NTNU – Trondheim
Norwegian University of
Science and Technology

# Mutagenic Potential of Spruce-derived fast Pyrolysis Oil measured by the Ames Salmonella assay

Nina Holteberg

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Supervisor: Åse Krøkje, IBI
Co-supervisor: Ingvar Eide, Statoil

Norwegian University of Science and Technology
Department of Biology

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Nina Holteberg

## Abstract

Biomass conversion to biofuels is promising for production of renewable energy. Biofuels are sustainable, $CO_2$-neutral, recognized to be cleaner than fossil fuels, but their combustion generates noxious gases and genotoxic substances and combustion of different types of biofuels has been associated with an increased incidence of lung cancer. Pyrolysis of biomass is the most efficient biofuel-producing conversion process and generates pyrolysis oil, or bio-oil, of which upgradation techniques are under development in order to produce green fuels, and similar to other biofuels genotoxic abilities have been shown in bio-oil.

The main purpose of this project was to measure the mutagenic potential of crude pyrolysis oil through application of the preincubation version of the Ames assay using bacterial strains TA98 and TA100 with and without the metabolically active S9-mix in order to assess bio-oil's ability to induce frameshift-mutations and base-pair substitutions in terms of primary or secondary mutagenicity. The Ames assay was chosen because of its ability to screen complex chemical mixtures for content of chemical mutagens. The sample material consisted of seven spruce-derived fast pyrolysis oils obtained from different relative proportions of wood, bark and needles, which created a unique opportunity to study the feedstock components' influence on mutagenic potential. Three of the oils were obtained from 100 % of wood, bark or needles while the remaining four oils derived from mixed feedstock compositions. With limited chemical data available on the oils, the aims of this project were to determine the types of mutations the bio-oils would induce and how the feedstock composition would influence the mutagenic potential, as well as trying to predict the mutagenic potential of the oils through a partial least square (PLS) regression model based solely on their feedstock composition.

The mutagenic potential of the concentration ranges of bio-oil test-solutions showed that all but the purely needle-derived oil induced positive test results under at least two of the four test conditions (TA98, TA98 + S9, TA100 and TA100 + S9) as well as evoking bacterial toxicity at different concentrations. Presence of primary and secondary mutagens inducing frameshift mutations and base-pair substitution were indicated in one or more of the oils, however with a higher frequency of positive test results for base-pair substitutions. The metabolically active S9 decreased the oil-evoked bacteriotoxic effect in both strains and generally lead to decreased reversion frequencies in TA98 and increased reversion

frequencies in TA100. The results demonstrated that both bacteriotoxic and mutagenic properties in spruce-derived fast pyrolysis oils were influenced by spruce feedstock composition and the statistical analyses showed that wood was the feedstock component contributing the most to the bio-oil-induced mutagenic potential.

The positive Ames assay results in this study suggest that crude spruce-derived bio-oils may contain hazardous compounds which in this case were unidentified. However, as crude pyrolysis oil needs upgradation in order to be utilized as a high quality fuel, the probability of potential hazard from exposure to crude bio-oils like the ones in this project is rather small. The results of this project demonstrated mutagenic potential in crude bio-oil, but it would be useful to conduct mutagenicity assessment on more refined pyrolysis oil in order to see if the mutagenic potential will decrease after performance of various upgradation techniques on pyrolysis oil.

## Sammendrag

Omdannelse av biomasse til biobrensel er lovende for produksjon av fornybar energi. Biobrensel er bærekraftig, $CO_2$-nøytralt, anerkjent for å være renere enn fossil brensel, men ved forbrenning genererer biobrensel skadelige gasser og genotoksiske substanser og forbrenning av flere typer biobrensel har blitt assosiert med økt forekomst av lungekreft. Pyrolyse av biomasse er den mest effektive biobrensel-produserende omdannelsesprosessen og produserer pyrolyseolje, eller bioolje, hvis oppgraderingsteknikker er under utvikling for å produsere "grønne" drivstoff, og i likhet med andre biobrensler har genotoksiske egenskaper blitt påvist i bioolje.

Hovedmålet med dette prosjektet var å måle det mutagene potensialet av uraffinert pyrolyseolje, gjennom anvendelse av Ames-testen med bakteriestammene TA98 og TA100 med og uten den metabolsk aktive S9-miksen, for å vurdere biooljes evne til å indusere frameshift-mutasjoner og basepar-substitusjoner i form av primær eller sekundær mutagenitet. Ames-testen ble valgt grunnet dens evne til å screene komplekse kjemiske blandinger for innhold av kjemiske mutagener. Prøvematerialet bestod av syv gran-baserte "fast" pyrolyseoljer generert fra ulike relative andeler av tre, bark og nåler, noe som skapte en unik anledning for å studere råstoffenes innflytelse på mutagent potensiale. Tre av oljene var produsert fra 100 % tre, bark eller nåler, mens de resterende fire oljene var generert fra ulike blandinger av råstoffene. Ettersom at de tilgjengelige kjemiske data på oljene var begrenset, var målsettingene i dette prosjektet å avdekke hvilke typer mutasjoner biooljene ville indusere og på hvilken måte sammensetningen av råstoffer ville virke inn på det mutagene potensialet, samt å forsøke å forutsi det mutagene potensialet i oljene ved bruk av en "partial least square" (PLS) regresjonsmodell basert kun på oljenes råstoffsammensetning.

Det mutagene potensialet av konsentrasjonsspennet av bioolje-testløsninger viste at alle unntatt den rent nålbaserte oljen induserte positive testresultater under minst to av de fire testbetingelsene (TA98, TA98 + S9, TA100 and TA100 + S9) og samtidig medførte bakteriell toksisitet ved ulike konsentrasjoner. Tilstedeværelse av primære og sekundære mutagener som induserer leseramme-forskyvninger og basepar-substitusjoner var indikert i en eller flere av oljene, imidlertid med en høyere frekvens av positive testresultater for basepar-substitusjoner. Den metabolsk aktive S9 minsket den oljeinduserte bakteriotoksiske effekten i

begge stammene og ga generelt lavere reversjonsfrekvens hos TA98 og høyere reversjonsfrekvens hos TA100. Resultatene demonstrerte at både bakteriotoksiske og mutagene egenskaper i granbaserte "fast" pyrolyseoljer var påvirket av råstoffsammensetningen, og de statistiske analysene viste at tre var det råstoffet som bidro mest til det bioolje-induserte mutagene potensialet.

De positive resultatene i Ames-testen i dette prosjektet antyder at uraffinerte granbaserte biooljer kan inneholde genotoksiske forbindelser, som i dette tilfellet var uidentifiserte. Imidlertid vil sannsynligheten for potensiell risiko for eksponering for uraffinerte biooljer, som ble benyttet i dette prosjektet, være ganske liten ettersom at uraffinerte pyrolyseoljer trenger oppgradering for å kunne benyttes som et høykvalitets drivstoff. Resultatene i dette prosjektet demonstrerte mutagent potensiale i uraffinert bioolje, men det ville være nyttig å utføre mutagenitetstesting på en mer raffinert pyrolyseolje for å avdekke om det mutagene potensialet vil minke etter at oljen er oppgradert ved ulike typer teknikker.

## Abbreviations

| | |
|---|---|
| BaP | Benzo[a]pyrene |
| BTL | Background Toxicity Level |
| CYP | Cytochrome P450 |
| DMSO | Dimethylsulphoxide |
| DNA | Deoxyribonucleic acid |
| ESI-MS | Electrospray Ionization Mass Spectrometry |
| GC-MS | Gas Chromatography Mass Spectrometry |
| GST | Glutathione-S-Transferase |
| $NO_x$ | Nitrogen Oxides |
| NPD | 4-nitro-*o*-phenylenediamine |
| PAH | Polycyclic Aromatic Hydrocarbon |
| PFI | The Paper and Fiber Research Institute |
| PLS | Partial Least Square regression |
| RMSE | Root of the Mean Square's Error |
| S.D. | Standard Deviation |
| S9 | Rat liver homogenate, the Supernatant at 9000 G |

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# 1. Introduction

## 1.1. Biofuels

### 1.1.1. The potential and future of biofuels

Renewable energy is predicted to play a crucial role in the future society of the 21st century (Blaschke et al., 2013). Biomass, solar, wind, hydroelectric and geothermal power are central renewable energy resources (Manzano-Agugliaro et al., 2013), and biomass conversion to biofuels is recognized as the potential solution to the depletion of fossil fuel reserves and increasing oil prices (Sharma et al., 2013). Combustion of fossil fuels contributes to global warming and increased levels of air pollutants (Ma et al., 2013). Biomass in contrast is a $CO_2$ neutral and sustainable energy resource (Ma et al., 2012) that is claimed to be cleaner and less pollutive than fossil fuels (Mohan et al., 2006). Biomass can either be applied directly or get converted into gaseous or liquid fuel by thermochemical, biochemical or agrochemical processes (Demirbaş, 2001). The most commonly applied biofuel is biodiesel (Bezergianni and Dimitriadis, 2013), but new technologies are developing rapidly to find new ways of utilizing biomass energy (Ramsurn and Gupta, 2013). Among the promising processes for biomass conversion to fuel, pyrolysis is the most efficient biomass conversion process (Demirbaş, 2001), and development of up-gradation techniques of pyrolysis liquids may result in production of green diesel and green gasoline (Hossain and Davies, 2013).

### 1.1.2. Biofuel health consequences and genotoxic activity

Biofuel combustion produces noxious gases and toxic substances; among others carbon monoxide, respirable particulates, nitrogen oxides ($NO_x$) and polycyclic aromatic hydrocarbons (PAHs) (Musthapa et al., 2004). The latter group of chemicals, the PAHs, have several toxicological properties (Collins et al., 1998) and are shown to be genotoxic (West et al., 1988, Audebert et al., 2012). This means that they are able to cause effects on the DNA and genetic processes potentially resulting in genetic disorders like cancer (Ehrenberg et al., 1983, Preston and Hoffman, 2008), and PAHs are indeed thought to induce various types of cancer tumours (Sinopoli et al., 1988, Jacob, 2008). Combustion products from different types of biofuels have shown genotoxic activity (Bell and Kamens, 1990) and several epidemiological studies have shown an increased incidence of lung cancer in populations using biofuels (Mumford et al., 1987, He et al., 1991, Zhong et al., 1999).

Biodiesel has been considered as the best alternative to fossil diesel (Bezergianni and Dimitriadis, 2013). With the exception of $NO_x$, it generates lower combustion emissions than petro-based diesel, it is sulphur free, biodegradable and claimed to be non-toxic (Luque et al., 2008). However, genotoxic effects from biodiesel pollution in soil (Leme et al., 2012b) and water (Leme et al., 2012a) have been shown and toxic biodiesel derived air pollutants have been detected (He et al., 2010). Genotoxic effects have also been associated with other biofuels; cow dung and wood (Musthapa et al., 2004), 2,5-dimetylfuran (Fromowitz et al., 2012) in addition to several types of biomass pyrolysis products; pyrolytic liquid from hazel nut shells (Pekol et al., 2012), pyrolysis oil from *Eucalyptus grandis* (Pimenta et al., 2000) and bio-oils from a range of different feedstocks and production systems (Gratson, 1994, Girard et al., 2005, Cordella et al., 2012).

### 1.1.3. Pyrolysis oil

#### 1.1.3.1. Pyrolysis of biomass

Pyrolysis oil is a liquid fuel obtained from biomass by rapid heating of the mass in the absence of oxygen to generate organic volatile components and then condensing the fuel vapours into an oil (Demirbaş, 2009). Charcoal, vapours and aerosols are generated during this thermal degradation of the biomass and cooling of the latter two products forms the pyrolysis oil, or bio-oil (Mohan et al., 2006). Differences in operating conditions generates different relative proportions of the products (Blin et al., 2007) and classifies the process as carbonization/very slow, conventional/slow or fast/flash pyrolysis (Maschio et al., 1992), which is the most intensively investigated pyrolysis process (Kleinert and Barth, 2008). The liquid product of biomass pyrolysis consists of two phases; an aqueous phase with various organo-oxygen compounds of low molecular weight and a non-aqueous phase which mainly contains insoluble aromatics of high molecular weight (Demirbaş, 2009). Unfortunately, this product needs further up-grading before it can be utilized as a motor fuel, and large-scale production of bio-oil is problematic because of the lack of an efficient up-grading technology (Kleinert and Barth, 2008). Production of bio-oil is nevertheless under development (Hossain and Davies, 2013) and nearly any type of biomass may be considered for the process, including sewage sludge, nutshells and algae (Mohan et al., 2006). Based on resource and process evaluations however, wood is claimed to be the preferred raw material (Kleinert and Barth, 2008).

### 1.1.3.2. Physical properties and hazardous components

The chemical structure of bio-oil changes completely from the original biomass during pyrolysis (Gellerstedt et al., 2008) and it is difficult to achieve a complete and detailed chemical characterization of pyrolysis oils (Cordella et al., 2012). Bio-oil is tar-like (Demirbaş, 2009), dark brown with a smoky odour, an acidity range from pH 2.0 – 3.0 caused by the high content of organic acids, and physical properties different from petroleum-derived oils (Czernik and Bridgwater, 2004). Many factors influence the nature of the pyrolysis oil; feed composition, particle size, temperature, heating rate and reaction time (Vamvuka, 2011). Bio-oil contains a highly complex mixture of oxygenated hydrocarbons of various size (Blin et al., 2007) produced mostly from depolymerisation and fragmentation of cellulose, hemicelluloses and lignin; key building blocks in biomass (Mohan et al., 2006). At higher temperatures during pyrolysis, aromatic compounds condense to generate PAHs (Mohan et al., 2006). It is shown that PAH concentration is greatly influenced by both temperature and residence time during production; higher temperatures and longer residence times seem to favour PAH formation (Williams and Besler, 1994). Concentration of the sum of 13 selected PAHs (∑13 PAH) in 21 different bio-oils was typically under 10 ppm, but could exceed 23 ppm for fast pyrolysis oil and 100 ppm for slow pyrolysis oil (Girard et al., 2005). However, PAH levels in slow pyrolysis oil have also been reported in the mg $kg^{-1}$ range by measurement of ∑16 PAHs (Cordella et al., 2012) and the total PAH concentration (Pimenta et al., 2000).

A study by Cordella et al. (2012) assessed the toxicological properties of slow pyrolysis oils and in order to screen the hazards associated with bio-oils they took a "macro-component" approach. This involved identification of similar groups of hazardous chemicals, referred to as macro-components, and selection of a key compound for each macro-component. The hazard profile of the key compounds was claimed to represent the hazards associated with the compounds belonging to their macro-components. Identified macro-components in slow pyrolysis oil and their respective key compounds are listed in Table 1.1.3.2.

**Table 1.1.3.2: Identified hazardous components in slow pyrolysis oil.** Macro-components consist of similar chemical groups of hazardous components, and for each macro-component a key compound was selected to represent the hazard profile of all the compounds belonging to their macro-component. Adapted from Cordella et al. (2012).

| **Macro-component** | **Key compound** |
|---|---|
| Anhydrosugars | Levoglucosan |
| Carboxylic acids | Acetic acid |
| Aldehydes | Hydroxyacetaldehyde |
| Ketons and alcohols | Hydroxyacetone |
| Furans | Furaldehyde |
| Phenols | Phenol |
| PAHs | Anthracene |

### 1.1.3.3. Pyrolysis oil applications

Without up-gradation, bio-oil is not a conventional oil/fuel and its utilization needs careful consideration because of factors like potential phase separation and viscosity (Kleinert and Barth, 2008). Other limitations are corrosiveness, presence of impurities and generation of aromatic toxicants (Luque et al., 2008). Thermal or catalytic up-gradation of bio-oil may be required to improve its properties (Luque et al., 2008) before it can be used for production of biofuels, energy or chemicals (Cordella et al., 2012), e.g. biopesticides (Hossain et al., 2012). Pyrolysis oil may substitute for diesel/fuel oil in several static applications including engines, furnaces and turbines for electricity generation (Zhang et al., 2007), but the burner and combustion characteristics need to be adapted to appropriately fit the unique characteristics of bio-oil (Vamvuka, 2011). The process of biomass pyrolysis and the applications of the generated products are still in early stages of development (Hossain and Davies, 2013), but recently the possibility to use biomass pyrolysis liquids as replacement for fossil oil in e.g. boilers or diesel engine generators has received increasing interest (Toven et al., 2012). The up-scaling of biomass pyrolysis techniques and the potential of bio-oil usage in large scale applications could expose many humans to potential hazards (Blin et al., 2007).

### 1.1.3.4. Exposure and environmental contamination

Health, Safety and Environment (HSE) aspects of bio-oil can be divided into environmental protection, health and safety at the work place and potential for incidents during bio-oil distribution and use (Bridgwater et al., 2000). The exposure routes after future commercialization of bio-oils will probably be dermal and inhalation exposure for the general public and mostly dermal exposure for plant workers (Oasmaa et al., 2012). Regarding potential accidental spillage bio-oil gets biodegraded more easily than fossil fuels, with values between 41 % and 50 % after 28 days, and has been classified as inherently biodegradable (Blin et al., 2007). Nevertheless, several compounds found in bio-oil have mutagenic and carcinogenic character and therefore have strong environmental impacts (Kaden et al., 1979, Gold et al., 1989). Bio-oil can contaminate the environment through accidental releases and routine loss during fuel usage (Leme et al., 2012b) and may represent a health hazard in itself and through its combustion products which may contain substantial amounts of PAHs (Williams and Besler, 1994).

### 1.1.3.5. Toxicological properties of pyrolysis oil

Cordella et al. (2012) assessed toxicity and carcinogenicity of bio-oils from three different feedstocks based on data on the single components of the oils and suggested that (1) chronic exposure to bio-oils could be hazardous to human health (2) bio-oil spillage may lead to acute toxic effects on humans as well as ecotoxic effects on aquatic systems and (3) the presence of carcinogenic compounds, e.g. PAHs and catechols, generates a marginal carcinogenic potential. Four hazardous components were identified regarding acute inhalation toxicity, six components with respect to acute dermal toxicity and 20 compounds in case of ingestion, in addition to 11 compounds with carcinogenic potential. In order to reach full understanding of the hazard potential from bio-oil production, storage and delivery, Cordella et al. (2012) underline that a broad spectrum of toxicological and carcinogenic properties need to be analysed.

Researchers within the Biotox Project assessed the toxicological properties of 21 different bio-oils almost exclusively obtained from wood (Girard et al., 2005). Their work included an ecotoxicological evaluation and assessment of acute dermal toxicity and genotoxicological endpoints like mutagenicity and micronuclei formation. The oils were produced from different types of feedstocks and under different production circumstances. One of these bio-oils, the spruce-derived BioTox-21, was further analysed and claimed to be a

reasonable representative for wood-derived fast pyrolysis oils, despite the fact that different biomass feedstocks and reactor systems may result in different composition and toxicity of the oils (Oasmaa et al., 2012). BioTox-21 was produced at 500 ºC in a fluidized bed reactor and had a ∑13 PAH concentration of 1.01 ppm (Girard et al., 2005). It was concluded that BioTox-21 evoked dermal irritation, slight acute oral toxicity to rats and lead to ambiguous results regarding mutagenic activity, and was not identified as environmentally hazardous (Table 1.1.3.4). Emphasis has been put on the need for carcinogenicity testing of bio-oil due to the PAH content and the fact that only about half of the material was chemically characterized (Lehto et al., 2013).

**Table 1.1.3.4: Toxicity of a wood-derived fast pyrolysis oil.** Bio-oil obtained by fast pyrolysis of spruce (BioTox-21) was regarded as representative for wood-derived fast pyrolysis oils and was therefore analysed to obtain toxicological data and ecotoxicological evaluation (Girard et al., 2005). The bio-oil was produced at 500 ºC in a fluidized bed reactor and the ∑13 polycyclic aromatic hydrocarbon (PAH) concentration was 1.01 ppm. Assessment of the mutagenic potential of the oil lead to ambiguous results, and due to the content of PAHs and the fact that only half of the material was chemically analysed, emphasis has been put on the need to conduct further testing to see if bio-oil is carcinogenic (Lehto et al., 2013).

| | **Endpoint** | **Test organism** | **Result** | **Conclusion** |
|---|---|---|---|---|
| Acute toxicity | Dermal irritation | Rabbits | Induce skin irritation | Corrosive |
| | Acute oral toxicity | Rats (females) | LD50 > 2500 mg/kg body weight | Slightly toxic |
| | 7-day oral toxicity | Rats (both sexes) | No premature deaths | Not classified as toxic |
| Genotoxicological evaluation | *In vitro* mutagenesis (Ames test) | *Salmonella typhimurium* (TA1535, TA1537, TA98, TA100, TA102) | Mutagenic with and without metabolic activation | Ambiguous results of the genotoxic activity leads to the recommendation to conduct further testing to determine if bio-oil is carcinogenic |
| | *In vitro* micronucleus | Mice (lymphoma cells) | No effect | |
| | *In vivo* micronucleus | Mice (bone marrow cells) | Little or no mutagenic activity | |
| Ecotoxicologal evaluation: | Algal growth inhibition | | No significant effect up to 100 mg/L | Not environmentally hazardous |
| | Toxicity to *Daphnia* | *Daphnia magna* | No significant effect up to 100 mg/L | Not environmentally hazardous |
| | Aerobal biodegradability in fresh water | | 42 % biodegradation after 28 days | Not environmentally hazardous |
| | Flammability | | Does not sustain combustibility | Nonflammable |

## 1.2. Genetic toxicology

### 1.2.1. Chemical genotoxicants and the importance of mutagenicity testing

The genetic material of cells is frequently damaged by naturally occurring events, and the cell repair machinery normally repairs these damages quite rapidly. Additional damage, referred to as genotoxic effects, is generated through physical or chemical agents' interaction with the DNA or genetic processes. Genotoxicity includes a variety of endpoints in the genetic material, e.g. DNA-adducts or -strand breaks, unscheduled DNA synthesis and chromosomal aberrations (Preston and Hoffman, 2008). The extent of genotoxic effects on the individual level depends on numerous factors, including rate of uptake, absorption, biotransformation and factors affecting DNA damage formation, e.g. cell turnover, adduct stability and DNA repair rates (Østby et al., 2005). If genetic damage is permanent and by cell division gets transferred to future generations of cells or individuals, the damage is called a mutation. Gene mutations are small changes in the DNA sequence of single genes and are generally classified as small base-pair additions or deletions or as base substitutions; replacements of correct nucleotides by incorrect ones. Addition/deletion of one or more base-pairs may have more severe consequences than base substitutions if it leads to a frame-shift of the whole nucleotide reading frame in protein-coding regions of the DNA. Chemical mutagens are referred to as primary or secondary mutagens; mutagenic as parent compounds or showing mutagenicity only after metabolic activation, respectively (Preston and Hoffman, 2008).

Genotoxicants are associated with disease states in both humans and experimental animals, such as acute toxicity and heritable diseases (Williams, 1989), and may pose a threat to natural populations by their potential to accelerate aging, alter reproduction and induce tumours (Roy et al., 1997, DeRosa et al., 1998). One of multiple cancer causes that have been established or suggested is genotoxic chemicals, which in this case are referred to as genotoxic carcinogens (Kamendulis and Klaunig, 2008). The concern for cancer is what drives most mutagenicity testing of chemicals (Mortelmans and Zeiger, 2000), as mutations are central in the development of cancer (Mahadevan et al., 2011). Chemical carcinogenesis usually has a long latency period between initial exposure and tumour observation, e.g. 20-30 years, and is considered as an irreversible toxic effect. The compounds involved in chemical carcinogenesis may as parent compounds or secondary metabolites exert effects in a genotoxic/DNA-reactive manner or a nongenotoxic/epigenetic manner (Eaton and Gilbert, 2008). The role of mutation is critical in chemically induced carcinogenesis and analysis of

chemically induced mutations is therefore essential in order to understand and predict chemical carcinogenesis (Preston and Hoffman, 2008).

### 1.2.2. Genetic toxicology testing

The aim of a genetic toxicology assay is to detect xenobiotics' potential to cause mutations or chromosomal damage (Lynch et al., 2011). There are several methods and systems available to measure genotoxicity both *in vivo* and *in vitro* for germinal and somatic cells by studying damage to the DNA, gene mutations and chromosomal alterations (Mahadevan et al., 2011). Genetic toxicology assays can be used for toxicological evaluation of chemicals in order to increase understanding of genetic and carcinogenic risk through: (1) identification of mutagens for hazard identification and (2) description of dose-response relationships and mutagenic mechanisms (Preston and Hoffman, 2008). Standard regulatory tests are often an *in vitro* genotoxicity assessment in bacterial and mammalian cells, like the Ames *Salmonella* assay, combined with rodent assays to detect chromosomal and DNA damage (Lynch et al., 2011). The Ames *Salmonella* assay is the most widely used *in vitro* primary screening test for gene mutation (Bajpayee et al., 2005) and is claimed to detect most human mutagens and carcinogens (Ames et al., 1975, Maron and Ames, 1983). This short-term test is well suited for testing complex mixtures other than urine samples and requires only small volumes of the test chemicals (Mortelmans and Zeiger, 2000).

## 1.3. Complex chemical mixtures

### 1.3.1. Challenges related to genotoxicity testing of complex mixtures

Bio-oil may contain thousands of constituents (Jarvis et al., 2012). Genotoxic risk assessment of complex chemical mixtures is challenged by the difficulty in identifying toxic compounds, finding sufficient toxicity information and a lack of knowledge about genotoxicant behavior in complex mixtures (Donnelly et al., 1995). Complex mixtures have led to nonadditive effects regarding genotoxicity and some studies suggest that nonmutagens may modulate the effects of mutagens such as benzo[a]pyrene (BaP) (White, 2002). Potential interactions between components in a mixture should be taken into account in the evaluation of the mixture's toxicological potency (Jarvis et al., 2013) and therefore when evaluating mutagenicity of a mixture it will be more representative to determine mutagenicity from the

whole mixture rather than estimating mutagenicity based on the individual components of the mixture (Hermann, 1981). This is referred to as a top down approach, contrasting the bottom up approach which focuses only on selected compounds from the mixture (Groten, 2000). Similar mutation spectra may be generated by different compounds either from different chemical classes or from agents with similarities (DeMarini, 1998). However, it is claimed that the mutation spectrum of a complex chemical mixture will reflect one or a few chemical classes dominating within the mixture, e.g. PAHs or nitroarenes, to create a range of mutational specificity.

