonic agitation (Aamot et al. 1987; Morin et al. 1987) and DCM as an organic extraction agent (White & Claxton 2004). Due to the toxicity of DCM the solvent was changed into dimethylsulfoxide (DMSO) (part 2.2.2), which is less toxic and commonly used as solvent for *in vitro* bioassays (Tillitt et al. 1991; Willett et al. 1997; Yu et al. 1997; Whyte et al. 2004). To eliminate interference or toxicity the DMSO concentration has been recommended to never exceed 0.5% of the total exposure solution in H4IIE assays (Whyte et al. 2004). In relation to this and earlier studies in our laboratory (Østby et al. 2006; Fugleneb 2007) the DMSO concentration never exceeded 0.1% for the H4IIE assays conducted in relation to this thesis.

All treatment of soil and extracts of soil were performed in reduced light to prevent photo degradation. Extracts were stored in the dark at 4°C, and the DMSO dissolved extracts were kept for up to one month. All glass equipment used for handling and storage of soil extracts were washed due to the following procedure: deacon wash for 24 hours followed by rinsing with 10x tap water, 10x distilled water, 1x acetone and 3x 96% ethanol. Larger glass equipment was washed in the dish washer at acid wash program for glass equipment, followed by rinse in 1x acetone and 3x 96% ethanol.

In addition to extracts of Soil 1- 5 a blank extract was included as a negative control for the extraction procedure. The blank was made by the same procedure as for the other extracts, without soil. Extracts of blank used in experiments will be referred to as Blank, in the same way as the soil samples are given their unique names.

2.2.1 Chemicals, solutions, equipment and commodities

Chemicals	Producer	Catalogue number
Acetone	MERCK	1.00014.1000
Dichloromethane (DCM)	MERCK	1.06050.1000
Dimethyl sulfoxide (DMSO)	MERCK	1.02950.0500
Ethanol 96 vol. %	SIGMA	24106
Nitrogen gas (N_2)	YARA	500743

Equipment and commodities	Producer	Catalogue number/Model
Centrifuge	MINOR	–
Glass equipment	-	–
Pasteur- pipette in glass (150 mm/ 230 mm) with balloon	VWR International	612- 1701/ 612- 1702
Scale	METTLER	AE260
Thermometer	-	-
Ultrasonic bath, with belonging equipment	NEY	300
Vortex- mixer Vibrofix	Janke & Kunkel	VF1
Water bath	Grant	Y22

2.2.2 Procedure for chemical extraction of soil and change of solvent

The soil extraction and solvent change was performed according to descriptions by Østby et al. (2006). Two parallels of 5 g soil were extracted in 30 mL DCM for each soil sample, under sonication in ultrasonic bath (degassed water) for 30 min. Samples were then centrifuged at low speed for 3- 30 minutes, depending on the deposition time of the soil, and the supernatant were removed by pipetting. Then 25 mL of DCM was added the soil, and the procedure repeated. The temperature of the water bath was cooled down to at least 20°C between each extraction, as the temperature could increase up to 10°C during the sonication. The parallel extracts were combined, and filtered on a glass- sinter filter, in order to remove smaller soil particles. The total volume of the raw extracts was measured, and calculated volumes were evaporated to dryness in a stream of pure nitrogen, preventing oxidizing of samples. Evaporation was performed in a water bath at 25 °C. The dried condensates were redissolved in DMSO and dilution series made from a stock solution. The blank extract was made by the same procedure as described above; giving a yield of "10 mg soil"/measured mL of DCM raw extract, and calculated into DMSO dissolved concentrations. For the dilution series of the blank extract, was the highest exposure concentration corresponding to the highest soil extract concentration in the specific exposure assays.

The DMSO dissolved test solutions were made from stocks of concentrations corresponding to x mg soil per 0.2 µL for the MTT assays, and x mg soil per 1 µL for the CYP1A induction assay, this to avoid exceeding 0.1% DMSO in the final exposure solutions. The exposure solutions for the Ames assay was x mg soil per 100 µL DMSO, made from a stock of the highest exposure concentration.

2.3 The Ames *Salmonella typhimurium* assay- measuring mutagenic potential of soil extracts

2.3.1 Principles of the method

The Ames *Salmonella* assay (Ames et al. 1975; Maron & Ames 1983) is an *in vitro* assay applicable for detecting chemical mutagens either singly or in mixtures, a test that is sensitive and easy to perform. The tester strains are mutant bacteria of the parent *Salmonella typhimurium* LT2, containing specific mutations in the histidine operon, making them auxotroph for histidine. In the Ames assay these bacteria are exposed to test solutions, and the number of bacteria reverted back to prototrophy after 48 h. incubation, meaning those that have regained the ability to synthesize histidine, gives an expression of the capacity of the exposure chemical/solution to induce mutations in the strain (Maron & Ames 1983).

Exposing bacterial tester strains that contain dissimilar types of histidine mutations makes it possible to detect presence of chemicals, causing different types of point mutations. The strain TA100 can be used to detect induced basepair substitutions, whereas strain TA98 is useful for detecting frameshift mutations (Ames et al. 1975). The strains contain additional mutations that make them more susceptible, thereby increasing the ability to detect mutagens. These are the rfa mutation, which increases the cell wall permeability, the uvrB mutation, which weaken the DNA excision repair system (Maron & Ames 1983), and the inserted R- factor plasmid pKM101, which carry antibiotic resistant genes and increases the strains sensitivity (McCann et al. 1975).

The *Salmonella* bacteria do not metabolize chemicals in the same way as mammalian cells, but this difference between prokaryotes and eukaryotes is reduced by performing the assay in presence and absence of S9 mix (S9 with cofactors). The S9 is a metabolic system containing biotransformation enzymes, normally derived from Arochlor induced rat liver homogenate, centrifuged for 10 min. at 9000 g. Exposing the bacteria both with and without the S9 mix makes it possible to detect if chemicals are primary or secondary mutagens (Maron & Ames 1983).

The Ames *Salmonelle typhimurium* assay was used in this master thesis to determine the mutagenic potential of organic extracts from soil in nursery schools in Oslo.

2.3.2 Chemicals, solutions, equipment and commodities

Chemicals	Producer	Catalogue number
Ampicillin tablet	ROSCO DIAGNOSTICA	–
Bacto- Agar	DIFCO	0140-01
Benzo[a]pyrene	SIGMA	B1760
Crystal violet	SIGMA	C0775
D- Biotin	SIGMA	B4501
D- Glucose- 6- phosphate sodium salt	SIGMA	G7879
Dimethyl sulfoxide (DMSO)	MERCK	1.02950.0500
di- Sodiumhydrogenphosphate- Dihydrat (Na_2HPO_4*$2H_2O$)	MERCK	K30532180225
L- Histidine monohydrochloride monohydrate ≥ 98%	SIGMA	H-8125
Magnesiumchloride- Hexahydrat ($MgCl_2$*$6H_2O$)	MERCK	1.05833.1000
Sodiumazid (NaN_3)	-	-
(β- Nicotinamide adenine dinucleotide phosphate sodium salt) NADP	SIGMA	077K7000
4- Nitro- o- phenylenediamine (NPD)	SIGMA- ALDRICH	73630
Nutrient broth No. 2	OXOID	59702
Sodium chloride (NaCl)	SIGMA	S3014
Sodium dihydrogen phosphate Monohydrate (NaH_2PO_4*H_2O)	MERCK	1.06346.0500
Rat- liver LS-9 (Aroclor 1254 induced male Sprague Dawley)	MOLTOX	-
Potassium chloride (KCl)	MERCK	1.04936.1000

Solutions	Appendix
Histidine- Biotin solution	B-1
Nutrient agar plates	B-1
Nutrition medium	B-1
Top agar	B-1
Co- factors for S9- mix:	B-1
- 0.4 M MgCl solution	B-1
- 1.65 M KCl solution	B-1
- 0.2 M Sodium di- hydrogen phosphate	B-1
- 0.2 M Di- sodium hydrogen phosphate	B-1
- 0.2 M Sodium phosphate buffer, pH = 7.4	B-1
S9- mix (50 µL S9/0.5 mL S9- mix)	B-1

Equipment and commodities	Producer	Catalogue number/Model
Automat pipette	Drummon	-
Automat pipette 5 mL	Eppendorf	88937
Conical flask 25 mL	SCHOTT DURAN	–
Conical flask 100 mL	PYREX	–
Cotton cap	VWR	-
e.pT.I.P.S. Standard/Bulk 100- 5000 µL (purple)	Eppendorf	022492080
Filter crystal violet	-	-
Glass pipette (10 mL)	Assistant	–
Glass tubes (for test solution)	BRAND	114110
Infrared CO_2 incubator	Forma Scientific Inc.	3194
Microscope	Zeiss	433044-9901
Minimal agarplates	St. Olavs HF	
Pipette 200 µL/1000 µL	GILSON	T67649H/ T64456H
Pipette tips 200 µL/ 1000 µL	SARSTEDT	70.760.502/70.762.100
Proline pipette 1- 5 mL	BIOHIT	ANO8926
Shaking incubator	Infors AG	CH-4103
Sterile filter (0.45 µm)	SARSTEDT	-

Sterile syringe (50 mL)	BD platipak™	-
Vortex	Labinco	L46
Water boiler	KOTTERMANN	3031
Water bath	KOTTERMANN	3047
Water bath	Grant	Y22

2.3.3 Procedure for the Ames *Salmonella* assay on extracts of soil

The plate incorporation test of the Ames assay was performed according to descriptions by Ames et al. (1975) and Maron and Ames (1983). Two independent experiments were performed, and the second included only repeated tests on exposure conditions showing mutagenic response in the first experiment. Those exposure conditions showing none, or low responses, were excluded to decrease the size of the assay. The applied tester strains were TA98 and TA100, earlier received from Dr. B. N. Ames, Berkley, California, and stored at -80 °C.

A suspension of approx. 10^9 bacteria/ mL was cultivated in nutrition medium by shaking at 120 rpm at 37°C for 12 hours. The S9 mix was freshly made right before use, at a concentration of 50 µL S9/0.5 mL S9- mix, the mixture was sterile filtrated (0.45 µm) and kept on ice. The S9 came from liver homogenate of Arochlor 1254 induced rats, and was stored at – 80 °C. Top agar containing 10% histidin- biotin were transferred to tubes, 2 mL in each, and kept at 45°C in water bath to prevent hardening. Just before plating the tubes were added 100 µL exposure solution/ B[a]P/ DMSO, 100 µL bacteria suspension and alternatively 500 µL of S9- mix, and the solution were poured onto minimal agar plates. The plates were incubated at 37°C, and bacteria colonies manually counted after 48 and 72 hours. Plates were examined in the microscope to check for potential toxic effects, as reduced background flora of non- reverted bacteria.

Quality of the tester strains were confirmed in tests performed in advance (results not shown), and from earlier experience and repeated use in our laboratory. These initial tests for confirming the strain integrity included spontaneous reversion (only tester strain to check the strains reversion frequency and physical conditions), positive controls of 4- nitro-o-phenylendiamine (NPD) (20 µg/ 100µL), sodiumazid (1 µg/ 100µL), and solvent controls of DMSO and distilled water, respectively. The soil extract exposure assays included tests of spontaneous reversion, DMSO control, positive control of the S9- mix together with B[a]P (1 µg/ 100µL) and dilution series of the soil extracts. The integrity of the strains were confirmed in the first assay and after the second, by incubating two plates of each strain in presence of crystal violet and an ampicillin tablet.

Choice of exposure concentrations were based on earlier studies (Krøkje & Gullvåg 1994), information on children's daily soil intake (Stanek & Calabrese 1995) and recommended intake values (U.S. EPA 2008). The first Ames experiment with soil extracts was conducted with all soil samples and the Blank at a concentration of 25 and 100 mg soil equivalents (eq.) per plate on both tester strains, with and without S9- mix. Additional concentration of 50 mg soil eq./ plate for Soil 3, Soil 4 and Soil 5 were included. The second experiment was

performed on TA98 with extract of Soil 3 at concentrations of 25, 50, 100 and 150 mg soil eq. per plate, and on TA98 + S9 and TA100 + S9 with the concentrations of 25, 50 and 100 mg soil eq. for the Blank and extract of Soil 1, Soil 2 and Soil 5. Exposure to extract of Soil 3 and Soil 4 were performed with the concentrations of 25, 50, 75 and 100 mg soil eq. per plate. Exposure solutions for the two experiments originated from the same DCM dissolved soil extracts, but were evaporated to dryness and solved in DMSO independently (explained in 2.2.2).

2.3.4 Treatment of raw data and interpretation of results

Number of revertants after 48 hours incubation was used for graphical and statistical presentation, using both Microsoft Office Excel 2007 and SigmaPlot 11.0.

Results were considered to indicate clear mutagenic effect if the following criterias were fulfilled: reproducibility between experiments, positive concentration- effect relationship, doubling of reverted bacterial colonies compared with the spontaneous reversion frequency (doubling criteria), and statistical significant difference between exposed and corresponding DMSO control (Krøkje et al. 1985), detected by Mann Whitney test ($p \leq 0.05$) in SigmaPlot 11.0. Because of a limited number of parallels (n= 3-5) the statistical analysis were interpreted as only indications on mutagenicity, together with the graphical presentation of the results. The DMSO + S9 control was mistakenly not included in the two soil exposure experiments, and an additional assay with the following controls; spontaneous, DMSO, DMSO + S9, B[a]P and B[a]P + S9, was conducted. The reversion frequency of the controls in the two first experiments was statistically compared with the controls of the last one. This to demonstrate the minor difference between controls in independently performed experiments, and thereby validating the choice of relating the DMSO + S9 control to the exposed in presence of S9 in the two first experiments. Mann Whitney test ($p \leq 0.05$) was used for this purpose.

2.4 Rat H4IIE hepatoma cell line

2.4.1 Characteristics of cell line

The H4IIE rat hepatoma cell line has shown advantageous for *in vitro* assays, screening environmental samples for the presence of AhR activating compounds, as some PCBs (Tillitt et al. 1991; Whyte et al. 2004) and PAHs (Willett et al. 1997), and for estimating the toxic potency of planar halogenated hydrocarbons (Whyte et al. 2004). The characteristics of having excellent growth properties, low basal CYP1A1 activity, high responsiveness toward AhR activating compounds, and high reproducibility and repeatability in assays, makes this cell line suitable for toxicity studies (Benedict et al. 1973; Tillitt et al. 1991; Whyte et al. 2004) and for biomonitoring purposes (Whyte et al. 2004). Hepatoma cells are also suited for studying toxic effect from organic pollutants due to the importance of the liver in processes of detoxification, bioactivation and excretion of chemicals (Parkinson & Ogilvie 2008). H4IIE has at earlier occasions shown usable in our laboratory, for examining toxic effects from exposure to chemical mixtures, at environmental relevant concentrations (Jensen & Krøkje 2008; Haldsrud & Krøkje 2009).

2.4.2 Chemicals, solutions, equipment and commodities

Chemicals	Producer	Catalogue number
CO_2	YARA	500204
Dimethyl sulfoxide (DMSO)	SIGMA	24106
di-Sodium hydrogen phosphate dihydrate ($Na_2HPO_4 * 2H_2O$)	MERCK	1.06580
Ethanol 96 vol. %	MERCK	1.04936
Ethylenediaminetetraacetic acid disodium salt dihydrate 99+%	SIGMA	E5134
Foetal Bovine Serum (FBS)	SIGMA	F3018
L- Glutamine 200mM	GIBCO	25030-032
Penicillin - Streptomycin (Pen Strep)	GIBCO	15070-063
Potassium chloride (KCl)	MERCK	1.02950
Potassium dihydrogen phosphate (KH_2PO_4)	MERCK	1.04873
RPMI-1640 medium	SIGMA	R0883
Sodium chloride (NaCl)	SIGMA	S3014
Trypsin 2.5%	GIBCO	15090- 046

Solutions	Appendix
Ethylenediaminetetraacetic acid (EDTA), 0.5M, pH 8.0	B-2
Growth medium	B-2
Phosphate buffered saline (PBS), pH 7.4	B-2
Trypsin 0.25 %	B-2

Equipment and commodities	Producer	Catalogue number/Model
Autoclave	TOMY	SX-700E
Automat pipette	Drummond	4-00-031
Automat pipette 5- 100 µL (multichannel)	Eppendorf	3238643
Centrifuge (Laborzentrifugen)	SIGMA	3-10
Centrifuge tube with apexbase 10 mL	NUNC	347856118559
CO_2 Water Jacketed Incubator	Forma Scientific	3111
Cryo tube round bottom (2mL)	VWR International	479- 0281
Cryo biological storage system (N_2- tank)	Thermolyne	LOCATOR 6+

e.pT.I.P.S. Standard/Bulk 100- 5000 µL (purple)	Eppendorf	022492080
Magnet stirrer	Gerhardt Bonn	02075125
Microscope	Nikon Eclipse	TS100
Pipette 200 µL	GILSON	T67617H
Pipette 1000 µL/ 5000 µL	Eppendorf	5.531.131/4375693
Pipette tips 200 µL/ 1000 µL	SARSTEDT	70.760.502/70.762.100
Scale	METTLER	AE260
Serological pipettes 25 mL	SARSTEDT	86.1685.001
Standard pH meter	Radiometer Copenhagen	PHM210
Sterile hood LaminAir	Heto- Holten AS	S- 2010 1.2
Tissue Culture Flask 75cm^2	SARSTEDT	831813

2.4.3 Procedure for cell culturing, harvesting and freezing

The H4IIE rat hepatoma cell line developed from the Reuber Hepatoma H- 35 cell line (Whyte et al. 2004) was purchased from The Centre for Applied Microbiology and Research (CAMR, ECACC, Salisbury, Wiltshire) and stored in liquid nitrogen. Cells applied in this project were taken up at passage number 10, and kept for up to 30 passages. By start- up was a cell containing cryo tube taken out of the N_2- tank, and the content thawn, centrifuged (1188 rpm for 5 min.) and resuspended in 1 mL of RPMI- 1640 medium, supplemented with 5% fetal bovine serum (FBS), 1% penicillin- streptomycin (Pen Strep) and 1% L- Glutamine (200mM) (Kopponen et al. 1992) with minor modifications. The content of the tube were then transferred to a 75cm^2 polystyrene culturing flask containing 20 mL medium, and incubated in humid atmosphere of 5% CO_2 at 37 °C. By start up were cells always given new medium the following day, and sub cultured at least one time before experimental use to assure that the cells were healthy and stably growing.

Sub cultivation was performed on 70- 90% monolayer confluent flasks every 3- 4 day. By sub cultivating the cells were first washed in 5 mL PBS, followed by incubation for 5 min in trypsin (0.0025%) and ethylenediaminetetraacetic acid (EDTA) (0.01%) in phosphate-buffered saline (PBS), to loosen the cells from the flask. Content of the flask was poured into a tube and centrifuged for 5 min at 1188 rpm. The pellet obtained was dissolved by flicking, and centrifuged in 8 mL PBS a second time. The cell pellet was again dissolved by flicking and suspended in 2 mL growth medium. The desired cell suspension volume was taken and used for culturing or experiment.

Deepfreezing of cells for storage in liquid nitrogen was performed by normal harvesting procedure, followed by suspending the pellet in 500 µL FBS, instead of medium. The suspension was transferred to a prepared cryo tube containing 450 µL FBS added 50 µL DMSO. The cryo tube was placed at -80°C for one hour, before transfer to a N_2- tank for storage.

2.5 MTT assay- viability of H4IIE exposed to extracts of soil

2.5.1 Principle of the method

The colorimetric 3-(4,5- dimethylthiazol-2-yl)- 2,5- diphenyl tetrazolium bromide (MTT) assay (Mosmann 1983) has been developed to quantitatively asses mammalian cell survival and proliferation. The amount of living cells can be measured spectrophotometrically and is based on reduction of the tetrazolium salt MTT, a pale yellow substrate, to a detectable dark blue formazan product. The amount of generated formazan is directly proportional to the quantity of living cells and is accepted as a suitable method for measuring cell viability or cytotoxicity, proliferation and activation (Mosmann 1983).

The MTT assay makes it possible to screen a large number of samples simultaneously in multiwell plates, and as no washing steps are needed the assay is rapid and precise (Mosmann 1983). The MTT assay was applied in this thesis to determine the highest non- cytotoxic concentrations of the soil extracts (i.e. H4IIE), so these concentrations could be further applied in the CYP1A induction assays.

2.5.2 Chemicals, solutions, equipment and commodities

Chemicals	Producer	Catalogue number
Thiazolyl Blue Tetrazolium Bromide, approx. 98 % TLC	SIGMA	M2128

Solutions	Appendix
MTT- solution	B-2

Equipment and commodities	Producer	Catalogue number/Model
Automat pipette 5- 100 µL (multichannel)	Eppendorf	3238643
Bürker counting chamber (depth 0.100mm) with cover slip	BRAND	-
COSTAR sterile 96 wells plate	Corning Incorporated	3599
Microscope	Nikon Eclipse	TS100
Multiscan Ascent plate reader	Labsystems	354-00578
Pipette 0.05- 10 µL	Eppendorf	4586413
Pipette 200 µL	GILSON	T67617H
Pipette tips 10 µL	Molecular BioProducts	3512
Pipette tips 200 µL	SARSTEDT	70.760.502
Sterile tubes (15 mL/ 50 mL)	SARSTEDT	62.554.502/ 62.547.254

2.5.3 Procedure for the MTT assay

The MTT assay (Mosmann 1983) was performed according to Kim et. al. (2003) and Haldsrud and Krøkje (2009) with modifications. The H4IIE cells were harvested according to procedure described in part 2.4.3, and plated in 96- well plates at a total volume of 200 µL per well. Counting of cells on a Bürker counting chamber was first conducted, finding the amount of cell suspension needed for plating. Cells were counted from a 1:10 dilution of the cell pellet, and three A- squares were counted from each of two to three independent drops of cell suspension on the counting chamber. Calculations were made to find number of cells per mL

from the average number of cells in the A squares [i.e cells/ mL = cells per A square * 10^4 (volume per A square is 0.1 µL)]. The plated cells were incubated for 24 h at 37°C under 5% CO_2 prior to exposure. Medium in each well was then changed with 200 µL of exposure solution mixed with medium, and the plate was incubated for additionally 46 hours. Then the exposure solution was replaced by medium containing MTT (0.5 mg/mL), and incubated for additionally 4 hours. The produced formazan crystals were dissolved by changing the solution with 200 µL DMSO, and absorbance measured spectrophotometrically at 550 nm.

2.5.3.1 Cell concentration in MTT

Before performing the MTT exposure studies with soil extracts, preliminary experiments were conducted to find a suitable cell concentration within the linear production of formazan. First some experiments were performed to find the range of concentrations to test (data not shown) and when a linear relationship was found, three replicate MTT experiments were conducted to check reproducibility of results. The results were treated in Microsoft Office Excel 2007 and transferred to SigmaPlot 11.0, where the average absorbance for each cell concentration ± standard deviation (S.D.) (n = 6) for four independent experiments was presented graphically. A linear regression line was made from all the data in the plot.

2.5.3.2 Exposure to soil extracts in MTT

The extracts of Soil 3, Soil 4, Soil 5 and the Blank were chosen for the MTT assays, based on the chemical data, table 2.1. For the first experiment concentrations corresponding to 0.1– 25 mg soil were applied for all the extracts. While the concentrations in the following experiments were based on results obtained, testing the concentrations around the turning point of viability. Three independent experiments were performed (four with extract of Soil 5), using the same DMSO dissolved soil extracts for each sample.