### 1.3.2. Polycyclic aromatic hydrocarbons (PAHs)

It has been indicated that PAHs are involved in the genotoxic activity of pyrolysis oil, as genotoxic effects have been found induced by the PAH fraction while no genotoxic effect was evoked from the total pyrolysis liquid (Pimenta et al., 2000). With the exception of phenol, Cordella et al. (2012) found that in slow pyrolysis oil all the individual chemical compounds with a maximum hazard score were PAHs. These compounds consist of condensed ring aromatic molecules (Manahan, 2010) which generally appear in complex mixtures. More than one hundred PAH compounds exist (Audebert et al., 2012) and most of them have shown genotoxicity *in vivo* and *in vitro* (Bostrom et al., 2002, Audebert et al., 2012) and several PAHs are classified as mutagens as well as animal carcinogens (White, 2002). The genotoxicity of PAHs is primarily caused by bioactivating metabolic pathways (Jacob, 2008, Audebert et al., 2012), as parent compound PAHs are relatively non-reactive toward biological macromolecules (Yu, 2002). The most relevant enzymes for PAH bioactivation into genotoxicants are cytochrome P450s (CYP450s) in the enzyme families CYP1-3 (Jacob, 2008) with CYP1A1 and CYP1B1 as the two most important enzymes (Baird et al., 2005). Further conjugation of reactive PAH intermediates may be catalyzed by enzymes such as glutathione-S-transferase (GST) resulting in their excretion (Ambrosone and Tang, 2009). Thus, various metabolic enzymes are involved in bioactivation/detoxification of PAHs such as benzo[a]pyrene (Figure 1.3.2A) and may lead to detoxification or subsequent production of DNA adducts (Figure 1.3.2B).

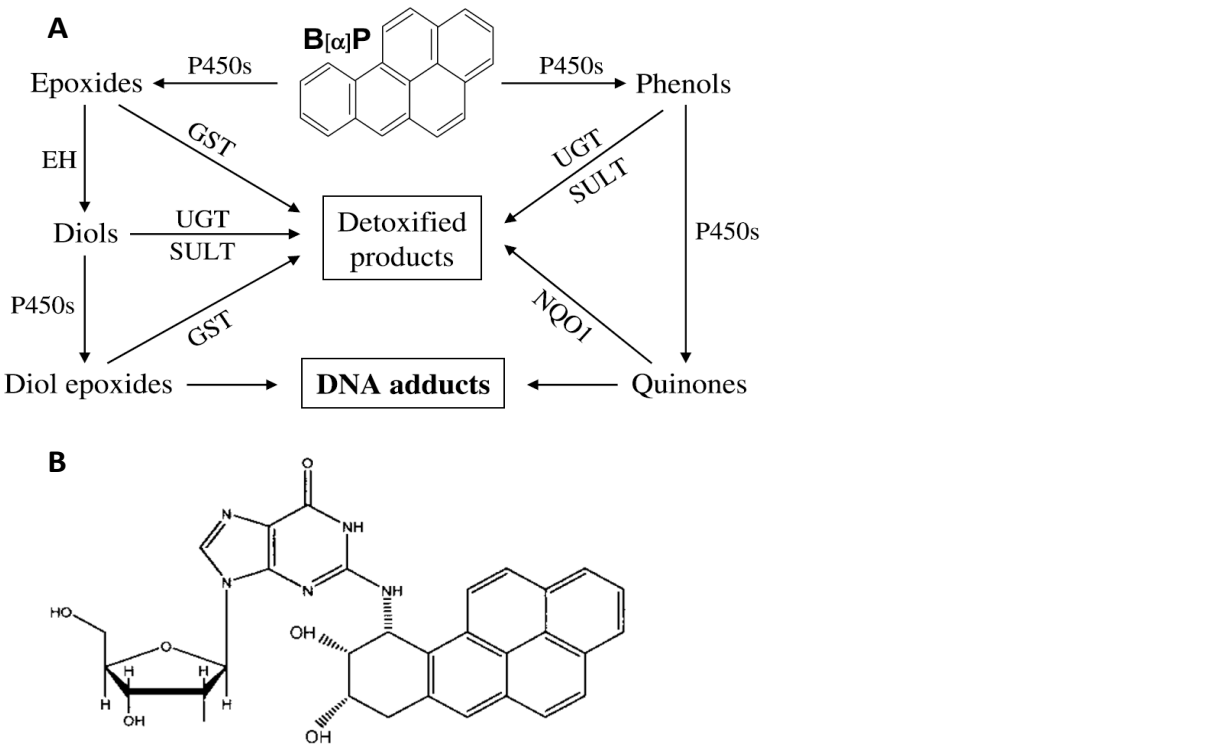


**Figure 1.3.2: A simplified illustration of polycyclic aromatic hydrocarbon metabolism.** Various enzymes are involved in polycyclic aromatic hydrocarbon (PAH) metabolism (A) and benzo[a]pyrene (BaP) is claimed to be a prototype of carcinogenic PAHs and may generate DNA adducts after cytochrome P450 mediated bioactivation into a diol epoxide (B). The epoxide is here adducted to DNA by a covalent binding to the exocyclic amino group of a purine base. P450s: cytochrome P450s, EH: epoxide hydrolase, GST: glutathione-S-transferase, SULT: sulfotransferase, NQO1: NAD(P)H-quinone oxidoreductase. A and B are adapted from Ambrosone and Tang (2009) and Baird et al. (2005), respectively.

## 1.4. Aim of the study

This study will assess the mutagenic potential of fast pyrolysis oil by application of the preincubation version of the Ames *Salmonella* assay. The bio-oils to be tested are obtained from Scandinavian forest residues of spruce wood, bark and needles, which are considered attractive resources for conversion into fast pyrolysis oil (Celaya et al., 2012). A previous attempt to evaluate the mutagenic potential of wood-derived fast bio-oils by the preincubation version of Ames assay has lead to relatively ambiguous results but nevertheless detection of mutagenic abilities in all tested bio-oils, and it is recommended to do further testing of the mutagenic potential of fast pyrolysis oil derived from spruce and other feedstocks (Oasmaa et al., 2012).

The overall goal of this project is to obtain information about mutagenic potential of pyrolysis oils with a focus on the oils' feedstock composition in relation to their results in the Ames assay. Multivariate data analyses will be performed in attempts to relate the feedstock composition of wood, bark and needles to their respective contributions to the mutagenic potential as well as to compare the feedstock-based predicted mutagenic potential against the measured mutagenic potential. The following questions to be addressed in the project are:

(1) Which types of mutations will be induced by the bio-oils and how much mutagenicity will arise from primary mutagens compared to the level induced by secondary mutagens?
(2) How will the different proportions of wood, bark and needles in the feedstock from which the oils were obtained influence the mutagenic responses?
(3) Will the predicted mutagenic potential based on feedstock composition be similar to the mutagenic potential measured by the Ames assay?

# 2. Materials and Methods

## 2.1. The pyrolysis oil samples

Pyrolysis oils were recieved from the Paper and Fiber Research Institute, Trondheim, Norway. The oils were produced at the University of Ashton using a fluidised bed reactor at 500 ºC. One batch was a generated in 2011 and a second batch in 2012, and the oils were stored at Centre for Research, Development and Innovation, Statoil ASA, at 4 ºC in absence of light. The material in this master project consisted of seven samples of fast pyrolysis oil obtained from forest residues of Norwegian spruce (*Picea abies*) in different relative proportions of wood, bark and needles (Table 2.1). Some oils were produced from brown (dried) as opposed to green forest residues. More detailed descriptions of the production system and conditions as well as the yields and bio-oil phase distribution and -characterization are described in (Celaya et al., 2012, Toven et al., 2013). After being received from PFI the oils were exposed to a minimum of light and stored at 4 ºC at NTNU.

**Table 2.1: The pyrolysis oil samples.** Seven spruce-derived fast pyrolysis oils recieved from the Paper and Fibre Research Institute (PFI) were in 2011/2012 produced from different relative proportions of wood, bark and needles (Celaya et al., 2012, Toven et al., 2013).

| Sample ID | Production Year | Spruce feedstock | Feedstock composition | | |
|---|---|---|---|---|---|
| | | | Wood (%) | Bark (%) | Needles (%) |
| 100-0-0 | 2011 | Wood | 100 | 0 | 0 |
| 0-100-0 | 2011 | Bark | 0 | 100 | 0 |
| 0-0-100 | 2011 | Needles | 0 | 0 | 100 |
| 80-15-5 | 2011 | Mixed brown forest residues | 80 | 15 | 5 |
| 60-40-0 | 2012 | Wood and bark | 60 | 40 | 0 |
| 60-30-10 | 2012 | Mixed brown forest residues | 60 | 30 | 10 |
| 43-22-35 | 2012 | Mixed green forest residues | 43 | 22 | 35 |

### 2.1.1. Chemical data of the pyrolysis oils

The characterization techniques applied to the pyrolysis oils were positive and negative electrospray ionization mass spectrometry (ESI-MS) and gas chromatography mass spectrometry (GC-MS); “fingerprinting” techniques providing detection of all compounds in the oils, however without identification and quantification of the individual compounds. The analyses took place at Statoil’s Centre for Research, Development and Innovation (Eide and Neverdal, 2014). Data from the ESI-MS with positive ionization applied on the wood-derived

100-0-0, the bark-derived 0-100-0 and the needle-derived 0-0-100 can be found in Figure 2.1.1.

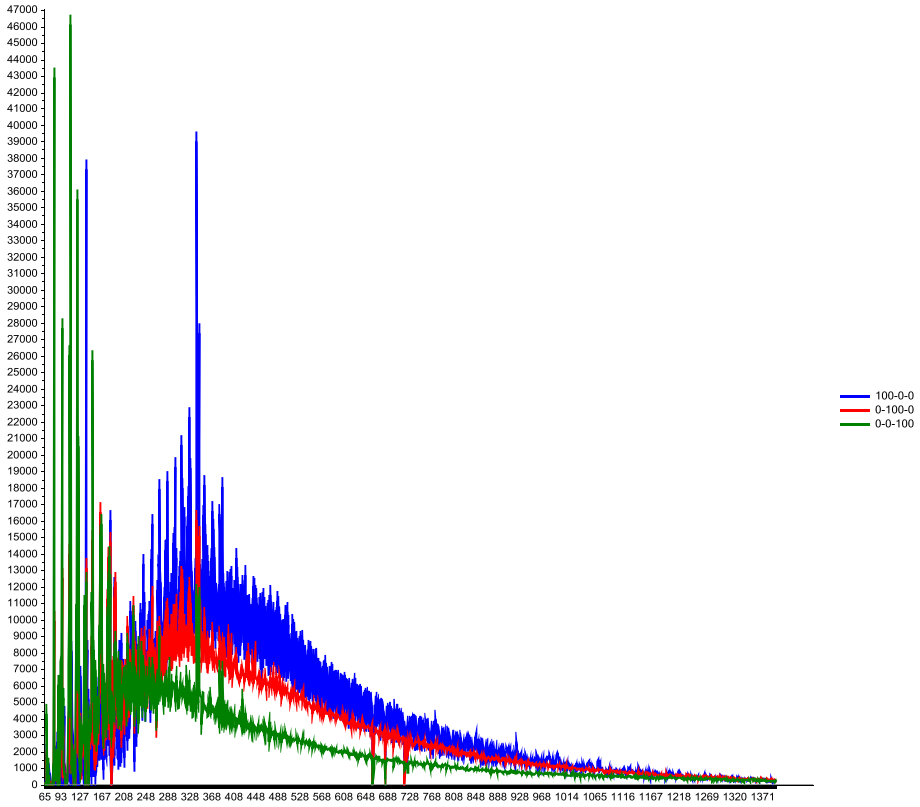


**Figure 2.1.1: Dominant mass numbers of pyrolysis oils produced from feedstocks of wood, bark and needles**. Electronspray ionization mass spectrometry (ESI-MS) was applied with positive ionization for analyzing spruce-derived fast pyrolysis oils produced from wood (blue), bark (red) and needles (green) as feedstock (Eide and Neverdal, 2014). The x-axis represents mass divided by charge (m/z) and the y-axis represents relative intensity.

## 2.2. The Ames *Salmonella* assay

### 2.2.1. Principle of the method

The Ames *Salmonella* assay (Ames et al., 1975, Maron and Ames, 1983) is an *in vitro* assay that measures the mutagenic potential of chemicals, either singly or in complex mixtures. The bacterial strains used in the assay are *Salmonella typhimurium* designed with specific types of point mutations in the histidine operon making them auxotroph to, or unable of synthesizing, histidine and therefore unable to grow into healthy bacterial colonies without addition of this essential amino acid. In the assay the bacterial strains are exposed for 48-72 hours to test chemicals in order to measure the level of back-mutations/reversions in the point mutations from histidine auxotrophy to prototrophy, meaning that the ability to synthesize histidine and grow into colonies is regained. The level of colonies/revertants is used as an expression for the test solution's capacity to induce mutations (Maron and Ames, 1983). Levels of back-mutations in exposed bacteria are compared with the strain-specific spontaneous revertant number in unexposed bacteria in order to distinguish between chemically induced point mutations and spontaneous mutations. Potential bacterial toxicity evoked by the test solutions can be revealed by a microscopic examination showing a thinner background lawn compared to the background lawn of negative controls, together with a decreased number of revertants.

Various strains of *S. typhimurium* are available for detection of different types of chemically induced mutations through the Ames assay. The strains differ from each other by the type of point mutation in the histidine operon, making it possible to discover various types of chemically induced mutations. For example, the strain TA98 is used for detection of frameshift mutations while TA100 detects base pair substitutions. Additional mutations in the strains increase their susceptibility to chemical mutagens and thereby increase the test's ability to detect this type of compounds. The *rfa* mutation weakens the polysaccharide barrier and results in increased cell wall permeability, and the *uvrB* mutation impairs the DNA excision repair system (Maron and Ames, 1983). Some strains, e.g. TA98 and TA100, are also inserted with the R-factor plasmid pKM101 which carries antibiotic resistance genes and contributes to an increased sensibility of the strains (McCann et al., 1975).

*Salmonella* bacteria differ from mammalian cells in their ability to metabolize chemicals and this difference is reduced in the Ames assay by the use of S9 mix; the metabolic system Supernatant at 9000G (S9) with cofactors. The S9 is usually derived from Arochlor 1254 induced rat liver homogenate which has been centrifuged for 10 min. at 9000 G, and contains substantial amounts of biotransformation enzymes. Applying the assay in

presence or absence of S9 mix makes it possible to identify if chemicals are primary or secondary mutagens (Maron and Ames, 1983).

It is possible to modify the Ames standard plate incorporation assay in order to allow testing of a broader range of chemicals, e.g. gases and volatile chemicals, or to increase the sensitivity of the test. The preincubation version of the Ames assay (Yahagi et al., 1975, Nagao et al., 1977) is claimed to be more sensitive in comparison to the standard plate-incorporation assay because the probability of short-lived mutagenic metabolites to react with the bacteria increases and the S9-mixture will get a higher effective concentration (Mortelmans and Zeiger, 2000).

### 2.2.2. Chemicals, solutions, equipment and commodities

| **Chemicals** | **Producer** | **Catalogue nr.** |
|---|---|---|
| Ampicillin tablet (33 µg) | 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 sulphoxide (DMSO) | MERCK | 1.02950.0500 |
| di-Sodiumhydrogenphosphate-Dihydrat ($Na_2HPO_4$ x $2H_2O$ | MERCK | K41725580101 |
| L-Histidine monohydrochloride Monohydrate (≥ 98 %) | SIGMA | H-8125 |
| Magnesiumchloride-Hexahydrat ($MgCl_2$ x $2H_2O$) | MERCK | 1.05833.1000 |
| Sodiumazid ($NaN_3$) | SIGMA | S-2002 |
| 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 ($NaH_2PO_4$ x $H_2O$) | MERCK | 1.06346.0500 |
| Rat-liver LS-9 (Arochlor 1254 induced male Sprague Dawley) | MOLTOX | - |
| Potassium chloride (KCl) | MERCK | 1.04936.1000 |

| **Solutions** | **Appendix** |
|---|---|
| Histidine-biotin solution | A-1 |
| Nutrient agar plates | A-1 |
| Nutrient medium | A-1 |
| Top agar | A-1 |
| S9-cofactor solutions: | A-1 |
| 0.4 M MgCl solution | |
| 0.165 M KCl solution | |
| 0.2 M Sodium di-hydrogen phosphate | |
| 0.2 M Di-sodium hydrogen phosphate | |
| 0.2 M Sodium phosphate buffer, pH 7.4 | A-1 |
| S9 mix (50 µL S9/0.5 mL S9 mix) | A-1 |

| **Equipment and commodities** | **Producer** | **Catalogue nr./Model** |
|---|---|---|
| Automat pipette | Drummon | - |
| Automat pipette (5 mL) | Eppendorf | 88937 |
| Conical flask | SCHOTT DURAN | - |
| Conical flask (100 mL) | PYREX | - |
| Cotton cap | VWR | - |

| | | |
|---|---|---|
| e.pT.I.P.S Standard/Bulk 100-5000 µL (purple) | Eppendorf | 22492080 |
| 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 agar plates | 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 |
| Shaking machine | Edmund Bühler | - |
| Small incubator (Brutschrank Incubat) | MELAG | - |
| Snap-cap vials with caps | - | - |
| Sterile filter (0.45 µm) | SARSTEDT | - |
| Sterile syringe (50 mL) | BD platipak™ | - |
| Vortex | Labinco | L46 |
| Water boiler | KOTTERMAN | 3031 |
| Water bath | KOTTERMAN | 3047 |
| Water bath | Grant | Y22 |

### 2.2.3. Preliminary test – comparing standard procedure to the preincubation version of the Ames assay on pyrolysis oil

In the first Ames experiment with pyrolysis oil a beech-derived fast pyrolysis oil (details not shown) was diluted with autoclaved distilled water to concentrations of 0.04 µL and 0.4 µL oil/plate and applied in the standard plate incorporation assay as well as to the preincubation version of the assay. Based on the results of this experiment (see Appendix B-1) and the fact that the preincubation version of the Ames test previously has been used for assessment of the mutagenic potential of various wood-derived pyrolysis oils (Girard et al., 2005), this version was chosen to test the spruce-derived pyrolysis oils.

### 2.2.4. Main experiment

#### 2.2.4.1. Test solutions

The spruce-derived pyrolysis oils were heated in a 50 ºC water bath for 1 hour to homogenize (G Neverdal, pers comm) before volumes of 750 µl were taken out and diluted with 750 µl of dimethylsulphoxide (DMSO) to stock concentrations of 50 µl oil/100 µl solution. The stock solutions were stored in the dark at room temperature and diluted immediately before application in the Ames test the following day. The concentration range of test solutions was prepared from the stock concentration (50 µL/100 µL) by diluting it with DMSO into a second stock solution which was further used to dilute the lower concentrations (>10-fold

diluting was avoided), and all solutions were mixed by pipetting the mixture up and down several times.

### 2.2.4.2. Procedure for the preincubation version of the Ames assay

The bacterial strains used for testing of the seven spruce-derived pyrolysis oils were TA98 and TA100, previously received from Dr. B. N. Ames at Berkley in California, stored at -80 ºC. The preincubation version of the Ames assay was applied according to descriptions by (Yahagi et al., 1975, Nagao et al., 1977), and for practical reasons one small modification was made; instead of preincubating solutions for each plate separately it was chosen to preincubate solutions in volumes sufficient for all parallel plates (3 or 5). The S9 originated from rat liver homogenate of Arochlor 1254 induced individuals and was stored at -80 ºC.

A bacteria suspension of approximately $10^9$ bacteria per mL was cultivated in nutrition medium using a shaking incubator at 120 rpm and 37 ºC for approximately 16 hours. The S9-mix was made at a concentration of 50 µL S9/0.5 mL S9-mix prepared right before use and kept on ice after sterile filtration (0.45 µm). The ranges of test concentrations (0.1 µL to 10 µL per plate) were then prepared. Top agar with 10 % histidine-biotin was transferred to glass tubes, 2 mL/tube, and kept in a 45.3 ºC water bath to prevent hardening of the top agar. The 0.2 M Na-phosphate buffer was diluted 1:1 with autoclaved distilled water. Depending on the number of parallels (n = 3 or 5), solutions for preincubation without S9-mix were made by adding 2500 or 3000 µL of 0.1 M Na-phosphate buffer, 500 or 600 µL of test solution and 500 or 600 µL of bacterial culture to snap-cap vials. When S9-mix was used 350 µL of test solution was added first, followed by S9-mix in a volume of 1750 µL and finally 350 µL bacterial culture was added before preincubation. The preincubation of the solutions was performed with shaking for 20 min at 37 ºC in the absence of light. Volumes of 700 µl of preincubation solution were added to the glass tubes containing histidine-biotin/top-agar, and the mixture was immediately poured onto minimal agar plates after mixing by the use of a vortex machine. Spontanous controls were applied without preincubation. The plates were incubated at 37 ºC and bacteria colonies were counted by hand after 48 and 72 hours. Microscope examination of the background flora of non-reverted bacteria was performed to check for potential toxic effects, measured by reduced background flora.

Quality of the test system was confirmed during the period lasting from the first to the last experiment, but had also been reassured by previous experience and frequent use in the

laboratory at the Department of Biology, NTNU. The integrity of the strains was confirmed repeatedly by several control tests; check of spontaneous reversion frequencies and physical conditions when applying only tester strains in the assay, positive controls of 4-nitro-*o*-phenylenediamine (NPD) (20 μg/100 μL), Na-azid (1 μg/100 μL), BaP (1 μg/100 μL) and solvent controls of DMSO with 0.1 M Na-phosphate buffer or S9-mix. Controls without preincubation consisting of BaP or DMSO were applied to each batch of S9-mix in the experiments involving S9-usage. Additionally, two nutrient plates of each strain were incubated in presence of an ampicillin tablet and crystal violet at both ends of the test period to ensure the presence of the R-factor plasmid and the *rfa* mutation, respectively.

### 2.2.4.3. Choice of test concentrations and S9-mix usage

The first part of mutagenicity testing of the seven spruce-derived oils aimed to estimate concentration-effect relationships, with mutagenicity as the response, in the absence of S9-mix. Both bacteria strains were exposed to oil concentrations ranging from 0.1 - 10 μL oil/plate (Table 2.2.4.3.1) with three parallel plates per concentration, in addition to DMSO with five parallel plates. Several experiments were applied per oil (Appendix C-1). Most of the oils were first tested one by one using various concentrations of oil per plate and for the oils not showing toxicity at the higher oil concentrations the concentration range was extended in a following experiment. Toxicity was defined as decreased background flora and in order to compare the toxicity between different oils and concentrations, background toxicity levels (BTLs) from 0 to 3 were defined as: (0) healthy background (1) thinner than a healthy background flora (2) more spread out and very thin background flora (3) extremely thin to almost invisible background flora.

**Table 2.2.4.3.1: Pyrolysis oil concentrations (µL/plate) applied in the Ames assay**. Establishment of concentration-effect relationships for the seven spruce-derived fast pyrolysis oils, with primary mutagenicity as the response, was obtained by application of different oil-DMSO dilutions.

| | 100-0-0 | | 0-100-0 | | 0-0-100 | | 80-15-5 | | 60-40-0 | | 60-30-10 | | 43-22-35 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| µL oil/plate | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 |
| 0.1 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 0.25 | + | + | + | + | | | | | | | | | | |
| 0.5 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 0.75 | + | + | + | + | | | | | | | | | | |
| 1 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 1.5 | | | | | | | + | + | | | | + | | |
| 2 | | | + | + | + | + | + | + | + | + | + | + | + | + |
| 3 | | | | + | | | | | | | + | | | |
| 4 | | | + | + | | | | | + | + | + | | + | + |
| 5 | + | + | + | + | + | + | + | + | | | + | + | | |
| 6 | | | + | | + | + | | | + | + | | | + | + |
| 8 | | | + | | + | + | | | | | | | | |
| 10 | | | + | | + | + | | | | | | | | |

After experiencing how the primary mutagenicity and toxicity turned out with the different doses in the first testing part, the choise of concentrations for testing with S9-mix fell on 0.1, 0.5, 1.0, 2.0 and 5.0 µL oil/plate for application on both bacteria strains, and the concentration range was extended in further tests for the oils not reaching toxicity with the first applied concentration range (Table 2.2.4.3.2 and Appendix C-1).

**Table 2.2.4.3.2: Pyrolysis oil concentrations (µL/plate) applied in the Ames assay with S9**. Establishment of concentration-effect relationships for the seven spruce-derived fast pyrolysis oils, with secondary mutagenicity as the response, was obtained by application of different oil-DMSO-dilutions and S9 in the Ames assay.

| | 100-0-0 | | 0-100-0 | | 0-0-100 | | 80-15-5 | | 60-40-0 | | 60-30-10 | | 43-22-35 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| µL oil/plate | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 |
| 0.1 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 0.5 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 1 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 1.5 | + | + | | | | | | + | | + | | + | | |
| 2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 3 | + | + | + | | | | + | + | + | + | + | + | + | |
| 4 | + | + | + | | | | + | + | + | + | + | + | + | |
| 5 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 6 | | | | + | + | | | | | | | | | + |
| 8 | | | | + | + | | | | | | | | | + |
| 10 | | | | + | + | | | | | | | | + | + |

### 2.2.5. Raw data treatment and interpretation of results

The numbers of revertants after 48 h incubation were used for graphical presentation by the use of Microsoft Office Excel 2007 and Sigmaplot version 12.5. Results were considered positive when fulfilling the criteria of being a reproducible and dose-related increase in the revertant number (de Serres and Shelby, 1979), as well as reaching a minimum of double the number of colonies seen in the spontaneous number (Krøkje et al., 1985).