Each experiment included two controls; cells in medium and cells in medium containing 0.1% DMSO. The results were presented graphically as average (n = 6) percent of the DMSO control, defined as 100% viability [i.e. viability (%) = 100 * (absorbance of treated sample)/(average absorbance of control)]. Results were treated in Microsoft Office Excel 2007, and graphically presented in SigmaPlot 11.0. For each soil extract an average data serie were made from all experiments, and applied for the graphical presentation and statistics. The cell concentration were changed from 11 500 to 13 000 cells/mL during the soil exposure experiments (still within the linear production of formazan), due to a drop in absorbance. The measured absorbance in the first experiment was therefore calculated as to relate to 13 000 cells/mL due to the average data serie that were made [i.e. absorbance = ((absorbance / 11 500 cells per mL) * 13 000 cells per mL)]. Mann-Whitney test was used to check for statistical significant ($p \leq 0.05$) change in the cell viability between the medium and DMSO control, and between extract exposures and DMSO control.

2.6 CYP1A induction in H4IIE cells exposed to extract of soil

The biotransformation enzymes CYP1A1 and CYP1A2 are important in the transformation of several organic pollutants into more hydrophilic and excretible products, but occasionally also into more genotoxic derivatives, having a detrimental effect on the current organism (explained in part 1.4 and 1.5). The induction of CYP1A enzymes in H4IIE from exposure to organic extracts of soil was measured immunologically by Western blotting. Techniques included were cell lysation, measurement of total protein concentration in cell lysate by Bradford- assay (Bradford 1976), separation of proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) (Laemmli 1970) and immunological detection of CYP1A by Western blotting (Towbin et al. 1979). The quantification of CYP1A induction in H4IIE has earlier been shown efficient in our laboratory for both environmental samples (Fugleneb 2007) and organic pollutants at known concentrations (Jensen & Krøkje 2008).

2.6.1 Principle of the methods; Bradford assay, SDS- PAGE and Western blotting

The Bradford assay can be used for quantifying total protein concentration in samples. The principle behind is a change in colour of the Coomassie Brilliant Blue G- 250 dye upon binding to proteins, changing from red to blue colour and subsequently causing a spectrophotometrically detected shift in maximum absorption from 456 to 595 nm. Inclusion of a standard set of bovine serum albumin (BSA) makes it possible to quantify the measured protein concentration in relation to the obtained standard curve (Bradford 1976).

Western blotting is a sensitive method, useful for identification and quantification of specific proteins. Proteins in the samples are first separated by SDS- PAGE according to size, before electrophoretically transferred to a solid support where proteins can be immunologically detected. Before loading on the SDS polyacrylamide gel, the proteins are denaturated and negatively charged by heating in presence of a sample buffer containing SDS, a strong anionic detergent, and dithiothreitol (DTT), a reducing agent. The SDS binds the polypeptide backbone, conferring the proteins with a negative charge, proportional to its length. An electric current makes the negatively charged proteins to move in the gel toward the positively charged electrode, resulting in small molecules moving further than the larger ones. The polyacrylamide gels are composed of chains of polymerized acrylamide, cross- linked by bisacrylamide, and the respective concentrations determine the pore size and the resulting sieving properties. The samples are loaded in a thin zone of stacking gel on top of the resolving gel, with the purpose of concentrating the sample before movement through the gel (Sambrook et al. 1989).

After separating proteins in the gel, they are electrophoretically transferred to a nitrocellulose membrane, and probed with reagents (antibodies) specific for a defined amino acid sequence, a method termed Western blotting. The membrane is first soaked in a blocking solution, e.g. nonfat dried milk, to reduce nonspecific binding between antibodies and irrelevant proteins, increasing the sensitivity of the method. The membrane is then incubated in a solution containing unlabeled, specific primary antibodies, binding the protein of interest, before the

solution is taken off and replaced with a solution containing labeled secondary antibody, e.g coupled to an enzyme as horseradish peroxidise (HRP). The incubation time for the solutions can vary with protocols. Substrate is added in the end, and left some minutes for the reaction to take place. A photograph, detecting luminescence, is in the end taken of the membrane. The photo can be used for presenting the induction of proteins qualitatively, or quatitatively by including desiometric analysis, measuring the amount of luminescence that is proportional with CYP1A (Sambrook et al. 1989).

2.6.2 Chemicals, solutions, equipment and commodities

Chemicals	Producer	Catalogue number
Albumin bovine serum (BSA)	SIGMA	B4287
Ammonium persulphate ≥ 98% (APS)	SIGMA	A-3678
Benzo(a)pyrene	SIGMA	B1760
Bio Rad Protein Assay	BIO- RAD	500- 0006
Bromophenol Blue Electrophoresis pure	BIO- RAD	161- 0404
CYP1A1 + OR (human)	BD Gentest™	455111
Dithiothreitol (DTT)	BIO- RAD	161- 0611
Glycerol	SIGMA	-
Glycine ≥ 99% ($C_2H_5NO_2$)	SIGMA	G8898
Goat pAB to cytochrome P450 1A1+1A2 (whole antiserum)	abcam	4227- 1
Hydrochloric acid (HCl) fuming 37 %	MERCK	1.00317.1000
Methanol (CH_3OH)	MERCK	1.06009.2500
N, N`- methylene- bis- acrylamide (30%)	BIO- RAD	161- 0156
N, N, N, N`- tetramethylene diamine (TEMED)	BIO- RAD	161- 0801
Polyclonal Rabbit Anti- Goat Immunoglobulins HRP	Dako Cytomation	P0160
Skim milk powder	OXOID	LP0031
Sodiumacetate ($C_2H_3NaO_2$)	MERCK	1.01539.0500
Sodium dodecyl sulphate (SDS)	BIO- RAD	161- 0301
Sucrose ($C_{12}H_{22}O_{11}$)	SIGMA	S9378
SuperSignal®West Pico Chemiluminiscent Substrate	Thermo scientific	34080
Trizma base ($C_4H_{11}NO_3$)	SIGMA	T6066
Tween 20	MERCK	-

Solutions	Appendix
Ammonium persulfat (10%)	B-2
Blocking solution (5%)	B-2
2M Dithithreitol (DTT)	B-2
Gel- loading buffer 5x	B-2
PBS- Tween (0.1%) (PBS see part 2.4.2)	B-2
Primary antibody solution (1:5000) in 3 % skim milk	B-2
Secondary antibody solution (1:5000) in 0.5 % skim milk	B-2
Separation gel (12%)	B-2
Skim milk solution (0.5%/ 3%)	B-2
0.01 M Sodium acetate (pH 5.2)	B-2
Sodium dodecyl sulphate (10%)	B-2
Stacking gel (4%)	B-2
Tris- glycine buffer 5x	B-2
1.5M Tris- HCl pH 8.8	B-2
0.05M Tris- 0.2M Sucrose pH 7.4	B-2
Western transfer buffer	B-2

Equipment and commodities	Producer	Catalogue number/Model
24 Well Cell Culture Cluster. Flat bottom with lid.	Corning Incorporated	3524
Bio Rad power supply (PowerPac Basic^{TM})	BIO RAD	041BR
Blotting paper, pure cellulose. Extra thick 20 cm x 20 cm	SIGMA- ALDRICH	P8171- 100EA
Conical flask with cap and vacuum opening (100 mL)	Pyrex	BS1739
ELISA Microplates 96- well	Greiner bio- one	650 001
Heating block Dri- Block	Techne	FDB02DD
Kodak Image Station 2000R	Kodak	2000R
Micro tube (0.5 mL/ 1.5 mL)	SARSTEDT	72.699/72.690.001
MiniProtean 3 (short plates)	BIO RAD	1653310
MiniProtean 3 (spacer plates) W/1 mm	BIO- RAD	-
Multiscan Ascent plate reader	Labsystems	354
Nitrocellulose membrane (0.45 µm)	Whatman Schleicher & Schull	10 439 196
Shaker- rotamax 120	Heidolph	544-41200-00-3
Stirring plate	Gerhardt Bonn	MAG- H
Tube (50mL)	SARSTEDT	62.547.254
Western blotting equipment- Mini- PROTEAN®3 Cell	BIO RAD	-

2.6.3 Procedure for measuring CYP1A induction from exposure to extract of soil

Cells were subcultured (part 2.4.3) at 70- 90% confluency, and plated in 24- well plates by adding 60 µL cell suspension (cell pellet dissolved in 2 mL medium) to each well, already containing 1 mL of preheated medium. Cells were incubated for 24 hours at 37 °C in humid atmosphere of 5% CO_2, before changing the medium into preheated medium containing exposure solution. Different concentrations of extracts from Soil 3, Soil 4, Soil 5 and the Blank were included, in addition to a 0.1% DMSO control, and a positive B[a]P control, with triplicates or quadruplicates of each exposure. After additionally 48 hours incubation the cells were lysed and harvested. Medium was aspirated from the wells, and each well washed in 1 mL PBS, before 160 µL of 0.05M Tris- 0.2M Sucrose (pH = 7.4) (Tysklind et al. 1994) were added, and plates freezed at -80 °C for 1 hour. Plates were then left to thaw for 15 min. before the solution in each well was transferred to tubes by scraping and pipetting. Bradford was conducted to measure total protein concentration in each sample, and samples were stored frozen at -80 °C until Western blotting.

Bradford assay was performed according to Bradford (1976) using Bio- Rad protein assay kit (1998). Unsterile 96- well plates were used, setting up a standard curve in the first three rows, followed by samples. As standard BSA (1 mg/mL) was applied. Triplicates of each concentration of the standard and the samples were used for the protein measures. First 40 µL of the Bio- Rad protein solution was added each well. The standard curve was then set up by adding 60- 50 µL milliQ water, then 0- 10 µL of standard, and at last 100 µL milliQ water to each well, giving a total volume of 200 µL before mixing by pipetting. The concentration of BSA in the standard curve corresponded to 0, 5, 10, 15, 20, 30, 40 and 50 µg/mL in each well. The remaining wells were added 56 µL milliQ water, 4 µL sample and 100 µL milliQ water, and the solutions in each well were mixed by pipetting. Absorbance was measured spectrophotometrically at 595 nm, and the total protein concentration (µg/mL) calculated in Ascent software program in relation to the standard curve.

SDS- PAGE was performed according to the method of Laemmli (1970) and Western blotting according to the method of Towbin et al. (1979), following the laboratory manual of Sambrook et al. (1989) and Bio- Rad instruction manual (1997a, 1997b), with minor modifications. Gels for the SDS- PAGE were made the day before and kept at 4 °C overnight, using discontinuous gels with 12% separation gel, 4% stacking gel, and 10 wells on each gel. Sample volumes corresponding to 7 µg total protein were boiled for 2 min in 5 µL gel-loading buffer and distilled water, giving a total volume of 20 µL. Two CYP1A standards at a concentration of 0.25 pmol were also included, together with two samples from each exposure on every gel. Proteins were separated with SDS- PAGE at 200 V for 1 hour (~ 300 mA) in tris-glycine buffer. The separated proteins were then transferred electrophoretically to a nitrocellulose membrane (0.45 µm) at 100 V for 1 hour (~ 300 mA) in western transfer buffer. Unspecific binding was blocked by leaving each gel on gentle shaking for 1 hour in 25 mL freshly made solution of 5% skim- milk in PBS- Tween (0.1%). Blocking solution was taken off and replaced by 25 mL of a 1:5000 dilution of polyclonal goat anti- rat CYP1A1/CYP1A2 antiserum in 3% skim milk, and left on shaking for 2 hours. The membranes were washed 6*5 min in 25 mL PBS- Tween (0.1%), and left on gentle shaking overnight at room temperature in a 1:5000 dilution of HPR- conjugated rabbit anti- goat antibody in 0.5% skim milk in PBS- Tween (0.1%). Membranes were washed 6*5 min in PBS- Tween (0.1%), before 10 mL of freshly mixed SuperSignal®West Pico Chemiluminiscent Substrate (1:1 from each bottle) were added and left on shaking for 5 min. A photograph was immediately taken of the Western blot by placing the membranes with the protein side down in the Kodak Image Station 2000R machine. Settings were corrected to standard exposure for 20 min, 2x binding of pixels in x- direction and luminescence. Desiometric analyses were manually performed using region of interest measurement (ROI), measuring the intensity of CYP1A proteins for each sample. The net intensities were thereby converted into picomole (pmol) quantities of CYP1A per mg total protein by employing a standard curve. The DMSO controls that gave negative values in the ROI analysis were interpreted as zero induction, and corrected to 0.01 to be compatible with the standard curve equation. A few clearly deviating standard values that were considered to be caused by human errors were excluded.

Preliminary tests had to be conducted for optimising the method, as the primary antibody serum and the lysing method had been changed from assays performed earlier in the laboratory (Fugleneb 2007; Jensen & Krøkje 2008). In the optimisation experiments samples of H4IIE exposed to 0.1, 1 and 10 µM B[a]P were used as positive controls, together with a negative 0.1% DMSO control. In addition to the above explained method, the membranes were left on shaking with the secondary antibody for 1 hour, after washing of the primary antibody as explained. A photo was taken, but due to low signals were results of a second overnight incubation used for calculating the CYP1A concentrations. Different incubation conditions and concentrations of the antibodies were tested, and the above explained method was the procedure giving the best results. Cleaning the samples from cell debris was tried by spinning, but resulted in loss of total protein. Because of time constrains the above explained procedure was applied, but further optimisation was considered necessary.

2.6.3.1 Standard curve for CYP1A

To quantify the induction of CYP1A (pmol/ mg total protein) was a standard curve earlier made by Søfteland (2005) utilised in this thesis. This standard curve was made with another primary antibody than the one used in the current study. A new standard curve should optimally have been made, but due to time constrains and lack of standard the utilisation of this standard curve was the only available option.

The general equation for the curve is given underneath (1), together with the values obtained for the curve (2).

$$X = (X_0^b(a/I) - X_0^b)^{1/b} \quad (1)$$

$$X = (0.5690^{-2.707}(4924000/I)-0.5690^{-2.707})^{1/-2.707} \quad (2)$$

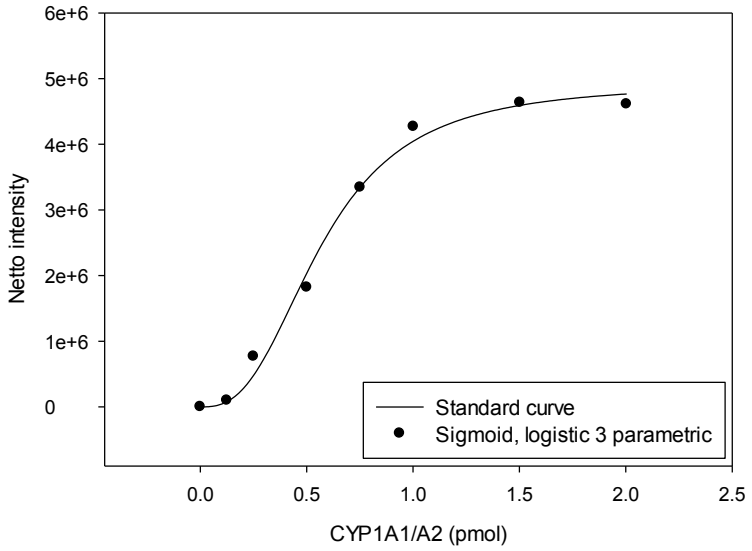

Figure 2.6.3.1 CYP1A1/A2 standard curve. Sigmoid, logistic 3 parametric, with $r^2 = 0.99$ (Søfteland 2005).

3 Results

Results are presented in the following order, Ames assay on *Salmonella* bacterias, and assays performed on H4IIE cells; MTT assay and CYP1A induction measured by Western blotting. Raw data and statistics can be found in C-1, C-2 and C-3, respectively.

3.1 Mutagenic potential of soil extracts- measured by the Ames assay

The results of the Ames assay are presented in the following table and figures. Table 3.1 present the average ± S.D. of controls in the assays, and results of statistical comparison. The figures illustrate the average number of colonies ± S.D. after 48 hours incubation. Figure 3.1.1. 3.1.2, 3.1.3, 3.1.4 and 3.1.5 present reverted colonies related to the DMSO control in presence and absence of S9 metabolic system, exposed to extract of Blank, Soil 1, Soil 2, Soil 3, Soil 4 and Soil 5, respectively. The number of revertants detected after 72 hours incubation are only given in appendix. A low increase in mutants was detected for some exposures from 48 to 72 hours incubation, but it did not seem to affect the overall mutagenic trend caused by the extracts. Raw data and results of statistical analysis, used for deciding presence of mutagenic effects, are given in appendix C-1.

Microscopic examination of the control plates with ampicillin revealed bacterial growth adjacent to the antibiotic tablet, while bacterial growth was absent adjacent to crystal violet.

Table 3.1 Average of controls ± S.D. (n = 3-5) from the Ames assay on strain TA98 and TA100. Mann Whitney statistical comparison ($p \leq 0.05$) between controls of experiment number one and two, in relation to controls of experiment number three. Significant difference between controls is marked by grey fill.

TA 98														
Exposure	Spontanous		DMSO		1 µg B[a]P + S9		Spontanous		DMSO		1 µg B[a]P + S9		DMSO + S9	1 µg B[a]P
Experiment	1	3	1	3	1	3	2	3	2	3	2	3	3	3
Mean	**16.4**	**22.8**	**14.7**	**19.6**	**142.3**	**150.7**	**17.2**	**22.8**	**16.3**	**19.6**	**128.0**	**150.7**	**20.0**	**20.7**
± S.D.	3.8	6.3	5.1	4.8	18.5	22.2	1.3	6.3	4.0	4.8	34.4	22.2	3.9	1.2
p- value	**0.095**		**0.393**		**0.400**		**0.151**		**0.571**		**0.400**			
TA 100														
Exposure	Spontanous		DMSO		1 µg B[a]P + S9		Spontanous		DMSO		1 µg B[a]P + S9		DMSO + S9	1 µg B[a]P
Experiment	1	3	1	3	1	3	2	3	2	3	2	3	3	3
Mean	**95.8**	**115.8**	**87.7**	**116.0**	**593.0**	**534.7**	**88.4**	**115.8**	**82.3**	**116.0**	**527.5**	**534.7**	**126.6**	**97.3**
± S.D.	19.7	15.8	17.5	22.4	127.4	36.4	5.0	15.8	13.7	22.4	69.5	36.4	17.3	6.4
p- value	**0.222**		**0.143**		**0.700**		**0.008**		**0.071**		**0.857**			

Statistical comparison performed between controls for the Ames assays (table 3.1) indicated significant difference only between the spontaneous reversion frequency for experiment two and three on TA100. Generally was the number of revertants some higher in the third experiment.

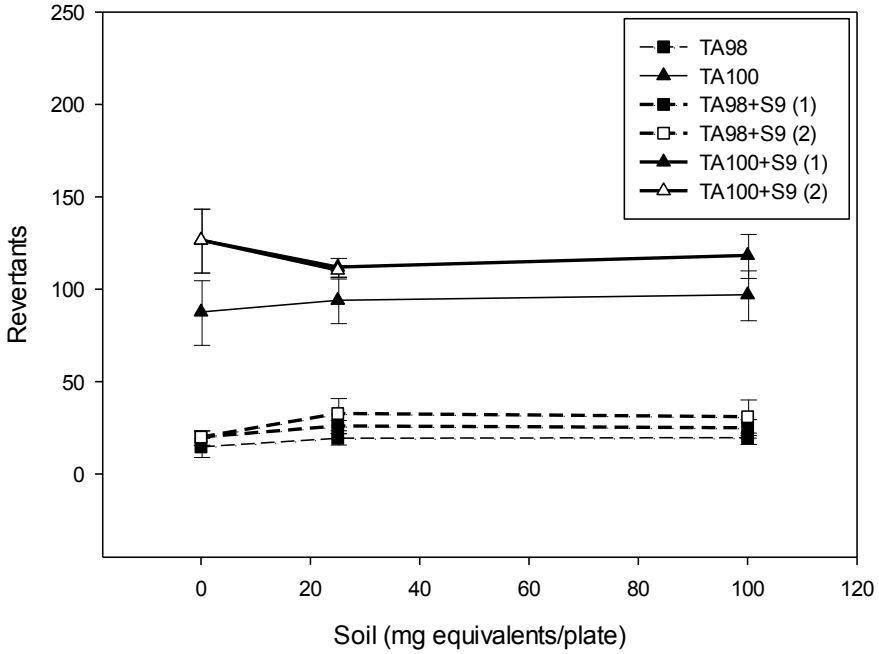

Figure 3.1.1 Average number of *S. typhimurium* revertants per plate ± S.D. (n = 3) induced in the Ames assay after exposure to extract of Blank under different exposure conditions. Extract concentration given as mg dry soil equivalents (eq.) per plate. Dashed line represents concentration- effect relationships for strain TA98, while solid lane denotes concentration- effect relationship for TA100. Thick line denote for both strains exposure in presence of S9- mix, while thin line is exposure without a metabolic system. Square symbols denote TA98 and triangle denote TA100, while black coloured symbols denote first experiment and white denote secondly performed experiment, also marked by number 1 and 2 in parenthesis in the figure.

The response from exposure to the Blank (figure 3.1.1) did not fulfil criteria for mutagenicity on either of the strains. No concentration- effects relationship was detected, and negative results of the doubling criteria and statistical analysis were obtained.

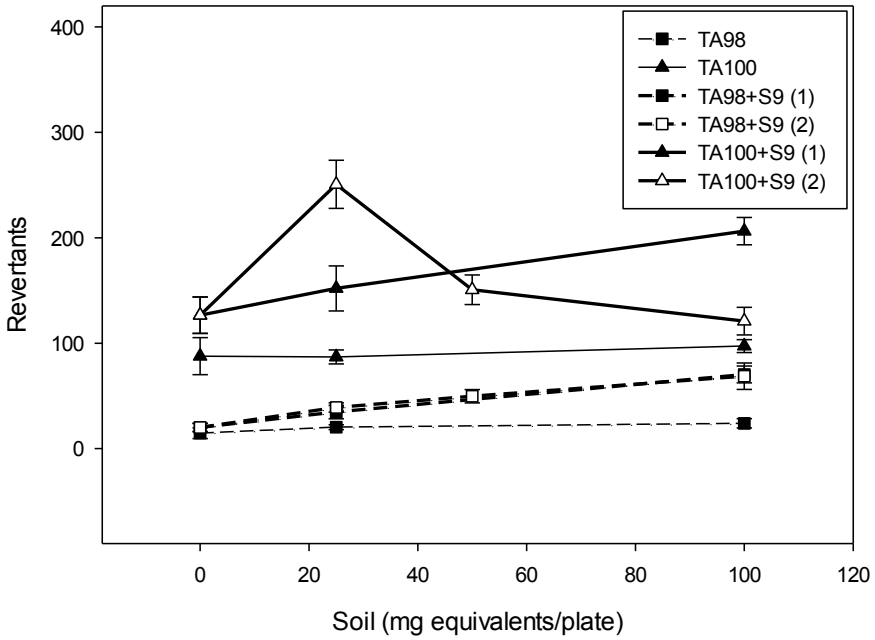

Figure 3.1.2 Average number of *S. typhimurium* revertants per plate ± S.D. (n = 3) induced in the Ames assay after exposure to extract of Soil 1 from a nursery school in Oslo, Norway. Extract concentration given as mg dry soil equivalents (eq.) per plate. Dashed line represents concentration- effect relationships for strain TA98, while solid lane denotes concentration- effect relationship for TA100. Thick line denote for both strains exposure in presence of S9- mix, while thin line denote exposure without a metabolic system. Square symbols denote TA98 and triangle denote TA100, while black coloured symbols denote first experiment and white denote secondly performed experiment, also marked by number 1 and 2 in parenthesis in the figure.

As illustrated in figure 3.1.2 no mutagenic effect of Soil 1 was detected on either of the strains in absence of S9. For TA98 in presence of S9 all the criterias for mutagenicity were fulfilled, except for the doubling criteria at the lowest concentration. The same trend was seen in the first experiment on TA100 in presence of S9, showing statistical difference from the control at 100 mg soil equivalents (eq.). The criteria for reproducibility were not met, by showing a negative concentration- response in the second experiment, having an induction peak at 25 mg soil eq.