#### 2.2.5.1. Statistical analysis

Multivariate data analysis relating mutagenic potential of the seven pyrolysis oils to their feedstock composition consisted of application of partial least square (PLS) regression and was performed by my co-supervisor, PhD Ingvar Eide, Centre for Research, Development and Innovation, Statoil ASA, using the Unscrambler X 10.3, Camo Software, Oslo, Norway. The statistical results were based on the numbers of reversions induced by the pyrolysis oils at nontoxic concentrations and which gave increasing mutagenic response with increasing concentration. The numbers of revertants from each parallel plate (n = 3 or 5) from all experiments performed with the same concentrations and under the same test conditions were used for linear regression; plotting oil concentration (μL/plate) against the number of induced revertants, where the slope of the line was used as a measure of the mutagenic potential in the statistical models. Values of mutagenic potential were based on both positive test results and results where the doubling criterion was not fulfilled. The PLS regression component analysis was based on an X-matrix consisting of three predictor variables (wood, bark and needles) and a Y-matrix consisting of four response variables (mutagenic responses TA98, TA98 + S9, TA100 and TA100 + S9), and the data set of seven observations (bio-oils).

# 3. Results

Results from the preincubation version of the Ames *Salmonella* assay are presented for one of the seven pyrolysis oils at a time, followed by statistical analysis relating feedstock composition to mutagenic potential of the oils. Raw data for the Ames assay results without and with S9 can be found in Appendix D-1 and D-2, respectively.

## 3.1 Controls

The number of spontaneous reversions, DMSO controls for all the 11 experiments as well as results on the positive controls NPD, Na-azid and BaP are given in Table 3.1.1. The results are presented as mean values ± SD of the number of revertants from the parallel plates (n = 3-5) of the controls from each experiment. Spontaneous reversions in the bacterial strain TA98 varied from 15.8 to 25 with a maximum SD of 7.66 and in TA100 the spontaneous reversion frequencies ranged from 98.4 to 116.8 with a maximum SD of 12.14. Controls with DMSO varied from 12 to 25.2 with SD values up to 7.27 in TA98 and the number of revertants ranged from 92.4 to 115.4 with SD values up to 12.78 in TA100. The number of revertants in NPD controls ranged from 1059 to 2186 with a maximum SD of 296.39, while the Na-azid controls gave reversions ranging from 650 to 939 and SD values up to 100.43. Controls with BaP induced reversions from 109 to 119 with SD values up to 18.36 in TA98, and in TA100 the number of revertants in BaP controls varied from 346.7 to 410 with a maximum SD of 51.07. Results on BaP and DMSO controls for each S9-batch can be found in Appendix D-2.

The ampicillin and crystal violet tests gave the same results at both ends of the experiment period; bacterial growth adjacent to the ampicillin tablet and absence of bacterial growth adjacent to crystal violet.

**Table 3.1.1: Controls of the Ames *Salmonella* assay test system with bacterial strains TA98 and TA100 used on the seven pyrolysis oils.** Spontaneous reversions, DMSO controls as well as BaP (1 µg/100 µL), NPD (20 µg/100 µL) and Na-azid (1 µg/100 µL) controls were registered in the 11 experiments (Exp.) assessing pyrolysis oil in the preincubation version of the Ames assay. Results are presented as mean values of the number of reversions ± SD (n = 3-5) observed after 48 hours incubation.

| TA98 | Spontaneous | | DMSO + buffer | | BaP + S9 | | NPD + buffer | |
|---|---|---|---|---|---|---|---|---|
| Exp. nr | **Mean** | ± SD | **Mean** | ± SD | **Mean** | ± SD | **Mean** | ± SD |
| 1 | **18,2** | 4,87 | **14** | 2,92 | - | - | - | - |
| 2 | **25** | 4,18 | **18,2** | 2,95 | - | - | - | - |
| 3 | **19,2** | 4,38 | **13,4** | 4,39 | - | - | **1682** | 46,67 |
| 4 | **19,2** | 7,66 | **12** | 1,87 | - | - | **1059** | 91,92 |
| 5 | **21,3** | 2,5 | **17,2** | 3,27 | - | - | **1971** | 1,41 |
| 7 | **20,8** | 4,82 | **24,6** | 7,27 | **109** | 11,14 | **1746,7** | 296,39 |
| 8 | **15,8** | 2,86 | **15,6** | 3,58 | - | - | **2186** | 48,08 |
| 11 | **22,8** | 4,82 | **25,2** | 3,11 | **119** | 18,36 | **1225,3** | 68,97 |
| | | | | | | | | |
| TA100 | Spontaneous | | DMSO + buffer | | BaP + S9 | | Na-azid + buffer | |
| Exp. nr | **Mean** | ± SD | **Mean** | ± SD | **Mean** | ± SD | **Mean** | ± SD |
| 1 | **108,6** | 11,67 | **115,4** | 9,04 | - | - | - | - |
| 2 | **100,2** | 3,11 | **92,4** | 6,31 | - | - | - | - |
| 3 | **98,4** | 7,33 | **95** | 9,38 | - | - | **681** | 49,5 |
| 4 | **110,2** | 7,33 | **113,2** | 11,65 | - | - | **939** | 12,73 |
| 5 | **110** | 12,14 | **105,6** | 12,78 | - | - | **847** | 12,73 |
| 6 | **107,6** | 5,94 | **109,2** | 4,44 | **410** | 51,07 | **682** | 81,17 |
| 9 | **110,6** | 9,07 | **107,6** | 8,14 | - | - | **650** | 26,91 |
| 10 | **116,8** | 8,35 | **108** | 5,34 | **346,7** | 26,63 | **850,7** | 100,43 |

## 3.2 Concentration-effect relationships for the oils' mutagenic potential

Mutagenic response in the bacterial strains TA98 and TA100 in both absence and presence of S9 is presented for each of the seven pyrolysis oils in the following order: 100-0-0, 0-100-0, 0-0-100, 80-15-5, 60-40-0, 60-30-10 and 43-22-35 (Figure 3.2.1-3.2.7). Lastly, concentration-effect relationships for all oils are included in the same illustration for comparison (Figure 3.2.8). Mutagenicity induced by the oils under the different test conditions is illustrated as a scatter-plot of the number of revertants registered against the DMSO controls and the concentration range of test solutions. Results obtained from more than one experiment are illustrated by different symbols representing the first, second and third experiments (circles, triangles and rectangles, respectively). Results for the total concentration ranges tested are illustrated in concentration-effect relationships, but only numbers of reversions within a linear range of concentration-effect relationship were used further on in the multivariate data analyses. Mean values of the numbers of revertants observed in parallel plates from the one to three experiments under the same concentrations and test conditions were used for drawing a line representing the average mutagenic response through the concentration ranges of test solutions. Background toxicity levels (BTLs), classified from one to three, are included in the concentration-effect relationships for each oil as crosses along the x-axes. Only reversions

after 48 hours of incubation are illustrated, as there was only a slight increase of reversions from 48 to 72 hours (Appendix D-1 and D-2), which did not seem to affect the overall trends in mutagenic responses. With few exceptions, the three or five parallel plates gave numbers of revertants within a relatively narrow range and no trends in the mutagenic response were seen related to the order in which the plates were poured. However, at concentrations evoking toxicity an increased variation in the number of reversions between parallel plates was often observed.

### 3.2.1. Mutagenic potential of pyrolysis oil 100-0-0

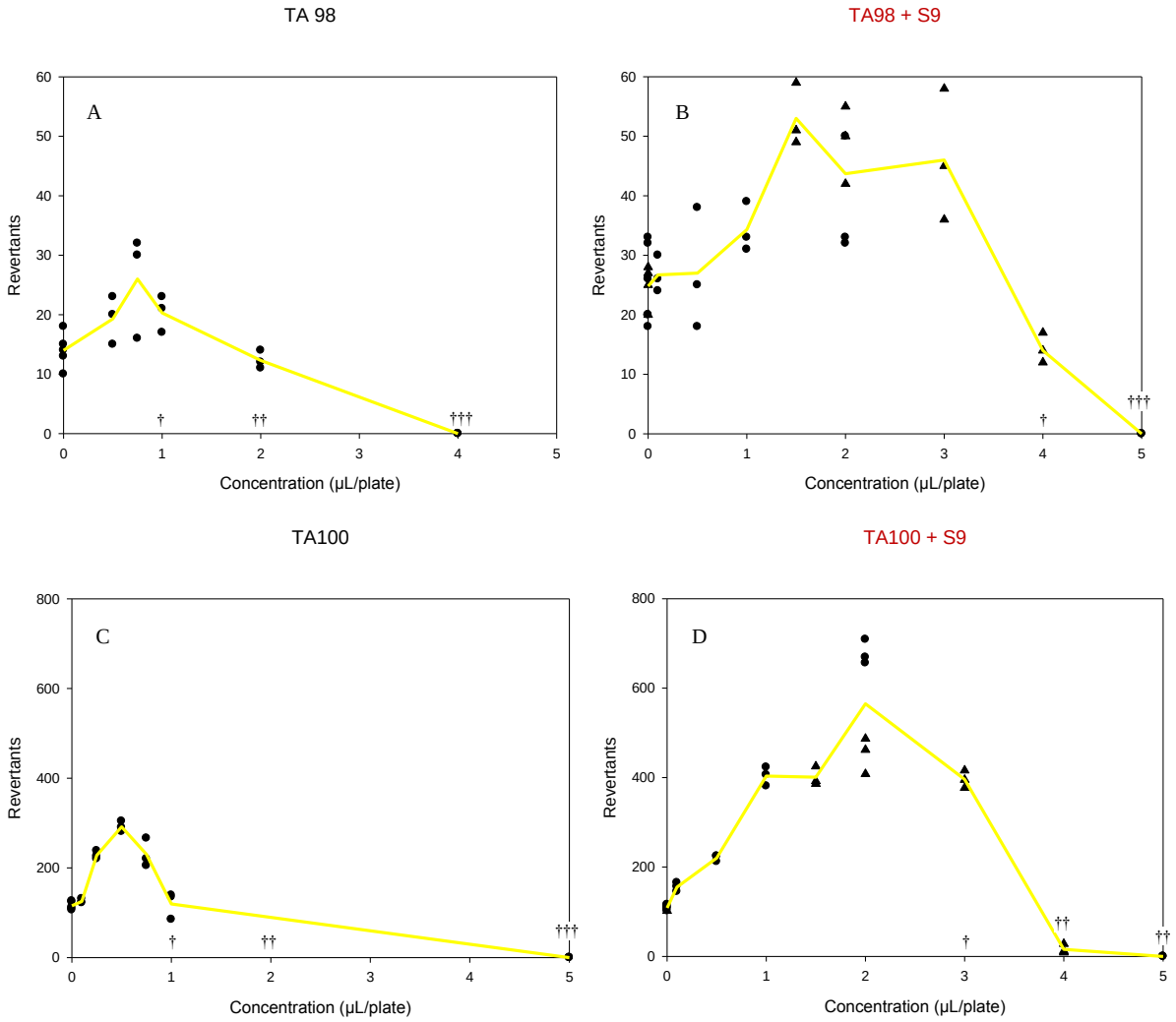


**Figure 3.2.1: Concentration-effect relationships for mutagenic response induced by pyrolysis oil 100-0-0**. The preincubation version of the Ames *Salmonella* assay was applied using bacteria strains TA98 (A), TA98 + S9 (B), TA100 (C) and TA100 + S9 (D) exposed to a concentration range (µL/plate) of spruce-derived fast pyrolysis oil obtained from a feedstock of 100 % wood for measuring levels of oil-induced revertants in order to evaluate the oil's mutagenic potential. Mean values of the number of revertants from parallel plates from all experiments under the same test conditions were used for graphical illustration of concentration-effect relationships. The results on relationships obtained from more than one experiment are represented by different symbols (circles and triangles, chronologically). Bacterial background toxicity levels (BTLs) from one to three are represented by crosses along the x-axis.

The test solutions from the wood derived oil 100-0-0 induced a positive mutagenic response in TA98 with S9 as well as TA100 with and without S9, while the doubling criterion for demonstrating a positive test result was not met in TA98 without S9 (Figure 3.2.1). Reversions increased for all four test conditions up to a certain concentration level which evoked toxicity, as illustrated by the BTLs. The most prominent mutagenic response was observed at 2 μL/plate in TA100 with S9, where numbers of reversions over fivefold the number of spontaneous reversions were induced. Both strains showed higher reversion levels and decreased toxicity in the presence of S9. Only the concentration-effect relationships with S9 were derived from two experiments, and the number of reversions increased and decreased in the second experiment with TA98 and TA100, respectively (presented as triangles in Figure 3.2.1B and D).

### 3.2.2. Mutagenic potential of pyrolysis oil 0-100-0

The bark-derived 0-100-0 induced positive test results for TA100 with and without S9 as well as for TA98 without S9, while the results for TA98 with S9 did not meet the criteria for a positive result as the number of revertants was relatively similar throughout the whole concentration range (Figure 3.2.2). The most prominent mutagenic response was seen in TA100 in the presence of S9 at 5 μL/plate, where above a threefold increase of reversions compared to spontaneous reversions was registered. The oil evoked toxicity only to TA100 without S9 at 10 μL/plate. Reproducibility between results from different experiments under the same circumstances was observed for TA98, but was not seen between the experiments with TA100. The second experiments applied to TA100 without and with S9 (presented as triangles in Figure 3.2.2C and D) showed an increase and decrease, respectively, in the number of revertants in comparison to the first experiment (presented as circles in Figure 3.2.2 C and D) with the number of reversions approximately doubling from the first to the second experiment in TA100 with S9. The third experiment applied to TA100 without S9 (presented as rectangles in Figure 3.2.2C) induced a decrease in the number of revertants in comparison to the second experiment. TA98 without S9 and TA100 with S9 were tested at concentrations up to 10 μL/plate; double the maximum concentration applied to TA98 with S9 and TA100 without S9.

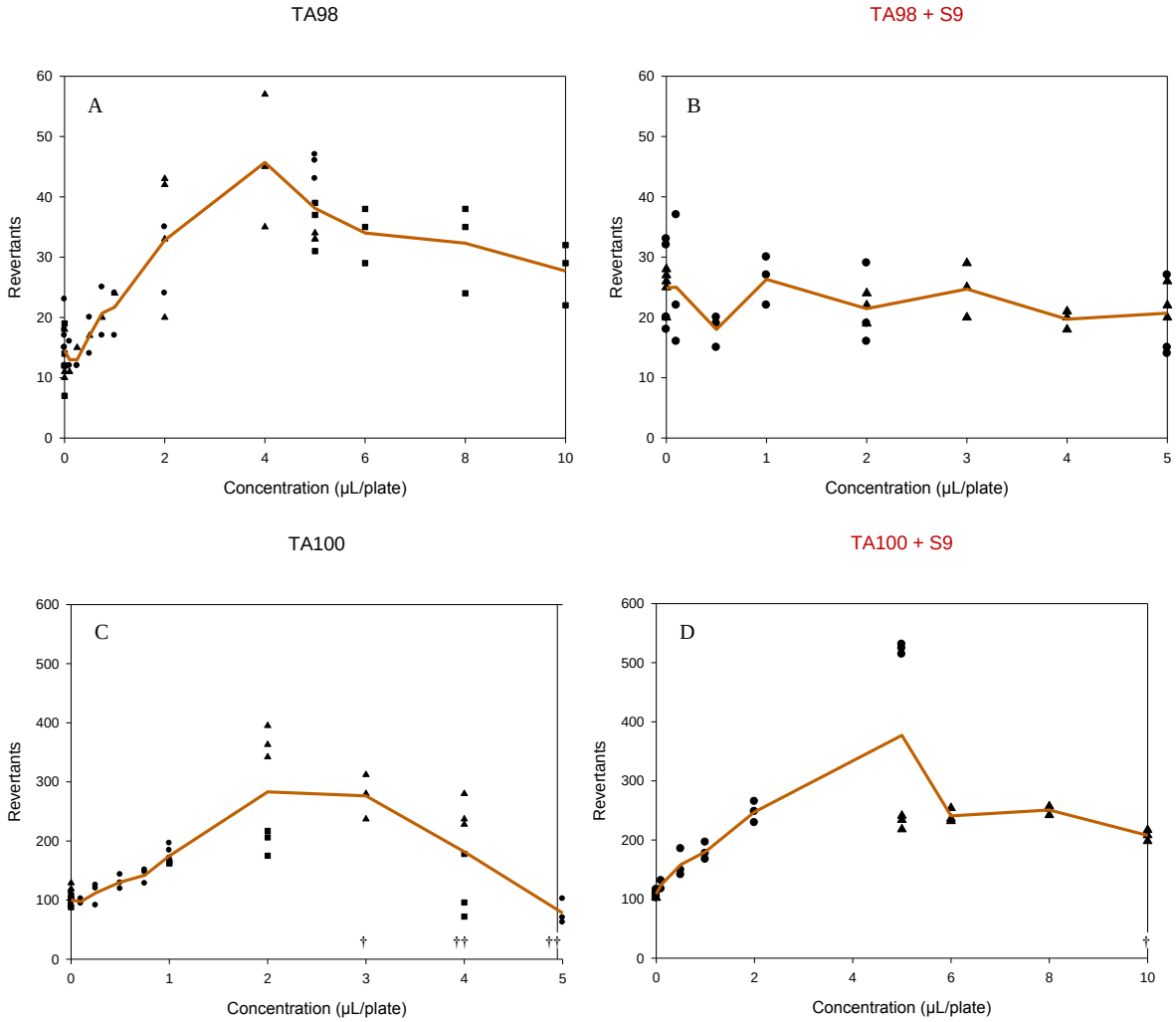


**Figure 3.2.2: Concentration-effect relationships for mutagenic response induced by pyrolysis oil 0-100-0**. The preincubation version of the Ames *Salmonella* assay was applied using bacterial strains TA98 (A), TA98 + S9 (B), TA100 (C) and TA100 + S9 (D) exposed to a concentration range (µL/plate) of spruce-derived fast pyrolysis oil obtained from a feedstock of 100 % bark for measuring levels of oil-induced revertants in order to evaluate the oil's mutagenic potential. Mean values of the number of revertants from parallel plates from all experiments under the same test conditions were used for graphical illustration of concentration-effect relationships. Results on concentration-effect relationships obtained from more than one experiment are represented by different symbols (circles, triangles and rectangles, chronologically). Bacterial background toxicity levels (BTLs) from one to three are represented by crosses along the x-axis.

### 3.2.3. Mutagenic potential of pyrolysis oil 0-0-100

The needle-derived 0-0-100 induced a slight concentration-dependent increase in reversions in TA98 with and without S9 as well as in TA100 without S9, a tendency which was not observed in TA100 with S9 (Figure 3.2.3). The criteria for a positive test result were not met under any of the four test conditions, as the doubling criterion was not fulfilled at any concentration, nor was toxicity observed in any group even at the highest concentrations. With close to a twofold increase in reversions compared to spontaneous reversions induced at 2 µL/plate, TA98 without S9 was the response reaching closest to fulfilling the doubling

criterion. Bacteria strain TA100 with S9 did not respond to the oil with a concentration-dependent increase in the number of revertants and was therefore not tested any further, while the follow-up experiments in the other groups (presented as triangles in Figure 3.2.3A-C) resulted in a slight decrease in the number of revertants.

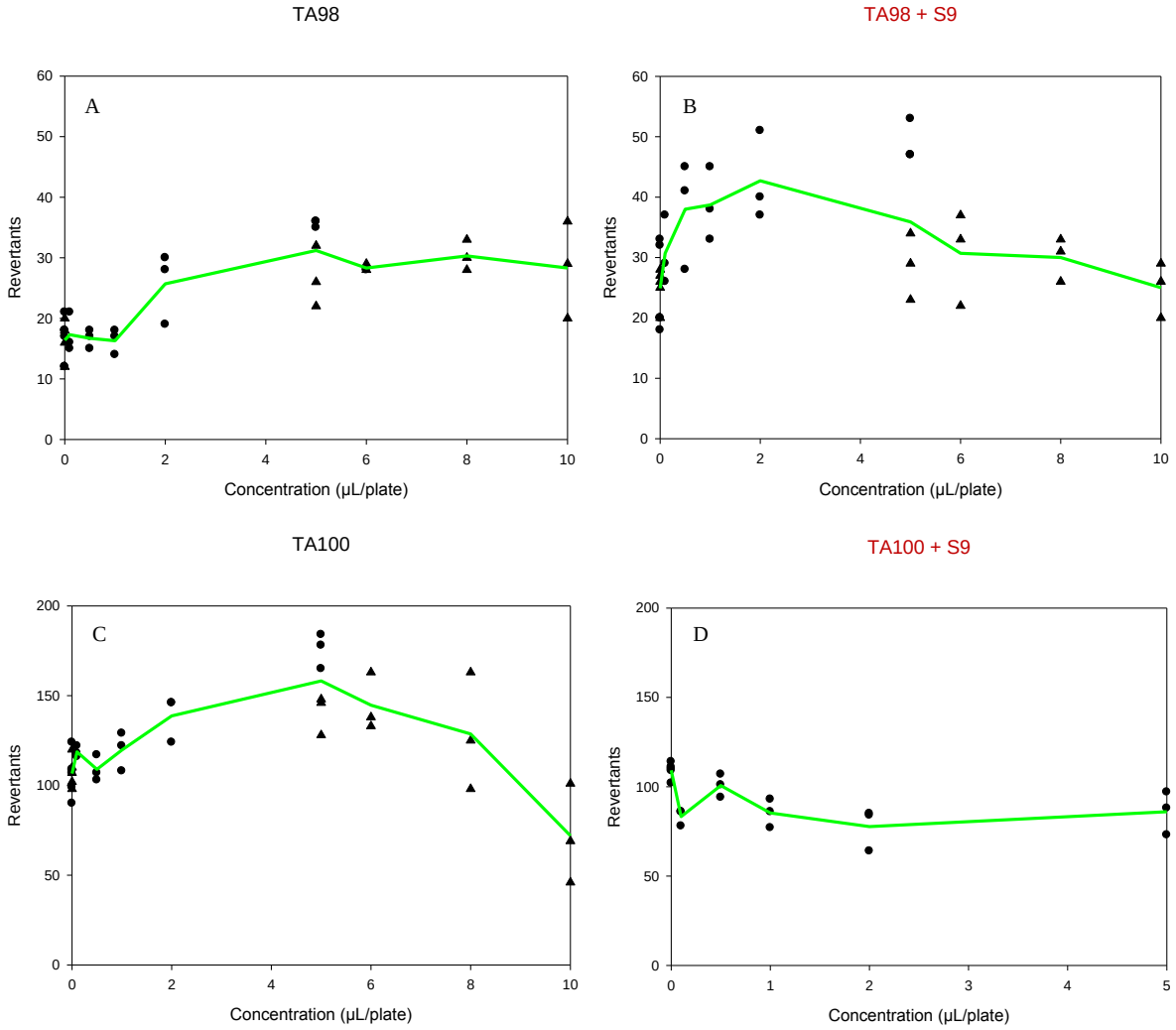


**Figure 3.2.3: Concentration-effect relationships for mutagenic response induced by pyrolysis oil 0-0-100**. The preincubation version of the Ames *Salmonella* assay was applied using bacterial strains TA98 (A), TA98 + S9 (B), TA100 (C) and TA100 + S9 (D) exposed to a concentration range (µL/plate) of spruce-derived fast pyrolysis oil obtained from a feedstock of 100 % needles for measuring levels of oil-induced revertants in order to evaluate the oil's mutagenic potential. Mean values of the number of revertants from parallel plates from all experiments under the same test conditions were used for graphical illustration of concentration-effect relationships. The results on relationships obtained from more than one experiment are represented by different symbols (circles and triangles, chronologically). Bacterial background toxicity levels (BTLs) from one to three are represented by crosses along the x-axis.

## 3.2.4. Mutagenic potential of pyrolysis oil 80-15-5

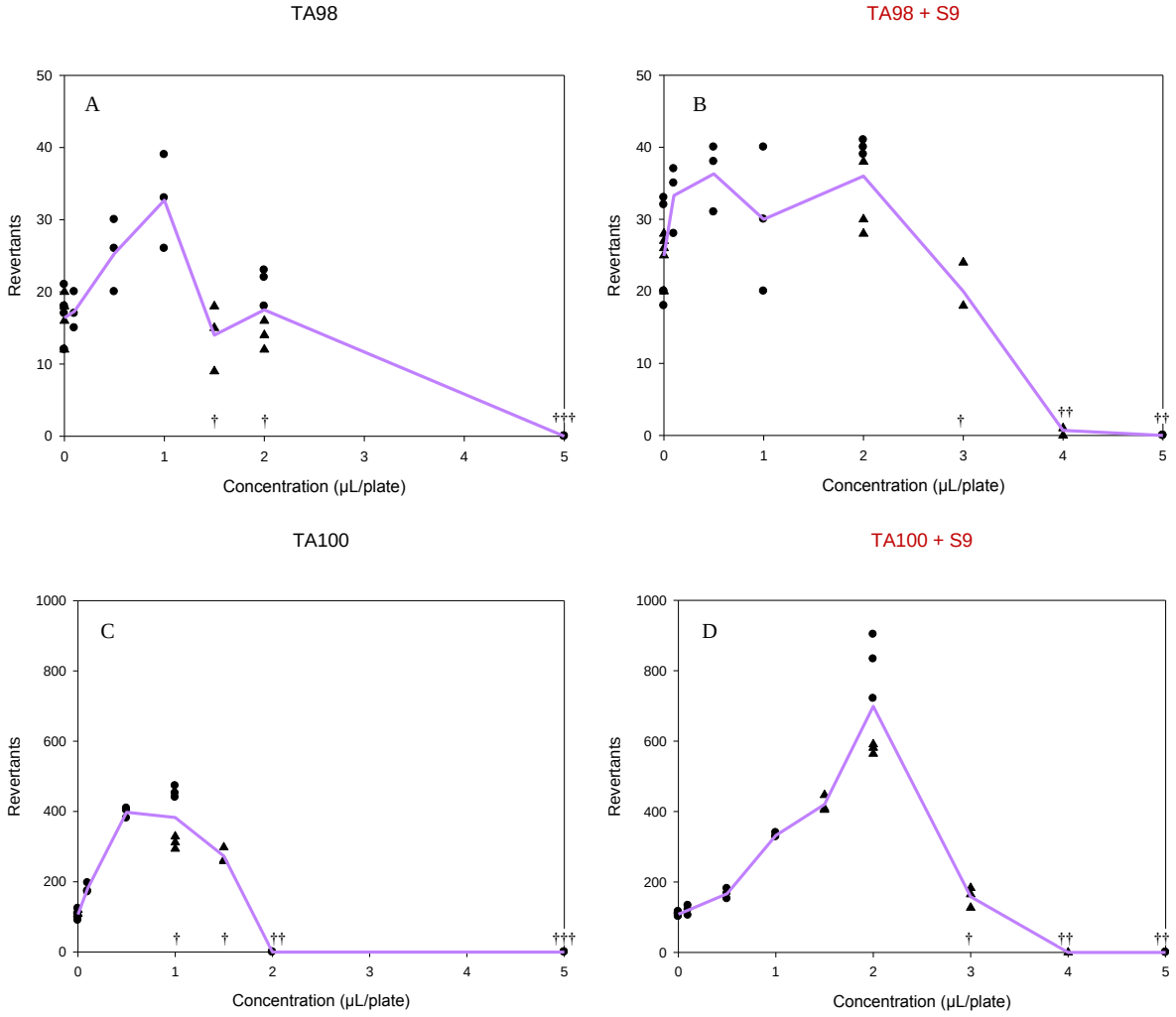


**Figure 3.2.4: Concentration-effect relationships for mutagenic response induced by pyrolysis oil 80-15-5**. The preincubation version of the Ames *Salmonella* assay was applied using bacterial strains TA98 (A), TA98 + S9 (B), TA100 (C) and TA100 + S9 (D) exposed to a concentration range (µL/plate) of spruce-derived fast pyrolysis oil obtained from a feedstock of 80 % wood, 15 % bark and 5 % needles for measuring levels of oil-induced revertants in order to evaluate the oil's mutagenic potential. Mean values of the number of revertants from parallel plates from all experiments under the same test conditions were used for graphical illustration of concentration-effect relationships. Results on concentration-effect relationships obtained from more than one experiment are represented by different symbols (circles and triangles, chronologically). Bacterial background toxicity levels (BTLs) from one to three are represented by crosses along the x-axis.