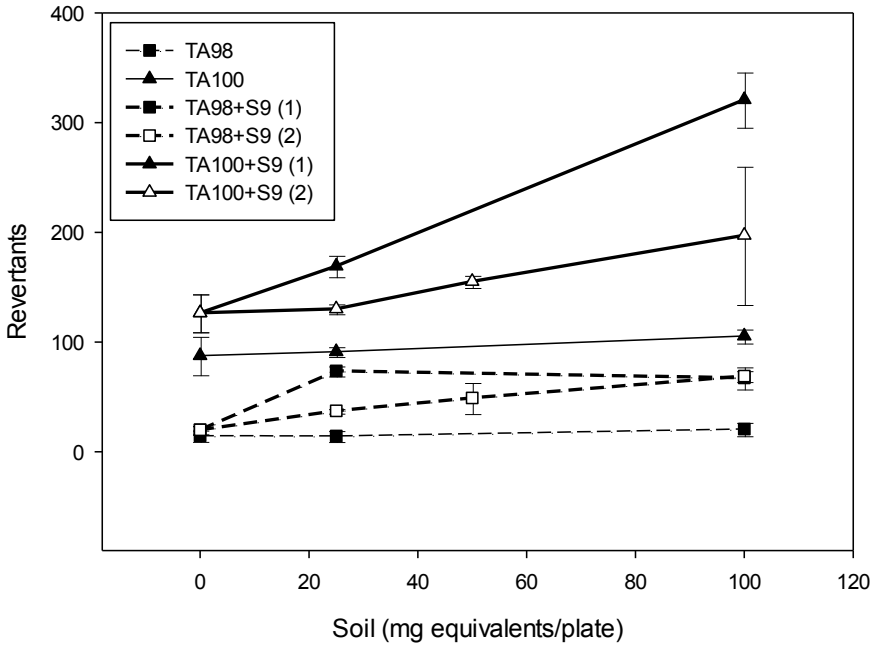

Figure 3.1.3 Average number of *S. typhimurium* revertants per plate ± S.D. (n = 3) induced in the Ames assay after exposure to extract of Soil 2 from a nursery school in Oslo, Norway. Extract concentration given as mg dry soil equivalents (eq.) per plate. Dashed line represents concentration- effect relationships for strain TA98, while solid lane denotes concentration- effect relationship for TA100. Thick line denote for both strains exposure in presence of S9- mix, while thin line denote exposure without a metabolic system. Square symbols denote TA98 and triangle denote TA100, while black coloured symbols denote first experiment and white denote secondly performed experiment, also marked by number 1 and 2 in parenthesis in the figure.

Soil 2 (fig. 3.1.3) did not induce mutagenic effect on either strain TA98 or TA100 in absence of S9, as the average number of colonies from exposure was within the standard deviation of the control. For TA98 in presence of S9 the extract of Soil 2 did induce mutations over the control level. In the first experiment the response was significantly higher than the control for both concentrations, while it was indicated a slight increase in the response with increasing concentrations in the second experiment. Results of the statistical analysis did indicate a positive deviation from the control for both experiments, with exception of a negative result of the doubling criteria at the lowest concentration in the second experiment. For TA100 in presence of S9 it was a high induction of mutants at the highest exposure concentration in the first experiment. In the second experiment the overall induction from extract of Soil 2 was lower than the first time. The response in this experiment also showed a positive concentration- effect relationship, but with a large standard deviation at the highest concentration.

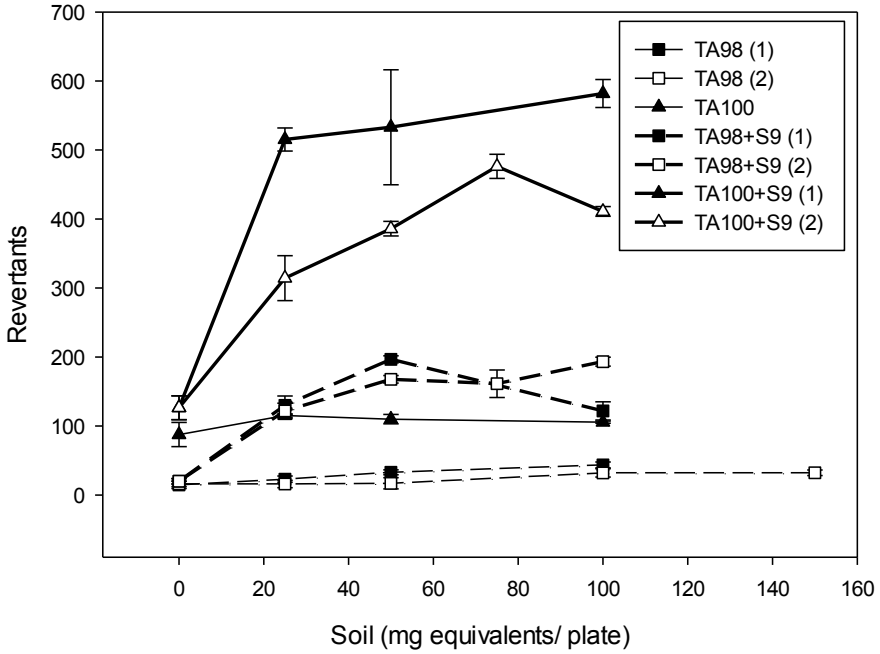

Figure 3.1.4 Average number of *S. typhimurium* revertants per plate ± S.D. (n = 3) induced in the Ames assay after exposure to extract of Soil 3 from a nursery school in Oslo, Norway. Extract concentration given as mg dry soil equivalents (eq.) per plate. Dashed line represents concentration- effect relationship for strain TA98, while solid lane denotes concentration- effect relationship for TA100. Thick line denote for both strains exposure in presence of S9- mix, while thin line denote exposure without a metabolic system. Square symbols denote TA98 and triangle denote TA100, while black coloured symbols denote first experiment and white denote secondly performed experiment, also marked by number 1 and 2 in parenthesis in the figure.

Soil 3 (fig. 3.1.4) did not induce mutagenic effect on neither TA98 nor TA100 in the absence of S9. For TA98 + S9 a clear positive concentration- effect relationship was detected up to a concentration of 50 mg soil eq. per plate. The number of reverted colonies seemed to decrease at the higher concentration, in relation to the mid exposure. This induction was even though still significantly increased from the control. The same concentration dependent trend was reproduced in the second experiment, with exception of a low increase in response for the highest exposure concentration of 100 mg soil eq. For TA100 + S9 the induction of mutants clearly increased from the control for all the concentrations tested, and statistical comparisons indicated significant increase from the controls for all exposure concentrations. The number of revertants was overall higher in the first experiment.

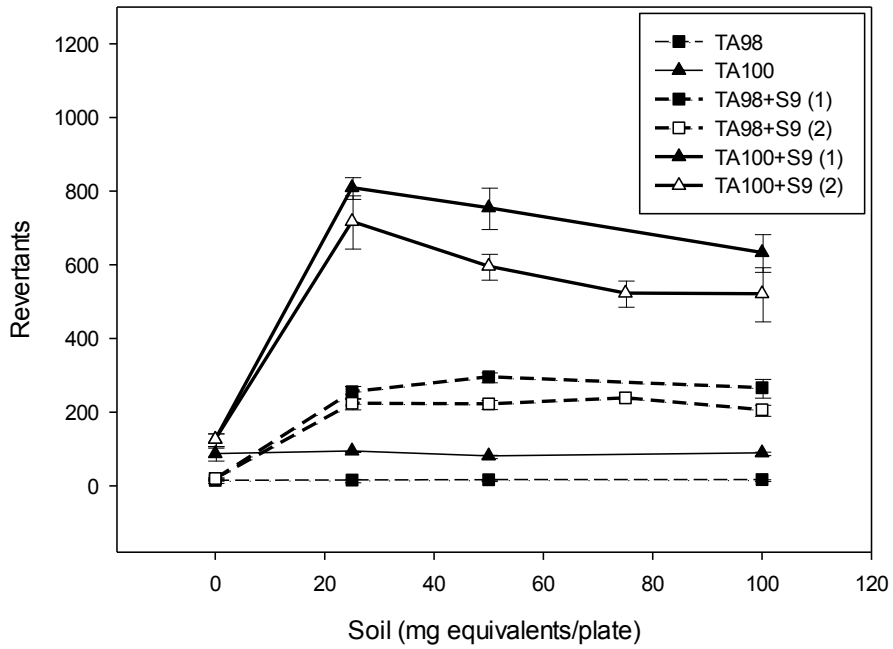

Figure 3.1.5 Average number of *S. typhimurium* revertants per plate ± S.D. (n = 3) induced in the Ames assay after exposure to extract of Soil 4 from a nursery school in Oslo, Norway. Extract concentration given as mg dry soil equivalents (eq.) per plate. Dashed line represents concentration- effect relationships for strain TA98, while solid lane denotes concentration- effect relationship for TA100. Thick line denote for both strains exposure in presence of S9- mix, while thin line denote exposure without a metabolic system. Square symbols denote TA98 and triangle denote TA100, while black coloured symbols denote first experiment and white denote secondly performed experiment, also marked by number one and two in parenthesis in the figure.

For Soil 4 (fig. 3.1.5) no signs of a mutagenic effect were detected on either of the strains in the absence of S9. In presence of the metabolic system the extract of Soil 4 induced the overall highest number of revertants in both strains, compared with the other soil extracts. All the criterias for mutagenicity were fulfilled for TA98 + S9, and a large increase in mutants for the lowest exposure concentration was shown compared with the control. The higher concentrations were more or less in the same range as the exposure concentration of 25 mg soil eq., showing only small variation in the inducing potential of the different concentrations, indicating levelling of effect. The microscopic studies may indicate weak signs of a reduced background flora in the current plates exposed to 100 mg soil eq.

For TA100 in presence of S9 a high induction of revertants was obtained from exposure to 25 mg soil eq. of Soil 4, followed by a negative concentration- effect relationship for the higher concentrations. The trend was the same in both experiments, but the overall number of revertants was highest in the first. The microscopic examinations may indicate weak signs of reduced background flora at an exposure concentration of 100 mg soil eq. in both experiments. The doubling criteria and the statistical analysis of control vs. exposed did support finding of a mutagenic response on both strains in the presence of S9, illustrated in figure 3.1.5.

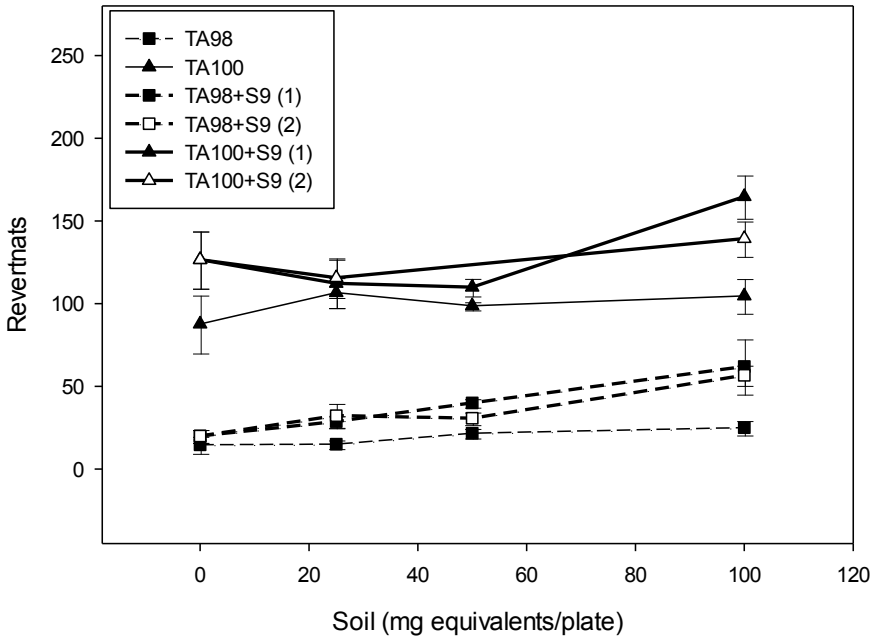

Figure 3.1.6 Average number of *S. typhimurium* revertants per plate ± S.D. (n = 3) induced in the Ames assay after exposure to extract of Soil 5 from a nursery school in Oslo, Norway. Extract concentration given as mg dry soil equivalents (eq.) per plate. Dashed line represents concentration- effect relationships for strain TA98, while solid lane denotes concentration- effect relationship for TA100. Thick line denote for both strains exposure in presence of S9- mix, while thin line denote exposure without a metabolic system. Square symbols denote TA98 and triangle denote TA100, while black coloured symbols denote first experiment and white denote secondly performed experiment, also marked by number 1 and 2 in parenthesis in the figure.

For Soil 5 (fig. 3.1.6) a mutagenic effect was not detected on either of the *S. typhimurium* strains in the absence of S9 mix, as none of the criterias for mutagenicity were fulfilled. For TA98 + S9 a concentration related increase in the number of revertants was detected, but the reversion frequency was low relative to the response induced from extracts of Soil 3 and Soil 4. The criterias for mutagenicity was confirmed at a concentration of 100 mg soil eq. in both experiments, and at 50 mg soil eq. the difference between control and exposure was detected as statistical significant. For TA100 in presence of S9 a mutagenic effect was not confirmed, and it was only for the exposure concentration of 100 mg soil eq. in the first experiment that a detectable, but relative low increase in number of reverted colonies was induced.

3.2 Viability of H4IIE exposed to extracts of soil- measured by MTT

Cell viability of H4IIE, measured by MTT, after exposure to extracts of Blank, Soil 3, Soil 4 and Soil 5 are presented in figure 3.2.2, 3.2.3, 3.2.4 and 3.2.5, respectively. The highest exposure concentration for the following CYP1A quantification assays were found by this assays. Raw data from cell counting, absorbance and calculated results as percent of the DMSO control can be found in appendix C-2.

3.2.1 Cell concentration in MTT

The average absorbance of formazan for each cell concentration ± S.D. (n = 6) from four independent MTT assays is presented in figure 3.2.1, showing a linear relationship between formazan and H4IIE cell concentration per mL.

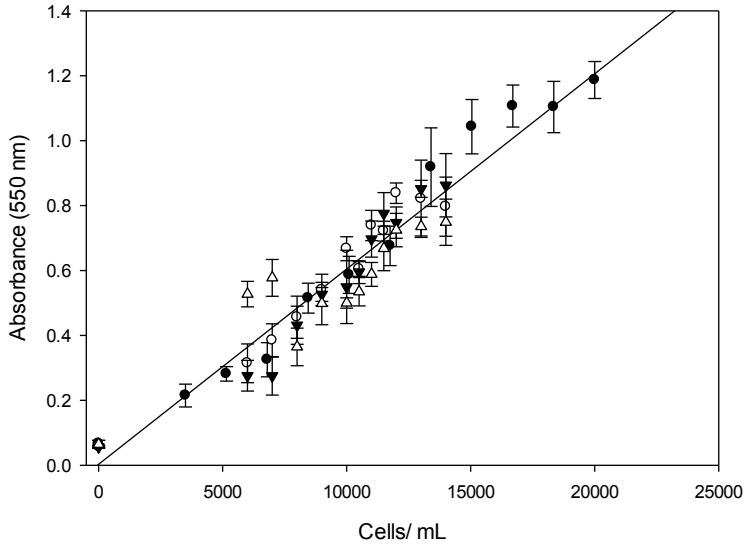

Figure 3.2.1. Scatter plot of absorbance (550 nm) from produced formazan as a function of H4IIE cells per mL. Average ± S.D. (n = 6) of four independently performed MTT experiments, each serie denoted by unique symbol. Linear regression line made from all data in the plot, $r^2 = 0.93$.

A concentration of 11 500 cells/ mL was chosen for the MTT exposure experiments, which was in the middle of the linear production of formazan, illustrated in figure 3.2.1. This cell concentration was later increased to 13 000 cells/ mL, still within the linear area of formazan production, due to a reduction in the absorbance of the medium control, further explained in the discussion part 4.4.1.

3.2.2 Exposure to extracts of soil in MTT

Results from MTT- assay after exposure to Blank and soil extracts are graphically presented in the following figures, 3.2.2, 3.2.3 and 3.2.4 and 3.2.5. Percent viability is calculated in relation to the DMSO control, and data presented as average ± S.D. (n = 6).

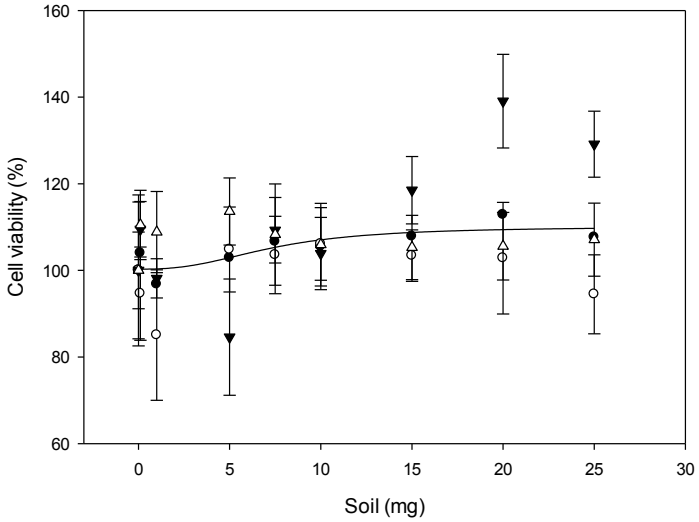

Figure 3.2.2 Viability of H4IIE cells as percent of DMSO control after exposure to Blank extract, measured by MTT- assay. Data given as average ± S.D. (n = 6) from each of three series of experiments, each serie denoted by a unique symbol. Extract concentration given as mg dried soil. Sigmodial Logistic 4 Parametric concentration- effect curve based on average of data series, $r^2 = 0.73$. No statistical significant difference detected between the average data serie and the control by Mann-Whitney ($p \leq 0.05$).

The MTT results illustrated in figure 3.2.2 did not show sign of cytotoxicity with any of the concentrations of the Blank extract. Percent viability of exposed cells was varying more or less in the same range as the DMSO control. The highest concentration of the Blank in the CYP1A assay was decided to correspond to 25 mg soil, due to current results and the highest exposure of soil extracts being 25 mg soil for extract of Soil 5 in the CYP1A assay.

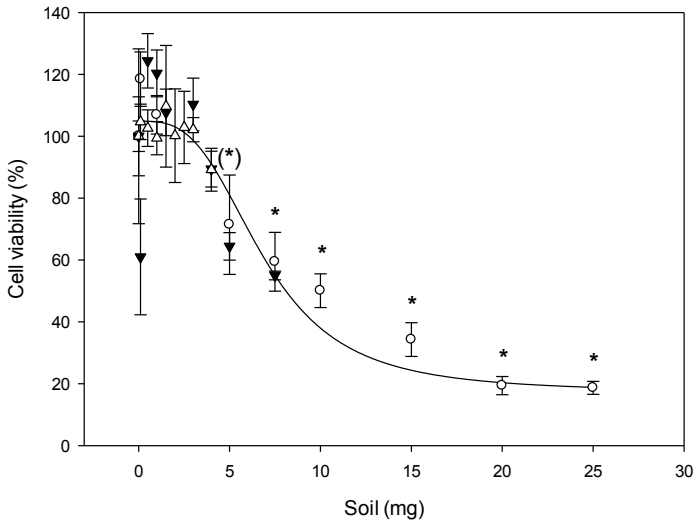

Figure 3.2.3 Viability of H4IIE cells as percent of DMSO control after exposure to extract of Soil 3, measured by MTT- assay. Data given as average ± S.D. (n = 6) from each of three series of experiments, each serie denoted by a unique symbol. Extract concentration given as mg dried soil. Sigmodial Logistic 4 Parametric concentration- effect curve based on average of data series, $r^2 = 0.98$. Asterisks denote statistical significant difference in the average data serie from the control, by Mann-Whitney ($p \leq 0.05$). Asterisk in parenthesis denote statistical difference in viability for one of the series.

Figure 3.2.3 of H4IIE exposure to extract of Soil 3 illustrates a negative sigmodial concentration- effect curve with increasing concentrations of the soil extract. The highest statistical significant concentration that was non- cytotoxic was 4 mg soil for the average serie, but due to high standard deviation, a statistical analysis was also performed on data from individual series at the concentrations of 5 and 7.5 mg soil. Of these concentrations 5 mg soil was not significantly different from the DMSO control in serie two, and mentioned concentration was consequently chosen as the highest exposure concentration for the CYP1A assay.

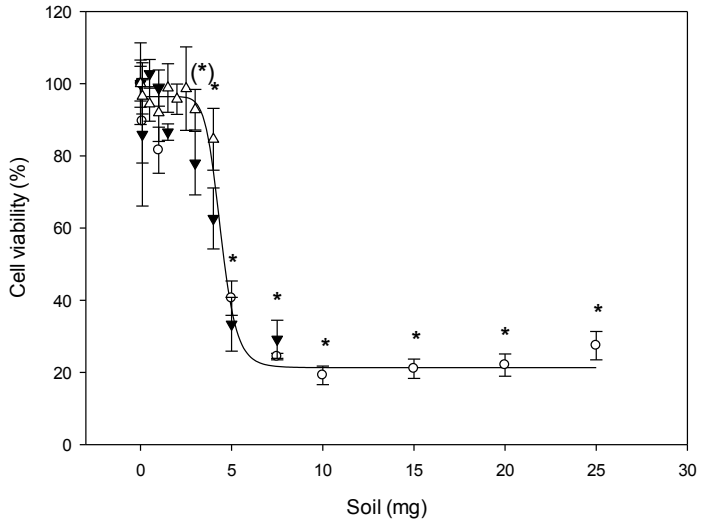

Figure 3.2.4 Viability of H4IIE cells as percent of DMSO control after exposure to extract of Soil 4, measured by MTT- assay. Data given as average ± S.D. (n = 6) from each of three series of experiments, each serie denoted by a unique symbol. Extract concentration given as mg dried soil. Sigmodial Logistic 4 Parametric concentration- effect curve based on average of data series, $r^2 = 0.99$. Asterisks denote statistical significant difference in the average data serie from the control, by Mann-Whitney ($p \leq 0.05$). Asterisk in parenthesis denote statistical difference in viability for one of the series.

Figure 3.2.4 illustrates a negative sigmodial concentration- effect relationship for the viability of H4IIE after exposure to increasing concentrations of extract of Soil 4. The highest non-cytotoxic concentration was 2.5 mg soil for the average serie, indicated by statistical analysis. Statistical analysis was additionally performed on single series at concentrations corresponding to 3 and 4 mg soil, due to variability between the series. These results indicated that 3 mg soil was not significantly cytotoxic, and was therefore decided as the highest exposure concentration in the CYP1A assay.

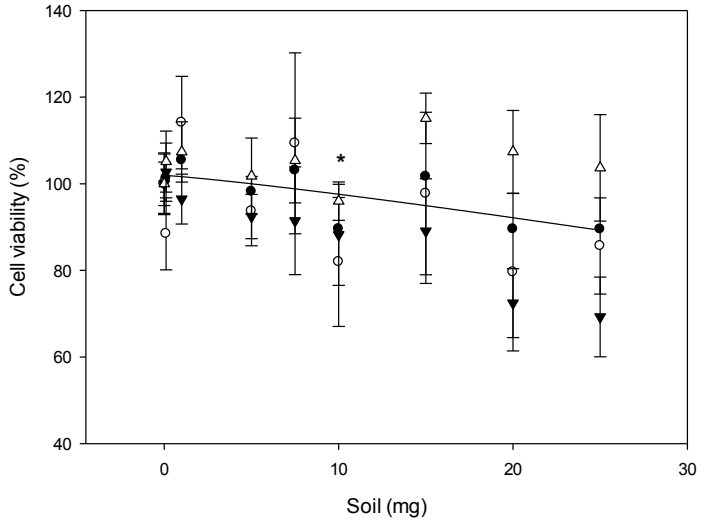

Figure 3.2.5 Viability of H4IIE cells as percent of DMSO control after exposure to extract of Soil 5, measured by MTT- assay. Data given as average ± S.D. (n = 6) from each of three series of experiment, each serie denoted by a unique symbol. Extract concentration given as mg dried soil. Sigmodial Logistic 4 Parametric concentration- effect curve based on average of data series, $r^2 = 0.52$. Asterisks denote statistical significant difference in the average data serie from the control, by Mann-Whitney ($p \leq 0.05$).

Figure 3.2.5 shows that the H4IIE viability from exposure to extract of Soil 5 did not show any clear trend of cytotoxicity with increasing exposure concentrations. The only extract concentration that showed statistical significant difference in cell viability was corresponding to 10 mg soil. As none of the exposure concentrations above or below 10 mg did induce any significant effect, the highest exposure concentration for the CYP1A assay was chosen to correspond to 25 mg of soil.

3.3 CYP1A induction in H4IIE exposed to extracts of soil

The relationship between exposure to soil extracts, corresponding to different concentrations of soil, and the induction of CYP1A biotransformation enzymes in the H4IIE cell line are presented in the following figures. Figure 3.3.1, 3.3.2, 3.3.3 and 3.3.4 illustrate concentration-effect relationship between CYP1A and exposure concentration for the Blank extract and extract of Soil 3, Soil 4 and Soil 5, respectively. Figure 3.3.5 illustrate the effect- relationship obtained in cells exposed to B[a]P. Raw data are given in appendix C- 3.

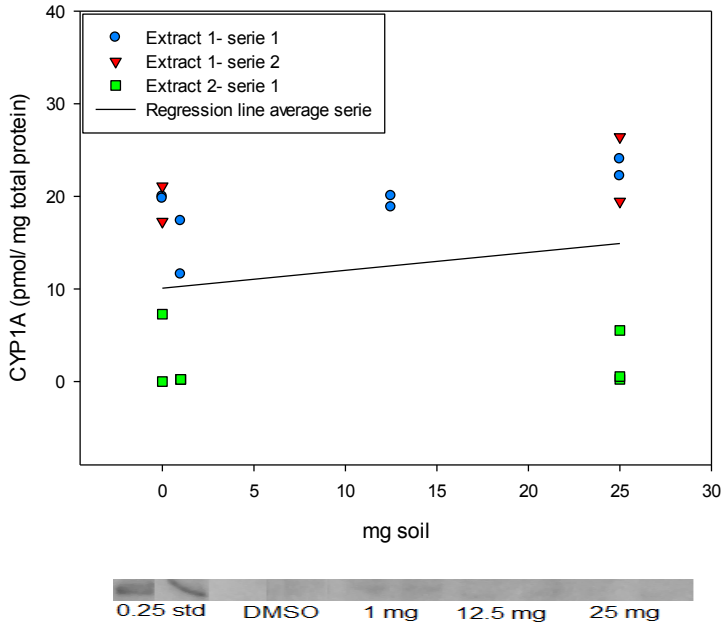

Figure 3.3.1. Induction of CYP1A (pmol/ mg total protein) in H4IIE cells from exposure to Blank extract, analysed by Western blotting. Concentration of extract is expressed in mg dried soil. The zero exposure concentration is a 0.1% DMSO control. Each experiment serie is denoted by a unique symbol and colour. Linear regression of average of all experiments, $r^2 = 0.30$. Together with a representative western blot of extract 1- serie 1.

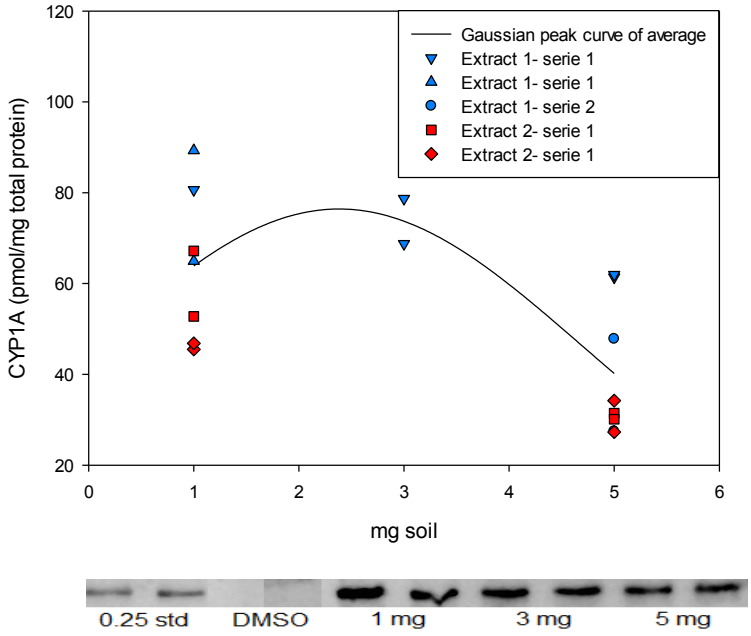

Figure 3.3.2. Induction of CYP1A (pmol/ mg total protein) in H4IIE cells from exposure to extract of Soil 3, analysed by Western blotting. Concentration of extract is expressed in mg dried soil. The CYP1A induction from the 0.1% DMSO control has been subtracted. Different symbols denote unique experiments, while each extract is denoted by different colours. 3 parametric Gaussian curve of average of all experiments, $r^2 = 1$. Together with a representative western blot of extract 1- serie 1.

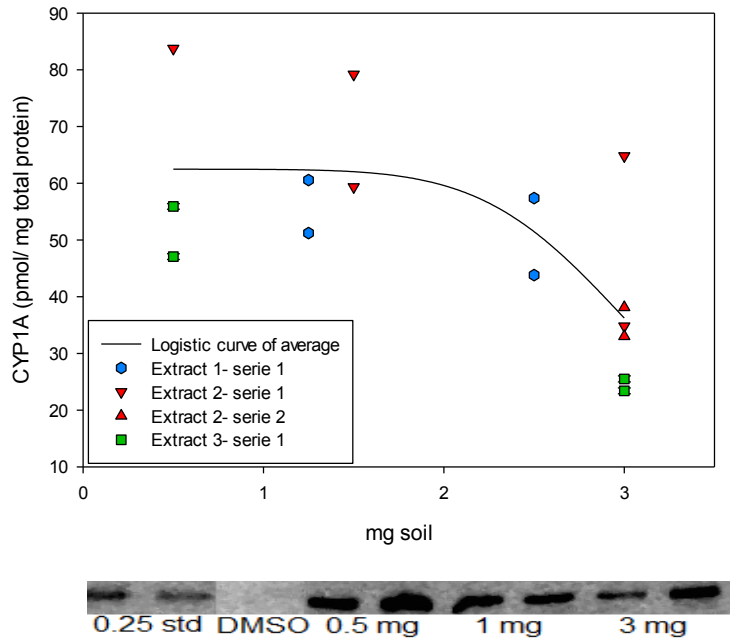

Figure 3.3.3. Induction of CYP1A (pmol/ mg total protein) in H4IIE cells from exposure to extract of Soil 4, analysed by Western blotting. Concentration of extract is expressed in mg dried soil. The CYP1A induction from the 0.1% DMSO control has been subtracted. Different symbols denote unique experiments, while each extract is denoted by different colours. Sigmoidal logistic 3 parametric curve of average of all experiments, $r^2 = 0.85$. Together with a representative blot of extract 2- serie 2.

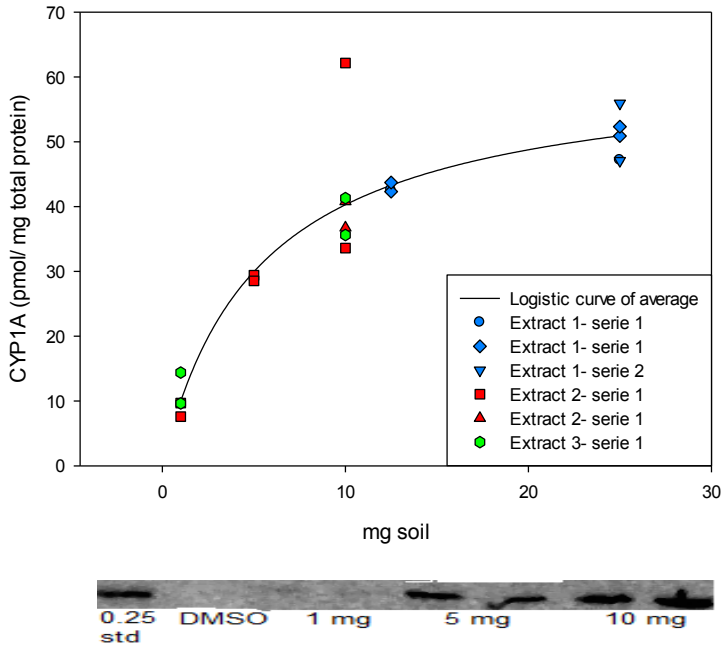

Figure 3.3.4. Induction of CYP1A (pmol/ mg total protein) in H4IIE cells from exposure to extract of Soil 5, analysed by Western blotting. Concentration of extract is expressed in mg dried soil. The CYP1A induction from the 0.1% DMSO control has been subtracted. Different symbols denote unique experiments, while each extract is denoted by different colours. Sigmoidal logistic 3 parametric curve of average of all experiments, r^2 = 0.996. Together with a representative western blot of extract 2- serie 1.

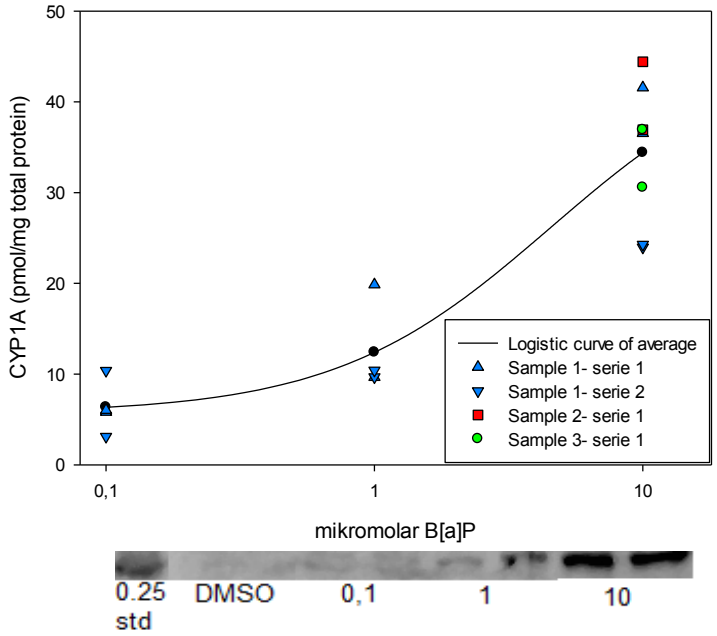

Figure 3.3.5. Induction of CYP1A (pmol/ mg total protein) in H4IIE from exposure to benzo[a]pyrene (µM), measured by Western blotting. The CYP1A induction from the 0.1% DMSO control has been subtracted. Different colours denote different samples of exposed H4IIE, while each experiment is denoted by a unique symbol. Sigmoidal logistic 4 parametric curve of average of all experiments, r^2 = 1. Together with a representative western blot of sample 1- serie 1.

Figure 3.3.1 illustrating the CYP1A induction in the cells exposed to Blank extract showed a response that was more or less in the same range as the DMSO control. A slight increase in CYP1A induction was obtained from increasing concentrations of extract 1, while it for extract 2 could not be detected any concentration- effect relationship. The linear regression of the average of all experiments showed a nearly horizontal line with a r^2 value of 0.30.

A clear induction of CYP1A in H4IIE was detected from exposure to extract of Soil 3 and Soil 4, illustrated in figure 3.3.2 and 3.3.3, respectively. The concentration- effect relationship obtained after exposure to extracts derived from these two soil samples was similar, and the CYP1A levels decreased at the highest concentration for both extracts. The opposite trend was detected with extracts of Soil 5, showing a positive relationship between CYP1A induction and all extract concentrations, figure 3.3.4. For extract of Soil 3 and Soil 4, the maximum average level of CYP1A was detected to be roughly 60- 70 pmol CYP1A/mg total protein. Extract of Soil 5 induced an average maximum level of 50 pmol CYP1A per mg total protein, at a concentration corresponding to 25 mg dried soil.

Figure 3.3.5 of the B[a]P exposed H4IIE showed an expected positive exposure- induction relationship, which was included to verify the procedure.

4 Discussion

In this chapter the soil samples and how the organic extraction and solvent change can lead to differences in bioassay endpoints will be discussed. Continuing with a discussion of the results from individual assays, and their relevance will be considered by looking at the; utility of assays in relation to *in vitro* vs. *in vivo* situations, bioavailability and toxicity of pollutants in soil, biological effects of exposure, and utility of current bioassays in assessing for potential hazards.

4.1 Soil samples and organic extraction

Chemical analysis of soil in nursery schools in Oslo revealed pollution levels exceeding the recommended quality limits at several places (Ottesen et al. 2008), causing a potential hazard for children upon exposure (U.S. EPA 2008). Extracts of selected soil samples (table 2.1) were assessed in the current project by screening for presence of potential hazards. The focus was on PAHs and PCBs, as these classes of chemicals were considered most relevant according to their genotoxic (Safe 1994; Schilderman et al. 2000; Meador 2008) and CYP1A inducing potentials (Mandal 2005; Nebert 2006; Nebert & Dalton 2006).

The utilisation of soil samples containing elevated levels of PCBs (i.e. Soil 5) and different amount of PAHs below, at and above the quality limits for soil (Alexander 2006) (i.e. Soil 1, Soil 2, Soil 3 and Soil 4) were found interesting in the assessment of differences in biological responses, and to check for a visible pattern between chemical content of soil and biological effects. Soil 4 (fig. 2.1) contained the highest level of PAHs, and was dominated by fluoranthene, pyrene and phenanthrene, followed by high levels of benzo[b]fluoranthene, chrysene, B[a]P and benzo[a]anthracene. No specific PAH compounds were designated as major constituents in Soil 2 and Soil 3 (fig 2.1). However, there was a general higher level of the high molecular weight PAHs, probably related to the slower degradation of these compounds (Johnsen et al. 2005). Soil 5 (fig 2.2) was the only sample containing a considerable amount of PCBs, and the congeners PCB 138, PCB 153 and PCB 180 seemed especially elevated.

The chemical content of the soil was expected to partly reflect the differences of the extract induced responses. However, since chemical analysis do not detect all chemical compounds, reveal the actual toxic effects, or the potential interacting effects (Courty et al. 2008), could an effect not be truly predicted. Supporting the effort of assessing the integrated biological response from a whole mixture (Loibner et al. 2004). The chemical analysis is also limited to selected compounds, and the presence and contribution from unknown compounds cannot be verified or excluded. For instance more than 100 different PAHs exist, but the analysed U.S. EPA selected PAHs are considered relevant for exposure and are expected to be harmful and representative of the effect induced by PAHs (ATSDR 1995).

Samples of soil taken from the same spot can have internal differences in relation to the chemical content, subsequently causing differences between independently made extracts. The nature of soil affects the chemical content of extracts (Courty et al. 2004), and the extraction is considered to result in some variation due to differences in extractability of chemicals. This is partly due to sorption between chemicals and soil organic content (Courty et al. 2008) (part 1.3). Implying that chemical profile of extracts does not necessarily reflect the chemical profile of the soil in an area.

4.2 Organic extracts of soil

Ultrasonic extraction with DCM was utilised for extracting compounds from soil (part 2.2), and it was reasonable to assume that the extracts mainly contained organic pollutants. Nevertheless the presence of inorganic pollutants could not be fully excluded, potentially affecting the overall toxicity. This effect was considered negligible because of expectedly low concentrations, and was consequently not given attention in the current thesis.

Ultrasonic extraction of compounds from soil has shown advantageous, as it gives consistent results (Morin et al. 1987) and have together with DCM been shown to give the best recovery of compounds (Aamot et al. 1987). Type of extraction procedure and solvent can directly influence the genotoxic potential of soil extracts (da Silva et al. 2009) along with parameters as temperature and duration, which by optimisation can increase the inducing potential of extracts (Courty et al. 2004). Ultrasonic agitation has shown effective for extracting mutagens from soil (Aamot et al. 1987) and DCM are frequently used as organic solvent. DCM has been found to often provide extracts that in the Ames assay are more mutagenic on TA98 than other strains in presence of S9, and differences in potency have been identified to depend on the polarity of the extraction agent (White & Claxton 2004). Changing solvent into DMSO is very common (Whyte et al. 2004), but has been asserted to give a underestimation of the real mutagenic potential of extracts, as DMSO has been claimed to "quenche" the genotoxic activity (Demarini et al. 1992). Based on theory it is reasonable that use of a different extraction procedure and solvents could have given slightly different biological responses, caused by different yield and proportion of chemicals. Assessing these differences was not an aim for this study and the utilised extraction procedure had earlier been shown suitable (Krøkje & Gullvåg 1994; Østby et al. 2006; Fugleneb 2007). Because of the low effect obtained from the DMSO solvent controls in all assays the DMSO was considered to have little impact on the results in this project.

The solvent change from DCM to DMSO (part 2.2.2) was considered to be an important source of variation between extracts, caused by variability in the chemical yield. Loss of chemicals was likely to occur by evaporation, spill or from insoluble parts. Variation in parallel DMSO dissolved extracts consequently gave variation in biological responses from different experiments within an assay. This variation would probably have been even higher if the DMSO dissolved extracts additionally originated from several DCM dissolved soil extracts for each soil sample.

4.2.1 The Blank extract- a negative control of the extraction procedure

The Blank was included to reveal if the extraction procedure affected the potential of the extracts to induce effects in the biological assays. There was not detected an induction of mutants in the Ames assay (fig. 3.1.1) and no sign of cytotoxicity in H4IIE (fig. 3.2.2) from exposure to the Blank. The CYP1A induction in H4IIE was also low (fig. 3.3.1), with the exception of some variation that were considered as negligible, as the response from both Blank extracts were considered to be low. In contrast to the soil extract exposures, was it neither for the Blank nor the DMSO control possible to visually see bands of CYP1A on the membranes. The low responses from exposure to the Blank in all the utilised assays indicated that the extraction procedure did not contribute to the inducing potential of the extracts, verifying the extraction procedure.

4.3 Mutagenic potential of soil extracts- measured by the Ames assay

The results of the Ames assay gave good indications on the presence of secondary mutagens in the extracts of soil. The number of induced mutants was overall high in presence of S9, suggesting a high content of secondary mutagens in some of the extracts. In contrast, there was no significant increase of mutated bacteria detected from exposure in absence of the metabolic S9 mix. However, the exposures without S9 was tested only ones (except for TA98 exposed to extract of Soil 3) and conclusions should be based on consistent results of at least two assays (de Serres & Shelby 1979). Therefore the results were interpreted as only indications on an absence or very low levels of primary mutagens.

In presence of S9 the extract of Soil 3 and Soil 4 induced high levels of frameshift mutations in strain TA98 and basepair substitutions in TA100 (fig. 3.1.4 and 3.1.5). An induction was also seen from extract of Soil 2 (fig. 3.1.3) in presence of S9, but at a relative lower level, and with some deviation in the responses from the experiments on TA100. High content of secondary mutagenic compounds is in accordance with expectations as the soil samples were known to contain PAHs. This is a group of chemicals recognized to be of a secondary toxic nature, whereby the toxicity can be increased by biotransformation enzymes (Klaunig & Kamendulis 2008; Meador 2008). Among the 16 PAHs analysed in the current soil samples B[a]P, benzo[b]fluoranthene (Nagai et al. 2002; White & Claxton 2004; Yan et al. 2004), benzo[a]anthracene, dibenzo[a,h]antracene (Nagai et al. 2002; White & Claxton 2004), benzo[k]fluoranthene (Nagai et al. 2002; Yan et al. 2004), fluoranthene (Nagai et al. 2002), indeno[1,2,3 cd]pyrene (White & Claxton 2004) and chrysene (Yan et al. 2004), have been identified as secondary mutagens in the Ames assay. The remaining has been detected as very low or non- mutagenic (Nagai et al. 2002; Yan et al. 2004). These results make it reasonable to suggest these selective compounds to be important for the total secondary mutagenic potential of the PAH containing extracts, especially those found in high amounts, such as fluoranthene in Soil 4 (fig. 2.1).

Soil 5 contained the highest level of PCBs, and exposure to extracts derived from this soil did not induce a specific increase in number of reverted bacteria, with exception of a low

response in TA98 + S9 (fig. 3.1.6). The mutagenic potential of this soil extract is suggested to be caused by other components than PCBs, and the low potential is considered to partly reflect the low detection of PAHs in this soil. Earlier have PCBs been claimed to be non-genotoxic in microbial assays (Demarini et al. 1992) and has been indicated to be non-mutagenic in the Ames assay (White & Claxton 2004).

It was expected to see an increased mutagenic response from exposure to extract of Soil 1 to Soil 4 according to the increasing concentration of PAHs, an expectation that were generally confirmed. Extracts of Soil 4 showed the overall highest number of revertants for the lowest exposure concentration, indicating a higher level of mutagenic compounds, in accordance with chemical analysis. The difficulty of truly predict a biological response from exposure to a mixture (part 4.1.), were seen for extract of Soil 1 (fig. 3.1.2). This extract induced a low, but positive response and indicated significant induction of revertants in TA98 + S9, suggesting presence of secondary frameshift mutagens. This is despite the chemical analysis revealing the soil as "clean", due to the organic pollutants being underneath limit of detection, along with very low levels of analysed inorganic compounds. The mutagenic potential of this soil extract can have been caused by the additive toxicity of chemicals, interactions increasing the toxic potential, or from unknown chemicals not included or detected in the chemical analysis. Current findings supported the inadequacy of applying only chemical data when assessing environmental samples for the presence of toxic compounds.

Negative concentration- effect relationships detected in this study may have several explanations. It could be suggested that it is caused by cytotoxicity from increasing exposure concentrations or non- homogenous exposure solutions, or by reduced toxicity related to deactivation of chemicals by biotransformation or antagonistic interactions. Moreover, it could also reflect the amount of S9 mix added or the enzyme activity in the S9- mix, which can be inhibited by constituents in the mixtures. The optimum amount of S9 for detecting inducing potentials can vary between chemical compounds and concentrations, affecting the sensitivity of the assay (Ames et al. 1975; Maron & Ames 1983). A depletion of biotransformation capacity is considered to be likely only for extracts with a large mutagenic potential (i.e Soil 3 and Soil 4). Cytotoxicity could only be a potential explanation if signs on cytotoxicity were detected in the microscopic studies, as for 100 mg soil eq. from extract of Soil 4 on TA98 + S9 and TA100 + S9. However this was considered less likely since indications on cytotoxicity were weak, and it can be a matter of subjective interpretation. The underlying reason for the negative response was uncertain for samples like extract of Soil 1 on TA 100 + S9 (fig. 3.1.2), and should ideally have been tested a third time.

The current results indicated presence of mainly secondary frameshift inducing mutagens, as the induction of mutants was only significant in presence of S9 and approx. twice as high in TA98 + S9, compared with TA100 + S9. The slightly higher mutation frequency that were generally seen after 72 hours incubation compared with 48 hours, has been suggested to be caused by chemicals causing a delayed appearance of mutants (de Serres & Shelby 1979). These results did not seem to indicate different trends in the mutagenic potential of extracts, and was consequently not given further attention. The overall lower response in the second

experiment (except for the Blank) is possible a result of a lower chemical yield of these extracts (part 4.2).