A positive test result was induced by 80-15-5 in TA98 without S9 as well as in TA100 with and without S9, but the criteria for a positive mutagenic response were not fulfilled in TA98 with S9, as the number of revertants did not reach double the number of spontaneous reversions nor did the mutagenic response seem to have a concentration-dependent increase in reversions before reaching toxicity-evoking concentrations (Figure 3.2.4). An above sixfold increase in reversions compared to spontaneous levels was observed with 2 µL/plate exposed to TA100 with S9, making it the most prominent mutagenic response observed in 80-15-5 and

the main experiment in total. Toxicity was observed in both strains with and without S9 and was decreased in the presence of S9. The second experiment in every group (presented as triangles in Figure 3.2.4A-D) induced a decreased number of revertants in comparison to the first experiment (presented as circles in Figure 3.2.4A-D).

## 3.2.5. Mutagenic potential of pyrolysis oil 60-40-0

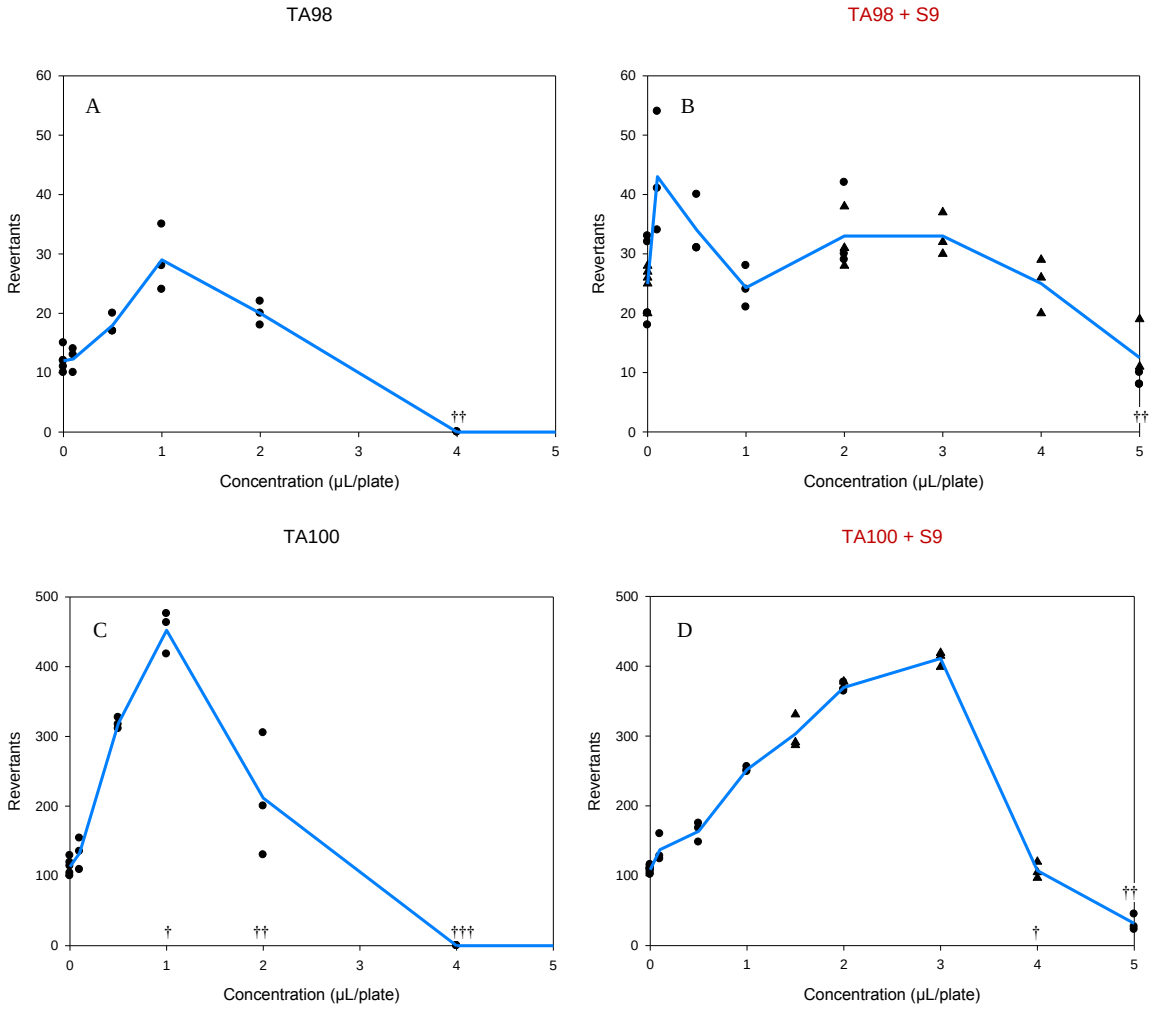


**Figure 3.2.5: Concentration-effect relationships for mutagenic response induced by pyrolysis oil 60-40-0**. The preincubation version of the Ames *Salmonella* assay was applied using bacterial strains TA98 (A), TA98 + S9 (B), TA100 (C) and TA100 + S9 (D) exposed to a concentration range (µL/plate) of spruce-derived fast pyrolysis oil obtained from a feedstock of 60 % wood and 40 % bark for measuring levels of oil-induced revertants in order to evaluate the oil's mutagenic potential. Mean values of the number of revertants from parallel plates from all experiments under the same test conditions were used for graphical illustration of concentration-effect relationships. The results on relationships obtained from more than one experiment are represented by different symbols (circles and triangles, chronologically). Bacterial background toxicity levels (BTLs) from one to three are represented by crosses along the x-axis.

Test solutions of pyrolysis oil 60-40-0 induced a positive test result in TA100 with and without S9, while the results for TA98 did not fulfill the criteria for a positive result, as fulfillment of the doubling criterion lacked in both absence and presence of S9 and no clear relationship between concentration and effect was observed in TA98 with S9 (Figure 3.2.5). The most pronounced mutagenic response was observed at 1 μL/plate in TA100 without S9, reaching numbers of revertants with over a fourfold increase in comparison to the spontaneous reversions, a slightly higher oil-induced increase in reversions than the increase observed in TA100 with S9. Toxicity was evoked in all four groups and seemed to decrease in the presence of S9. The experiments on both strains with S9 seemed to give reproducible results.

### 3.2.6. Mutagenic potential of pyrolysis oil 60-30-10

A positive test result was induced with testsolutions of 60-30-10 in TA100 both with and without S9, while in bacterial strain TA98 only a slight increase in number of revertants was observed without S9 and a relatively flat concentration-effect relationship was seen before toxic levels were reached in presence of S9 (Figure 3.2.6). Toxicity was evoked in both strains and decreased in the presence of S9. Above a threefold increase in reversions compared to spontaneous levels was registered at 2 μL/plate in TA100 with S9, which was the strongest registered mutagenic response induced by 60-30-10. Follow-up experiments in all groups (presented as triangles in Figure 3.2.6A-D) gave a slight decrease in the number of revertants in comparison to the first experiments (presented as circles in Figure 3.2.6A-D).

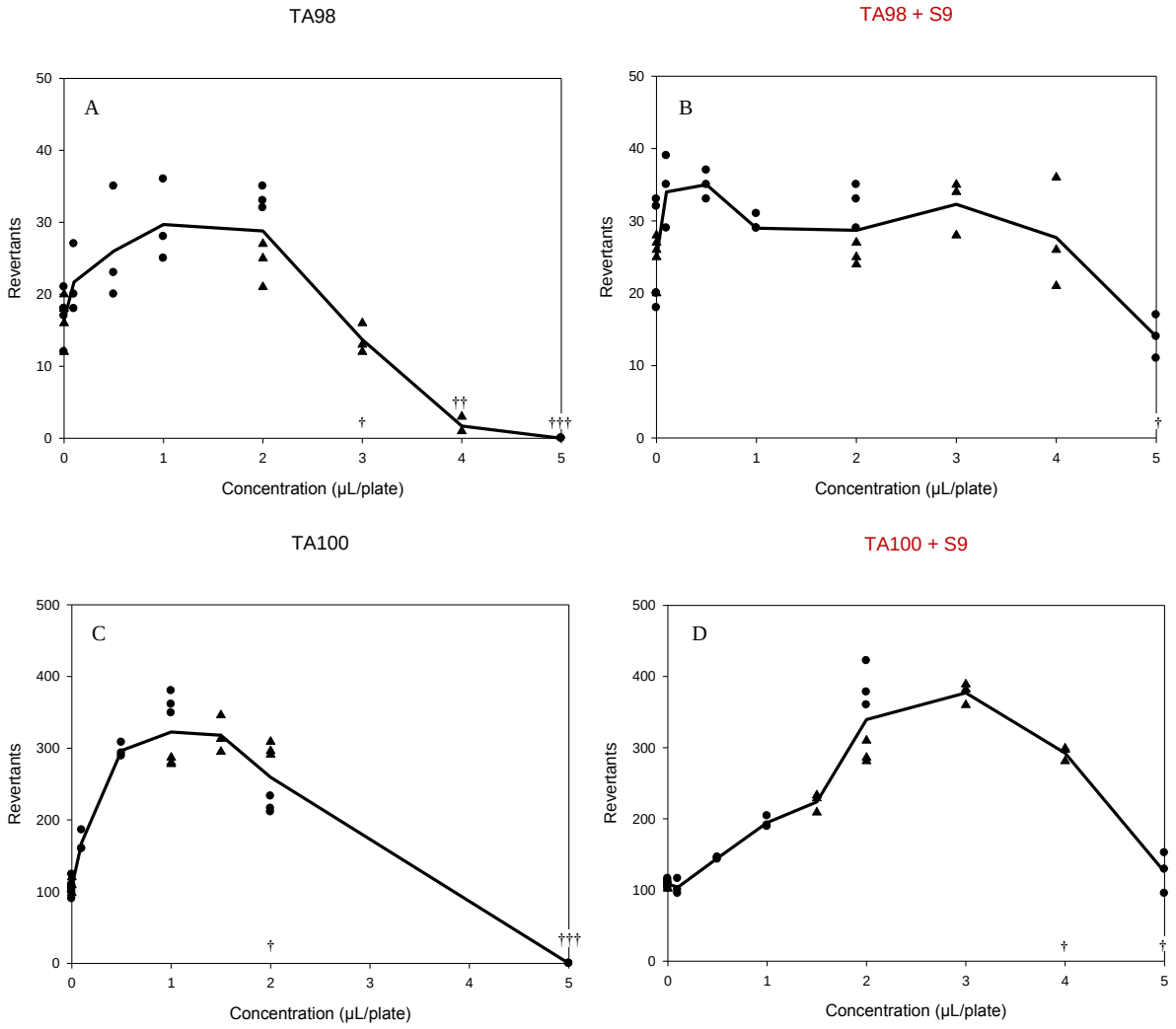


**Figure 3.2.6: Concentration-effect relationships for mutagenic response induced by pyrolysis oil 60-30-10**. The preincubation version of the Ames *Salmonella* assay was applied using bacterial strains TA98 (A), TA98 + S9 (B), TA100 (C) and TA100 + S9 (D) exposed to a concentration range (µL/plate) of spruce-derived fast pyrolysis oil obtained from a feedstock of 60 % wood, 30 % bark and 10 % needles for measuring levels of oil-induced revertants in order to evaluate the oil's mutagenic potential. Mean values of the number of revertants from parallel plates from all experiments under the same test conditions were used for graphical illustration of concentration-effect relationships. The results on relationships obtained from more than one experiment are represented by different symbols (circles and triangles, chronologically). Bacterial background toxicity levels (BTLs) from one to three are represented by crosses along the x-axis.

## 3.2.7. Mutagenic potential of pyrolysis oil 43-22-35

Pyrolysis oil 43-22-35 induced a positive mutagenic response in TA100 in both absence and presence of S9 before reaching concentrations evoking toxicity, while in TA98 in the absence of S9 the doubling criterion was not met and the presence of S9 in TA98 gave a rather flat concentration-effect relationship and thereby not fulfilling the criteria for a positive test result (Figure 3.2.7). The most pronounced mutagenic response was observed at 2 µL/plate in TA100 without S9, reaching a threefold increase in reversions compared to the spontaneous level. Reproducibility was observed between results from the first and second experiments in

the presence of S9 (presented as circles and triangles in Figure 3.2.7B and D, chronologically), while follow-up experiments without S9 was not performed. Toxicity was evoked in both strains without S9 while toxicity was not reached in TA98 with S9 and decreased in TA100 with the addition of S9.

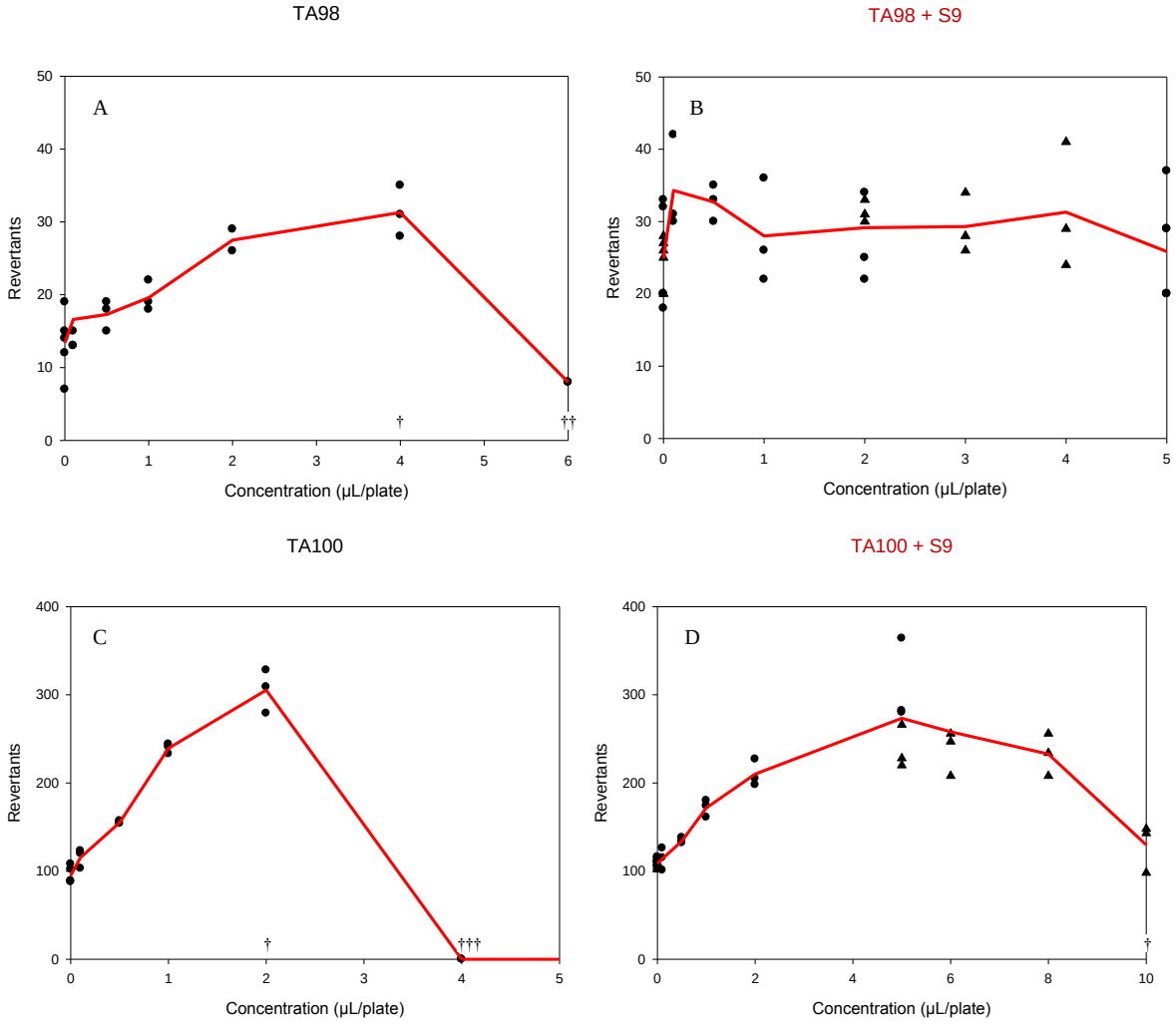


**Figure 3.2.7: Concentration-effect relationships for mutagenic response induced by pyrolysis oil 43-22-35.** The preincubation version of the Ames *Salmonella* assay was applied using bacterial strains TA98 (A), TA98 + S9 (B), TA100 (C) and TA100 + S9 (D) exposed to a concentration range (µL/plate) of spruce-derived fast pyrolysis oil obtained from a feedstock of 43 % wood, 22 % bark and 35 % needles for measuring levels of oil-induced revertants in order to evaluate the oil's mutagenic potential. Mean values of the number of revertants from parallel plates from all experiments under the same test conditions were used for graphical illustration of concentration-effect relationships. The results on relationships obtained from more than one experiment are represented by different symbols (circles and triangles, chronologically). Bacterial background toxicity levels (BTLs) from one to three are represented by crosses along the x-axis.

### 3.2.8. Comparison of mutagenic responses between pyrolysis oils

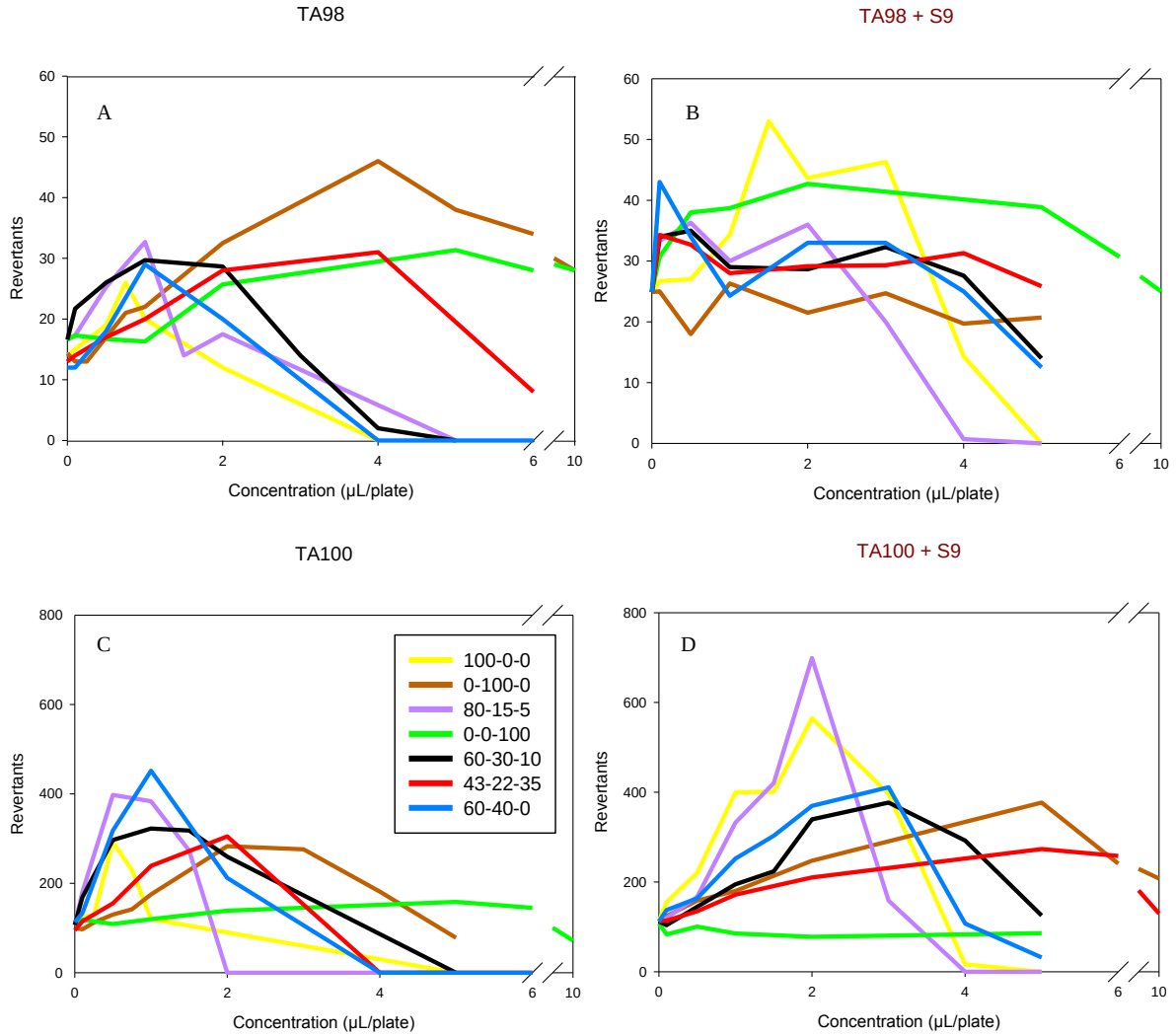


**Figure 3.2.8: Concentration-effect relationships for mutagenic response in the Ames assay induced by the seven pyrolysis oils**. A preincubation version of the Ames *Salmonella* assay was applied using bacterial strains TA98 (A), TA98 + S9 (B), TA100 (C) and TA100 + S9 (D) exposed to a concentration range (µL/plate) of spruce-derived fast pyrolysis oil obtained from a feedstock of different proportions of wood, bark and needles for measuring levels of oil-induced revertants in order to evaluate the oils' mutagenic potential. Mean values of the number of revertants from parallel plates from one to three experiments performed under the same test conditions were used for graphical illustration of concentration-effect relationships.

Both TA98 and TA100 gave positive test results for several fast pyrolysis oils in the preincubation Ames assay with and without S9, but at different concentrations of oil test solutions (Figure 3.2.8). Generally, the presence of S9 resulted in a decreased number of revertants in TA98 while presence of S9 in TA100 resulted in increased numbers of revertants, a trend that can be seen by the fact that for several oils the number of revertants compared to spontaneous activity seemed to decrease and increase more with the presence of S9 in TA98 and TA100 compared to the number of revertants observed in absence of S9, respectively. The most pronounced mutagenic response observed in TA100 with S9 reaching

above a sixfold increase in the number of revertants compared to the spontaneous activity was induced by oil 80-15-5 (purple line in Figure 3.2.8D) and the second most prominent mutagenic response under the same circumstances was a greater than fivefold increase in the number of reversions induced by 100-0-0 (yellow line in Figure 3.2.8D). In TA98 without S9 the greatest pyrolysis oil-induced increase in the number of revertants was observed with 0-100-0 (brown line in Figure 3.2.8A) reaching a threefold increase in the number of revertants.

## 3.3. Relating feedstock composition to mutagenic potential of pyrolysis oil

Partial least square (PLS) regression of the seven pyrolysis oils' mutagenic potential was applied, and the score plot and correlation loadings plot of the PLS model is found in Figure 3.3.1. The PLS model was based on results on mutagenic potential, acquired by the preincubation version of the Ames *Salmonella* assay using bacterial strains TA98 and TA100 in both absence and presence of S9, of bio-oils obtained from different relative proportions of wood, bark and needles. Calculated values of mutagenic potentials of the oils under the different test conditions can be found in Appendix E-1. The score plot illustrates similarities between oils based on their feedstock composition and individual mutagenic potential observed in TA98 and TA100 in absence and presence of S9 (Figure 3.3.1A), while the correlation loadings plot gives an illustration of potential correlations between the feedstock components wood, bark and needles together with mutagenic potential in TA98 and TA100 in absence and presence of S9 (Figure 3.3.1B). The PLS model based on mutagenic potential calculated from the Ames assay results of the seven pyrolysis oils in relation to their feedstock components was applied in an attempt to predict the mutagenic potential of the oils based on their feedstock composition, and the predicted mutagenic potential versus the measured mutagenic potential of the oils acquired by the Ames assay is illustrated in Figure 3.3.2.