4.3.1 Validity of results

The relative consistent results from exposure to extracts and the confirming results from the controls were considered to validate the measured effect. The integrity of the bacterial strains was confirmed in the tests with ampicillin and crystal violet, demonstrating presence of the R-factor by bacterial growth adjacent to ampicillin, and presence of the rfa mutation by absence of bacteria adjacent to crystal violet (Maron & Ames 1983). The results from exposure to 1 µg B[a]P + S9 was for both TA98 and TA100 at the same level as reported in other studies (Maron & Ames 1983; Krøkje et al. 1985) and was considered to validate the activity of the S9 mixtures.

The spontaneous reversion of the strains were reported to be 30- 50 colonies per plate for TA98 and 120- 200 colonies per plate for TA100, after 48 hours incubation (Maron & Ames 1983), but this might vary between laboratories (de Serres & Shelby 1979). The reversion frequencies were lower in the current assays (13- 31 for TA98 and 78- 140 for TA100), although considered to be within an acceptable range. Deviating ranges might occur and is regarded not to be diminishing as long as the same reversion frequency are seen over a longer period in the same laboratory (Maron & Ames 1983; Mortelmans & Zeiger 2000), which is the case here.

Because the DMSO + S9 control was mistakenly not included in the two first experiments, an additional experiment was performed, including only controls. Mann Whitney statistical comparison between the controls in these assays (table 3.1) supported the choice of relating the DMSO + S9 control of the last experiment to the exposure in presence of S9 for the two first. Due to indications of a significant difference detected only for the spontaneous reversion of TA100 in the second experiment. Mann Whitney is a non- parametric test, and was chosen because of the low sample size. Low sample sizes diminish the statistical power, and the statistical analysis could therefore only be interpreted as a support for observable trends, which apply to the statistic used in the entire project. The controls of the third experiment were overall slightly higher than the other controls, a result that would cause a relative underestimation of the mutagenic potential of the soil extracts. This difference was considered as minor and not affecting the overall presentation of mutagenicity.

4.4 Cell viability in H4IIE exposed to extracts of soil, and choice of concentrations to use in the CYP1A induction assays

When performing induction studies on cells it is important that the exposure concentrations do not affect the viability. The highest non- cytotoxic extract concentrations were consequently found for the CYP1A induction assays. Reduced cell viability could potentially result in false negatives, manifested as negative or flattening of the CYP1A induction response. The H4IIE

cell viability from exposure to extract of Blank, Soil 3, Soil 4 and Soil 5 gave fairly consistent results in relation to pattern of induction (part 3.2.2).

The results from extract of Soil 3 and Soil 4 (fig. 3.2.3 and 3.2.4) showed a clear negative relationship between cell viability and increasing exposure concentrations. The reproducibility between experiments was considered good as the same pattern and low variation was obtained between series of experiments. The standard deviation within a serie was also relative small. Together with trends and statistical analysis on single series, showing that 5 mg of Soil 3 and 3 mg of Soil 4 were the highest non- cytotoxic concentrations, this was considered to support the choice of using current concentrations for the CYP1A induction assays. In contrast, the average series for these concentrations were detected as significantly reduced from the control, potentially implying that differences in chemical yield of the extracts (part 4.2) can cause variation in the cytotoxic response.

Extract of Soil 5 did not induce clear signs of cytotoxicity (fig. 3.2.5), and according to the statistical analysis, 10 mg soil was the only concentration showing a significant decrease in cell viability. As cytotoxicity was not observed for any of the lower or higher concentrations, it was interpreted as variation due to the specific exposure solution and not as a result of a cytotoxic concentration. The highest exposure concentration of 25 mg soil was consequently used in the CYP1A induction assay. The relative high variability within and between series was likely to be caused by non- homogenous exposure solutions, supported by difficulties of dissolving the extract when preparing exposure solutions in growth medium. Another likely explanation for variation could be potential non- homogenous cell suspensions used for plating the cells. Moreover, the counting of cells is considered to be a subjective method which can cause differing results. Potential remains of RPMI- 1640 growth medium in the wells is also suggested to cause interference with the measures, as the medium has similar absorbance spectrum as formazan (Nikkhah et al. 1992).

Cytotoxicity of cells is generally dependent on concentration of toxicant and duration of exposure. Shorter exposure times might result in an underestimation of toxicity, potentially caused by exclusion of delayed apoptosis. Longer exposure times (e.g. 48 and 72 h.) have shown to give more consistent results between cytotoxicity assays (Komissarova et al. 2005). The long term effect can be considered as more interesting in relation to environmental samples, and a 48 hours exposure was considered suitable for this study.

4.4.1 Validity of results

The change of cell concentration and calculations of the absorbance values, explained in part 2.5.3.2, was expected not to have any effect on the overall results, as the concentration change was very small and within the linear area of formazan production (fig. 3.2.1). The reason for the observed absorbance drop could be a reflection of cells physiological state since the production of formazan is dependent on cell activity (Mosmann 1983), or by other mechanism interfering with formazan production (Liu et al. 1997).

The results were presented as percent viability from the DMSO control and not the medium control, as the aim was to find the effect induced from the soil extract, and not from the DMSO itself. The DMSO concentration of 0.1% was expected not to have any negative effect on viability (Whyte et al. 2004; Østby et al. 2006), on the other side deviating effects on cultured cells have been published (Doostdar et al. 1991; Xie et al. 2003). In a study by Doostdar et al. (1991) 2% DMSO increased the mixed function- oxidase enzyme activity in human HepG2 hepatoma cells, while the general metabolic activity was shown to decrease by 38% in H4IIE exposed to 0.1% DMSO (Xie et al. 2003). Since the amount of formazan depends on cell activity (Mosmann 1983) a DMSO induced reduction in metabolic activity could consequently be expected to result in false negatives in the MTT assay. Statistical comparison was therefore conducted between the medium and DMSO controls (only presented in appendix C-2), indicating a significant reduction in the DMSO exposed in only four of the assays. This effect was considered as minor and no clear indication on a reduction caused by DMSO. The MTT assay is a widely used method and has been argued as suitable for measuring cell viability (Berridge & Tan 1993; Liu et al. 1997). This assay was consequently considered adequate for the current purpose.

4.5 CYP1A inducing potential in H4IIE exposed to extracts of soil

The graphical presentation of CYP1A induction in H4IIE cells clearly illustrated inductive response- trends for all of the soil extracts, though with variation both within and between experiments, and relative low intensities of luminescence (proportional to CYP1A).

A negative trend of CYP1A induction in relation to increasing concentrations of soil extracts was detected for Soil 3 (fig. 3.3.2) and Soil 4 (fig.3.3.3.). The gaussian bell shaped curve illustrating the response from extract of Soil 3, indicated induction at the low concentration and inhibition at the highest concentration. The intermediate concentration of 3 mg that induced the highest level of CYP1A was tested only once, questioning the reproducibility of response. The CYP1A induction from extract of Soil 4 was best illustrated by a sigmoidal logistic curve, with highest induction at the lowest concentration. Extract of Soil 5 (fig. 3.3.4) did oppositely induce CYP1A in a positive concentration- dependent manner.

Dose- dependent responses, as obtained from extract of Soil 5, have been measured in other studies for CYP1A catalytic activity (EROD) (Tillitt et al. 1991; Sanderson et al. 1998) and immunoquantified CYP1A proteins (Søfteland 2005; Jensen & Krøkje 2008), in H4IIE from exposure to PCBs. Of the seven PCBs analysed in the soil PCB28 and PCB118 are distinguished from the other by having a mono- *ortho* coplanar configuration that is known to resemble AhR activity (part 1.4.1), while the remaining are di- *ortho* PCBs (Schmitz et al. 1995). The stereochemistry of the di- *ortho* PCBs are expected to give a low or absent affinity for the Ah- receptor and a consequent reflection in the CYP1A inducing potential (Safe et al. 1985). For instance, PCB 153 has been reported not to induce CYP1A activity *in vitro* (Bruschweiler et al. 1996) nor protein (Bruschweiler et al. 1996; Søfteland 2005), while the similar congener PCB 138 oppositely induced CYP1A protein at higher concentrations (Søfteland 2005). Studies exposing H4IIE to mixtures of PCB congeners have reported both

additive (Sawyer & Safe 1985; Schmitz et al. 1995) and non- additive effects (Schmitz et al. 1995), in relation to EROD activity. While mixtures of PCBs with TCDD also have been shown to induce both non- additive and additive EROD activity in H4IIE, depending on the exposure concentration (Vamvakas et al. 1996). In the study by Schmitz and co-workers (1995) did a mixture of six potent PCB congeners induce EROD activity in an additive manner. When adding a tenfold surplus of a mono- and di- *ortho* PCB mixture (28, 52, 101, 138 and 180), which is the same congeners analysed for in the current soil samples, the detected potency was almost three times higher than predicted. These findings suggested a moderate synergistic enhancement in the potency of the CYP1A inducing congeners by less potent PCBs, illustrating the complexity of mixtures. The complexity of PAHs and other constituents in the extracts is suggested to give rise to interactions, mediating the biological response. Furthermore, it is known that the dose level of mixtures can be important for the outcome of effect. It has to be pointed out that the CYP1A activity and protein levels do not necessarily reflect each other (Willett et al. 1998), but to my knowledge the literature about immunoquantified CYP1A induction from mixtures is limited.

Decreased induction of CYP1A at higher inducer concentrations, as for extract of Soil 3 and Soil 4, have been immunologically detected from exposure to a mixture (Østby et al. 2006) and inducers at known concentrations (Hahn et al. 1996; Lorenzen et al. 1997). Biphasic CYP1A activity from single PAHs or environmental fractions has also been detected in H4IIE cells (Willett et al. 1997; Willett et al. 1998; Gale et al. 2000), a mouse Hepa- 1 cell line (Matlova et al. 1995) and in a fish PLHC- 1 hepatoma cell line (Fent & Batscher 2000). Willett and co-workers (1998) demonstrated an inhibitive interaction on CYP1A activity in H4IIE induced by the PAH compound fluoranthene, a major constituent of the PAHs in Soil 4. Fluoranthene did not induce CYP1A activity when cells were singly exposed, but seemed to inhibit the TCDD- induced activity in a non-competitive manner when co- exposed. No significant reduction in mRNA or immunoreactive CYP1A protein levels were detected, suggesting the inhibition to occur at the level of enzyme activity (Willett et al. 1998).

High content of CYP1A inducers have been indicated to reduce total protein synthesis and result in negative concentration related responses (Østby et al. 2006), or induce CYP1A in an increasing manner up to an certain exposure concentration, before levelling of effect (Østby & Krøkje 2002; Jensen 2005; Søfteland 2005). To check if the change in CYP1A levels in this project could be linked to changes in the total protein synthesis, the total protein levels for the extract exposed cells was calculated as percent of the corresponding DMSO control. These results did not detect clearly different trends in protein levels and thereby rejected this theory (appendix C-3). Neither was the negative concentration- effect relationship for CYP1A (i.e. Soil 3 and Soil 4) regarded to be caused by cytotoxicity (part 4.4), also partly confirmed by the relative steady measures of total protein quantities. It was therefore suggested that the reduction in CYP1A levels could be caused by inhibitory interactions, possible linked to antagonistic interference at the Ah- receptor (part 1.4.1), caused by constituents in the mixtures (part 1.6).

Both deviation (Hahn et al. 1996; Lorenzen et al. 1997; Willett et al. 1998; Fent & Batscher 2000) and covariance (Wilson et al. 2000) between EROD and CYP1A protein levels have

been registered. The CYP1A inductions in the current study do not necessarily correlate with the activity of the enzymes, confining results to indicate presence of CYP1A inducing compounds. In relation to the highly cytotoxic effect detected in the MTT assay (i.e. Soil 3 and Soil 4) (part 3.2.2) and the CYP1A inducing potential, is it clear that the extracts did have a toxic potential upon the hepatoma cells. A simultaneously measure of both EROD activity and CYP1A induction could have been interesting, in order to get a better picture of the potency of the extracts (Hahn et al. 1996).

4.5.1 Factors potentially contributing to variability

The CYP1A induction showed some varying results and was only considered to show good reproducibility for H4IIE exposed to extracts of Soil 5. The variation within and between Western blots was low for this soil sample, while it was considerably higher for Soil 3 and Soil 4. Some minor variation was considered non- problematic in relation to this being a biological assay and was potentially caused by factors as yield of extracts or physiological state of cells. Clearly deviation between parallel samples and variations obtained from the same samples at two different blots, were considered to question the reliability of results (i.e exposure to 10 mg of Soil 5 from extract number two- serie one, 1 mg of Soil 3 from extract one- serie one and 0.5 mg of Soil 4 of extract two- serie one, given in appendix C-3).

The above mentioned exposures, 1 mg of Soil 3 and 0.5 mg of Soil 4, seemed to induce CYP1A in one of the parallel samples to levels so high that the net intensities detected by ROI could not be transformed into pmol CYP1A, as it deviated from the range of the standard curve. The current sample exposed to extract of Soil 4 was included a second time in a new blot, giving more consistent results. The reason for these variations was suggested to be a reflection of the many steps in the procedure, increasing the likelihood of errors. Crucial steps seemed to be preparation of samples before loading on the gel and the loading itself, as it included working with small volumes that increase the importance of accuracy. Generally, the variation was higher between assays than between parallels on the same blot. This could be suggested to potentially reflect different separation of proteins on the SDS gel and transfer to the nitrocellulose membrane, connected to variations in the electrophoresis conditions and gels. Variation between parallels could also be due to the lysing method, resulting in different yield of proteins and contamination of samples by cell debris. A further optimisation by including several freeze/ thaw cycles and a softer centrifugation is considered to reduce this variation.

4.5.2 Validity of results

The quantification of CYP1A induced from exposure to B[a]P was included as a positive control of the method (fig. 3.3.5). A positive concentration- effect relationship with a positive sigmodial logistic curve of the average illustrated the response. Earlier has B[a]P shown similar inductive patterns in H4IIE (Fugleneb 2007; Jensen & Krøkje 2008) and was subsequently interpreted as a good indication on a working induction and detection of CYP1A. Some variability was also seen for the B[a]P induced quantities of CYP1A,

dismissing a suspicion of a very sensitive and varying induction caused only by the complexity of the extracts.

The reliability of the quantified CYP1A levels might be questioned, due to the use of a standard curve made with another primary antibody, together with the net intensities being relatively low for both samples and the CYP1A standard (Søfteland 2005; Fugleneb 2007). The low intensities of luminescence were considered to be caused by non- optimal antibody concentrations and a lower sensitivity. The low intensities of the standard might be caused by low sensitivity of the anti- rat raised CYP1A antibody against the human CYP1A1 standard. These differences were causing a higher correction factor for the standard curve adjustment, increasing uncertainties and diminishing the reliability of the absolute measures of pmol CYP1A. The relative values were considered more reliable as the net intensities, beside for the above mentioned exceptions, showed quite good consistency and the average intensity of the standard was in the same range at the different blots.

Because of uncertainties, the method was considered not to be reliable enough for scientific research at the current state. It did however give a good indication on the relative potency of the soil extracts. By using more optimal antibody concentrations and a standard curve made with the current antibodies, the intensities and the accuracy of quantified pmol CYP1A are expected to increase. With these corrections the immunoquantitative detection by Western blotting is considered to be a suitable method for detecting induction of CYP1A also for the absolute measures.

4.6 Utility value of *in vitro* test systems

The results obtained in the current project gave good indications on the mutagenic potential of organic soil extracts and the potential of the extracts to induce CYP1A biotransformation enzymes, by utilising *in vitro* bioassays. But the relevance of such *in vitro* assays has been questioned, and a following discussion will pinpoint the utility and challenges of such assays.

4.6.1 *In vitro* vs. *in vivo* test systems

Differences between *in vitro* and *in vivo* context do exist, and can complicate the extrapolation of results as parameters found *in vitro* not necessarily reflect the process and effect of the intact animal (Nebert et al. 2004; Nebert & Dalton 2006; Uno et al. 2009).

4.6.1.1 Mutagenic potential studied *in vitro* in *Salmonella* bacteria

The mutant prokaryotic *S. typhimurium* bacteria utilised in the Ames assay (Maron & Ames 1983) has several practical and theoretical advantages, such as a small genome (4×10^4 base pairs), a simultaneous exposure of many bacteria and positive selection for mutants (Ames 1974). The additional mutations that the bacterial strains inhabit (Ames et al. 1975; McCann et al. 1975; Maron & Ames 1983) increase the likelihood of detecting *in vitro* mutagens. Mutagenicity is dependent on the DNA repair system (Lagerqvist et al. 2008) and as

considerable variability exist between prokaryote and eukaryote repair systems (Aravind et al. 1999) in addition to the induced mutations, is this considered to raise differences in mutagenic susceptibility.

The *Salmonella* bacteria do not inhabit the metabolic capability (Maron & Ames 1983; Mortensen et al. 2006) of activating compounds into mutagens or carcinogens, such as eukaryotes do (Parkinson & Ogilvie 2008). The metabolic S9- mix derived from arochlor 1254 induced rat liver were consequently included, taking secondary mutagens into account. Arochlor 1254 contain multiple PCB congeners and polychlorinated dibenzofurans, inducing CYP1A activity both *in vitro* and *in vivo* (Schmitz et al. 1996). These enzymes are likely to be among the main contributors of the S9 metabolic activity. The S9- metabolism is considered to be aberrant from *in vivo* situations, caused by a difference in the amount of phase II-enzymes (part 1.4) and/or a loose coupling between biotransformation enzymes. The resultant enhanced toxicity by adduct formation, oxidative stress and cellular damage (Nebert et al. 2004; Nebert & Dalton 2006) is likely to overestimate the *in vivo* mutagenic potential. The role of CYP1A in detoxification *in vivo* is furthermore suggested to be dependent on sub cellular content and location of CYP1A, along with kinetic and dynamic factors (Nebert & Dalton 2006) of toxicity. Differences among prokaryotes vs. eukaryotes and *in vitro* vs. *in vivo* are likely to interfere with the mutagenic capacity of compounds. Positive results of the Ames assay are considered to detect presence of compounds that in the current assay are mutagenic, but the mutagenic potential of *in vivo* exposure in other organisms cannot be suggested without further studies.

4.6.1.2 CYP1A induction studied *in vitro* in H4IIE rat hepatoma cells

The cell line H4IIE was considered suitable for CYP1A induction assays due to a low basal CYP1A activity and a high responsiveness toward AhR activating compounds (Tillitt et al. 1991; Whyte et al. 2004). This was reflected in the current study by low detection of CYP1A from the DMSO controls and inducing potential of soil extracts.

The CYP1A enzymes have proven to be a sensitive biomarker of exposure for *in vitro* screening environmental samples for presence of AhR ligands and inducing potentials (Whyte et al. 2004; Fugleneb 2007), and for assessing the potential of known mixtures and chemicals (Willett et al. 1998; Gale et al. 2000). Good agreement between *in vitro* and *in vivo* findings has earlier regarded value of such assays (Safe 1989), but as pointed out in part 4.6.1.1 have the role of CYP1A between *in vitro* and *in vivo* conditions been questioned by studies showing contradictory results (Nebert et al. 2004; Nebert 2006; Nebert & Dalton 2006; Uno et al. 2006).

Inconsistency of findings can question the relevance of *in vitro* conducted hazard assessment in relation to organism's actual hazard upon exposure. Differences in sensitivity of rodent and human derived cell lines (Vamvakas et al. 1996; Baird et al. 2005) and deviations in CYP1A substrate affinity and metabolic rate (Uno et al. 2009), illustrate among other factors the importance of careful extrapolation when evaluating human risk of exposure.

4.6.2 Bioavailability and toxicity of pollutants in soil

For a toxic effect to occur, it has to be established contact between the toxic compound and target at the toxic site of action. Toxicity in organisms is therefore obviously connected to exposure, bioavailability, absorption and distribution of chemical compounds to the site of action (Lidman 2005).

The bioavailability and toxicity of pollutants in soil seems to be organism and species specific (Reid et al. 2000) and can be highly affected by chemical properties (Ellickson et al. 2001; Cave et al. 2010), soil type (Ruby et al. 1999), soil organic carbon content (Cave et al. 2010) and processes in the soil such as physical and chemical weathering, biological processes, water infiltration, anthropogenic activity (Ruby et al. 1999) and soil- pollution contact time (aging) (Kelsey et al. 1997; White et al. 1997; Reid et al. 2000) (part 1.2 and 1.3). When assessing toxicity of organic pollutants in soil, it can be problematic that organic extraction is needed for solubilising compounds, not taking the above mentioned factors into consideration.

Solvents can affect assays (Reid et al. 2000) and the obtained extracts do not always reflect the bioaccessible and bioavaliable fractions (part 1.3), which especially question the relevance of harsh extraction techniques. Mild extraction procedures that better reflect bioavailability can therefore be preferable when assessing organism's actual exposure and risk. On the other side the appropriate solvent for predictive purposes can vary with compounds and species (Kelsey et al. 1997), complicating the use of a standard procedure. The extracts made in the current project can be assumed to not necessarily give a good reflection of the real bioavaliable fraction. A milder extraction agent would therefore be advisable if aiming on taking bioavailability into account.

4.6.3 Biological effects caused by exposure to soil extracts

The *in vitro* biological responses obtained in the current assays were a result of the total quantitative and qualitative composition of the soil extracts. The measured endpoints reflected the integrated effect of the mixture, potentially mediated by agonist and antagonists. It was not possible to detect the type of interactions by the utilised procedure, but indications on interactions could be seen. For example the reduction in CYP1A induction for extracts of Soil 3 and Soil 4 at the higher concentrations, considered to potentially reflect an inhibition of CYP1A induction by antagonistic interactions at the Ah- receptor (part 4.5). Interactions are known to sometimes affect toxicity of mixtures, and combinations of non- carcinogenic and weak carcinogenic PAHs has shown to induce CYP1A and reduce the genotoxic potential of B[a]P both *in vitro* (Mahadevan et al. 2004) and *in vivo* (Marston et al. 2001).

It was not possible to verify the inducers by the methods used in the current project. The detected responses were expected to be caused by several compounds present in the extracts. Based on theory and chemical analysis, assumptions about importance of PCBs in extract of Soil 5 and PAHs in extract of Soil 3 and Soil 4 could only be suggested. The importance of constituents could have been assessed by including chemical analyses of the extracts, along with correlation tests between individual compounds and biological responses. Østby and co-

workers (2006) detected a positive correlation between B[a]P and CYP1A induction, but no correlation to $\sum PAH_{16}$, in Fao cells exposed to extracts of soil. These results indicated B[a]P to be an important contributor for CYP1A induction. It is likely that similar correlations could have been seen in the present study, due to presence of B[a]P and knowledge about the inducing potential of this compound, but this could only be speculated. In this study the extract of Soil 4 was identified as most potent in regard to mutagenicity and CYP1A induction at the lower concentration, probably reflecting the chemical detection of high levels of B[a]P and $\sum PAH_{16}$ in the soil.

Assessing toxicity of mixtures is valuable since a complex composition of chemicals represent the real exposure situation (Cassee et al. 1998). In the current thesis, a so called top-down approach was applied, studying the effect from a whole mixture (Feron & Groten 2002). Simple mixtures at known concentrations can give information about interactions between chemicals (Cassee et al. 1998). Inclusion of such a mixture design with compounds known to be present in the soil could have been interesting, in order to get a better understanding of potential interactions in the extracts.

4.6.4 Utility of current bioassays in assessing for environmental hazards

Screening environmental samples in relation to a biomarker can be valued as a first and cost-effective measure, intending to detect presence or effect from pollutants (den Besten 1998). Bioassays are considered to have value in regard to chemical analysis of soil, as the chemical interactions are incorporated in the biological endpoints (Ragnvaldsson et al. 2007). The Ames assay and the CYP1A induction assay showed to be useful in the current project for screening soil extracts for presence of mutagens and CYP1A inducers. Genotoxicity assays might fail to detect potentially harmful compounds or mixtures (Kopponen et al. 1994), and this cannot be verified using only one assay. Addition of CYP1A assay in H4IIE can therefore be considered as valuable in relation to biotransformation capability (Kopponen et al. 1994).

It was useful to assess both mutagenicity and CYP1A induction, due to potential genotoxic increase caused by CYP1A catalyzed biotransformation. The high mutagenicity detected only in presence of the S9 metabolic system, indicated presence of secondary mutagens and an importance of biotransformation enzymes for increasing *in vitro* genotoxicity of mixtures. Strong correlation between EROD activity, CYP1A1 levels and DNA adduct formation have been detected in a study by Wilson and co- workers (2000). Relations between CYP1A induction and mutagenicity could be speculated in the present study, and PAHs were considered as likely contributors for the inducing potential of the extracts in both assays. Since the metabolic and toxicodynamic behaviour of PAHs is only partly accounted for *in vitro* (Fent & Batscher 2000) and differences in responses between species can occur (Vamvakas et al. 1996; Fent & Batscher 2000), single *in vitro* assays can be limited for predicting toxicity in other species (Fent & Batscher 2000). The measured endpoints should thus preferably been conducted in the same biological system if aiming on revealing the *in vitro* connection between CYP1A and genotoxicity. Environmental samples can also contain genotoxic compounds that work by other mechanism than CYP1A induction (Stegeman &

Lech 1991), and negative results in the applied tests would not necessarily mean absence of toxic compounds.

The analysis in this project clearly detected presence of secondary mutagens and CYP1A inducers in the soil, and demonstrate the *in vitro* potential the soil might have upon exposure. The present compounds might be tightly bound in the soil and the fractions that are extractable do not necessarily represent the bioavaliable compounds. It is difficult to forecast bioavailability and *in vivo* effects. Further tests are needed if aiming on assessing the actual hazard for children upon exposure to polluted soil.

5 Conclusion

The main purpose of this project was to utilise *in vitro* methods for measuring the mutagenic and CYP1A inducing potential of organic extracts of soil, sampled in nursery schools in Oslo.

- The mutagenic potential of soil extracts, measured by the Ames assay, showed a secondary toxic nature. This was detected by frameshift mutations in TA98 and base-pair substitutions in TA100, only in presence of the S9 metabolic system. The high mutation frequency in presence of S9 was suggested to mainly reflect the content of PAHs in the soil, based on metabolic activation and increasing content of PAHs in the soil (e.g. Soil 2, Soil 3 and Soil 4).

- The CYP1A inducing potential of extracts in the rat H4IIE hepatoma cells suggested presence of CYP1A inducing compounds in Soil 3, Soil 4 and Soil 5. Negative concentration- effect relationship detected at the higher concentrations of extracts derived from Soil 3 and Soil 4 indicated an inhibitory effect. The inhibition of CYP1A may be linked to antagonistic interactions at the Ah- receptor.

- A positive relation between organic pollutant levels in soil and the inducing potential of extracts was indicated by a general increase in the mutagenic potential from Soil 1 to Soil 4, ranked according to increasing content of $\sum PAH_{16}$.

- Similar responses were detected by extract of Soil 3 and Soil 4, inducing mutants in the Ames assay in presence of S9 and CYP1A enzymes in H4IIE. Soil 5 did only show a clear potential upon CYP1A induction. These differences are suggested caused by varying constituents in the extracts. PAHs are suggested to be important in extract of Soil 3 and Soil 4. The high induction of CYP1A enzymes suggests a content of AhR activating compounds. These enzymes are possible important in the bioactivation of constituents in the extracts, causing a mutagenic potential. This relation could only be speculated due to the utilisation of two different biological systems and the complexity of enzymes present in the S9 mix. The low induction of mutants from extracts of Soil 5 was suggested linked to a low content of PAHs. The PCBs which were considered as the main constituents in Soil 5 have been reported not to give effect in Ames test.

Both methods seemed valuable as first screening assays for assessing the potential of environmental samples to induce biological effects *in vitro*. The responses reflected the integrated effect from exposure, potentially mediated by interactions. Differences between *in vitro* and *in vivo* conditions can affect the toxicity of chemicals and mixtures, along with several biological and environmental parameters that are important for availability and toxicity. The current study indicate presence of potential hazards, and further assessment is needed for assessing if the pollutants in the soil represent an actual risk for human upon exposure.

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Appendix

Appendix A-1: Chemical data of soil

Table A1. Chemical data of organic and inorganic analysis of soil samples asses in this master project.

Sample ID	**1**	**2**	**3**	**4**	**5**
Naphthalene	<0.01	0.013	0.17	0.19	<0.01
Acenaphthylene	<0.01	0.1	0.074	1.5	0.01
Acenaphthene	<0.01	<0,01	0.14	0.27	<0.01
Fluorene	<0.01	0.019	0.13	1.2	0.01
Phenanthrene	<0.01	0.44	1.9	12	0.15
Anthracene	<0.01	0.099	0.46	1.8	0.03
Fluoranthene	<0.01	1.4	2.8	18	0.33
Pyrene	<0.01	1.3	2.1	15	0.25
Benzo[a]anthracene	<0.01	0.69	2	5.9	0.13
Chrysene	<0.01	0.73	2	7	0.11
Benzo[b]fluoranthene	<0.01	1	2.8	8.4	0.1
Benzo[k]fluoranthene	<0.01	0.32	1.1	3.1	0.09
Benzo[a]pyrene	<0.01	0.8	3.1	6.4	0.11
Dibenzo[a,h]anthracene	<0.01	0.11	0.24	0.55	0.03
Benzo[g,h,i]perylene	<0.01	0.49	0.48	2.6	0.08
Indeno[1,2,3,cd]pyrene	<0.01	0.57	0.77	3.3	0.08
Sum PAH_{16}	<0.20	8.1	20	87	1.5
PCB 28	<0.001	<0,001	<0,001	<0,001	<0.001
PCB 52	<0.001	<0,001	<0,001	<0,001	0.013
PCB 101	<0.001	<0,001	<0,001	<0,001	0.15
PCB 118	<0.001	<0,001	<0,001	0.0012	0.079
PCB 138	<0.001	0.0013	<0,001	0.0024	0.591
PCB 153	<0.001	0.0016	<0,001	0.0016	0.581
PCB 180	<0.001	<0,001	<0,001	0.0017	0.565
Sum PCB_7	<0.004	0.0054	<0,004	0.0084	1.97
Pb	2.5	90.7	40.7	80.8	42.3
Ni	7.2	30.1	29.1	21.2	32.7
Cd	<0.1	0.36	0.29	0.45	0.14
Cr	7.40	32.4	28.9	19.3	27.6
As	2.2	4.9	4.5	6.2	6.1
Hg	< 0.01	0.234	0.079	0.283	0.082

Appendix B-1: Solutions for the Ames assay

Histidin-Biotin
0.0309 g D-Biotin
0.0240 g L-Histidin
250 mL distilled water

Transferred D- Biotin to volumetric flask (250mL), added some of the water. Heated solution to boiling point, continuing to all the biotin was dissolved. Cooled down to room temperature. Added L-Histidine, the rest of the water and mixed with a magnet stirrer. Filtrated solution to sterilised flask by using a 50 mL syringe and a 0.22 µm filter.

Nutrient agar plates
8g Difco Bacto Nutrienth Broth*
5g NaCl
15g Agar
1000mL distilled water

Autoclaved at 121˚C for 30 min. After cooling to approx. 50˚C, transferred the agar to petri dishes (9cm) ~20 mL in each. Made by Grethe Stavik Eggen.

Nutrient medium
4 g Nutrient broth
160 mL distilled water

The solution was transferred to 25 mL conical flasks, 5 mL in each. Cotton tops with alu- foil were used to seal the flask. Autoclaved at 120 °C for 20 min.

Top agar
6. 6 g Difco-Bacto-Agar
5. 5 g NaCl
1100 mL distilled water

Agar, NaCl and water were mixed in a 3 L conical flask. The flask was closed with alu- foil and mixture boiled in water bath for 1 ½ hour. Transferred to glass flasks (200 mL), and autoclaved at 120 °C for 20 min.

Co- factors for S9- mix
1. 0.4 M Magnesium chlorid (MgCl)-solution
   20.3505 g $MgCl_2x6H_20$
   250 mL distilled water
2. 1.65 M Potassium chloride (KCl)-solution
   30.75 g KCl
   250 mL distilled water
3. 0.2 M Sodium di-hydrogen phosphate ($NaH_2PO_4xH_2O$)
   5.52 g $NaH_2PO_4xH_2O$
   200 mL distilled water
4. 0.2 M Di-sodium hydrogenphosphate ($Na_2HPO_4x2H_2O$)
   35.598 g $Na_2HPO_4x2H_2O$
   1000 mL distilled water

5. 0.2 M Sodium phosphate buffer, pH= 7.4
   880 mL of solution 4 added 180 mL of solution 3. Then added solution 3 until pH = 7.4.

All solutions autoclaved at 120 °C for 40-45 min.

S9-mix (50 µL S9/0.5 mL S9- mix) Pr. 20 mL:
2 mL $MgCl_2$- dilution (1 mL 0.4 M $MgCl_2$-solution diluted to 5 mL with autoclaved water) .
4 mL KCl-dilution (0.5 mL 1.65 M KCl-solution diluted to 5 mL with autoclaved water).
2 mL autoclaved distilled water
10 mL Sodium phosphate buffer 0.2 M (pH=7.4)
28.21 mg Glucose-6-phosphate
69.0 mg NADP
2 mL S9-homogenat

Mixed solution in sterile conical flask (100 mL). S9 homogenat was thawed a bit, added 2.1 mL autoclaved distilled water. Mixed gently, and then transferred to the rest of the solution. The S9- mix was (0.45 µm) filtered using a syringe (50 mL). Placed on ice.

Appendix B-2: Solutions for cell culturing, MTT- assay and CYP1A induction assay measured by Western blotting

Cell culturing:

Ethylenediaminetetraacetic acid (EDTA), 0.5M, pH 8.0
186.1 g Na_2ethylen diamine tetraacetate * $2H_2O$
1000 mL distilled H_2O

Growth medium
500 mL RPMI- 1640
5% FBS
1% Pen Strep
1% L- Glutamine 200mM
Stored at 4 °C

Phosphate buffered saline (PBS), pH 7.4
8 g NaCl
0.2 g KCl
1.80 g Na_2HPO_4 * $2H_2O$
0.24 g KH_2PO_4
in 800 mL distilled H_2O
Adjusted to pH 7.4 with HCl, added H_2O to 1 liter. Autoclaved for 20 minutes at 120 °C. Stored at 4 °C

Trypsin 0.25%, 10mL
1 mL 2.5% Trypsin
0.537 mL EDTA
8.462 mL PBS, pH 7.4
The solution were distributed in 1.5 mL eppendorf tubes and stored at -20°C.

MTT- assay:

MTT (5 mg/ mL)
0.1 mg MTT (Thiazolyl Blue Tetrazolium Bromide)
20 mL PBS, pH 7.4
MTT solution was filtered and kept in dark at 4 °C for one month

CYP1A induction assay:

Ammonium persulphate 10% (0.5 mL)
0.05 g Ammonium persulphate (APS)
0.5 mL distilled water

5% Blocking solution (50 mL)
2.5 g Skim milk powder
50 mL PBS- Tween (0.1%)

2 M Dithiothreitol (DTT)
1.543 g DTT
5 mL sodium acetate 0.01 M, pH 5.2
Filtrated and aliquoted in 500 µL eppendorf tubes, stored at - 20 °C.

Gel- loading buffer
0.5% bromphenol blue
500 mM DTT
10% glycerol
10% SDS
250 mM Tris- HCl, pH 6.8

PBS-Tween 20 (0.1 %) (1L)
1 mL Tween 20
1 L PBS

Primary antibody solution (1:5000) (50 mL)
10 µL Primary goat anti- rat antibody
50 mL 0.5% skim milk

Secondary antibody solution (1:5000) (50 mL)
10 µL Secondary rabbit anti- goat antibody
50 mL 0.5% skim milk

Separation gel (12%)
5 mL 1.5 M Tris- HCl pH 8.8
200 µL SDS (10 %)
6.7 mL distilled water
8 mL Acrylamide (30%)
150 µL APS (10%)
10 µL TEMED
Solution were degassed for 15 min. before APS and TEMED were added.

Skim milk 0.5% and 3% (50 mL)
0.25 g/ 1.5 g skim milk powder
50 mL PBS- Tween (0.1%)

0.01 M Sodium acetate, pH 5.2
0.082 g sodium acetate
100 mL distilled water
Autoclaved 120 °C, 20 min.

Stacking gel (4%)
2.5 mL 0.5 M Tris- HCl pH 6.8
100 µL SDS (10%)
6 mL distilled water
1.33 mL Acrylamide (30%)
100 µL APS (10%)
10 µL TEMED
Solution was degassed for 15 min before APS and TEMED were added.

Sodium dodecyl sulphate 10% (SDS) (100 mL)
10 g SDS
100 mL distilled H_2O
Heated to 68 °C to dissolve and adjust pH to 7.2.

Tris-Glycinen electrophoresisbuffer, 5x (1L)
15.1 g Tris base
94 g Glycine
in 900 mL distilled water
Added then 50 mL of 10% SDS, and adjusted volume to 1000 mL with distilled water. Stored at 4°C, 5x stock solution had to be diluted to 1x solution before use

1.5 M Tris- HCl pH 8.8 (100 mL)
18.1714 g Tris
100 mL distilled water
Adjusted to pH 8.8 with HCl

0.05 M Tris- 0.2 M Sucrose pH 7.4 (200 mL)
1.2114 g Tris
13.692 g Sucrose
200 mL distilled water
Adjusted pH to 7.4 with HCl

Western transfer buffer (1L)
2.9 g Glycine
5.8 g Tris
0.37 g SDS
200 mL methanol
700 mL distilled water
After adding everything was the volume adjusted to 1000 mL with distilled water. Stored at 4 °C until use.

Appendix C-1: Raw data and statistical analysis of Ames results

Table C 1.1. Results of Ames assay on TA98 exposed to extracts of Blank, soil and controls; spontaneous, DMSO, DMSO + S9[3], 1 µg B[a]P+ S9. Presented as individual number of reverted colonies per plate, along with mean ± S.D. (n = 3-5), after 48 hours incubation (grey field) and 72 hours (white field). Statistical analysis performed by Mann Whitney ($p \leq 0.05$) on results from 48 hours incubation.

TA 98																					
			Experiment 1									Experiment 2									
Exposure	Dose	Hours	Individual values					Mean	S.D.	2x Revert. p^1	Ho:$R^T = R^K$ p^2	Individual values					Mean	S.D.	2x Revert. p^1	Ho:$R^T = R^K$ p^2
Spontanous		48	18	13	16	22	13	**16**	**3.8**			16	17	18	19	16	**17**	**1.3**		
		72	25	20	24	25	22	23	2.2			28	23	22	27	21	24	3.1		
DMSO		48	9	16	19			**15**	**5.1**	0.036		20	17	12			**16**	**4.0**	0.036	
		72	18	16	24			19	4.2			23	26	15			21	5.7		
DMSO + S9 [3]		48	22	26	18	17	17	**20**	**3.9**	0.016		22	26	18	17	17	**20**	**3.9**	0.008	
		72	35	39	33	31	28	33	4.1			35	39	33	31	28	33	4.1		
1 µg B[a]P + S9		48	154	121	152			**142**	**18.5**	**0.036**	**0.036**	120	164	144	84		**128**	**34.4**	**0.016**	**0.016**
		72	138	126	166			143	20.5			124	170	159	88		135	37.1		
Blank	25	48	16	20	22			**19**	**3.1**	0.036	0.200									
		72	23	26	27			25	2.1											
	100	48	19	17	23			**20**	**3.1**	0.036	0.200									
		72	24	23	25			24	1.0											
Soil 1	25	48	19	19	23			**20**	**2.3**	0.036	0.200									
		72	25	26	32			28	3.8											
	100	48	27	19	26			**24**	**4.4**	0.250	0.100									
		72	29	23	33			28	5.0											
Soil 2	25	48	20	12	11			**14**	**4.9**	0.036	0.250									
		72	29	17	16			21	7.2											
	100	48	27	31	18			**25**	**6.7**	0.393	0.200									
		72	22	26	14			21	6.1											
Soil 3	25	48	18	24	27			**23**	**4.6**	0.143	0.200	10	20	18			**16**	**5.3**	0.036	1.000
		72	22	26	34			27	6.1			14	24	26			21	6.4		
	50	48	36	29	34			**33**	**3.6**	0.786	0.100	26	10	15			**17**	**8.2**	0.036	1.000
		72	37	38	38			38	0.6			29	15	17			20	7.6		
	100	48	47	39	45			**44**	**4.2**	0.071	0.100	26	32	38			**32**	**6.0**	0.571	0.100
		72	53	44	50			49	4.6			31	40	30			34	5.5		
	150	48										33	28	36			**32**	4.0	0.571	0.100
		72										38	33	41			37	4.0		
Soil 4	25	48	13	13	21			**16**	**4.6**	0.036	1.000									
		72	17	16	22			18	3.2											
	50	48	14	18	17			**16**	**2.1**	0.036	1.000									
		72	16	26	27			23	6.1											
	100	48	18	19	14			**17**	**2.6**	0.036	0.700									
		72	30	20	18			23	6.4											
Soil 5	25	48	13	14	18			**15**	**2.6**	0.036	1.000									
		72	20	22	25			22	2.5											
	50	48	20	20	25			**22**	**2.9**	0.036	0.100									
		72	21	26	32			26	5.5											
	100	48	20	27	28			**25**	**4.4**	0.939	0.100									
		72	24	33	36			31	6.2											

[1] Statistical comparison by Mann Whitney ($p \leq 0.05$) between values of the spontaneous reversion multiplied by two, and the other exposures. Bold values in dark grey field indicate significantly higher values of the exposed compared with the two times spontaneous reversion (doubling criteria).

[2] Statistical comparison by Mann Whitney ($p \leq 0.05$) between exposed bacteria and the corresponding DMSO control. Bold values in dark grey field indicate significantly higher values in the exposed compared with the DMSO control.

[3] Control from experiment number three.

Table C1.2. Results of Ames assay on TA100 exposed to extracts of Blank, soil and controls; spontaneous, DMSO, DMSO + S9[3], 1 µg B[a]P+ S9. Presented as individual number of reverted colonies per plate, along with mean ± S.D. (n= 3-5), after 48 hours incubation (grey field) and 72 hours (white field). Statistical analysis performed by Mann Whitney ($p \leq 0.05$) on results from 48 hours incubation.

TA 100													
			Experiment 1										
Exposure	Dose	Hours	Individual values					Mean	S.D.	2x Revert. p^1	$Ho:R^T = R^K$ p^2
Spontanous		48	123	78	87	81	110	**96**	**19.7**		
		72	126	83	91	86	112	100	18.6		
DMSO		48	73	83	107			**88**	**17.5**	0.036	
		72	79	88	114			94	18.2		
DMSO + S9 [3]		48	149	128	110	137	109	**127**	**17.3**	0.008	
		72	156	138	117	145	119	135	16.8		
1 µg B[a]P + S9		48	644	687	448			**593**	**127.4**	0.036	**0.036**
		72	646	687	449			594	127.2		
Blank	25	48	101	101	80			**94**	**12.1**	0.036	1.000
		72	109	103	82			98	14.2		
	100	48	83	110	98			**97**	**13.5**	0.036	0.400
		72	88	120	103			104	16.0		
Soil 1	25	48	88	93	80			**87**	**6.6**	0.036	1.000
		72	92	97	84			91	6.6		
	100	48	92	104	96			**97**	**6.1**	0.036	0.700
		72	96	109	97			101	7.2		
Soil 2	25	48	96	91	87			**91**	**4.5**	0.036	0.700
		72	99	92	90			94	4.7		
	100	48	110	101				**106**	**6.4**	0.095	0.400
		72	111	102				107	6.4		
Soil 3	25	48	115	118	113			**115**	**2.5**	0.036	0.100
		72	120	124	119			121	2.6		
	50	48	114	114	102			**110**	**6.9**	0.036	0.200
		72	117	115	112			115	2.5		
	100	48	104	107	106			**106**	**1.5**	0.036	0.400
		72	108	113	111			111	2.5		
Soil 4	25	48	96	95	93			**95**	**1.5**	0.036	0.700
		72	113	103	101			106	6.4		
	50	48	85	83	76			**81**	**4.7**	0.036	1.000
		72	90	86	82			86	4.0		
	100	48	88	95	86			**90**	**4.7**	0.036	0.700
		72	96	106	95			99	6.1		
Soil 5	25	48	117	103	100			**107**	**9.1**	0.036	0.400
		72	121	106	104			110	9.3		
	50	48	99	101	96			**99**	**2.5**	0.036	0.700
		72	105	107	101			104	3.1		
	100	48	94	105	115			**105**	**10.5**	0.036	0.400
		72	107	108	117			111	5.5		

[1] Statistical comparison by Mann Whitney ($p \leq 0.05$) between values of the spontaneous reversion multiplied by two, and the other exposures. Bold values in dark grey field indicate significantly higher values of the exposed compared with the two times spontaneous reversion (doubling criteria).

[2] Statistical comparison by Mann Whitney ($p \leq 0.05$) between exposed bacteria and the corresponding DMSO control. Bold values in dark grey field indicate significantly higher values in the exposed compared with the DMSO control.

[3] Control from experiment number three.

Table C1.3. Results of Ames assay on TA98 + S9 exposed to extracts of Blank, soil and controls; spontaneous, DMSO, DMSO + S9[3], 1 µg B[a]P+ S9. Presented as individual number of reverted colonies per plate, along with mean ± S.D. (n= 3- 5), after 48 hours incubation (grey field) and 72 hours (white field). Statistical analysis by Mann Whitney ($p \leq 0.05$) on results from 48 h. incubation.