### 3.3.1 Correlations between feedstock components and mutagenic potential

The mutagenic potentials of the three oils deriving from a feedstock of pure wood (100-0-0), pure bark (0-100-0) and pure needles (0-0-100) were found in different areas of the score plot, while the oils produced from mixed feedstock compositions were clustered more together within the area where the oils derived from pure feedstock components were found (Figure 3.3.1A). The proportion of the three feedstock components in a bio-oil deriving from a mixture generally seemed to affect its proximity to the three bio-oils obtained from pure

feedstock components, showing a trend that the higher the proportion of a feedstock component in a mixture, the closer is the mixture-derived oil's proximity to the bio-oil derived purely from that feedstock component. The explained variation in PLS regression component 1 was ascribed to feedstock by 53 % and to mutagenic potential by 60 %, while 47 % and 5 % of the variation in PLS regression component 2 could be explained by feedstock and mutagenic potential, respectively. Wood was correlated with TA98, TA100 and TA100 + S9 in the correlation loadings plot (Figure 3.3.1B) as opposed to the lack of correlation between bark and needles to any of the four mutagenic responses. A larger fraction of the observed mutagenic response in TA100 + S9, TA100 and TA98 could be explained by the feedstock, while rather little of the mutagenic response in TA98 + S9 could be explained by the feedstock, as seen by the proximities between the four mutagenic responses and the inner/outer circles explaining 50%/100% of the results.

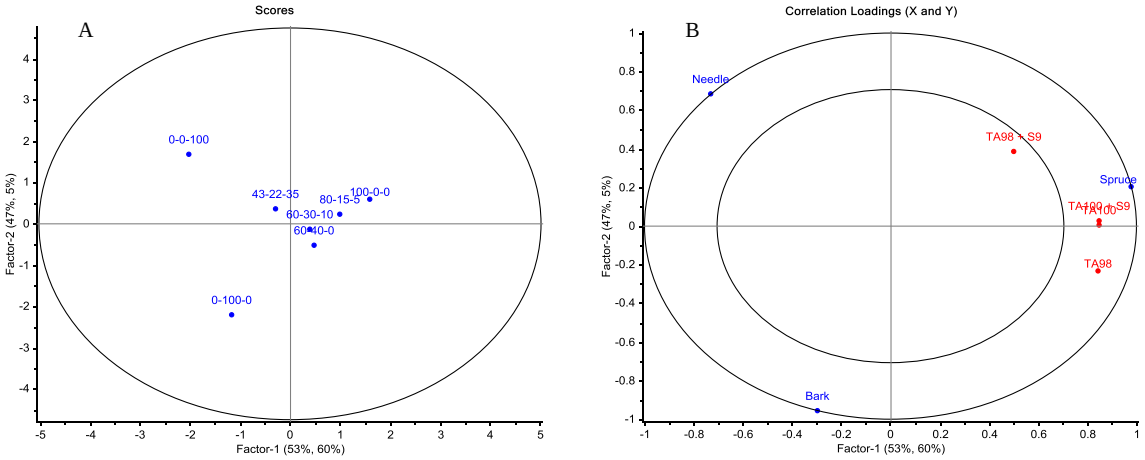


**Figure 3.3.1: Score plot and correlation loadings plot based on PLS regression relating the mutagenic potential of seven spruce-derived pyrolysis oils to their feedstock components.** The score plot (A) is based on results on the oils' mutagenic potential derived from results obtained by the preincubation version of the Ames *Salmonella* assay applied with bacterial strains TA98 and TA100 in absence and presence of S9. The correlation loadings plot (B) is based on the relative proportions of the feedstock components Spruce (wood), bark and needle from which the oils were produced in order to compare the feedstock components' influence on the oils' mutagenic potential observed in the Ames assay. The explained variation of the results in the score plot and correlation loadings plot are listed for Factor 1 and Factor 2 as one percent number explaining variation in feedstock and a second percent number explaining the variation in mutagenic response. The outmost circle in the correlation loadings plot illustrates 100 % explanation of the results while the inner circle represents an explanation of 50 %.

### 3.3.2. Predicting mutagenic potential based on feedstock composition

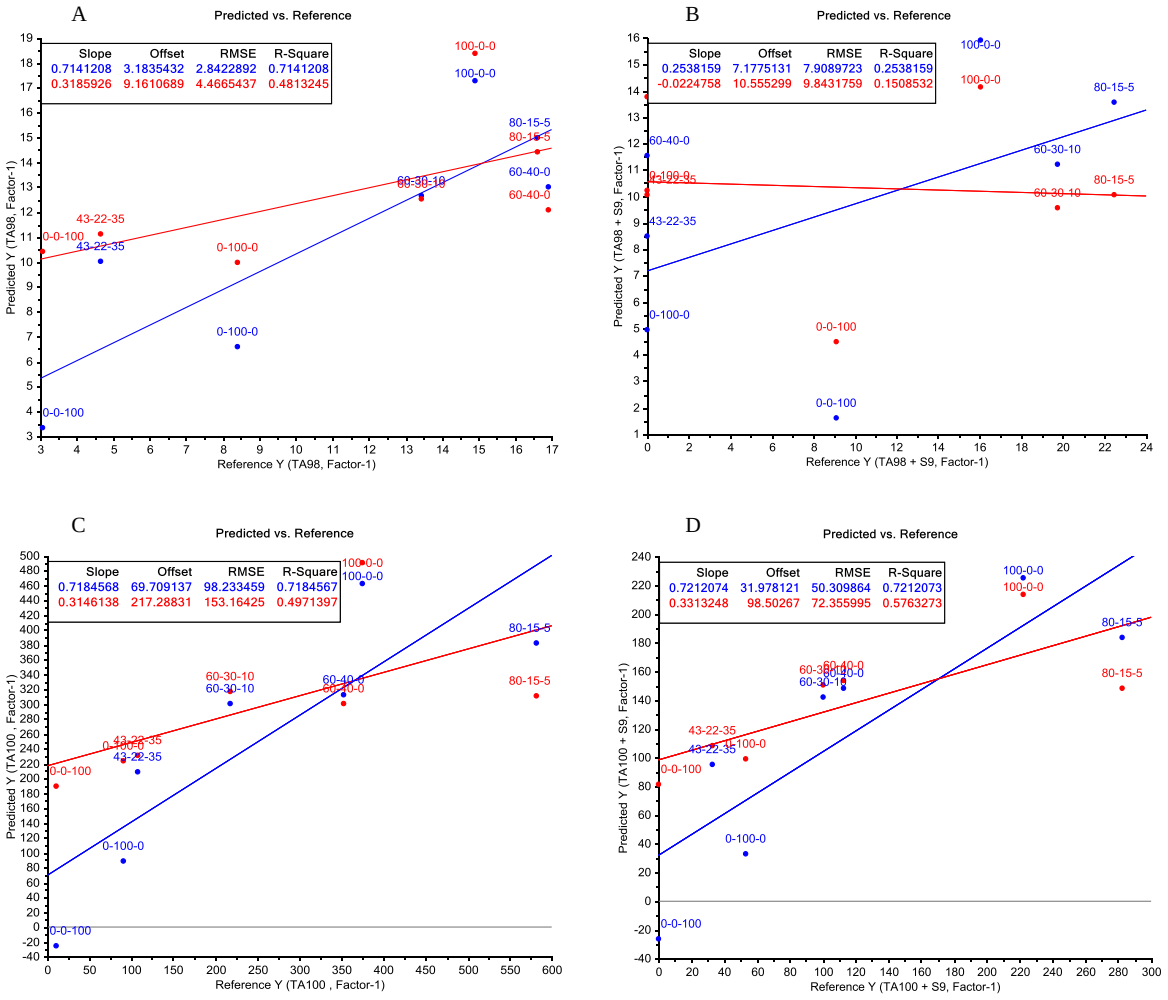


**Figure 3.3.2: Predicted versus observed mutagenic potential of the seven spruce-derived fast pyrolysis oils by the PLS model.** The preincubation version of the Ames *Salmonella* assay was applied using bacterial strains TA98 and TA100 in absence and presence of S9 for assessment of the mutagenic potentials of seven spruce-derived pyrolysis oils generated from different relative proportions of wood, bark and needles as the feedstock. Partial Least Square (PLS) regression was used for modeling of the mutagenic potential of the oils observed in TA98 (A), TA98 + S9 (B), TA100 (C) and TA100 + S9 (D) based on the feedstock composition (red lines) and is represented together with regression lines of the measured mutagenic potential (blue lines). $R^2$: correlation coefficient for goodness of prediction (red) or fit (blue). RMSE: root of the mean square's error.

The PLS regression modeled plots and lines and the corresponding regression plots and lines based on the measured results illustrate different proximities between plots and lines for the four mutagenic responses TA98, TA98 + S9, TA100 and TA100 + S9 (Figure 3.3.2A-D). The measured against the predicted results could explain approximately 72 % against 48 % of the mutagenic potential in TA98, 25 % against 15 % in TA98 with S9, 72 % against 50 % in TA100 and 72 % against 58 % of the mutagenic potential in TA100 with S9. The proximity between measured and predicted mutagenic potential for the pyrolysis oils deriving from wood or mixtures of wood/bark/needles was generally closer in comparison to the distance

between measured and predicted mutagenic potential in the purely bark- or needle-derived oils (0-100-0 and 0-0-100).

# 4. Discussion

In this chapter the bio-oil material and the bio-oil-induced mutagenic responses will be discussed and compared to previous results on pyrolysis-oil induced mutagenicity detected through the Ames assay. The influence of feedstock composition and chemical content on mutagenic response will be looked into, followed by a discussion about the Ames assay's role in obtaining information about chemical mutagens. Some toxicological aspects of bio-oil combustion will also be looked into, as well as some factors which could be important in further mutagenic evaluation of bio-oil.

## 4.1 The pyrolysis oil samples

Pyrolysis oil may be used as a future biofuel by generation of green diesel or green gasoline (Hossain and Davies, 2013) after suitable upgradation (Bridgwater, 2012). Recommendations for mutagenicity and toxicity assessment of bio-oil have been made because of its content of PAHs (Cordella et al., 2012, Lehto et al., 2013, Oasmaa et al., 2012). Seven spruce-derived fast pyrolysis oils (Table 2.1) generated from different relative proportions of wood, bark and needle forest residues were in this project chosen for assessment of wood-derived bio-oils' mutagenic potential by application of the Ames *Salmonella* assay with bacterial strains TA98 and TA100 in absence and presence of S9. Production of biofuels may lead to ecological effects, like reduced biodiversity, which can be mitigated through selection of e.g. forest residues for the biofuel production (Fargione et al., 2010), suggesting that the selected sample material in this project in an ecological view is a good choice of feedstock. Previous application of the Ames assay has lead to controversial results (Girard et al., 2005) and mutagenic potential has been detected in bio-oils deriving from different types of wood and operating systems (Gratson, 1994, Girard et al., 2005), whereof several spruce-derived bio-oil assessed in the Biotox Project induced positive test result in the Ames assay in TA98 and TA100 as well as in other *S. typhimurium* strains. Although different feedstocks and reactor systems used for pyrolysis oil production may lead to variation in chemical content and toxicity (Oasmaa et al., 2012), the type of sample material in this project, consisting of spruce-derived fast pyrolysis oil produced in a fluidised bed reactor at 500 ºC, has been claimed to be a representative sample material for wood-derived bio-oil because the fluidised bed is the most common process for bio-oil production and a soft wood like spruce is a typical European biomass (Girard et al., 2005). The fact that the bio-oils in this project were

generated from different feedstock proportions of wood, bark and needles created a unique opportunity to study how differences in feedstock composition within a particular wood-species could influence the mutagenic potential in the bio-oil.

From the ESI-MS analysis (Figure 2.1.1) one can see that the dominant masses (m/z) are different for the purely wood-, bark- and needle-based oils, a fact that was thought possibly to cause some variation in the Ames assay results, without having any specific expectations about how these differences could influence mutagenic potential. It was challenging to anticipate any details about the Ames assay results, as no scientific studies were found to focus on mutagenicity assessment of bio-oils derived from different feedstock components from the same wood species, a fact that makes this project quite exceptional. The results obtained by this project were nevertheless expected to be somewhat comparable to the results observed in the previous mutagenicity assessment performed in the Biotox Project on spruce-derived bio-oils, which were found to induce bacteriotoxic and a variety of mutagenic responses. Similarly, this project indeed found that different spruce-derived bio-oils lead to differences in bacteriotoxic and mutagenic abilities.

## 4.2 Results of the Ames assay applied to spruce-derived pyrolysis oil

The preliminary mutagenicity assessment on beech-derived pyrolysis oil did not detect any oil-induced mutagenic responses using the standard plate incorporation assay while a clear mutagenic response was detected in bacterial strain TA100 using the preincubation version of the Ames assay (Appendix B-1). This observation, which contributed to the decision of applying the preincubation version of the Ames assay in the main experiment, and the fact that this version was applied on pyrolysis oils in the Biotox Project suggest that the preincubation version of the Ames assay is more sensitive and could be more appropriate for mutagenicity assessment of bio-oil than the standard plate incorporation assay.

Conclusions drawn from results obtained by the Ames assay should be based on at least two experiments (de Serres and Shelby, 1979) and all oils were assessed at least twice under minimum two of the four test conditions, whereof six out of the seven pyrolysis oils were found to possess mutagenic potential. The mutagenicity results obtained from one experiment (100-0-0, 60-40-0 and 43-35-22 applied to TA98 and TA100 without S9) should be interpreted as somewhat weaker indications of presence of mutagens in contrast to the results from two or three experiments, but whether assessed in one or more experiments the results strongly suggest that mutagens are present in six out of the seven bio-oils. The positive

Ames assay results indicate presence of both primary and secondary mutagens inducing base-pair substitutions as well as lower levels of primary and secondary frameshift-inducing mutagens in the pyrolysis oil sample material. Storage of spruce-derived bio-oil for 12 months has been shown to lead to weaker mutagenic potential (Girard et al., 2005), and whether the one year difference in storage time for some of the oils in this project had any influence on the results is unknown, but it is assumed that the differences in mutagenic potential between the oils were attributed to feedstock composition more than storage time.

The spruce-derived bio-oils clearly induced differences in the results of the Ames assay both in terms of bacterial toxicity and mutagenicity, which shows that bacteriotoxic and mutagenic properties in bio-oils may differ between oils obtained from different relative proportions of wood, bark and needles of the same wood species. Prominent background toxicity was evoked under one or more of the four test conditions for all oils except for the needle-based oil (0-0-100). It is unknown whether the mutagenic responses could have increased with increasing concentrations to even higher numbers of revertants than observed in this assessment if toxicity had been evoked at higher oil concentrations and in this way would have extended the upper nontoxic concentration limits to be tested. It is nevertheless possible that extending the upper concentration limits could lead to precipitation and thereby hinder the possibility to obtain results by the assay, as observed to happen for some bio-oils in the Biotox Project.

### 4.2.1 Types of mutations induced by spruce-derived bio-oils

The Biotox Project assessed mutagenicity of 21 bio-oils obtained mostly from wood-species, whereof 10 oils derived from spruce, using bacterial strains TA98 and TA1537 (detect frameshift-mutagens) as well as TA100, TA102 and TA1535 (detect base-pair substitution-inducing agents) in the Ames assay and found that all 21 oils gave positive test results in at least one of the five strains, making them all considered as mutagenic. In this way it was demonstrated that variation between and within wood species can induce different patterns of mutagenic responses.

Positive test results induced by the pyrolysis oils in this project were observed to be both more pronounced and to happen more frequently in TA100 in comparison to TA98, suggesting that mutagens which induce base pair-substitutions could be either more potent or exist in higher amounts than frameshift-inducing agents in the bio-oils. All oils except the purely needle-based oil (0-0-100) induced a positive mutagenic response in TA100 both in

absence and presence of S9, as opposed to only two of the oils (100-0-0 and 0-100-0) giving a positive test result in TA98 either with or without S9. Out of the 10 spruce-derived bio-oils assessed by Girard and co-workers (2005) nine oils were found to induce frameshift mutations and nine oils induced base-pair substitutions, an observation which contrasts this project's finding of a higher frequency of oil-induced basepair substitutions than frameshift mutations. The differences between the results on mutagenic potential of spruce-derived pyrolysis oil observed in this project and those observed in the Biotox Project further support this project's demonstration that mutagenic abilities may vary in different bio-oils obtained from the same wood species. The oils in this project were produced under equal production parameters, while the production of the spruce-derived bio-oils in the Biotox Project varied in temperature, reaction time and reactor type. Altogether the Ames assay results from this project and the Biotox Project shows that production parameters as well as feedstock composition may influence the pattern of mutagenic responses induced by spruce-derived fast pyrolysis oils.

The mutagenic response in TA100 was often increased by the presence of S9, as four of the oils had a higher mutagenic potency when S9 was added (Figure 3.2.8A-B). In contrast, S9 seemed to lower the difference between spontaneous and oil-induced reversions in TA98 for all but one bio-oil (100-0-0) (Figure 3.2.8C-D). Similar to the Biotox Project this assessment found that all but one (0-0-100) of the tested spruce-derived bio-oils contained secondary base-pair substituting agents. However, this project's finding of only one bio-oil which induced secondary frameshift mutagenesis contrasts the finding of seven out of the 10 spruce-derived bio-oils in the Biotox Project inducing secondary frameshift mutations. Although the S9-mixture can be claimed to differ from the *in vivo* situation, it is clear that potential bioactivation of compounds in spruce-derived bio-oils may differ between oils which may therefore induce different responses in biological systems,

### 4.2.2 Bacterial toxicity

Bacterial toxicity was evoked by all but the needle-based pyrolysis oil (0-0-100) and was observed to increase with increasing concentrations of test solution, shown by an increasingly thinner background lawn of bacterial growth and consequently increasing BTLs with increasing concentrations. As observed in this project, the Biotox Project detected background toxicity in TA98 and TA100 evoked by spruce-derived bio-oil, but additionally found that precipitation occurred at high concentrations of some bio-oils obtained from wood species

other than spruce and in this way restricted the concentration range to be tested, as opposed to the situation in this project where background toxicity was the determining factor for setting the upper concentration limits of the pyrolysis oils to be assessed. The concentrations of test solution evoking toxicity varied greatly between the oils, suggesting that differences in toxicological properties between the oils also apply to properties other than mutagenic potential. Bacterial toxicity was often observed at higher concentrations in TA98 than in TA100, which demonstrates that there are differences in the two strains' tolerance for pyrolysis oil. It is likely to believe that the prominent background toxicity evoked at quite low concentrations for some of the oils may have lead to an underestimation of the mutagenic response, as bacterial toxicity leads to false negatives in the Ames assay (Maron and Ames, 1983). The ability to evoke toxicity at low concentrations was however often combined with a positive test result, most often in TA100, and with this combination the possible underestimated mutagenic response would only underestimate the mutagenic potency of oils already shown to give positive test results. Most oils gave a negative test result in TA98 at the same time as toxicity was evoked, and the influence of toxicity could therefore have played a more important role in the evaluation of the oils' mutagenic potential in TA98 compared to in TA100. The observed toxicity in both TA98 and TA100 decreased in the presence of S9, which indicates that toxic compounds in the bio-oils may be metabolized to less bacteriotoxic compounds by enzymes in the S9-mix. This decrease in bio-oil evoked bacterial toxicity observed with S9 was similarly found for the spruce-derived bio-oil in the Biotox Project, and was in fact a trend found to occur for 19 out of the 21 bio-oils assessed. Altogether the effect of S9 was beneficial for the survival of bacteria, but the S9-mediated increase in mutagenic potency of bio-oils in TA100 indicates a potential hazard of crude bio-oil exposed to organisms with high metabolic capacity.

### 4.2.3 Factors potentially contributing to variation

Although the viscosity of the bio-oils decreased after homogenization, the oils' stickiness was still high after homogenization and might have lead to inaccurate pipetting in the preparation of stock solutions, a fact which may subsequently have contributed to inaccurate oil dilutions and differences in the results between the experiments with the same oils under equal test conditions. The variation in numbers of revertants between parallel plates observed at oil concentrations evoking BTLs > 0 was observed, as expected since bacterial toxicity leads to increased variance (Agnese et al., 1984).

### 4.2.4 Validity of the results

The integrity of TA98 and TA100 was assured through the ampicillin and crystal violet tests; bacterial growth adjacent to the ampicillin tablet demonstrates presence of the R-factor and absence of bacteria adjacent to crystal violet shows that the bacteria contain the *rfa* mutation (Maron and Ames, 1983).

The spontaneous reversion frequenciess of the bacterial strains after 48 hours incubation were reported to vary between 30-50 colonies per plate for TA98 and 120-200 colonies per plate for TA100 (Maron and Ames, 1983), which are higher levels than the reversion frequencies observed in this project (10-30 for TA98 and 87-124 for TA100). This rather large range of observed numbers of spontaneous reversions in both strains was considered not to influence the test results. The numbers of spontaneous reversions may vary between laboratories (de Serres and Shelby, 1979), and deviating ranges are not considered to diminish the validity of the assay as long as the reversion frequencies remain constant over a longer period at the same laboratory (Maron and Ames, 1983, Mortelmans and Zeiger, 2000), which was the case here. Consequently, the somewhat lower reversion frequencies observed in this project were regarded to be within acceptable ranges.

Solubility trials performed on bio-oils have suggested DMSO to be among the best vehicles for bio-oils (Girard et al., 2005) and the small difference in reversion frequencies observed between the DMSO solvent controls and the spontaneous levels made DMSO considered to have a negligible impact on the results in this project. The high numbers of revertants induced by NPD and Na-azid could confirm that TA98 and TA100 were able to respond to known mutagenic agents. The reversion frequencies induced by BaP in presence of S9 were somewhat lower than previously reported (Maron and Ames, 1983) but the activity of the S9-mixtures was nevertheless considered to be validated through the observation of higher mutagenic potential of oils in the presence of S9 compared to when S9 was in absent.

## 4.3 Relating the Ames assay results to feedstock composition

Neither bacterial toxicity nor any positive mutagenic response was found for the purely needle-based bio-oil (0-0-100), although the doubling criterion was close to being met, and this oil was thereby the oil showing in total the weakest bacteriotoxic and mutagenic potential of the bio-oils studied in this project. The oils deriving purely from wood (100-0-0) and bark (0-100-0) induced mutagenic responses in both strains, while the oils obtained from mixed feedstock compositions induced positive test results only in TA100. The purely wood-derived

oil evoked toxicity at lower concentrations of test solutions than the lowest bacteriotoxic test-solutions of bark-derived bio-oil, suggesting that wood has stronger bacteriotoxic properties than bark as bio-oil feedstock, and it seemed that decreasing the proportion of wood in a bio-oil derived from mixed feedstocks generally decreased the bacterial toxicity. The most potent test solution concerning mutagenic potential was 2 μL/plate of pyrolysis oil obtained from a mixture of the three feedstock components (80-15-5), which in TA100 with S9 induced over a sixfold increase in the number of revertants compared to the spontaneous levels. There is thus a possibility that bio-oil from mixed feedstock composition may induce a higher mutagenic potency than bio-oil deriving from 100 % of either feedstock components. On the other hand, only the wood- and bark-derived oils (100-0-0 and 0-100-0) induced a mutagenic response in TA98 but similar to the mixed feedstock-based bio-oils these two oils gave positive results in TA100 both in presence and absence of S9. The influence of feedstock composition on bio-oil's mutagenic properties is evidently hard to predict but may possibly relate to chemical compounds in the prepyrolysis feedstock components or compounds generated from reactions between chemicals in the components during pyrolysis. Information about potential differences in mutagenic abilities in oils obtained from brown and green forest residues is impossible to obtain by this project, as the number of samples is too limited.

### 4.3.1 Mutagenic potential and feedstock composition

The differences in mutagenic potential between bio-oils deriving from different feedstock compositions was shown by their differing concentration-effect relationships and were further confirmed by statistical analysis (Figure 3.3.1). The PLS score plot (Figure 3.3.1A) shows that the oils from feedstocks of pure wood (100-0-0), bark (0-100-0) and needles (0-0-100) had the highest degree of dissimilarity to each other based on information about both feedstock composition and mutagenic potential, and these three oils created a triangle in the score plot; a triangle which also reflects the oils' mixture design as described by Celaya et al. (2012)2012). The score plot also indicates that an oil produced from mixed feedstock composition was most similar to the purely feedstock-based oils of highest proportions in the feedstock mixture. Thus, the oils were distributed in a fairly logical pattern in the view of their differences in feedstock proportions also when their differences in mutagenic potential was taken into account. A bigger proportion of difference could be ascribed to feedstock (53 % in PLS component 1 and 47 % in PLS component 2) compared to the proportion explained by mutagenic potential (60 % in PLS component 1 and 5 % in PLS component 2), which

indicates that the feedstock composition had a higher degree of influence on the overall differences between the oils than the mutagenic potential.

The statistically measured influence of the three feedstock components on mutagenic potential (Figure 3.3.1B) showed that wood was the only feedstock component correlating with mutagenic responses and was consequently the feedstock component indicated to contribute the most to the mutagenic potential of the bio-oils, despite the fact that the purely bark-derived oil was observed to induce mutagenic responses in both strains (Figure 3.2.2). Three of the mutagenic responses (TA98, TA100 and TA100 + S9) could be explained quite well (approximately 71-72 %) by the content of wood in contrast to bark or needles, while one response (TA98 + S9) was rather poorly explained (25 %) by the PLS-model. The number of obtained mutagenic potential values differed between the four responses, and the fact that the mutagenic response most poorly explained by feedstock composition was based on fewer mutagenic potential values (4 values) than the other responses (6-7 values) may have had an influence on the poor explanation of this response.

The ability of the PLS model to predict mutagenic potential of the pyrolysis oils was rather limited (Figure 3.3.2), made obvious by the different slopes in the measured and corresponding predicted regression lines and the significantly lower $R^2$ values for predicted regression lines compared to $R^2$ values for measured regression lines. However, wood generally seemed to be the feedstock component best capable of predicting mutagenic response, as increased wood content in the oils seemed to increase the predictive value; making the proximity of corresponding measured and predicted scores closer for oils with a high proportion of wood as opposed to those made from pure bark or needles. Accordingly, an increased proportion of wood in a pyrolysis oil made the mutagenic potential of this oil more predictable. The oils obtained from pure bark (0-100-0) or needles (0-0-100) however, were the oils which were the least predicted by the PLS-model and therefore contributed the most to the deviation between the measured and predicted regression lines. As pure needles did not induce any mutagenic response, it was rational to expect that this feedstock component had the least influence on prediction of mutagenic potential, while the observation of bark having little influence on mutagenic potential prediction of the oils was more unforeseen.