TA 98 + S9																					
			Experiment 1									Experiment 2									
Exposure	Dose	Hours	Individual values					Mean	S.D.	2x Revert. p^1	Ho:$R^T = R^K$ p^2	Individual values					Mean	S.D.	2x Revert. p^1	Ho:$R^T = R^K$ p^2	
Spontanous		48	18	13	16	22	13	**16**	**3.8**			16	17	18	19	16	**17**	**1.3**			
		72	25	20	24	25	22	23	2.2			28	23	22	27	21	24	3.1			
DMSO		48	9	16	19			**15**	**5.1**			20	17	12			**16**	**4.0**			
		72	18	16	24			19	4.2			23	26	15			21	5.7			
DMSO + S9 [3]		48	22	26	18	17	17	**20**	**3.9**	0.016		22	26	18	17	17	**20**	**3.9**	0.008		
		72	35	39	33	31	28	33	4.1			35	39	33	31	28	33	4.1			
1 µg B[a]P + S9		48	154	121	152			**142**	**18.5**	**0.036**	**0.036**	120	164	144	84		**128**	**34.4**	**0.016**	**0.016**	
		72	138	126	166			143	20.5			124	170	159	88		135	37.1			
Blank	25	48	25	23	30			**26**	**3.6**	0.143	0.143	40	23	30			**31**	**8.5**	0.571	0.071	
		72	41	45	47			44	3.1			54	38	46			46	8.0			
	100	48	25	20	30			**25**	**5.0**	0.143	0.250	20	38	35			**31**	**9.6**	1.000	0.143	
		72	40	35	39			38	2.6			27	48	48			41	12.1			
Soil 1	25	48	42	31	31			**35**	**6.4**	1.000	**0.036**	37	44	36			**39**	**4.4**	0.143	**0.036**	
		72	53	44	38			45	7.5			55	55	49			53	3.5			
	50	48										47	57	45			**50**	**6.4**	**0.036**	**0.036**	
		72										57	68	51			59	8.6			
	100	48	62	78	71			**70**	**8.0**	**0.036**	**0.036**	83	60	63			**69**	**12.5**	**0.036**	**0.036**	
		72	80	94	78			84	8.7			94	72	70			79	13.3			
Soil 2	25	48	79	71	71			**74**	**4.6**	**0.036**	**0.036**	38	39	35			**37**	**2.1**	0.143	**0.036**	
		72	88	83	84			85	2.6			48	48	40			45	4.6			
	50	48										44	38	65			**49**	**14.2**	**0.036**	**0.036**	
		72										55	50	83			63	17.8			
	100	48	66	58	78			**67**	**10.1**	**0.036**	**0.036**	74	69	64			**69**	**5.0**	**0.036**	**0.036**	
		72	78	73	93			81	10.4			79	83	74			79	4.5			
Soil 3	25	48	131	116	143			**130**	**13.5**	**0.036**	**0.036**	110	124	132			**122**	**11.1**	**0.036**	**0.036**	
		72	139	126	151			139	12.5			115	133	144			131	14.6			
	50	48	202	195	192			**196**	**5.1**	**0.036**	**0.036**	168	173	162			**168**	**5.5**	**0.036**	**0.036**	
		72	212	201	198			204	7.4			177	181	170			176	5.6			
	75	48										171	141	162			**158**	**15.4**	**0.036**	**0.036**	
		72										187	144	167			166	21.5			
	100	48	137	117	112			**122**	**13.2**	**0.036**	**0.036**	198	186	196			**193**	**6.4**	**0.036**	**0.036**	
		72	143	128	117			129	13.1			204	194	200			199	5.0			
Soil 4	25	48	236	269	261			**255**	**17.2**	**0.036**	**0.036**	215	242	216			**224**	**15.3**	**0.036**	**0.036**	
		72	241	274	262			259	16.7			219	247	225			230	14.7			
	50	48	307	281	300			**296**	**13.5**	**0.036**	**0.036**	235	222	210			**222**	**12.5**	**0.036**	**0.036**	
		72	311	283	307			300	15.1			236	225	210			224	13.1			
	75	48										237	238	241			**239**	**2.1**	**0.036**	**0.036**	
		72										241	242	244			242	1.5			
	100	48	237	279	283			**266**	**25.5**	**0.036**	**0.036**	224	198	198			**207**	**15.0**	**0.036**	**0.036**	
		72	246	283	285			271	22.0			227	204	201			211	14.2			
Soil 5	25	48	25	32	29			**29**	**3.5**	0.393	0.071	24	35	38			**32**	**7.4**	1.000	0.071	
		72	39	45	45			43	3.5			34	50	52			45	9.9			
	50	48	42	41	37			**40**	**2.6**	0.250	**0.036**	28	29	35			**31**	**3.8**	0.250	**0.036**	
		72	51	57	56			55	3.2			35	33	45			38	6.4			
	100	48	44	65	77			**62**	**16.7**	**0.036**	**0.036**	50	58	62			**57**	**6.1**	**0.036**	**0.036**	
		72	54	72	89			72	17.5			63	73	73			70	5.8			

[1] Statistical comparison by Mann Whitney ($p \leq 0.05$) between values of the spontaneous reversion multiplied by two, and the other exposures. Bold values in dark grey field indicate significantly higher values of the exposed compared with the two times spontaneous reversion (doubling criteria).

[2] Statistical comparison by Mann Whitney ($p \leq 0.05$) between exposed bacteria and the corresponding DMSO control. Bold values in dark grey field indicate significantly higher values in the exposed compared with the DMSO control.

[3] Control from experiment number three.

Table C1.4. Results of Ames assay on TA100 + S9 exposed to extracts of Blank, soil and controls; spontaneous, DMSO, DMSO + S9[3], 1 µg B[a]P+ S9. Presented as individual number of reverted colonies per plate, along with mean ± S.D. (n = 3- 5), after 48 hours incubation (grey field) and 72 hours (white field). Statistical analysis by Mann Whitney ($p \leq 0.05$) on results from 48 h. incubation.

TA 100 + S9														
			Experiment 1					Experiment 2						
Exposure	Dose	Hours	Individual values	Mean	S.D.	2x Revert. p^1	Ho:$R^T = R^K$ p^2	Individual values	Mean	S.D.	2x Revert. p^1	Ho:$R^T = R^K$ p^2
Spontanous		48	123 78 87 81 110	**96**	**19.7**			83 94 93 88 84	**88**	**5.0**		
		72	126 83 91 86 112	100	18.6			88 97 100 95 84	93	6.6		
DMSO		48	73 83 107	**88**	**17.5**			70 80 97	**82**	**13.7**		
		72	79 88 114	94	18.2			72 84 99	85	13.5		
DMSO + S9 [3]		48	149 128 110 137 109	**127**	**17.3**	0.008		149 128 110 137 109	**127**	**17.3**	0.008	
		72	156 138 117 145 119	135	16.8			156 138 117 145 119	135	16.8		
1 µg B[a]P + S9		48	644 687 448	**593**	**127.4**	**0.036**	**0.036**	432 593 560 525	**528**	**69.5**	**0.016**	**0.016**
		72	646 687 449	594	127.2			434 593 560 525	528	68.5		
Blank	25	48	109 109 118	**112**	**5.2**	0.036	0.250	107 111 113	**110**	**3.1**	0.036	0.393
		72	115 115 127	119	6.9			109 114 122	115	6.6		
	100	48	122 105 128	**118**	**11.9**	0.036	0.393					
		72	124 107 131	121	12.3							
Soil 1	25	48	128 159 169	**152**	**21.4**	0.250	0.143	277 238 237	**251**	**22.8**	**0.036**	**0.036**
		72	132 159 174	155	21.3			282 240 237	253	25.2		
	50	48						164 152 136	**151**	**14.0**	0.036	0.143
		72						167 159 137	154	15.5		
	100	48	221 197 201	**206**	**12.9**	0.571	**0.036**	135 119 109	**121**	**13.1**	0.036	0.571
		72	221 199 202	207	11.9			143 122 113	126	15.4		
Soil 2	25	48	180 167 161	**169**	**9.7**	0.786	**0.036**	130 126 135	**130**	**4.5**	0.036	1.000
		72	187 174 161	174	13.0			132 129 140	134	5.7		
	50	48						159 149 158	**155**	**5.5**	0.036	**0.036**
		72						160 152 163	158	5.7		
	100	48	350 309 304	**321**	**25.2**	**0.036**	**0.036**	240 125 227	**197**	**63.0**	0.571	0.250
		72	352 311 305	323	25.6			240 215 228	228	12.5		
Soil 3	25	48	534 502 510	**515**	**16.7**	**0.036**	**0.036**	350 286 307	**314**	**32.6**	**0.036**	**0.036**
		72	534 505 510	516	15.5			350 286 309	315	32.4		
	50	48	525 454 620	**533**	**83.3**	**0.036**	**0.036**	394 374 390	**386**	**10.6**	**0.036**	**0.036**
		72	527 454 622	534	84.2			394 376 391	387	9.6		
	75	48						476 459 494	**476**	**17.5**	**0.036**	**0.036**
		72						476 459 496	477	18.5		
	100	48	600 560 586	**582**	**20.3**	**0.036**	**0.036**	403 414 416	**411**	**7.0**	**0.036**	**0.036**
		72	602 560 589	584	21.5			404 414 416	411	6.4		
Soil 4	25	48	786 843 801	**810**	**29.5**	**0.036**	**0.036**	702 797 655	**718**	**72.3**	**0.036**	**0.036**
		72	787 843 804	811	28.7			702 799 657	719	72.6		
	50	48	814 702 749	**755**	**56.2**	**0.036**	**0.036**	636 570 583	**596**	**35.0**	**0.036**	**0.036**
		72	814 702 752	756	56.1			639 570 583	597	36.7		
	75	48						484 533 553	**523**	**35.5**	**0.036**	**0.036**
		72						484 533 553	523	35.5		
	100	48	692 596 613	**634**	**51.2**	**0.036**	**0.036**	601 508 456	**522**	**73.5**	**0.036**	**0.036**
		72	692 596 613	634	51.2			601 508 456	522	73.5		
Soil 5	25	48	97 126 114	**112**	**14.6**	0.036	0.393	104 128 115	**116**	**12.0**	0.036	0.393
		72	99 129 115	114	15.0			107 128 117	117	10.5		
	50	48	104 112 114	**110**	**5.3**	0.036	0.393					
		72	114 118 117	116	2.1							
	100	48	150 175 169	**165**	**13.1**	0.571	**0.036**	146 145 127	**139**	**10.7**	0.036	0.571
		72	152 179 169	167	13.7			148 145 127	140	11.4		

[1] Statistical comparison by Mann Whitney ($p \leq 0.05$) between values of the spontaneous reversion multiplied by two, and the other exposures. Bold values in dark grey field indicate significantly higher values of the exposed compared with the two times spontaneous reversion (doubling criteria).

[2] Statistical comparison by Mann Whitney ($p \leq 0.05$) between exposed bacteria and the corresponding DMSO control. Bold values in dark grey field indicate significantly higher values in the exposed compared with the DMSO control.

[3] Control from experiment number three.

Table C1.5. Results from Ames assay on controls; spontaneous, DMSO, DMSO + S9, 1µg B[a]P, 1 µg B[a]P + S9. Average ± S.D. (n = 3- 5) after 48 hours incubation (grey field) and 72 hours (white field).

TA98										
Exposure	Spontanous		DMSO		DMSO + S9		1 µg B[a]P		1 µg B[a]P + S9	
Hours	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
Individual values	27	35	25	32	22	35	20	24	169	175
	31	36	19	27	26	39	22	29	126	152
	22	29	24	29	18	33	20	24	157	167
	19	25	15	24	17	31				
	15	23	15	25	17	28				
Average	**23**	30	**20**	27	**20**	33	**21**	26	**151**	165
± S.D.	**6.3**	5.8	**4.8**	3.2	**3.9**	4.1	**1.2**	2.9	**22.2**	11.7
TA100										
Exposure	Spontanous		DMSO		DMSO + S9		1 µg B[a]P		1 µg B[a]P + S9	
Hours	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
Individual values	122	133	90	94	149	156	100	108	551	556
	140	150	99	107	128	138	90	100	493	494
	100	106	132	139	110	117	102	107	560	562
	112	115	144	151	137	145				
	105	108	115	126	109	119				
Average	**116**	122	**116**	123	**127**	135	**97**	105	**535**	537
± S.D.	**15.8**	18.7	**22.4**	23.2	**17.3**	16.8	**6.4**	4.4	**36.4**	37.6

Appendix C-2: Raw data and statistical analysis of MTT results

Table C2.1.Absorbance of formazan (550 nm) in relation to H4IIE cell concentration measured in MTT assay. Cell counting from a 1:10 dilution transformed into cells/mL by multiplying average cells per A square with 10^4 (volume per A square is 0.1 µL). Seeded in concentrations per well. Absorbance values, average ± S.D (n = 6) are given.

Cell counting		Measured absorbance (550 nm) in relation to cell concentration									
Drop	**A square**	**Cells/mL**	**Cells/ well**	**Absorbance**						**Average**	**S.D.**
1	83	0	0	0.055	0.054	0.065	0.073	0.075	0.077	0.067	0.01
	76	3500	700	0.207	0.162	0.222	0.209	0.217	0.272	0.215	0.04
	70	5150	1030	0.279	0.279	0.252	0.264	0.308	0.306	0.281	0.02
2	73	6800	1360	0.324	0.255	0.367	0.314	0.401	0.290	0.325	0.05
	91	8450	1690	0.432	0.542	0.552	0.491	0.546	0.526	0.515	0.05
	81	10100	2020	0.519	0.574	0.542	0.682	0.605	0.597	0.587	0.06
Average	79	11750	2350	0.661	0.610	0.616	0.747	0.748	0.673	0.676	0.06
S.D.	7.0	13400	2680	0.787	0.801	0.975	0.956	1.109	0.883	0.919	0.12
Cells/ mL	790000	15050	3010	0.977	1.018	0.998	0.981	1.189	1.096	1.043	0.08
		16700	3340	1.012	1.126	1.148	1.154	1.161	1.038	1.107	0.06
		18350	3670	1.057	1.071	1.202	1.012	1.080	1.200	1.104	0.08
		20000	4000	1.163	1.186	1.255	1.255	1.121	1.142	1.187	0.06
Drop	**A square**	**Cells/mL**	**Cells/ well**	**Absorbance**						**Average**	**S.D.**
1	75	0	0	0.060	0.058	0.055	0.064	0.054	0.053	0.057	0.00
	63	6000	1200	0.302	0.260	0.395	0.295	0.254	0.379	0.314	0.06
	79	7000	1400	0.345	0.337	0.355	0.398	0.397	0.475	0.385	0.05
2	95	8000	1600	0.531	0.504	0.491	0.405	0.445	0.360	0.456	0.06
	76	9000	1800	0.531	0.540	0.461	0.536	0.559	0.612	0.540	0.05
	80	10000	2000	0.653	0.599	0.684	0.682	0.702	0.682	0.667	0.04
Average	78	10500	2100	0.570	0.598	0.636	0.620	0.605	0.599	0.605	0.02
S.D.	9.4	11000	2200	0.702	0.821	0.742	0.710	0.757	0.699	0.739	0.05
Cells/ mL	780000	11500	2300	0.735	0.712	0.708	0.728	0.768	0.677	0.721	0.03
		12000	2400	0.844	0.815	0.878	0.826	0.797	0.869	0.838	0.03
		13000	2600	0.816	0.941	0.915	0.628	0.742	0.884	0.821	0.12
		14000	2800	0.938	0.771	0.798	0.836	0.778	0.659	0.797	0.09
Drop	**A square**	**Cells/mL**	**Cells/ well**	**Absorbance**						**Average**	**S.D.**
1	58	0	0	0.054	0.055	0.055	0.058	0.063	0.054	0.057	0.00
	67	6000	1200	0.311	0.253	0.267	0.294	0.331	0.199	0.276	0.05
	64	7000	1400	0.357	0.314	0.310	0.219	0.222	0.230	0.275	0.06
2	79	8000	1600	0.487	0.450	0.359	0.355	0.464	0.472	0.431	0.06
	101	9000	1800	0.527	0.541	0.508	0.512	0.561	0.509	0.526	0.02
	79	10000	2000	0.649	0.541	0.632	0.542	0.594	0.338	0.549	0.11
3	83	10500	2100	0.559	0.563	0.584	0.636	0.640	0.587	0.595	0.04
	68	11000	2200	0.709	0.694	0.670	0.639	0.799	0.670	0.697	0.06
	94	11500	2300	0.790	0.710	0.896	0.758	0.746	0.754	0.776	0.06
Average	77	12000	2400	0.731	0.718	0.755	0.701	0.742	0.838	0.748	0.05
S.D.	13.4	13000	2600	0.852	0.834	0.820	0.842	0.894	0.867	0.852	0.03
Cells/ mL	770000	14000	2800	0.883	0.815	1.003	0.839	0.920	0.716	0.863	0.10
Drop	**A square**	**Cells/mL**	**Cells/ well**	**Absorbance**						**Average**	**S.D.**
1	71	0	0	0.049	0.055	0.068	0.061	0.082	0.073	0.065	0.01
	60	6000	1200	0.528	0.519	0.483	0.514	0.601	0.518	0.527	0.04
	60	7000	1400	0.604	0.560	0.626	0.527	0.502	0.645	0.577	0.06
2	98	8000	1600	0.364	0.313	0.345	0.300	0.442	0.424	0.365	0.06
	89	9000	1800	0.434	0.422	0.557	0.465	0.563	0.549	0.498	0.07
	84	10000	2000	0.503	0.482	0.502	0.489	0.528	0.495	0.500	0.02
3	98	10500	2100	0.597	0.473	0.530	0.547	0.558	0.502	0.535	0.04
	77	11000	2200	0.629	0.634	0.542	0.560	0.582	0.581	0.588	0.04
	85	11500	2300	0.550	0.719	0.662	0.751	0.668	0.658	0.668	0.07
Average	80	12000	2400	0.705	0.661	0.770	0.701	0.802	0.709	0.725	0.05
S.D.	13.6	13000	2600	0.728	0.699	0.763	0.769	0.707	0.747	0.736	0.03
Cells/ mL	802222	14000	2800	0.702	0.726	0.640	0.795	0.815	0.814	0.749	0.07

Table C2.2. Raw data, average ± S.D. (n= 6) from cell counting on Bürker counting chamber from a 1:10 diluted H4IIE cell suspension.

Cell counting from 1:10 dilution of cell suspension								
	Drop 1			Drop 2				
Assays	Square 1	Square 2	Square 3	Square 1	Square 2	Square 3	Average	± S.D.
Serie 1 for blank & Soil 5	69	80	78	76	54	79	**73**	**10.0**
Serie 1 for Soil 3 & Soil 4	66	50	55	82	83	57	**66**	**14.2**
Serie 2 for blank & Soil 5	61	61	82	98	95	84	**80**	**16.1**
Serie 2 for Soil 3 & Soil 4, Serie 3 for blank & Soil Soil 5	92	87	109	98	86	96	**95**	**8.5**
Serie 3 for Soil 3 & Soil 4, Serie 4 for Soil 5	84	92	91	81	90	91	**88**	**4.5**

Table C2.3. Cell viability of H4IIE after exposure to different concentrations of Blank extract and extract of Soil 3, Soil 4 and Soil 5, measured by MTT assay. Absorbance of formazan (550 nm) and calculated cell viability as percent of 0.1% DMSO control are given in addition to average ± S.D (n =6).

Blank																		
Serie	Cells/ mL	Exposure	Absorbance						Average	S.D.	Cell viability (percent of control)[1]						Average	S.D.
1	11500	Medium	0.591	0.507	0.682	0.579	0.622	0.673	0.609	0.07	109	93	126	107	114	124	**112**	**12.0**
		DMSO	0.557	0.453	0.436	0.511	0.667	0.636	0.543	0.09	103	83	80	94	123	117	**100**	**17.4**
		0.1	0.563	0.426	0.542	0.460	0.524	0.570	0.514	0.06	104	78	100	85	96	105	**95**	**10.8**
		1	0.472	0.370	0.367	0.468	0.525	0.571	0.462	0.08	87	68	68	86	97	105	**85**	**15.1**
		5	0.518	0.577	0.632	0.553	0.508	0.629	0.570	0.05	95	106	116	102	93	116	**105**	**9.8**
		7.5	0.574	0.524	0.512	0.552	0.565	0.649	0.563	0.05	106	96	94	102	104	119	**104**	**8.9**
		10	0.531	0.586	0.583	0.519	0.597	0.643	0.577	0.05	98	108	107	96	110	118	**106**	**8.4**
		15	0.515	0.533	0.577	0.598	0.561	0.587	0.562	0.03	95	98	106	110	103	108	**103**	**5.9**
		20	0.474	0.574	0.546	0.537	0.685	0.536	0.559	0.07	87	106	100	99	126	99	**103**	**12.9**
		25	0.500	0.492	0.486	0.492	0.496	0.614	0.513	0.05	92	91	89	91	91	113	**94**	**9.1**
2	11500	Medium	0.324	0.304	0.343	0.358	0.397	0.456	0.364	0.06	94	88	100	104	115	133	**106**	**16.1**
		DMSO	0.321	0.356	0.366	0.262	0.332	0.426	0.344	0.05	93	104	106	76	97	124	**100**	**15.8**
		0.1	0.373	0.348	0.412	0.374	0.363	0.389	0.377	0.02	108	101	120	109	106	113	**110**	**6.4**
		1	0.340	0.347	0.322	0.357	0.316	0.343	0.338	0.02	99	101	94	104	92	100	**98**	**4.5**
		5	0.255	0.285	0.272	0.266	0.285	0.382	0.291	0.05	74	83	79	77	83	111	**85**	**13.4**
		7.5	0.330	0.401	0.369	0.369	0.392	0.393	0.376	0.03	96	117	107	107	114	114	**109**	**7.6**
		10	0.375	0.330	0.331	0.349	0.405	0.353	0.357	0.03	109	96	96	102	118	103	**104**	**8.3**
		15	0.391	0.434	0.436	0.387	0.423	0.374	0.408	0.03	114	126	127	113	123	109	**119**	**7.8**
		20	0.436	0.510	0.437	0.476	0.483	0.527	0.478	0.04	127	148	127	138	140	153	**139**	**10.8**
		25	0.459	0.460	0.476	0.412	0.443	0.414	0.444	0.03	133	134	138	120	129	120	**129**	**7.6**
3	13000	Medium	0.613	0.583	0.628	0.610	0.590	0.661	0.614	0.03	111	106	114	110	107	120	**111**	**5.1**
		DMSO	0.545	0.494	0.600	0.590	0.495	0.591	0.553	0.05	99	89	109	107	90	107	**100**	**8.8**
		0.1	0.613	0.613	0.685	0.561	0.570	0.621	0.611	0.04	111	111	124	102	103	112	**110**	**8.0**
		1	0.532	0.644	0.633	0.646	0.541	0.612	0.601	0.05	96	117	115	117	98	111	**109**	**9.4**
		5	0.653	0.600	0.685	0.570	0.605	0.653	0.628	0.04	118	109	124	103	110	118	**114**	**7.8**
		7.5	0.541	0.548	0.702	0.624	0.546	0.628	0.598	0.06	98	99	127	113	99	114	**108**	**11.7**
		10	0.576	0.604	0.658	0.500	0.566	0.608	0.585	0.05	104	109	119	90	102	110	**106**	**9.5**
		15	0.638	0.599	0.605	0.571	0.522	0.555	0.582	0.04	115	108	110	103	94	100	**105**	**7.4**
		20	0.633	0.609	0.623	0.546	0.554	0.535	0.583	0.04	115	110	113	99	100	97	**106**	**7.8**
		25	0.582	0.540	0.674	0.576	0.612	0.566	0.592	0.05	105	98	122	104	111	102	**107**	**8.4**
Soil 3																		
Serie	Cells/ mL	Exposure	Absorbance						Average	S.D.	Cell viability (percent of control)[1]						Average	S.D.
1	2300	Medium	0.396	0.345	0.363	0.492	0.426	0.547	0.428	0.08	106	92	97	132	114	147	115	20.9
		DMSO	0.358	0.409	0.406	0.381	0.400	0.284	0.373	0.05	96	110	109	102	107	76	100	12.8
		0.1	0.451	0.422	0.401	0.420	0.484	0.473	0.442	0.03	121	113	108	113	130	127	118	8.8
		1	0.404	0.401	0.361	0.389	0.432	0.406	0.399	0.02	108	108	97	104	116	109	107	6.2
		5	0.228	0.227	0.236	0.253	0.270	0.384	0.266	0.06	61	61	63	68	72	103	71	16.1
		7.5	0.203	0.191	0.185	0.234	0.239	0.278	0.222	0.04	54	51	50	63	64	75	59	9.5
		10	0.176	0.159	0.179	0.186	0.211	0.210	0.187	0.02	47	43	48	50	57	56	50	5.5
		15	0.114	0.112	0.123	0.120	0.131	0.167	0.128	0.02	31	30	33	32	35	45	34	5.5
		20	0.064	0.064	0.067	0.071	0.075	0.093	0.072	0.01	17	17	18	19	20	25	19	2.9
		25	0.064	0.065	0.064	0.068	0.073	0.084	0.070	0.01	17	17	17	18	20	23	19	2.1

Continue table C2.3

2	2600	Medium	0.667	0.616	0.450	0.679	0.592	0.674	0.613	0.09	140	129	94	142	124	141	128	18.2
		DMSO	0.518	0.567	0.617	0.541	0.272	0.352	0.478	0.13	108	119	129	113	57	74	100	28.3
		0.1	0.458	0.253	0.231	0.235	0.330	0.242	0.292	0.09	96	53	48	49	69	51	61	18.7
		0.5	0.570	0.534	0.647	0.601	0.634	0.580	0.594	0.04	119	112	135	126	133	121	124	8.8
		1	0.565	0.564	0.609	0.626	0.527	0.559	0.575	0.04	118	118	127	131	110	117	120	7.6
		1.5	0.525	0.554	0.508	0.466	0.483	0.552	0.515	0.04	110	116	106	98	101	116	108	7.5
		3	0.515	0.494	0.601	0.498	0.508	0.546	0.527	0.04	108	103	126	104	106	114	110	8.5
		4	0.415	0.436	0.428	0.453	0.379	0.452	0.427	0.03	87	91	90	95	79	95	89	5.8
		5	0.335	0.308	0.310	0.289	0.279	0.326	0.308	0.02	70	64	65	60	58	68	64	4.4
		7.5	0.267	0.261	0.261	0.274	0.255	0.259	0.263	0.01	56	55	55	57	53	54	55	1.4
3	2600	Medium	0.596	0.538	0.561	0.580	0.536	0.598	0.568	0.03	120	108	113	117	108	120	114	5.5
		DMSO	0.473	0.485	0.485	0.499	0.543	0.502	0.498	0.02	95	97	97	100	109	101	100	4.9
		0.1	0.546	0.479	0.537	0.552	0.502	0.510	0.521	0.03	110	96	108	111	101	102	105	5.7
		0.5	0.474	0.514	0.564	0.506	0.505	0.502	0.511	0.03	95	103	113	102	101	101	103	5.9
		1	0.512	0.506	0.528	0.463	0.463	0.497	0.495	0.03	103	102	106	93	93	100	99	5.4
		1.5	0.446	0.719	0.559	0.473	0.505	0.574	0.546	0.10	90	144	112	95	101	115	110	19.7
		2	0.488	0.363	0.576	0.514	0.493	0.559	0.499	0.08	98	73	116	103	99	112	100	15.1
		2.5	0.458	0.462	0.480	0.507	0.567	0.598	0.512	0.06	92	93	96	102	114	120	103	11.7
		3	0.477	0.508	0.518	0.510	0.501	0.536	0.508	0.02	96	102	104	102	101	108	102	3.9
		4	0.406	0.413	0.464	0.423	0.468	0.490	0.444	0.03	82	83	93	85	94	98	89	6.9

Soil 4

Serie	Cells/ mL	Exposure	Absorbance						Average	S.D.	Cell viability (percent of control)[1]						Average	S.D.
1	2300	Medium	0.390	0.380	0.417	0.431	0.355	0.488	0.410	0.05	108	105	115	119	98	135	113	12.9
		DMSO	0.357	0.343	0.347	0.361	0.380	0.386	0.362	0.02	99	95	96	100	105	107	100	4.8
		0.1	0.335	0.315	0.322	0.294	0.281	0.400	0.325	0.04	92	87	89	81	78	110	90	11.5
		1	0.289	0.288	0.277	0.310	0.274	0.335	0.296	0.02	80	79	76	86	76	92	82	6.4
		5	0.133	0.150	0.166	0.167	0.125	0.141	0.147	0.02	37	41	46	46	34	39	41	4.8
		7.5	0.088	0.086	0.088	0.084	0.091	0.093	0.088	0.00	24	24	24	23	25	26	24	0.9
		10	0.064	0.062	0.064	0.066	0.075	0.086	0.070	0.01	18	17	18	18	21	24	19	2.6
		15	0.068	0.067	0.074	0.074	0.081	0.093	0.076	0.01	19	18	20	20	22	26	21	2.7
		20	0.079	0.073	0.069	0.074	0.084	0.100	0.080	0.01	22	20	19	20	23	28	22	3.1
		25	0.086	0.091	0.086	0.100	0.115	0.118	0.099	0.01	24	25	24	28	32	33	27	3.9
2	2600	Medium	0.552	0.549	0.471	0.540	0.453	0.581	0.524	0.05	112	111	95	109	92	117	106	10.2
		DMSO	0.566	0.401	0.486	0.529	0.476	0.509	0.495	0.06	114	81	98	107	96	103	100	11.3
		0.1	0.371	0.492	0.467	0.505	0.249	0.466	0.425	0.10	75	99	94	102	50	94	86	19.9
		0.5	0.473	0.505	0.523	0.509	0.508	0.530	0.508	0.02	96	102	106	103	103	107	103	4.0
		1	0.459	0.457	0.494	0.497	0.514	0.510	0.489	0.02	93	92	100	101	104	103	99	5.0
		1.5	0.429	0.418	0.412	0.435	0.442	0.433	0.428	0.01	87	85	83	88	89	88	87	2.3
		3	0.409	0.359	0.447	0.319	0.389	0.391	0.386	0.04	83	73	90	65	79	79	78	8.8
		4	0.335	0.243	0.344	0.300	0.285	0.352	0.310	0.04	68	49	70	61	58	71	63	8.5
		5	0.194	0.131	0.142	0.164	0.135	0.223	0.165	0.04	39	26	29	33	27	45	33	7.5
		7.5	0.145	0.169	0.129	0.110	0.133	0.180	0.144	0.03	29	34	26	22	27	36	29	5.3
3	2600	Medium	0.560	0.420	0.527	0.417	0.554	0.518	0.499	0.06	122	91	114	91	120	112	108	14.0
		DMSO	0.504	0.441	0.424	0.480	0.441	0.473	0.461	0.03	109	96	92	104	96	103	100	6.5
		0.1	0.409	0.461	0.467	0.459	0.444	0.427	0.445	0.02	89	100	101	100	96	93	97	4.9
		0.5	0.416	0.475	0.431	0.424	0.419	0.443	0.435	0.02	90	103	94	92	91	96	94	4.8
		1	0.408	0.412	0.476	0.381	0.403	0.460	0.423	0.04	89	89	103	83	88	100	92	7.9
		1.5	0.432	0.449	0.439	0.462	0.434	0.514	0.455	0.03	94	98	95	100	94	112	99	6.7
		2	0.418	0.476	0.435	0.434	0.445	0.436	0.441	0.02	91	103	94	94	97	95	96	4.2
		2.5	0.393	0.410	0.473	0.433	0.477	0.539	0.454	0.05	85	89	103	94	104	117	99	11.6
		3	0.427	0.409	0.418	0.474	0.401	0.435	0.427	0.03	93	89	91	103	87	94	93	5.6
		4	0.335	0.392	0.394	0.383	0.376	0.457	0.390	0.04	73	85	86	83	82	99	85	8.6

Continue table C2.3

Soil 5																			
Serie	**Cells/ mL**	**Exposure**	**Absorbance**						**Average**	**S.D.**	**Cell viability (percent of control)[1]**						**Average**	**S.D.**
1	2300	Medium	0.515	0.430	0.443	0.564	0.634	0.570	0.526	0.08	106	88	91	116	130	117	108	16.2
		DMSO	0.496	0.452	0.492	0.470	0.468	0.547	0.488	0.03	102	93	101	96	96	112	100	6.9
		0.1	0.463	0.440	0.439	0.400	0.368	0.477	0.431	0.04	95	90	90	82	75	98	88	8.3
		1	0.556	0.480	0.527	0.552	0.592	0.631	0.556	0.05	114	98	108	113	121	129	114	10.7
		5	0.472	0.467	0.455	0.449	0.508	0.389	0.457	0.04	97	96	93	92	104	80	94	8.0
		7.5	0.458	0.472	0.478	0.462	0.656	0.672	0.533	0.10	94	97	98	95	135	138	109	20.9
		10	0.386	0.355	0.394	0.384	0.337	0.541	0.400	0.07	79	73	81	79	69	111	82	14.9
		15	0.473	0.422	0.391	0.404	0.546	0.623	0.477	0.09	97	87	80	83	112	128	98	18.8
		20	0.366	0.390	0.265	0.338	0.449	0.520	0.388	0.09	75	80	54	69	92	107	80	18.2
		25	0.391	0.373	0.384	0.384	0.477	0.496	0.418	0.05	80	77	79	79	98	102	86	11.1
2	2300	Medium	0.390	0.395	0.392	0.507	0.436	0.430	0.425	0.04	100	101	100	129	111	110	108	11.5
		DMSO	0.367	0.391	0.378	0.416	0.365	0.434	0.392	0.03	94	100	96	106	93	111	100	7.1
		0.1	0.381	0.377	0.381	0.424	0.412	0.439	0.402	0.03	97	96	97	108	105	112	103	6.7
		1	0.355	0.370	0.371	0.420	0.368	0.384	0.378	0.02	91	94	95	107	94	98	96	5.8
		5	0.362	0.368	0.383	0.380	0.329	0.351	0.362	0.02	92	94	98	97	84	90	92	5.1
		7.5	0.297	0.342	0.319	0.366	0.424	0.402	0.358	0.05	76	87	81	93	108	103	91	12.4
		10	0.319	0.317	0.345	0.358	0.430	0.305	0.346	0.05	81	81	88	91	110	78	88	11.7
		15	0.323	0.369	0.405	0.379	0.347	0.271	0.349	0.05	82	94	103	97	89	69	89	12.1
		20	0.274	0.234	0.318	0.270	0.312	0.295	0.284	0.03	70	60	81	69	80	75	72	8.0
		25	0.289	0.275	0.231	0.227	0.286	0.320	0.271	0.04	74	70	59	58	73	82	69	9.2
3	2600	Medium	0.584	0.549	0.717	0.615	0.557	0.561	0.597	0.06	110	103	135	116	105	106	112	11.9
		DMSO	0.550	0.519	0.511	0.572	0.500	0.534	0.531	0.03	104	98	96	108	94	101	100	5.0
		0.1	0.570	0.510	0.621	0.564	0.547	0.537	0.558	0.04	107	96	117	106	103	101	105	7.1
		1	0.603	0.530	0.610	0.532	0.549	0.596	0.570	0.04	114	100	115	100	103	112	107	7.0
		5	0.525	0.557	0.595	0.515	0.469	0.581	0.540	0.05	99	105	112	97	88	109	102	8.8
		7.5	0.485	0.533	0.610	0.627	0.553	0.549	0.560	0.05	91	100	115	118	104	103	105	9.8
		10	0.466	0.523	0.514	0.532	0.520	0.503	0.510	0.02	88	98	97	100	98	95	96	4.4
		15	0.656	0.592	0.627	0.624	0.568	0.600	0.611	0.03	124	111	118	118	107	113	115	5.8
		20	0.482	0.565	0.610	0.550	0.594	0.620	0.570	0.05	91	106	115	104	112	117	107	9.5
		25	0.530	0.496	0.631	0.507	0.503	0.636	0.551	0.07	100	93	119	95	95	120	104	12.3
4	2600	Medium	0.532	0.527	0.521	0.504	0.473	0.471	0.505	0.03	113	112	111	107	100	100	107	5.7
		DMSO	0.421	0.449	0.509	0.487	0.480	0.481	0.471	0.03	89	95	108	103	102	102	100	6.6
		0.1	0.500	0.527	0.564	0.493	0.538	0.508	0.522	0.03	106	112	120	105	114	108	111	5.7
		1	0.456	0.455	0.461	0.475	0.489	0.536	0.479	0.03	97	97	98	101	104	114	102	6.6
		5	0.478	0.475	0.507	0.509	0.493	0.503	0.494	0.01	101	101	108	108	105	107	105	3.1
		7.5	0.484	0.470	0.471	0.496	0.522	0.501	0.491	0.02	103	100	100	105	111	106	104	4.2
		10	0.439	0.423	0.446	0.417	0.428	0.460	0.436	0.02	93	90	95	89	91	98	92	3.4
		15	0.485	0.482	0.513	0.492	0.495	0.442	0.485	0.02	103	102	109	104	105	94	103	5.0
		20	0.461	0.466	0.469	0.431	0.452	0.464	0.457	0.01	98	99	100	91	96	98	97	3.0
		25	0.473	0.425	0.446	0.456	0.447	0.497	0.457	0.02	100	90	95	97	95	105	97	5.3

[1] Calculated from the absorbance values after transformation as to relate to 13 000 cells/mL (i.e. (absorbance/ 11 500)* 13 000).

Table C2.4. Mann Whitney statistical comparison ($p \leq 0.05$) of medium and 0.1% DMSO control in MTT assay on H4IIE. Bold values in dark grey field indicate significant decrease in cell viability of the DMSO control.

Sample	Serie	Control	Average	± S.D.	p- value	Sample	Serie	Control	Average	± S.D.	p- value
Blank	1	Medium	0.688	0.074	0.279	Soil 4	1	Medium	0.464	0.053	**0.050**
		DMSO	0.614	0.107				DMSO	0.410	0.020	
	2	Medium	0.411	0.062	0.645		2	Medium	0.524	0.051	0.505
		DMSO	0.389	0.061				DMSO	0.495	0.056	
	3	Medium	0.614	0.028	**0.050**		3	Medium	0.499	0.065	0.328
		DMSO	0.553	0.049				DMSO	0.461	0.030	
Soil 3	1	Medium	0.484	0.088	0.279	Soil 5	1	Medium	0.595	0.089	0.382
		DMSO	0.422	0.054				DMSO	0.551	0.038	
	2	Medium	0.613	0.087	0.105		2	Medium	0.480	0.051	0.130
		DMSO	0.478	0.135				DMSO	0.443	0.032	
	3	Medium	0.568	0.028	**0.015**		3	Medium	0.597	0.063	**0.028**
		DMSO	0.498	0.025				DMSO	0.531	0.027	
							4	Medium	0.505	0.027	0.195
								DMSO	0.471	0.031	

Appendix C-3: Data from CYP1A induction assay

Table C3.1. Results of measured total protein concentration (mg/mL) in H4IIE exposed to Blank extract and extract of Soil 3, Soil 4 and Soil 5, measured by Bradford assay. Bovine serum albumin used as standard curve (results not included). Results presented as average ± S.D. (n=6) from two parallel samples that are pooled here for easier calculating the percent of the exposed compared with the DMSO control. Grey and white field denote the different samples, measured in triplicates. Red numbers were excluded.

Blank									
	Extract 1- serie 1				Extract 1- serie 2		Extract 2- serie 1		
Exposure	0	1	12.5	25	0	25	0	1	25
Individual values	772	791	705	816	719	917	894	751	752
	753	830	757	817	751	861	867	816	760
	708	805	766	810	711	698	867	745	668
	717	703	742	809	766	809	942	691	721
	792	796	759	817	780	845	949	667	691
	736	756	790	799	769	663	965	634	642
Average	**746**	**780**	**753**	**811**	**749**	**858**	**914**	**717**	**706**
S.D.	32	45	28	7	28	99	44	66	47
%	**100**	**105**	**101**	**109**	**100**	**115**	**100**	**78**	**77**
Soil 3									
	Extract 1- serie 1				Extract 1- serie 2		Extract 2- serie 1		
Exposure	0	1	3	5	0	5	0	1	5
Individual values	832	692	735	680	719	758	761	748	710
	873	779	740	686	751	748	746	790	773
	873	740	729	654	711	703	745	767	718
	806	700	770	460	766	666	736	812	722
	882	698	796	627	780	691	757	864	729
	830	837	765	721	769	625	716	801	674
Average	**849**	**741**	**756**	**638**	**749**	**698**	**743**	**797**	**721**
S.D.	31	58	26	93	28	50	16	40	32
%	**100**	**87**	**89**	**75**	**100**	**93**	**100**	**107**	**97**
Soil 4												
	Extract 3- serie 1			Extract 2- serie 2		Extract 2- serie 1				Extract 3- serie 1		
Exposure	0	1.25	2.5	0	3	0	0.5	1.5	3	0	0.5	3
Individual values	843	921	919	719	971	760	860	717	740	761	890	853
	870	908	927	751	964	870	892	925	856	746	853	780
	885	927	848	711	833	832	910	916	1008	745	760	754
	899	897	880	766	963	738	931	753	736	736	898	782
	907	892	912	780	978	872	961	894	842	757	854	799
	891	854	992	769	828	868	932	947	1005	716	773	792
Average	**883**	**900**	**913**	**749**	**969**	**823**	**914**	**859**	**864**	**743**	**838**	**793**
S.D.	23	26	49	28	72	60	35	98	121	16	58	33
%	**100**	**102**	**103**	**100**	**129**	**100**	**111**	**104**	**105**	**100**	**108**	**111**

Continue table C3.1

Soil 5												
	Extract 1- serie 1			Extract 1- serie 2		Extract 2- serie 1				Extract 3- serie 1		
Exposure	0	12.5	25	0	25	0	1	5	10	0	1	10
Individual values	843	757	821	719	922	795	710	852	913	894	793	870
	870	770	900	751	932	824	911	839	911	867	828	792
	885	718	846	711	782	867	845	898	1019	867	764	756
	899	883	828	766	1023	762	708	1007	859	942	903	897
	907	916	854	780	1027	861	848	933	976	949	839	803
	891	840	802	769	891	787	826	996	948	965	817	803
Average	**883**	**814**	**842**	**749**	**976**	**816**	**808**	**921**	**938**	**914**	**824**	**820**
S.D.	23	78	34	28	91	42	82	71	56	44	47	53
%	**100**	**92**	**95**	**100**	**130**	**100**	**99**	**113**	**115**	**100**	**90**	**90**

Benzo[a]pyrene								
	Sample 1				Sample 2		Sample 3	
Exposure	0	0.1	1	10	0	10	0	10
Individual values	720	756	818	892	843	685	761	765
	791	827	888	862	870	744	746	718
	883	818	904	862	885	743	745	725
	725	927	812	872	899	712	736	685
	863	965	943	866	907	777	757	696
	861	920	881	898	891	747	716	728
Average	**807**	**869**	**874**	**876**	**883**	**735**	**743**	**720**
S.D.	73	80	51	16	23	32	16	28
%	**100**	**108**	**108**	**109**	**100**	**83**	**100**	**97**

Table C3.2. Results of CYP1A induction immunoquantified by Western blotting after H4IIE exposure to organic extracts of Blank, Soil 3, Soil 4 and Soil 5 and B[a]P. Net intensity of luminescence is proportional to CYP1A and measured by region of interest measures (ROI) and transformed into pmol CYP1A/ mg total protein, utilising following equation from the CYP1A standard curve, $X = (0.5690^{-2.707}(4924000/I)-0.5690^{-2.707})^{1/-2.707}$. Numbers marked with red are excluded and blue numbers are negative measures interpreted as zero and corrected to 0.01 to be compatible with the standard curve. Standard is of 0.25 pmol CYP1A.

Blank				
Serie	**Exposure**	**Net Intensity**	**CYP1A (pmol/mg tot. protein)**	**Corr. factor**
Extract 1- serie 1	std	28202		27
	std	29338		
	0	4039	20.0	
	0	3927	19.8	
	1	2777	17.3	
	1	938	11.6	
	12.5	3449	18.8	
	12.5	4077	20.0	
	25	5333	22.2	
	25	6568	24.0	
Extract 1- serie 2	std	25252		31
	std	23840		
	0	3983	21.1	
	0	2341	17.3	
	25	7172	26.4	
	25	3209	19.4	
Extract 2- serie 1	std	14753		61
	std	10225		
	0	116	7.3	
	0	116	0.0	
	1	0.01	0.2	
	1	0.01	0.2	
	1	0.01	0.2	
	25	0.01	0.2	
	25	55	5.5	
	25	181	0.5	
Soil 3				
Serie	**Exposure**	**Net Intensity**	**CYP1A (pmol/mg tot. protein)**	**Corr. Factor**
Extract 1- serie 1	std	20059		35
	std	23561		
	0	0.01	0.2	
	1	159029		
	1	69463	80.6	
	3	67150	78.7	
	3	54660	68.8	
	5	44848	61.4	
	5	45615	62.0	

Continue table C3.2

Extract 1- serie 1	std	29235		30
	std	21885		
	0	0.01	0.2	
	1	92645	89.3	
	1	58142	65.0	
Extract 1- serie 2	std	22136		40
	std	16201		
	0	739	12.3	
	0	2373	19.0	
	5	18738	27.4	
	5	41640	47.8	
Extract 2- serie 1	std	23686		29
	std	29228		
	0	1067	12.5	
	0	454	9.1	
	1	80159	67.2	
	1	57672	52.8	
	5	24720	31.5	
	5	22940	30.1	
Extract 2- serie 1	std	29235		30
	std	21885		
	0	0.01	0.2	
	1	28487	45.5	
	1	30366	46.8	
	5	8255	27.3	
	5	14572	34.2	
Soil 4				
Serie	**Exposure**	**Net Intensity**	**CYP1A (pmol/mg tot. protein)**	**Corr. factor**
Extract 1- serie 1	std	17614		60
	std	7978		
	0	1912	20.4	
	0	16	3.5	
	1.25	27570	51.2	
	1.25	34776	60.6	
	2.5	32371	57.4	
	2.5	21770	43.8	
Extract 2- serie 1	std	18174		50
	std	12377		
	0	580	12.2	
	0	714	13.2	
	0.5	60206	83.8	
	0.5	118804		
	1.5	57111	79.2	
	1.5	41113	59.3	
	3	18594	34.8	
	3	45911	64.8	

Continue table C3.2

Extract 2- serie 2	std	22136		40
	std	16201		
	0	739	12.3	
	0	2373	19.0	
	3	24586	33.0	
	3	30339	38.1	
Extract 3- serie 1	std	14316		54
	std	3693		
	0	2698	22.3	
	0	0.01	0.2	
	0.5	34357	55.9	
	0.5	26604	47.1	
	3	8319	23.4	
	3	9616	25.5	
Soil 5				
Serie	**Expsoure**	**Net Intensity**	**CYP1A (pmol/mg tot. protein)**	**Corr. factor**
Extract 1- serie 1	std	23437		33
	0	252	7.7	
	12.5	22224	42.5	
	25	28047	47.2	
Extract 1- serie 1	std	23551		38
	std	16385		
	0	963	13.4	
	0	0.01	0.2	
	12.5	26068	42.3	
	12.5	27707	43.7	
	25	36275	50.9	
	25	38043	52.3	
Extract 1- serie 2	std	14316		54
	std	3693		
	0	2698	22.3	
	0	0.01	0.2	
	25	34357	55.9	
	25	26604	47.1	
Extract 2- serie 1	std	25940		30
	std	5589		
	0	979	12.2	
	0	1699	15.0	
	1	5469	9.7	
	1	4267	7.6	
	5	25229	29.4	
	5	24080	28.5	
	10	31110	33.6	
	10	75320	62.1	

Continue table C3.2

Extract 2- serie 1	std	34925		26
	std	23488		
	0	2	1.2	
	0	1568	13.9	
	10	30373	36.7	
	10	36892	40.8	
Extract 3- serie 1	std	34925		26
	std	23488		
	0	2	1.2	
	0	1568	13.9	
	1	2749	9.6	
	1	5263	14.4	
	10	37743	41.3	
	10	28624	35.6	
Benzo[a]pyrene				
Serie	**Samples**	**Net Intensity**	**CYP1A (pmol/mg tot. protein)**	**Corr. factor**
Sample 1- serie 1	std	18442		42
	0	2346	19.2	
	0	1289	15.4	
	0.1	3813	5.8	
	0.1	3903	6.0	
	1	5684	9.7	
	1	12707	19.9	
	10	29232	36.5	
	10	34860	41.6	
Sample 1- serie 2	std	11769		65
	0	2055	21.7	
	0	870	15.7	
	0.1	4396	10.4	
	0.1	2085	3.1	
	1	4132	9.7	
	1	4407	10.4	
	10	11226	24.0	
	10	11417	24.3	
Sample 2- serie 1	std	23551		38
	std	16385		
	0	963	13.4	
	0	0.01	0.2	
	10	20149	36.9	
	10	28519	44.4	
Sample 3- serie 1	std	23686		29
	std	29228		
	0	1067	12.5	
	0	454	9.1	
	10	32497	36.9	
	10	23516	30.6	