Prediction of health and environmental risk from chemical mixtures through use of prediction models is desirable (Kim et al., 2013), but extremely challenging when it comes to predicting the combined toxicological effects of complex mixtures (Eide et al., 2002). This project's attempt to predict mutagenic potential of pyrolysis oil through a statistical model based solely on feedstock composition turned out to give a fairly decent level of predictability

of mutagenic potential in three of the responses (TA98, TA100 and TA100 + S9) while the last response (TA98 + S9) was poorly predictable by the PLS model. The PLS model also gained some insight to how the variation in feedstock composition influenced the predictability of mutagenic potential. It is likely that the ability of the model to predict mutagenic potential would have improved with a higher number of oils of different feedstock composition to increase the number of observations for the model to base on.

## 4.4 Relating the Ames assay results to chemical content

Bio-oils may contain thousands of different chemical compounds (Jarvis et al., 2012), whereof undesired compounds, impurities and residuals from unconverted biomass can be found (Eide and Neverdal, 2014). Differences in feedstock and reactor parameters during bio-oil production may influence chemical composition and toxicity of the generated bio-oils (Oasmaa et al., 2012) and furthermore, storage of bio-oils may lead to changes in physical properties and chemical composition (Toven et al., 2013). Hence, the outcome of toxicity assessment of crude bio-oil depends on several factors. Moreover, the fact that a detailed identification of the chemical constituents in complex mixtures is hard to obtain and may in fact be impossible (Eide et al., 2001) makes it challenging trying to attribute toxicological effects to specific components in e.g. bio-oil. As the chemical information about the oils in this project is restricted to data on molecular weight (Eide and Neverdal, 2014, Toven et al., 2013), only speculations can be done trying to relate the Ames assay results to the chemical nature of the oils.

The ESI-MS of the pyrolysis oils containing pure wood (100-0-0), bark (0-100-0) and needles (0-0-100) showed that their chemical content was quite different and that the mass numbers of components in the oils, ranged from highest to lowest molecular weights, was as follows: wood > bark > needles (Figure 2.1.1). Unfortunately, ESI-MS generally applies to polar compounds while nonpolar hydrocarbons, e.g. aromatics, largely remain unionized and consequently not detected in the analysis (Eide et al., 2011). The differences detected by ESI-MS between the three oils are therefore assumed to be attributed only to polar compounds in the bio-oils and not to gain any information about differences in content of e.g. PAHs or other nonpolar hydrocarbons which may have contributed to mutagenic response. Over 400 organic compounds have been detected in bio-oils and gas chromatography mass spectrometry (GC-MS) has been applied to the oils of this project to identify organic compounds, however without quantification, (Eide and Neverdal, 2014).

Regarding the observed bacterial toxicity it was indicated in the Biotox Project that PAH concentration might correlate with toxicity, but having no data on PAH content of the oils in this project it is impossible to say whether PAHs influenced the bacterial toxicity or not. It is possible that the oils' content of a variety of phenols originating from depolymerization of lignin (Mohan et al., 2006) may have had an impact on bacteriotoxic properties in the oils, as phenols have been shown to inhibit bacterial growth in *S. typhimurium* (Kumar et al., 2013). The lacking toxicity evoked by 0-0-100 suggests that polar low molecular weight compounds in bio-oil do not play a prominent role in the bio-oil-evoked bacterial toxicity observed. The chemical groups containing the compounds of the highest mass proportions in bio-oil, e.g. acetic acid, hydroxyacetaldehyde and anhydrosugars, are all known to have low toxicity and were in the Biotox Project not found to impact toxicity through potential synergistic or antagonistic effects (Girard et al., 2005). Analysis of content of PAHs and phenols in the bio-oils could have gained some information about their potential impact on bacteriotoxic properties.

Genotoxic effects of pyrolysis oil have previously been suggested to be attributed to PAHs (Pimenta et al., 2000) and the content of PAHs in bio-oils has been a factor rushing the need for further mutagenicity and carcinogenic data on pyrolysis oils (Lehto et al., 2013). Several PAHs have been identified as secondary mutagens in the Ames assay (Nagai et al., 2002, White and Claxton, 2004, Yan et al., 2004) and their potential presence in this project's sample material may have influenced the results on secondary mutagenic responses. The primary mutagenic responses induced are considered as unlikely to correlate with PAH content, as several *in vitro* studies with bacteria have demonstrated that numerous PAHs require metabolic activation in order to exert mutagenic effects (Jacob, 2008). It has been suggested that strong adsorption of PAHs to char particles in bio-oil may decrease PAHs' bioavailability to bacteria and may consequently inhibit potential mutagenic effects of PAHs in bio-oil, and another issue which may influence genotoxic assessment of bio-oil is the possibility for antagonistic interactions between PAHs and other chemicals in bio-oil which has in fact been detected (Pimenta et al., 2000). Although predicting the combined toxic or mutagenic effects of PAH-containing mixtures based on PAH data is extremely difficult (Eide et al., 2001), having some data on potential PAH content in the bio-oils could have given some clues about the responsible mutagens for the mutagenic activity observed in this project. Concentration of ∑13 PAHs in spruce-derived fast pyrolysis oils in the Biotox Project ranged from 0.25 – 23.43 ppm, and it is possible that the oils in this project had PAH levels around the same levels as two of these oils which were produced under similar conditions as the oils

in this project and had ∑13 PAH concentrations of 1.01 and 2.70 ppm. Despite of the rather low PAH concentrations detected in bio-oils, their presence in bio-oil has lead to recommendations for further mutagenicity and carcinogenicity assessment of bio-oil (Lehto et al., 2013).

## 4.5 Bio-oil combustion – some toxicological aspects

It is well known that PAHs contribute to health and safety concerns in conventional petroleum derived fuels (Girard et al., 2005) and emissions from diesel engines are classified as probably carcinogenic to humans (Bünger et al., 2007). Use of biodiesel as diesel fuel replacement has been detected to increase the mutagenic effect induced by the emitted particles (Bünger et al., 2007), and a large fraction of the mutagenic activity of biodiesel exhaust is claimed to be attributed to PAHs (Mauderly, 1997). The potential for human health hazards from biodiesel usage has been paid less attention (Swanson et al., 2007), and perhaps other biofuels as well may generate unforeseen health hazards which receive less focus than their benefits to the environment as fossil fuel replacements.

There is unfortunately little published data on PAH emission levels through bio-oil combustion (Lehto et al., 2013). Although relatively few burner models for fast pyrolysis oil are commercially available, careful adaptation to the unique properties in bio-oil might lead to bio-oil combustion on an industrial scale. Emission levels of combustion products of environmental concern from bio-oil typically rank between those of light fuel oil and the lightest heavy fuel oil, however with a possibility for higher particulate emissions; mainly small particles (<10 μm) of incombustible matter originating from content of solids in bio-oil. Consequently it is recommended to reduce solids content and lower presence of ash and sand in the oils (Lehto et al., 2013).

The content of char particles in crude bio-oil, which increases with increasing amount of bark and needles as the forest residue feedstock, is desirable to remove by char-separation, as presence of char particles weakens the ability for using pyrolysis liquids as high quality fuels (Toven et al., 2012). The bio-oil sample material assessed in this project consisted of crude bio-oils which all had solid contents exceeding the recommendations for pyrolysis liquid fuels for heat and power applications (Toven et al., 2013). Although the oils have not been upgraded and would become more usable and likely to be exposed to humans and the environment through further upgradation, this project's demonstration of their mutagenic potential showed that material which can further be upgraded for production of high-quality biofuel contained hazardous compounds, and it is yet to reveal how different upgradation

techniques may impact the mutagenic potential of the material. Char-separation of the bio-oils in this project has been performed by ultracentrifugation and lead to efficient removed of char particles, and the production of char-free pyrolysis oils may become possible in the future (Toven et al. 2013). Wood, bark and needles generate varying yields of bio-oil, with wood being the feedstock component yielding the highest amounts of bio-oil and bark the lowest (Toven et al., 2012). This variation in bio-oil yields can probably impact the choice of forest residue feedstock components for future pyrolysis oil production. Through the results of this project wood turned out to be the feedstock component contributing the most to the mutagenic responses induced by the bio-oils, and wood's higher bio-oil yield could make wood viewed as a more attractive feedstock component than bark or needles for bio-oil production, a view that could subsequently influence the mutagenic potential of the oil. However, it is unknown how different types of upgradation techniques applied to wood-derived bio-oil could impact its mutagenic potential.

## 4.6 From positive Ames test results to further toxicological evaluation of bio-oils

The Ames assay is a screening test used for hazard identification as it correlates with health end points, and its central role in the field of genetic toxicology since the 1970s has created an extensive database with information about tested samples (Claxton et al., 2010). The potential hazards to humans or the natural environment caused by *Salmonella* mutagens might be posed through the risk of chemical carcinogenesis, but also through the risk of induction of deleterious mutations in natural populations which may reduce survival and reproductive rates (Lynch and Gabriel, 1990). The scientific literature seems to have often focused on the risk for chemical carcinogenesis by *S.* mutagens, but potential risk for the natural environment should not be neglected. A test chemical inducing a positive result in the Ames assay is claimed to have an approximately 80 % probability of being a chemical carcinogen in rodents, making the Ames assay the most predictive mutagenicity-based assay for chemical carcinogenicity (Benigni et al., 2010). Detection of mutagenic activity through the assay is recommended to be further confirmed and characterized by more complex assessments (Claxton et al., 2010). However, the appropriate approach for further assessing *Salmonella* mutagens detected through the Ames assay is not straight forward; *in vitro* mutagenicity assays do not complement the Ames assay when it comes to predicting chemical carcinogenesis, *in vivo* mutagenicity assays give rise to numerous false negatives and e.g. the

modern "-omics" technologies do not seem promising for following up positive Ames assay results (Benigni et al., 2010).

Although it has been claimed that chemical mixtures may lead to inhibition of S9-mediated bioactivation (Haugen and Peak, 1983), the Ames assay is claimed to be the superior assay for evaluating mutagenic potential of complex mixtures containing unknown compounds (Benigni et al., 2010), as was the case in this project. The observed prominent bacterial toxicity evoked by some of the bio-oils may raise a question about the applicability of the Ames assay to mixtures where bacteriotoxicity is a property dominating over mutagenic properties, but the Ames assay was nevertheless shown to be a useful tool for the purpose of achieving information about mutagenic potential of the bio-oils in this project.

The results of this project demonstrated positive mutagenic responses induced by crude spruce-derived fast pyrolysis oils through application of the Ames assay and support the emphasis on the need for obtaining more toxicity and carcinogenic data on bio-oils (Lehto et al., 2013). Differences in both bacteriotoxic and mutagenic properties were observed between bio-oils obtained from different feedstock compositions of spruce. Whether the mutagenic properties in the bio-oils of this project are attributed to PAHs or other compounds is unknown, but bio-oil's content of PAHs, e.g. the known carcinogens BaP and benzo(a)anthracene, is a reason behind the recommendation for conducting a life span skin painting assay to determine whether the test material is carcinogenic (Lehto et al., 2013).

The focus on environmental, health and safety aspects of bio-oil will increase as bio-oil becomes more widely available (Bridgwater, 2012). It would be useful to perform mutagenicity assessment on upgraded wood-derived fast pyrolysis oil in order to evaluate the mutagenic potential of a product that could probably be exposed to humans and the environment on a larger scale than the type of crude bio-oils in this project. The observed variation in mutagenic properties within and between wood species from which crude bio-oils were obtained is possible to be influenced by further upgradation techniques, e.g. removal of char particles, which may affect the mutagenic potential of bio-oils. Crude bio-oil contains numerous reactive species (Mohan et al., 2006) and potential reduction or removal of these constituents through upgradation may lead to a lower mutagenic potential of the oils compared to the observed mutagenic potentials of the oils in this project. However, this project indicates that crude bio-oils have mutagenic potential and may therefore pose a risk through e.g. occupational exposure or spillage in the natural environment.

# 5. Conclusions

The main purpose of this project was to assess the mutagenic potential of crude spruce-derived pyrolysis oils obtained from different relative proportions of wood, bark and needles. Comparative application of the Ames assay showed that the preincubation version of the assay was more sensitive to bio-oil-induced mutagenicity than the standard plate incorporation assay, and the different bio-oils were demonstrated to have differences in their bacteriotoxic and mutagenic abilities.

- All but the purely needle-based bio-oil induced positive test results under at least two of the four test conditions and evoked bacterial toxicity at different concentrations. Presence of primary and secondary mutagenic agents inducing frameshift mutations and base-pair substitution were indicated in one or more of the oils, but with a higher frequency of positive test results and generally a higher mutagenic potency of mutagens inducing base-pair substitutions. The oil-induced reversion frequencies when S9 was added generally decreased in TA98 and increased in TA100, indicating a domination of primary frameshift-inducing mutagens over secondary frameshift-inducing agents and secondary base-pair substitution-inducing mutagens over primary base-pair-inducing agents in the bio-oil sample material.
- Both bacteriotoxic and mutagenic properties in spruce-derived fast pyrolysis oils were demonstrated to be influenced by spruce feedstock composition. Wood demonstrated bacteriotoxic properties and was the feedstock component shown by the statistical analyses to contribute the most to the bio-oil-induced mutagenic activity, yet it was a bio-oil derived from mixed feedstock composition which demonstrated the highest mutagenic potency of the sample material. The needle-derived oil did not evoke bacterial toxicity nor did it induce any mutagenic responses while bark showed to possess both bacteriotoxic properties and mutagenic abilities.
- The PLS model based solely on data on feedstock composition was not able to predict mutagenic potential with a high accuracy, but was more capable of predicting the mutagenic potential of bio-oils obtained from high proportions of wood than of the oils deriving from pure bark or needles.

As the crude bio-oils in this project turned out to possess mutagenic abilities, it would be useful to further perform mutagenicity assessment on more refined/upgraded bio-oils to see if the different upgradation techniques of bio-oil, e.g. char removal, will have any effect on the mutagenic potential of pyrolysis oil.

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# Appendix

Appendix A-1: Solutions applied in the Ames assay

Appendix B-1: Results of the preliminary Ames assay comparing the standard plate-incorporation and the preincubation version of the assay on pyrolysis oil

Appendix C-1: Overview of the oil concentrations applied in the 11 Ames experiments with spruce-derived pyrolysis oils

Appendix D-1: Raw data of the Ames results for spruce-derived pyrolysis oils in absence of S9

Appendix D-2: Raw data of the Ames results for spruce-derived pyrolysis oils in the presence of S9

Appendix E-1: Calculated mutagenic potentials of the spruce-derived oils

Appendix A-1: Solutions applied in the Ames *Salmonella* assay

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

D-Biotin was transferred to volumetric flask (250 mL), added some of the water and the solution was heated and kept at boiling point until all the biotin was dissolved. The solution was cooled down to room temperature. L-Histidine and the rest of the water was added and mixed with a magnet stirrer. Filtrated solution was put into a sterilized flask by the use of a syringe (50 mL) and a filter (0.22 μm).

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

The components were mixed, autoclaved at 121 ˚C for 30 min, and cooled down to about 50 ˚C. The solution was transferred to Petri dishes (9 cm) ~ 20 mL in each dish. Made by Grethe Stavik Eggen.

**Nutrient medium**
4 g Nutrient Broth
160 mL distilled water

A magnetic stirrer was used for mixing of the solution. 5 mL was transferred to conical flasks (25 mL) and cotton tops with alu-foil sealed the flasks. The solutions were autoclaved at 121 ºC for 20 min.

**Top agar**
6.6 g Difco-Bacto-Agar
5.5 g NaCl
1100 mL distilled water

NaCl, Difco-Bacto Agar and water were mixed in a conical flask (3L) closed with alu-foil. The mixture was kept at boiling point in a water bath for 1 ½ hour, transferred to glass flasks (200 mL) and autoclaved at 121 ºC for 20 min.

**S9-cofactor solutions**

1. 0.4 M Magnesium chloride (MgCl)-solution
   20.3505 g $MgCl_2$ x $6H_2O$ was diluted to 250 mL distilled water.
2. 1.65 M Potassium chloride (KCl)-solution
   30.7500 g KCl (Merck) was diluted to 250 ml distilled water
3. 0.2 M Sodium di-hydrogen phosphate ($NaH_2PO_4$ x $H_2O$)
   5.5200 g $NaH_2PO_4xH_2O$ was diluted to 200 mL distilled water
4. 0.2 M Di-sodium hydrogenphosphate ($Na_2HPO_4$ x $2H_2O$)
   35.598 g $Na_2HPO_4x2H_2O$ was diluted to 1000 mL distilled water

0.2 M Sodium phosphate buffer (pH 7.4)
880 mL of solution 4 was added 180 mL of solution 3. Then solution 3 was added until pH 7.4 was reached.

All solutions were autoclaved at 120 ºC for 40-45 min.

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

The components were added into a sterile conical flask (100 mL) in the above order. S9-homogenat was defrosted a bit, added 2.1 mL cooled autoclaved distilled water and mixed gently. This mixture was added to the rest of the solution, filtered by the use of a syringe (50 mL) and placed on ice.

Appendix B-1: Results of the preliminary Ames assay comparing the standard plate-incorporation and the preincubation version of the assay on pyrolysis oil

**Table B 1.1: Results of the Ames assay on TA98 and TA100 exposed to beech-derived pyrolysis oil**. The standard plate-incorporation assay and the preincubation version of the Ames assay was applied exposing TA98 and TA100 to two concentrations (0.04 µL/plate and 0.4 µL/plate) of a beech-derived pyrolysis oil (n = 3) together with distilled water controls and registration of spontaneous reversions (n = 5) after 48 and 72 hours incubation. Individual numbers of revertants, mean values and ± SD are listed.

| | TA98 | | | | TA100 | | | |
|---|---|---|---|---|---|---|---|---|
| | Standard | | Preincubation | | Standard | | Preincubation | |
| Exposure | 48 h | 72 h | 48 h | 72 h | 48 h | 72 h | 48 h | 72 h |
| Spontane | 21 | 29 | | | 69 | 75 | | |
| | 25 | 32 | | | 69 | 74 | | |
| | 21 | 34 | | | 58 | 63 | | |
| | 12 | 21 | | | 79 | 79 | | |
| | 22 | 28 | | | 48 | 48 | | |
| | 18 | 25 | | | 43 | 46 | | |
| Mean | 20 | 28 | | | 61 | 64 | | |
| ± SD | 4 | 5 | | | 14 | 14 | | |
| Water | 17 | 23 | 21 | 28 | 60 | 65 | 65 | 68 |
| | 16 | 26 | 20 | 32 | 56 | 63 | 49 | 51 |
| | 20 | 35 | 23 | 37 | 58 | 61 | 48 | 57 |
| Mean | 18 | 28 | 21 | 32 | 58 | 63 | 54 | 59 |
| ± SD | 2 | 6 | 2 | 5 | 2 | 2 | 10 | 9 |
| 0.04 µL oil/plate | 16 | 25 | 22 | 29 | 52 | 56 | 56 | 62 |
| | 23 | 30 | 16 | 21 | 74 | 79 | 86 | 88 |
| | 25 | 31 | 16 | 24 | 59 | 61 | 67 | 73 |
| Mean | 21 | 29 | 18 | 25 | 62 | 65 | 70 | 74 |
| ± SD | 5 | 3 | 3 | 4 | 11 | 12 | 15 | 13 |
| 0.4 µL oil/plate | 15 | 20 | 36 | 54 | 84 | 89 | 242 | 245 |
| | 26 | 31 | 17 | 28 | 77 | 81 | 240 | 241 |
| | 20 | 26 | 19 | 25 | 71 | 78 | 271 | 277 |
| Mean | 20 | 26 | 24 | 36 | 77 | 83 | 251 | 254 |
| ± SD | 6 | 6 | 10 | 16 | 7 | 6 | 17 | 20 |

Appendix C-1: Overview of the oil concentrations applied in the 11 Ames experiments with spruce-derived pyrolysis oils

**Table C 1.1: The concentrations of pyrolysis oil applied in the Ames assay on TA98 and TA100 in absence of S9**. Mutagenic assessment of seven spruce-derived fast pyrolysis oils obtained from different relative proportions of wood, bark and needles as feedstock was performed through application of the preincubation version of the Ames assay without S9 in several experiments (Experiment 1-5 and 8-9) exposing TA98 and TA100 to different concentrations of the pyrolysis oils.

| | 100-0-0 | | 0-100-0 | | 0-0-100 | | 80-15-5 | | 60-40-0 | | 60-30-10 | | 43-22-35 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| µL oil/plate | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 |
| 0.1 | 1 | 1 | 2 | 2 | 5 | 5 | 5 | 5 | 4 | 4 | 5 | 5 | 3 | 3 |
| 0.25 | 1 | 1 | 2 | 2 | | | | | | | | | | |
| 0.5 | 1 | 1 | 2 | 2 | 5 | 5 | 5 | 5 | 4 | 4 | 5 | 5 | 3 | 3 |
| 0.75 | 1 | 1 | 2 | 2 | | | | | | | | | | |
| 1 | 1 | 1 | 2 | 2+3 | 5 | 5 | 5 | 5+9 | 4 | 4 | 5 | 5+9 | 3 | 3 |
| 1.5 | | | | | | | 8 | 9 | | | | 9 | | |
| 2 | | | 2+4 | 3+4 | 5 | 5 | 5+8 | 5 | 4 | 4 | 5+8 | 9 | 3 | 3 |
| 3 | | | | 4 | | | | | | | 8 | | | |
| 4 | | | 4 | 3+4 | | | | | 4 | 4 | 8 | | 3 | 3 |
| 5 | 1 | 1 | 2+3+4 | 2 | 5+8 | 5+9 | 5 | 5 | | | 5 | 5 | | |
| 6 | | | 3 | | 8 | 9 | | | 4 | 4 | | | 3 | 3 |
| 8 | | | 3 | | 8 | 9 | | | | | | | | |
| 10 | | | 3 | | 8 | 9 | | | | | | | | |

**Table C 1.2: The concentrations of pyrolysis oil applied in the Ames assay on TA98 and TA100 in the presence of S9**. Mutagenic assessment of seven spruce-derived fast pyrolysis oils obtained from different relative proportions of wood, bark and needles as feedstock was performed through application of the preincubation version of the Ames assay with S9 in several experiments (Experiment 6-7 and 10-11) exposing TA98 and TA100 to different concentrations of the pyrolysis oils.

| | 100-0-0 | | 0-100-0 | | 0-0-100 | | 80-15-5 | | 60-40-0 | | 60-30-10 | | 43-22-35 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| µL oil/plate | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 | TA98 | TA100 |
| 0.1 | 7 | 6 | 7 | 6 | 7 | 6 | 7 | 6 | 7 | 6 | 7 | 6 | 7 | 6 |
| 0.5 | 7 | 6 | 7 | 6 | 7 | 6 | 7 | 6 | 7 | 6 | 7 | 6 | 7 | 6 |
| 1 | 7 | 6 | 7 | 6 | 7 | 6 | 7 | 6 | 7 | 6 | 7 | 6 | 7 | 6 |
| 1.5 | 11 | 10 | | | | | | 10 | | 10 | | 10 | | |
| 2 | 7+11 | 6+10 | 7+11 | 6 | 7 | 6 | 7+11 | 6+10 | 7+11 | 6+10 | 7+11 | 6+10 | 7+11 | 6 |
| 3 | 11 | 10 | 11 | | | | 11 | 10 | 11 | 10 | 11 | 10 | 11 | |
| 4 | 11 | 10 | 11 | | | | 11 | 10 | 11 | 10 | 11 | 10 | 11 | |
| 5 | 7 | 6 | 7+11 | 6+10 | 7+11 | 6 | 7 | 6 | 7+11 | 6 | 7 | 6 | 7+11 | 6 +10 |
| 6 | | | | 10 | 11 | | | | | | | | | 10 |
| 8 | | | | 10 | 11 | | | | | | | | | 10 |
| 10 | | | | 10 | 11 | | | | | | | | | 10 |

Appendix D-1: Raw data of the Ames results for spruce-derived pyrolysis oils in absence of S9

**Table D 1.1: Results on primary mutagenicity observed in TA98 and TA100 induced by 100-0-0 in Experiment 1 of the Ames assay.** The preincubation version of the Ames assay was applied exposing TA98 and TA100 to a concentration range (0.1-5 µL/plate) of the spruce-derived fast pyrolysis oil 100-0-0 (n = 3) together with DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | | TA98 | | | TA100 | | |
|---|---|---|---|---|---|---|---|
| Exposure | | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | | 23 | 35 | 0 | 103 | 110 | 0 |
| | | 13 | 24 | | 120 | 126 | |
| | | 20 | 31 | | 119 | 122 | |
| | | 13 | 23 | | 92 | 96 | |
| | | 22 | 29 | | 109 | 111 | |
| | Mean | 18 | 28 | | 109 | 113 | |
| | ± SD | 5 | 5 | | 12 | 12 | |
| DMSO | | 14 | 29 | 0 | 126 | 132 | 0 |
| | | 13 | 19 | | 109 | 115 | |
| | | 15 | 22 | | 112 | 118 | |
| | | 10 | 17 | | 124 | 131 | |
| | | 18 | 24 | | 106 | 119 | |
| | Mean | 14 | 22 | | 115 | 123 | |
| | ± SD | 3 | 5 | | 9 | 8 | |
| 0.1 µl | | | | | 122 | 127 | 0 |
| | | | | | 131 | 135 | |
| | | | | | 123 | 127 | |
| | Mean | | | | 125 | 130 | |
| | ± SD | | | | 5 | 5 | |
| 0.25 µl | | | | | 238 | 240 | 1 |
| | | | | | 220 | 222 | |
| | | | | | 226 | 229 | |
| | Mean | | | | 228 | 230 | |
| | ± SD | | | | 9 | 9 | |
| 0.5 µl | | 20 | 27 | 0 | 304 | 304 | 1 |
| | | 23 | 29 | | 290 | 290 | |
| | | 15 | 24 | | 281 | 282 | |
| | Mean | 19 | 27 | | 292 | 292 | |
| | ± SD | 4 | 3 | | 12 | 11 | |
| 0.75 µl | | 32 | 44 | 1 | 205 | 209 | 1 |
| | | 30 | 37 | | 220 | 226 | |
| | | 16 | 23 | | 266 | 271 | |
| | Mean | 26 | 35 | | 230 | 235 | |
| | ± SD | 9 | 11 | | 32 | 32 | |
| 1 µl | | 17 | 23 | 1 | 135 | 140 | 2 |
| | | 23 | 34 | | 139 | 148 | |
| | | 21 | 25 | | 85 | 94 | |
| | Mean | 20 | 27 | | 120 | 127 | |
| | ± SD | 3 | 6 | | 30 | 29 | |
| 2 µl | | 14 | 18 | 2 | | | |
| | | 12 | 26 | | | | |
| | | 11 | 18 | | | | |
| | Mean | 12 | 21 | | | | |
| | ± SD | 2 | 5 | | | | |
| 4 µl | | 0 | 0 | 3 | | | |
| | | 0 | 0 | | | | |
| | | 0 | 0 | | | | |
| | Mean | 0 | 0 | | | | |
| | ± SD | 0 | 0 | | | | |
| 5 µl | | | | | 0 | 0 | 3 |
| | | | | | 0 | 0 | |
| | | | | | 0 | 0 | |
| | Mean | | | | 0 | 0 | |
| | ± SD | | | | 0 | 0 | |

**Table D 1.2 A: Results on primary mutagenicity observed in TA98 induced by 0-100-0 in Experiment 2-4 of the Ames assay.** The preincubation version of the Ames assay was applied exposing TA98 to a concentration range (0.1-10 µL/plate) of the spruce-derived fast pyrolysis oil 0-100-0 (n = 3) together with DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | | Experiment 2 | | | Experiment 3 | | | Experiment 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Exposure | | 48 h | 72 h | BTL | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | | 29 | 37 | 0 | 14 | 22 | 0 | 10 | 15 | 0 |
| | | 24 | 34 | | 18 | 26 | | 18 | 27 | |
| | | 19 | 31 | | 26 | 41 | | 15 | 22 | |
| | | 24 | 33 | | 20 | 30 | | 23 | 29 | |
| | | 29 | 34 | | 18 | 24 | | 30 | 36 | |
| | Mean | 25 | 34 | | 19 | 29 | | 19 | 26 | |
| | ± SD | 4 | 2 | | 4 | 8 | | 8 | 8 | |
| DMSO | | 23 | 29 | 0 | 14 | 20 | 0 | 12 | 23 | 0 |
| | | 18 | 27 | | 7 | 19 | | 12 | 18 | |
| | | 17 | 26 | | 12 | 18 | | 11 | 18 | |
| | | 18 | 24 | | 15 | 25 | | 15 | 27 | |
| | | 15 | 19 | | 19 | 29 | | 10 | 18 | |
| | Mean | 18 | 25 | | 13 | 22 | | 12 | 21 | |
| | ± SD | 3 | 4 | | 4 | 5 | | 2 | 4 | |
| 0.1 µl | | 16 | 26 | 0 | | | | | | |
| | | 11 | 18 | | | | | | | |
| | | 12 | 19 | | | | | | | |
| | Mean | 13 | 21 | | | | | | | |
| | ± SD | 3 | 4 | | | | | | | |
| 0.25 µl | | 12 | 20 | 0 | | | | | | |
| | | 15 | 19 | | | | | | | |
| | | 12 | 18 | | | | | | | |
| | Mean | 13 | 19 | | | | | | | |
| | ± SD | 2 | 1 | | | | | | | |
| 0.5 µl | | 20 | 31 | 0 | | | | | | |
| | | 17 | 24 | | | | | | | |
| | | 14 | 18 | | | | | | | |
| | Mean | 17 | 24 | | | | | | | |
| | ± SD | 3 | 7 | | | | | | | |
| 0.75 µl | | 25 | 30 | 0 | | | | | | |
| | | 20 | 32 | | | | | | | |
| | | 17 | 24 | | | | | | | |
| | Mean | 21 | 29 | | | | | | | |
| | ± SD | 4 | 4 | | | | | | | |
| 1 µl | | 24 | 31 | 0 | | | | | | |
| | | 24 | 33 | | | | | | | |
| | | 17 | 25 | | | | | | | |
| | Mean | 22 | 30 | | | | | | | |
| | ± SD | 4 | 4 | | | | | | | |
| 2 µl | | 35 | 41 | 0 | | | | 42 | 51 | 0 |
| | | 20 | 25 | | | | | 43 | 53 | |
| | | 24 | 34 | | | | | 33 | 44 | |
| | Mean | 26 | 33 | | | | | 39 | 49 | |
| | ± SD | 8 | 8 | | | | | 6 | 5 | |
| 4 µL | | | | | | | | 45 | 50 | 0 |
| | | | | | | | | 35 | 43 | |
| | | | | | | | | 57 | 65 | |
| | Mean | | | | | | | 46 | 53 | |
| | ± SD | | | | | | | 11 | 11 | |
| 5 µl | | 43 | 52 | 0 | 37 | 46 | 0 | 33 | 36 | 0 |
| | | 46 | 57 | | 39 | 47 | | 33 | 39 | |
| | | 47 | 60 | | 31 | 37 | | 34 | 39 | |
| | Mean | 45 | 56 | | 36 | 43 | | 33 | 38 | |
| | ± SD | 2 | 4 | | 4 | 6 | | 1 | 2 | |
| 6 µL | | | | | 38 | 44 | 0 | | | |
| | | | | | 35 | 46 | | | | |
| | | | | | 29 | 34 | | | | |
| | Mean | | | | 34 | 41 | | | | |
| | ± SD | | | | 5 | 6 | | | | |
| 8 µL | | | | | 38 | 44 | 0 | | | |
| | | | | | 35 | 40 | | | | |
| | | | | | 24 | 35 | | | | |
| | Mean | | | | 32 | 40 | | | | |
| | ± SD | | | | 7 | 5 | | | | |
| 10 µL | | | | | 22 | 32 | 0 | | | |
| | | | | | 32 | 39 | | | | |
| | | | | | 29 | 33 | | | | |
| | Mean | | | | 28 | 35 | | | | |
| | ± SD | | | | 5 | 4 | | | | |

**Table D 1.2 B: Results on primary mutagenicity observed in TA100 induced by 0-100-0 in Experiment 2-4 of the Ames assay.** The preincubation version of the Ames assay was applied exposing TA100 to a concentration range (0.1-5 µL/plate) of the spruce-derived fast pyrolysis oil 0-100-0 (n = 3) together with DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | | Experiment 2 | | | Experiment 3 | | | Experiment 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Exposure | | 48 h | 72 h | BTL | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | | 103 | 109 | 0 | 101 | 113 | 0 | 118 | 124 | 0 |
| | | 102 | 113 | | 100 | 109 | | 108 | 114 | |
| | | 95 | 102 | | 87 | 93 | | 115 | 120 | |
| | | 100 | 110 | | 107 | 115 | | 111 | 117 | |
| | | 101 | 107 | | 97 | 105 | | 99 | 103 | |
| | Mean | 100 | 108 | | 98 | 107 | | 110 | 116 | |
| | ± SD | 3 | 4 | | 7 | 9 | | 7 | 8 | |
| DMSO | | 90 | 98 | 0 | 102 | 108 | 0 | 104 | 113 | 0 |
| | | 89 | 95 | | 88 | 95 | | 100 | 111 | |
| | | 103 | 109 | | 108 | 120 | | 129 | 139 | |
| | | 87 | 95 | | 88 | 101 | | 119 | 123 | |
| | | 93 | 104 | | 89 | 92 | | 114 | 120 | |
| | Mean | 92 | 100 | | 95 | 103 | | 113 | 121 | |
| | ± SD | 6 | 6 | | 9 | 11 | | 12 | 11 | |
| 0.1 µl | | 102 | 108 | 0 | | | | | | |
| | | 94 | 103 | | | | | | | |
| | | 96 | 106 | | | | | | | |
| | Mean | 97 | 106 | | | | | | | |
| | ± SD | 4 | 3 | | | | | | | |
| 0.25 µl | | 125 | 132 | 0 | | | | | | |
| | | 120 | 133 | | | | | | | |
| | | 91 | 99 | | | | | | | |
| | Mean | 112 | 121 | | | | | | | |
| | ± SD | 18 | 19 | | | | | | | |
| 0.5 µl | | 143 | 149 | 0 | | | | | | |
| | | 129 | 131 | | | | | | | |
| | | 119 | 125 | | | | | | | |
| | Mean | 130 | 135 | | | | | | | |
| | ± SD | 12 | 12 | | | | | | | |
| 0.75 µl | | 151 | 160 | 0 | | | | | | |
| | | 128 | 131 | | | | | | | |
| | | 147 | 152 | | | | | | | |
| | Mean | 142 | 148 | | | | | | | |
| | ± SD | 12 | 15 | | | | | | | |
| 1 µl | | 171 | 173 | 0 | 165 | 176 | 0 | | | |
| | | 196 | 196 | | 162 | 167 | | | | |
| | | 184 | 188 | | 170 | 171 | | | | |
| | Mean | 184 | 186 | | 166 | 171 | | | | |
| | ± SD | 13 | 12 | | 4 | 5 | | | | |
| 2 µL | | | | | 175 | 176 | 0 | 342 | 344 | 0 |
| | | | | | 206 | 209 | | 363 | 368 | |
| | | | | | 217 | 221 | | 395 | 403 | |
| | Mean | | | | 199 | 202 | | 367 | 372 | |
| | ± SD | | | | 22 | 23 | | 27 | 30 | |
| 3 µL | | | | | | | | 237 | 246 | 1 |
| | | | | | | | | 280 | 288 | |
| | | | | | | | | 312 | 322 | |
| | Mean | | | | | | | 276 | 285 | |
| | ± SD | | | | | | | 38 | 38 | |
| 4 µL | | | | | 228 | 235 | 1 | 72 | 136 | 2 |
| | | | | | 280 | 284 | | 96 | 171 | |
| | | | | | 237 | 242 | | 178 | 196 | |
| | Mean | | | | 248 | 254 | | 115 | 168 | |
| | ± SD | | | | 28 | 27 | | 56 | 30 | |
| 5 µl | | 70 | 101 | 2 | | | | | | |
| | | 102 | 133 | | | | | | | |
| | | 62 | 110 | | | | | | | |
| | Mean | 78 | 115 | | | | | | | |
| | ± SD | 21 | 17 | | | | | | | |

**Table D 1.3 A: Results on primary mutagenicity observed in TA98 induced by 0-0-100 in Experiment 5 and 8 of the Ames assay.** The preincubation version of the Ames assay was applied exposing TA98 to a concentration range (0.1-10 µL/plate) of the spruce-derived fast pyrolysis oil 0-0-100 (n = 3) together with NPD controls (n = 2), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | | Experiment 5 | | | Experiment 8 | | |
|---|---|---|---|---|---|---|---|
| Exposure | | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | | 18 | 26 | 0 | 20 | 23 | 0 |
| | | 21 | 33 | | 16 | 24 | |
| | | 24 | 37 | | 16 | 38 | |
| | | 22 | 34 | | 15 | 28 | |
| | | | | | 12 | 23 | |
| | Mean | 22 | 33 | | 16 | 27 | |
| | ± SD | 3 | 5 | | 3 | 6 | |
| DMSO | | 12 | 21 | 0 | 18 | 25 | 0 |
| | | 21 | 34 | | 20 | 24 | |
| | | 18 | 24 | | 12 | 19 | |
| | | 18 | 26 | | 16 | 21 | |
| | | 17 | 22 | | 12 | 19 | |
| | Mean | 17 | 25 | | 16 | 22 | |
| | ± SD | 3 | 5 | | 4 | 3 | |
| 0.1 µL | | 15 | 22 | 0 | | | |
| | | 16 | 20 | | | | |
| | | 21 | 21 | | | | |
| | Mean | 17 | 21 | | | | |
| | ± SD | 3 | 1 | | | | |
| 0.5 µL | | 17 | 22 | 0 | | | |
| | | 18 | 22 | | | | |
| | | 15 | 21 | | | | |
| | Mean | 17 | 22 | | | | |
| | ± SD | 2 | 1 | | | | |
| 1 µL | | 18 | 23 | 0 | | | |
| | | 17 | 26 | | | | |
| | | 14 | 23 | | | | |
| | Mean | 16 | 24 | | | | |
| | ± SD | 2 | 2 | | | | |
| 2 µL | | 30 | 37 | 0 | | | |
| | | 28 | 35 | | | | |
| | | 19 | 31 | | | | |
| | Mean | 26 | 34 | | | | |
| | ± SD | 6 | 3 | | | | |
| 5 µL | | 36 | 39 | 0 | 32 | 38 | 0 |
| | | 36 | 46 | | 26 | 36 | |
| | | 35 | 43 | | 22 | 29 | |
| | Mean | 36 | 43 | | 27 | 34 | |
| | ± SD | 1 | 4 | | 5 | 5 | |
| 6 µl | | | | 0 | 28 | 36 | 0 |
| | | | | | 29 | 39 | |
| | | | | | 28 | 35 | |
| | Mean | | | | 28 | 37 | |
| | ± SD | | | | 1 | 2 | |
| 8 µl | | | | | 30 | 37 | 0 |
| | | | | | 28 | 32 | |
| | | | | | 33 | 37 | |
| | Mean | | | | 30 | 35 | |
| | ± SD | | | | 3 | 3 | |
| 10 µl | | | | | 29 | 31 | 0 |
| | | | | | 20 | 26 | |
| | | | | | 36 | 45 | |
| | Mean | | | | 28 | 34 | |
| | ± SD | | | | 8 | 10 | |
| NPD | | 1972 | | | 2220 | | |
| | | 1970 | | | 2152 | | |
| | Mean | 1971 | | | 2186 | | |
| | ± SD | 1 | | | 48 | | |

**Table D 1.3 B: Results on primary mutagenicity observed in TA100 induced by 0-0-100 in Experiment 5 and 9 of the Ames assay.** The preincubation version of the Ames assay was applied exposing TA100 to a concentration range (0.1-10 µL/plate) of the spruce-derived fast pyrolysis oil 0-100-0 (n = 3) together with Na-azid controls (n = 2-3), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | | Experiment 5 | | | Experiment 9 | | |
|---|---|---|---|---|---|---|---|
| Exposure | 48 h | 72 h | BTL | | 48 h | 72 h | BTL |
| Spontane | | 115 | 119 | 0 | 105 | 122 | 0 |
| | | 105 | 108 | | 104 | 121 | |
| | | 124 | 126 | | 121 | 133 | |
| | | 96 | 101 | | 120 | 131 | |
| | | | | | 103 | 119 | |
| | Mean | 110 | 114 | | 111 | 125 | |
| | ± SD | 12 | 11 | | 9 | 6 | |
| DMSO | | 98 | 103 | 0 | 120 | 130 | 0 |
| | | 109 | 115 | | 110 | 126 | |
| | | 124 | 132 | | 107 | 120 | |
| | | 107 | 112 | | 102 | 118 | |
| | | 90 | 95 | | 99 | 117 | |
| | Mean | 106 | 111 | | 108 | 122 | |
| | ± SD | 13 | 14 | | 8 | 6 | |
| 0.1 µL | | 122 | 124 | 0 | | | |
| | | 116 | 120 | | | | |
| | | 118 | 124 | | | | |
| | Mean | 119 | 123 | | | | |
| | ± SD | 3 | 2 | | | | |
| 0.5 µL | | 103 | 105 | 0 | | | |
| | | 117 | 119 | | | | |
| | | 107 | 107 | | | | |
| | Mean | 109 | 110 | | | | |
| | ± SD | 7 | 8 | | | | |
| 1 µL | | 108 | 111 | 0 | | | |
| | | 129 | 133 | | | | |
| | | 122 | 124 | | | | |
| | Mean | 120 | 123 | | | | |
| | ± SD | 11 | 11 | | | | |
| 2 µL | | 146 | 149 | 0 | | | |
| | | 146 | 149 | | | | |
| | | 124 | 127 | | | | |
| | Mean | 139 | 142 | | | | |
| | ± SD | 13 | 13 | | | | |
| 5 µL | | 165 | 166 | 0 | 128 | 149 | 0 |
| | | 178 | 181 | | 146 | 163 | |
| | | 184 | 185 | | 148 | 166 | |
| | Mean | 176 | 177 | | 141 | 159 | |
| | ± SD | 10 | 10 | | 11 | 9 | |
| 6 µl | | | | 0 | 133 | 141 | 0 |
| | | | | | 138 | 142 | |
| | | | | | 163 | 169 | |
| | Mean | | | | 145 | 151 | |
| | ± SD | | | | 16 | 16 | |
| 8 µl | | | | | 98 | 112 | 0 |
| | | | | | 125 | 136 | |
| | | | | | 163 | 173 | |
| | Mean | | | | 129 | 140 | |
| | ± SD | | | | 33 | 31 | |
| 10 µl | | | | | 46 | 87 | 1 |
| | | | | | 69 | 84 | |
| | | | | | 101 | 108 | |
| | Mean | | | | 72 | 93 | |
| | ± SD | | | | 28 | 13 | |
| Na-azid | | 838 | | | 620 | | |
| | | 856 | | | 672 | | |
| | | | | | 658 | | |
| | Mean | 847 | | | 650 | | |
| | ± SD | 13 | | | 27 | | |

**Table D 1.4 A: Results on primary mutagenicity observed in TA98 induced by 80-15-5 in Experiment 5 and 8 of the Ames assay.** The preincubation version of the Ames assay was applied exposing TA98 to a concentration range (0.1-5 µL/plate) of the spruce-derived fast pyrolysis oil 80-15-5 (n = 3) together with NPD controls (n = 2), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | | Experiment 5 | | | Experiment 8 | | |
|---|---|---|---|---|---|---|---|
| Exposure | | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | | 18 | 26 | 0 | 20 | 23 | 0 |
| | | 21 | 33 | | 16 | 24 | |
| | | 24 | 37 | | 16 | 38 | |
| | | 22 | 34 | | 15 | 28 | |
| | | | | | 12 | 23 | |
| | Mean | 21 | 33 | | 16 | 27 | |
| | ± SD | 3 | 5 | | 3 | 6 | |
| DMSO | | 12 | 21 | 0 | 18 | 25 | 0 |
| | | 21 | 34 | | 20 | 24 | |
| | | 18 | 24 | | 12 | 19 | |
| | | 18 | 26 | | 16 | 21 | |
| | | 17 | 22 | | 12 | 19 | |
| | Mean | 17 | 25 | | 16 | 22 | |
| | ± SD | 3 | 5 | | 4 | 3 | |
| 0.1 µL | | 17 | 20 | 0 | | | |
| | | 15 | 18 | | | | |
| | | 20 | 26 | | | | |
| | Mean | 17 | 21 | | | | |
| | ± SD | 3 | 4 | | | | |
| 0.5 µL | | 26 | 38 | 0 | | | |
| | | 30 | 35 | | | | |
| | | 20 | 25 | | | | |
| | Mean | 25 | 33 | | | | |
| | ± SD | 5 | 7 | | | | |
| 1 µL | | 39 | 44 | 0 | | | |
| | | 26 | 32 | | | | |
| | | 33 | 37 | | | | |
| | Mean | 33 | 38 | | | | |
| | ± SD | 7 | 6 | | | | |
| 1,5 µl | | | | | 9 | 19 | 1 |
| | | | | | 15 | 25 | |
| | | | | | 18 | 23 | |
| | Mean | | | | 14 | 22 | |
| | ± SD | | | | 5 | 3 | |
| 2 µL | | 18 | 20 | 1 | 16 | 20 | 1 |
| | | 23 | 26 | | 12 | 16 | |
| | | 22 | 34 | | 14 | 21 | |
| | Mean | 21 | 27 | | 14 | 19 | |
| | ± SD | 3 | 7 | | 2 | 3 | |
| 5 µL | | 0 | 0 | | | | |
| | | 0 | 0 | | | | |
| | | 0 | 0 | | | | |
| | Mean | 0 | 0 | | | | |
| | ± SD | 0 | 0 | | | | |
| NPD | | 1972 | | 0 | 2220 | | |
| | | 1970 | | | 2152 | | |
| | Mean | 1971 | | | 2186 | | |
| | ± SD | 1 | | | 48 | | |

**Table D 1.4 B: Results on primary mutagenicity observed in TA100 induced by 80-15-5 in Experiment 5 and 9 of the Ames assay.** The preincubation version of the Ames assay was applied exposing TA100 to a concentration range (0.1-5 µL/plate) of the spruce-derived fast pyrolysis oil 80-15-5 (n = 3) together with Na-azid controls (n = 2-3), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | | Experiment 5 | | | Experiment 9 | | |
|---|---|---|---|---|---|---|---|
| Exposure | | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | | 115 | 119 | 0 | 105 | 122 | 0 |
| | | 105 | 108 | | 104 | 121 | |
| | | 124 | 126 | | 121 | 133 | |
| | | 96 | 101 | | 120 | 131 | |
| | | | | | 103 | 119 | |
| | Mean | 110 | 114 | | 111 | 125 | |
| | ± SD | 12 | 11 | | 9 | 6 | |
| DMSO | | 98 | 103 | 0 | 120 | 130 | 0 |
| | | 109 | 115 | | 110 | 126 | |
| | | 124 | 132 | | 107 | 120 | |
| | | 107 | 112 | | 102 | 118 | |
| | | 90 | 95 | | 99 | 117 | |
| | Mean | 106 | 111 | | 108 | 122 | |
| | ± SD | 13 | 14 | | 8 | 6 | |
| 0.1 µL | | 174 | 175 | 0 | | | |
| | | 171 | 171 | | | | |
| | | 197 | 197 | | | | |
| | Mean | 181 | 181 | | | | |
| | ± SD | 14 | 14 | | | | |
| 0.5 µL | | 381 | 384 | 0 | | | |
| | | 409 | 411 | | | | |
| | | 403 | 406 | | | | |
| | Mean | 398 | 400 | | | | |
| | ± SD | 15 | 14 | | | | |
| 1 µL | | 440 | 440 | 1 | 312 | 319 | 1 |
| | | 473 | 474 | | 329 | 333 | |
| | | 452 | 453 | | 294 | 307 | |
| | Mean | 455 | 456 | | 312 | 320 | |
| | ± SD | 17 | 17 | | 18 | 13 | |
| 1.5 µl | | | | | 259 | 274 | 1 |
| | | | | | 262 | 273 | |
| | | | | | 298 | 316 | |
| | Mean | | | | 273 | 288 | |
| | ± SD | | | | 22 | 25 | |
| 2 µL | | 0 | 0 | 2 | | | |
| | | 0 | 0 | | | | |
| | | 0 | 3 | | | | |
| | Mean | 0 | 1 | | | | |
| | ± SD | 0 | 2 | | | | |
| 5 µL | | 0 | 0 | 3 | | | |
| | | 0 | 0 | | | | |
| | | 0 | 0 | | | | |
| | Mean | 0 | 0 | | | | |
| | ± SD | 0 | 0 | | | | |
| Na-azid | | 838 | | 0 | 620 | | |
| | | 856 | | | 672 | | |
| | | | | | 658 | | |
| | Mean | 847 | | | 650 | | |
| | ± SD | 13 | | | 27 | | |

**Table D 1.5: Results on primary mutagenicity observed in TA98 and TA100 induced by 60-40-0 in Experiment 4 of the Ames assay.** The preincubation version of the Ames assay was applied exposing TA98 and TA100 to a concentration range (0.1-6 µL/plate) of the spruce-derived fast pyrolysis oil 60-40-0 (n = 3) together with NPD and Na-azid controls (n = 2), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | | TA98 | | |
|---|---|---|---|---|
| Exposure | | 48 h | 72 h | BTL |
| Spontane | | 10 | 15 | 0 |
| | | 18 | 27 | |
| | | 15 | 22 | |
| | | 23 | 29 | |
| | | 30 | 36 | |
| | Mean | 19 | 26 | |
| | ± SD | 8 | 8 | |
| DMSO | | 12 | 23 | 0 |
| | | 12 | 18 | |
| | | 11 | 18 | |
| | | 15 | 27 | |
| | | 10 | 18 | |
| | Mean | 12 | 21 | |
| | ± SD | 2 | 4 | |
| 0.1 µl | | 13 | 23 | 0 |
| | | 14 | 22 | |
| | | 10 | 17 | |
| | Mean | 12 | 21 | |
| | ± SD | 2 | 3 | |
| 0.5 µl | | 17 | 26 | 0 |
| | | 20 | 29 | |
| | | 17 | 24 | |
| | Mean | 18 | 26 | |
| | ± SD | 2 | 3 | |
| 1 µl | | 35 | 45 | 0 |
| | | 24 | 36 | |
| | | 28 | 37 | |
| | Mean | 29 | 39 | |
| | ± SD | 6 | 5 | |
| 2 µl | | 20 | 29 | 0 |
| | | 22 | 28 | |
| | | 18 | 25 | |
| | Mean | 20 | 27 | |
| | ± SD | 2 | 2 | |
| 4 µl | | 0 | 0 | 2 |
| | | 0 | 0 | |
| | | 0 | 0 | |
| | Mean | 0 | 0 | |
| | ± SD | 0 | 0 | |
| 6 µl | | 0 | 0 | 3 |
| | | 0 | 0 | |
| | | 0 | 0 | |
| | Mean | 0 | 0 | |
| | ± SD | 0 | 0 | |
| NPD | | 1124 | | 0 |
| | | 994 | | |
| | Mean | 1059 | | |
| | ± SD | 92 | | |

| | | TA100 | | |
|---|---|---|---|---|
| Exposure | | 48 h | 72 h | BTL |
| Spontane | | 118 | 124 | 0 |
| | | 108 | 114 | |
| | | 115 | 120 | |
| | | 111 | 117 | |
| | | 99 | 103 | |
| | Mean | 110 | 116 | |
| | ± SD | 7 | 8 | |
| DMSO | | 104 | 113 | 0 |
| | | 100 | 111 | |
| | | 129 | 139 | |
| | | 119 | 123 | |
| | | 114 | 120 | |
| | Mean | 113 | 121 | |
| | ± SD | 12 | 11 | |
| 0.1 µl | | 109 | 112 | 0 |
| | | 154 | 163 | |
| | | 135 | 143 | |
| | Mean | 133 | 139 | |
| | ± SD | 23 | 26 | |
| 0.5 µl | | 311 | 313 | 0 |
| | | 317 | 320 | |
| | | 327 | 331 | |
| | Mean | 318 | 321 | |
| | ± SD | 8 | 9 | |
| 1 µl | | 476 | 478 | 1 |
| | | 463 | 465 | |
| | | 418 | 420 | |
| | Mean | 452 | 454 | |
| | ± SD | 30 | 30 | |
| 2 µl | | 130 | 147 | 2 |
| | | 200 | 207 | |
| | | 305 | 312 | |
| | Mean | 212 | 222 | |
| | ± SD | 88 | 84 | |
| 4 µl | | 0 | 0 | 3 |
| | | 0 | 0 | |
| | | 0 | 0 | |
| | Mean | 0 | 0 | |
| | ± SD | 0 | 0 | |
| 6 µl | | 0 | 0 | 3 |
| | | 0 | 0 | |
| | | 0 | 0 | |
| | Mean | 0 | 0 | |
| | ± SD | 0 | 0 | |
| Na-azid | | 948 | | 0 |
| | | 930 | | |
| | Mean | 939 | | |
| | ± SD | 13 | | |

**Table D 1.6 A: Results on primary mutagenicity observed in TA98 induced by 60-30-10 in Experiment 5 and 8 of the Ames assay.** The preincubation version of the Ames assay was applied exposing TA98 to a concentration range (0.1-5 µL/plate) of the spruce-derived fast pyrolysis oil 60-30-10 (n = 3) together with NPD controls (n = 2), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | | Experiment 5 | | | Experiment 8 | | |
|---|---|---|---|---|---|---|---|
| Exposure | | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | | 18 | 26 | 0 | 20 | 23 | 0 |
| | | 21 | 33 | | 16 | 24 | |
| | | 24 | 37 | | 16 | 38 | |
| | | 22 | 34 | | 15 | 28 | |
| | | | | | 12 | 23 | |
| | Mean | 21 | 33 | | 16 | 27 | |
| | ± SD | 3 | 5 | | 3 | 6 | |
| DMSO | | 12 | 21 | 0 | 18 | 25 | 0 |
| | | 21 | 34 | | 20 | 24 | |
| | | 18 | 24 | | 12 | 19 | |
| | | 18 | 26 | | 16 | 21 | |
| | | 17 | 22 | | 12 | 19 | |
| | Mean | 17 | 25 | | 16 | 22 | |
| | ± SD | 3 | 5 | | 4 | 3 | |
| 0.1 µL | | 27 | 31 | 0 | | | |
| | | 20 | 26 | | | | |
| | | 18 | 29 | | | | |
| | Mean | 22 | 29 | | | | |
| | ± SD | 5 | 3 | | | | |
| 0.5 µL | | 23 | 27 | 0 | | | |
| | | 35 | 40 | | | | |
| | | 20 | 25 | | | | |
| | Mean | 26 | 31 | | | | |
| | ± SD | 8 | 8 | | | | |
| 1 µL | | 25 | 32 | 0 | | | |
| | | 36 | 52 | | | | |
| | | 28 | 38 | | | | |
| | Mean | 30 | 41 | | | | |
| | ± SD | 6 | 10 | | | | |
| 2 µL | | 33 | 40 | 0 | 25 | 36 | 0 |
| | | 32 | 38 | | 27 | 35 | |
| | | 35 | 44 | | 21 | 25 | |
| | Mean | 33 | 41 | | 24 | 32 | |
| | ± SD | 2 | 3 | | 3 | 6 | |
| 3 µl | | | | 2 | 12 | 19 | 1 |
| | | | | | 13 | 18 | |
| | | | | | 16 | 22 | |
| | Mean | | | | 14 | 20 | |
| | ± SD | | | | 2 | 2 | |
| 4 µl | | | | 0 | 1 | 13 | 2 |
| | | | | | 1 | 11 | |
| | | | | | 3 | 13 | |
| | Mean | | | | 2 | 12 | |
| | ± SD | | | | 1 | 1 | |
| 5 µL | | 0 | 0 | | | | |
| | | 0 | 0 | | | | |
| | | 0 | 3 | | | | |
| | Mean | 0 | 1 | | | | |
| | ± SD | 0 | 2 | | | | |
| NPD | | 1972 | | | 2220 | | |
| | | 1970 | | | 2152 | | |
| | Mean | 1971 | | | 2186 | | |
| | ± SD | 1 | | | 48 | | |

**Table D 1.6 B: Results on primary mutagenicity observed in TA100 induced by 60-30-10 in Experiment 5 and 8 of the Ames assay.** The preincubation version of the Ames assay was applied exposing TA98 to a concentration range (0.1-5 µL/plate) of the spruce-derived fast pyrolysis oil 60-30-10 (n = 3) together with Na-azid controls (n = 2-3), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | | Experiment 5 | | | Experiment 9 | | |
|---|---|---|---|---|---|---|---|
| Exposure | | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | | 115 | 119 | 0 | 105 | 122 | 0 |
| | | 105 | 108 | | 104 | 121 | |
| | | 124 | 126 | | 121 | 133 | |
| | | 96 | 101 | | 120 | 131 | |
| | | | | | 103 | 119 | |
| | Mean | 110 | 114 | | 111 | 125 | |
| | ± SD | 12 | 11 | | 9 | 6 | |
| DMSO | | 98 | 103 | 0 | 120 | 130 | 0 |
| | | 109 | 115 | | 110 | 126 | |
| | | 124 | 132 | | 107 | 120 | |
| | | 107 | 112 | | 102 | 118 | |
| | | 90 | 95 | | 99 | 117 | |
| | Mean | 106 | 111 | | 108 | 122 | |
| | ± SD | 13 | 14 | | 8 | 6 | |
| 0.1 µL | | 160 | 161 | 0 | | | |
| | | 160 | 162 | | | | |
| | | 186 | 188 | | | | |
| | Mean | 169 | 170 | | | | |
| | ± SD | 15 | 15 | | | | |
| 0.5 µL | | 308 | 310 | 0 | | | |
| | | 289 | 292 | | | | |
| | | 293 | 295 | | | | |
| | Mean | 297 | 299 | | | | |
| | ± SD | 10 | 10 | | | | |
| 1 µL | | 361 | 363 | 0 | 280 | 282 | 0 |
| | | 380 | 384 | | 278 | 282 | |
| | | 349 | 352 | | 287 | 290 | |
| | Mean | 363 | 366 | | 282 | 285 | |
| | ± SD | 16 | 16 | | 5 | 5 | |
| 1.5 µl | | | | | 313 | 321 | 0 |
| | | | | | 295 | 302 | |
| | | | | | 346 | 349 | |
| | Mean | | | | 318 | 324 | |
| | ± SD | | | | 26 | 24 | |
| 2 µL | | 216 | 222 | 1 | 291 | 301 | 1 |
| | | 211 | 213 | | 309 | 317 | |
| | | 233 | 237 | | 296 | 307 | |
| | Mean | 220 | 224 | | 299 | 308 | |
| | ± SD | 12 | 12 | | 9 | 8 | |
| 5 µL | | 0 | 0 | 3 | | | |
| | | 0 | 0 | | | | |
| | | 0 | 0 | | | | |
| | Mean | 0 | 0 | | | | |
| | ± SD | 0 | 0 | | | | |
| Na-azid | | 838 | | 0 | 620 | | |
| | | 856 | | | 672 | | |
| | | | | | 658 | | |
| | Mean | 847 | | | 650 | | |
| | ± SD | 13 | | | 27 | | |

**Table D 1.7: Results on primary mutagenicity observed in TA98 and TA100 induced by 43-22-35 in Experiment 3 of the Ames assay.** The preincubation version of the Ames assay was applied exposing TA98 and TA100 to a concentration range (0.1-6 µL/plate) of the spruce-derived fast pyrolysis oil 43-22-35 (n = 3) together with NPD and Na-azid controls (n = 2), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | | TA98 | | | | | TA100 | | |
|---|---|---|---|---|---|---|---|---|---|
| Exposure | | 48 h | 72 h | BTL | Exposure | | 48 h | 72 h | BTL |
| Spontane | | 14 | 22 | 0 | Spontane | | 101 | 113 | 0 |
| | | 18 | 26 | | | | 100 | 109 | |
| | | 26 | 41 | | | | 87 | 93 | |
| | | 20 | 30 | | | | 107 | 115 | |
| | | 18 | 24 | | | | 97 | 105 | |
| | Mean | 19 | 29 | | | Mean | 98 | 107 | |
| | ± SD | 4 | 8 | | | ± SD | 7 | 9 | |
| DMSO | | 14 | 20 | 0 | DMSO | | 102 | 108 | 0 |
| | | 7 | 19 | | | | 88 | 95 | |
| | | 12 | 18 | | | | 108 | 120 | |
| | | 15 | 25 | | | | 88 | 101 | |
| | | 19 | 29 | | | | 89 | 92 | |
| | Mean | 13 | 22 | | | Mean | 95 | 103 | |
| | ± SD | 4 | 5 | | | ± SD | 9 | 11 | |
| 0.1 µl | | 15 | 19 | 0 | 0.1 µl | | 120 | 127 | 0 |
| | | 13 | 23 | | | | 103 | 110 | |
| | | 13 | 20 | | | | 123 | 131 | |
| | Mean | 14 | 21 | | | Mean | 115 | 123 | |
| | ± SD | 1 | 2 | | | ± SD | 11 | 11 | |
| 0.5 µl | | 18 | 26 | 0 | 0.5 µl | | 157 | 162 | 0 |
| | | 19 | 26 | | | | 154 | 155 | |
| | | 15 | 22 | | | | 154 | 158 | |
| | Mean | 17 | 25 | | | Mean | 155 | 158 | |
| | ± SD | 2 | 2 | | | ± SD | 2 | 4 | |
| 1 µl | | 18 | 28 | 0 | 1 µl | | 241 | 244 | 0 |
| | | 22 | 32 | | | | 233 | 235 | |
| | | 19 | 30 | | | | 244 | 248 | |
| | Mean | 20 | 30 | | | Mean | 239 | 242 | |
| | ± SD | 2 | 2 | | | ± SD | 6 | 7 | |
| 2 µl | | 29 | 39 | 0 | 2 µl | | 279 | 280 | 1 |
| | | 26 | 35 | | | | 328 | 331 | |
| | | | | | | | 309 | 321 | |
| | Mean | 28 | 37 | | | Mean | 305 | 311 | |
| | ± SD | 2 | 3 | | | ± SD | 25 | 27 | |
| 4 µl | | 28 | 33 | 1 | 4 µl | | 0 | 0 | 3 |
| | | 35 | 42 | | | | 0 | 0 | |
| | | 31 | 36 | | | | 0 | 0 | |
| | Mean | 31 | 37 | | | Mean | 0 | 0 | |
| | ± SD | 4 | 5 | | | ± SD | 0 | 0 | |
| 6 µl | | 8 | 22 | 2 | 6 µl | | 0 | 0 | 3 |
| | | 8 | 22 | | | | 0 | 0 | |
| | | | | | | | 0 | 0 | |
| | Mean | 8 | 22 | | | Mean | 0 | 0 | |
| | ± SD | 0 | 0 | | | ± SD | 0 | 0 | |
| NPD | | 1715 | 1748 | 0 | Na-azid | | 716 | 735 | 0 |
| | | 1649 | 1674 | | | | 646 | 669 | |
| | Mean | 1682 | 1711 | | | Mean | 681 | 702 | |
| | ± SD | 47 | 52 | | | ± SD | 49 | 47 | |

Appendix D-2: Raw data of the Ames results for spruce-derived pyrolysis oils in the presence of S9

**Table D 2.1: Results on secondary mutagenicity observed in TA98 and TA100 induced by 100-0-0 in Experiment 6-7 and 10-11 of the Ames assay.** The preincubation version of the Ames assay was applied with S9 exposing TA98 and TA100 to a concentration range (0.1-5 µL/plate) of the spruce-derived fast pyrolysis oil 100-0-0 (n = 3) together with NPD, Na-azid and BaP controls (n = 3), S9 batch controls with DMSO and BaP (n = 1), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | TA98 | | | | | | TA100 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Experiment 7 | | | Experiment 11 | | | Experiment 6 | | | Experiment 10 | | |
| Exposure | 48 h | 72 h | BTL | 48 h | 72 h | BTL | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | 15 | 21 | 0 | 18 | 27 | 0 | 116 | 125 | 0 | 111 | 117 | |
| | 23 | 31 | | 28 | 32 | | 101 | 117 | | 105 | 113 | |
| | 27 | 33 | | 20 | 29 | | 103 | 119 | | 124 | 130 | |
| | 22 | 27 | | 20 | 29 | | 108 | 121 | | 122 | 124 | |
| | 17 | 22 | | 28 | 38 | | 110 | 124 | | 122 | 126 | |
| Mean | 21 | 27 | | 23 | 31 | | 108 | 121 | | 117 | 122 | |
| ± SD | 5 | 5 | | 5 | 4 | | 6 | 3 | | 8 | 7 | |
| DMSO | 20 | 31 | 0 | 27 | 40 | 0 | 114 | 126 | 0 | 102 | 113 | |
| | 33 | 46 | | 25 | 41 | | 109 | 115 | | 107 | 118 | |
| | 18 | 34 | | 20 | 31 | | 111 | 128 | | 110 | 118 | |
| | 32 | 44 | | 26 | 30 | | 102 | 118 | | 105 | 116 | |
| | 20 | 25 | | 28 | 38 | | 110 | 121 | | 116 | 126 | |
| Mean | 25 | 36 | | 25 | 36 | | 109 | 122 | | 108 | 118 | |
| ± SD | 7 | 9 | | 3 | 5 | | 4 | 5 | | 5 | 5 | |
| 0.1 µL | 30 | 47 | 0 | | | | 156 | 161 | 0 | | | |
| | 24 | 35 | | | | | 165 | 172 | | | | |
| | 26 | 37 | | | | | 145 | 154 | | | | |
| Mean | 27 | 40 | | | | | 155 | 162 | | | | |
| ± SD | 3 | 6 | | | | | 10 | 9 | | | | |
| 0.5 µL | 38 | 49 | 0 | | | | 224 | 233 | 0 | | | |
| | 25 | 33 | | | | | 224 | 229 | | | | |
| | 18 | 30 | | | | | 212 | 219 | | | | |
| Mean | 27 | 37 | | | | | 220 | 227 | | | | |
| ± SD | 10 | 10 | | | | | 7 | 7 | | | | |
| 1 µL | 33 | 44 | 0 | | | | 381 | 383 | 0 | | | |
| | 31 | 37 | | | | | 412 | 417 | | | | |
| | 39 | 44 | | | | | 406 | 408 | | | | |
| Mean | 34 | 42 | | | | | 400 | 403 | | | | |
| ± SD | 4 | 4 | | | | | 16 | 18 | | | | |
| 1,5 µL | | | | 49 | 63 | 0 | | | | 392 | 395 | |
| | | | | 51 | 61 | | | | | 425 | 428 | |
| | | | | 59 | 75 | | | | | 386 | 387 | |
| Mean | | | | 53 | 66 | | | | | 401 | 403 | |
| ± SD | | | | 5 | 8 | | | | | 21 | 22 | |
| 2 µL | 33 | 42 | 0 | 50 | 58 | 0 | 669 | 672 | 0 | 408 | 411 | |
| | 32 | 46 | | 42 | 53 | | 656 | 657 | | 462 | 464 | |
| | 50 | 65 | | 55 | 64 | | 709 | 713 | | 487 | 488 | |
| Mean | 38 | 51 | | 49 | 58 | | 678 | 681 | | 452 | 454 | |
| ± SD | 10 | 12 | | 7 | 6 | | 28 | 29 | | 40 | 39 | |
| 3 µL | | | 3 | 45 | 60 | 0 | | | | 395 | 396 | |
| | | | | 36 | 46 | | | | | 377 | 380 | |
| | | | | 58 | 67 | | | | | 416 | 420 | |
| Mean | | | | 46 | 58 | | | | | 396 | 399 | |
| ± SD | | | | 11 | 11 | | | | | 20 | 20 | |
| 4 µL | | | | 14 | 21 | 1 | | | | 9 | 17 | |
| | | | | 17 | 27 | | | | | 12 | 19 | |
| | | | | 12 | 26 | | | | | 28 | 41 | |
| Mean | | | | 14 | 25 | | | | | 16 | 26 | |
| ± SD | | | | 3 | 3 | | | | | 10 | 13 | |
| 5 µL | 0 | 0 | | | | | 0 | 0 | 2 | | | |
| | 0 | 0 | | | | | 0 | 0 | | | | |
| | 0 | 0 | | | | | 0 | 0 | | | | |
| Mean | 0 | 0 | | | | | 0 | 0 | | | | |
| ± SD | 0 | 0 | | | | | 0 | 0 | | | | |
| NPD | 1528 | | | 1212 | | | Na-azid | 676 | | 0 | 936 | |
| | 1628 | | | 1164 | | | | 604 | | | 876 | |
| | 2084 | | | 1300 | | | | 766 | | | 740 | |
| Mean | 1747 | | | 1225 | | | Mean | 682 | | | 851 | |
| ± SD | 296 | | | 69 | | | ± SD | 81 | | | 100 | |
| Bap | 121 | | | 106 | | | Bap | 466 | | 0 | 376 | |
| | 107 | | | 140 | | | | 398 | | | 340 | |
| | 99 | | | 111 | | | | 366 | | | 324 | |
| Mean | 109 | | | 119 | | | Mean | 410 | | | 347 | |
| ± SD | 11 | | | 18 | | | ± SD | 51 | | | 27 | |
| | | | | | | | S9 1 | | | | | |
| S9 1 DMSO | | 33 | | | 28 | | DMSO | | 127 | | 143 | |
| | | | | | | | S9 2 | | | | | |
| S9 2 DMSO | | 35 | | | 30 | | DMSO | | 112 | | 136 | |
| | | | | | | | S9 3 | | | | | |
| S9 3 DMSO | | 29 | | | 32 | | DMSO | | 143 | | | |
| | | | | | | | S9 4 | | | | | |
| S9 4 DMSO | | 47 | | | 27 | | DMSO | | 128 | | | |
| Mean | | 36 | | | 29 | | Mean | | 128 | | 140 | |
| ± SD | | 8 | | | 2 | | ± SD | | 13 | | 5 | |
| S9 1 BaP | | 68 | | | 130 | | S9 1 BaP | | 372 | | 572 | |
| S9 2 BaP | | 122 | | | 128 | | S9 2 BaP | | 448 | | 532 | |
| S9 3 BaP | | 109 | | | 65 | | S9 3 BaP | | 536 | | | |
| S9 4 BaP | | 91 | | | 125 | | S9 4 BaP | | 632 | | | |
| Mean | | 98 | | | 112 | | Mean | | 497 | | 552 | |
| ± SD | | 23 | | | 31 | | ± SD | | 112 | | 28 | |

**Table D 2.2: Results on secondary mutagenicity observed in TA98 and TA100 induced by 0-100-0 in Experiment 6-7 and 10-11 of the Ames assay.** The preincubation version of the Ames assay was applied with S9 exposing TA98 and TA100 to a concentration range (0.1-10 µL/plate) of the spruce-derived fast pyrolysis oil 0-100-0 (n = 3) together with NPD, Na-azid and BaP controls (n = 3), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| TA98 | | | | | | | TA100 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Experiment 7 | | | Experiment 11 | | | | Experiment 6 | | | Experiment 10 | | |
| Exposure | 48 h | 72 h | BTL | 48 h | 72 h | BTL | Exposure | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | 15 | 21 | 0 | 18 | 27 | 0 | Spontane | 116 | 125 | 0 | 111 | 117 | 0 |
| | 23 | 31 | | 28 | 32 | | | 101 | 117 | | 105 | 113 | |
| | 27 | 33 | | 20 | 29 | | | 103 | 119 | | 124 | 130 | |
| | 22 | 27 | | 20 | 29 | | | 108 | 121 | | 122 | 124 | |
| | 17 | 22 | | 28 | 38 | | | 110 | 124 | | 122 | 126 | |
| Mean | 21 | 27 | | 23 | 31 | | Mean | 108 | 121 | | 117 | 122 | |
| ± SD | 5 | 5 | | 5 | 4 | | ± SD | 6 | 3 | | 8 | 7 | |
| DMSO | 20 | 31 | 0 | 27 | 40 | 0 | DMSO | 114 | 126 | 0 | 102 | 113 | 0 |
| | 33 | 46 | | 25 | 41 | | | 109 | 115 | | 107 | 118 | |
| | 18 | 34 | | 20 | 31 | | | 111 | 128 | | 110 | 118 | |
| | 32 | 44 | | 26 | 30 | | | 102 | 118 | | 105 | 116 | |
| | 20 | 25 | | 28 | 38 | | | 110 | 121 | | 116 | 126 | |
| Mean | 25 | 36 | | 25 | 36 | | Mean | 109 | 122 | | 108 | 118 | |
| ± SD | 7 | 9 | | 3 | 5 | | ± SD | 4 | 5 | | 5 | 5 | |
| 0.1 µL | 37 | 48 | 0 | | | | 0.1 µL | 131 | 143 | 0 | | | |
| | 16 | 29 | | | | | | 117 | 127 | | | | |
| | 22 | 34 | | | | | | | | | | | |
| Mean | 25 | 37 | | | | | Mean | 124 | 135 | | | | |
| ± SD | 11 | 10 | | | | | ± SD | 10 | 11 | | | | |
| 0.5 µL | 19 | 28 | 0 | | | | 0.5 µL | 149 | 155 | 0 | | | |
| | 20 | 31 | | | | | | 185 | 192 | | | | |
| | 15 | 25 | | | | | | 141 | 147 | | | | |
| Mean | 18 | 28 | | | | | Mean | 158 | 165 | | | | |
| ± SD | 3 | 3 | | | | | ± SD | 23 | 24 | | | | |
| 1 µL | 30 | 47 | 0 | | | | 1 µL | 177 | 184 | 0 | | | |
| | 22 | 29 | | | | | | 167 | 173 | | | | |
| | 27 | 37 | | | | | | 196 | 204 | | | | |
| Mean | 26 | 38 | | | | | Mean | 180 | 187 | | | | |
| ± SD | 4 | 9 | | | | | ± SD | 15 | 16 | | | | |
| 2 µL | 16 | 23 | 0 | 24 | 36 | 0 | 2 µL | 265 | 270 | 0 | | | |
| | 19 | 30 | | 22 | 39 | | | 229 | 232 | | | | |
| | 29 | 35 | | 19 | 36 | | | 248 | 254 | | | | |
| Mean | 21 | 29 | | 22 | 37 | | Mean | 247 | 252 | | | | |
| ± SD | 7 | 6 | | 3 | 2 | | ± SD | 18 | 19 | | | | |
| 3 µL | | | | 29 | 40 | 0 | 5 µL | 514 | 519 | 0 | 234 | 240 | 0 |
| | | | | 20 | 26 | | | 524 | 528 | | 241 | 246 | |
| | | | | 25 | 38 | | | 531 | 535 | | 218 | 224 | |
| Mean | | | | 25 | 35 | | Mean | 523 | 527 | | 231 | 237 | |
| ± SD | | | | 5 | 8 | | ± SD | 9 | 8 | | 12 | 11 | |
| 4 µL | | | | 21 | 30 | 0 | 6 µL | | | 0 | 254 | 259 | 0 |
| | | | | 18 | 27 | | | | | | 236 | 241 | |
| | | | | 20 | 31 | | | | | | 232 | 234 | |
| Mean | | | | 20 | 29 | | Mean | | | | 241 | 245 | |
| ± SD | | | | 2 | 2 | | ± SD | | | | 12 | 13 | |
| 5 µL | 27 | 38 | 0 | 22 | 35 | 0 | 8 µL | | | 0 | 242 | 250 | 0 |
| | 15 | 26 | | 20 | 30 | | | | | | 253 | 260 | |
| | 14 | 34 | | 26 | 42 | | | | | | 257 | 257 | |
| Mean | 19 | 33 | | 23 | 36 | | Mean | | | | 251 | 256 | |
| ± SD | 7 | 6 | | 3 | 6 | | ± SD | | | | 8 | 5 | |
| NPD | 1528 | | 0 | 1212 | | 0 | 10 µL | | | | 217 | 223 | 1 |
| | 1628 | | | 1164 | | | | | | | 208 | 213 | |
| | 2084 | | | 1300 | | | | | | | 198 | 206 | |
| Mean | 1747 | | | 1225 | | | Mean | | | | 208 | 214 | |
| ± SD | 296 | | | 69 | | | ± SD | | | | 10 | 9 | |
| Bap | 121 | | 0 | 106 | | 0 | Na-azid | 676 | | 0 | | 936 | |
| | 107 | | | 140 | | | | 604 | | | | 876 | |
| | 99 | | | 111 | | | | 766 | | | | 740 | |
| Mean | 109 | | | 119 | | | Mean | 682 | | | | 851 | |
| ± SD | 11 | | | 18 | | | ± SD | 81 | | | | 100 | |
| | | | | | | | Bap | 466 | | 0 | | 376 | |
| | | | | | | | | 398 | | | | 340 | |
| | | | | | | | | 366 | | | | 324 | |
| | | | | | | | Mean | 410 | | | | 347 | |
| | | | | | | | ± SD | 51 | | | | 27 | |

**Table D 2.3: Results on secondary mutagenicity observed in TA98 and TA100 induced by 0-0-100 in Experiment 6-7 and 11 of the Ames assay.** The preincubation version of the Ames assay was applied with S9 exposing TA98 and TA100 to a concentration range (0.1-10 µL/plate) of the spruce-derived fast pyrolysis oil 0-0-100 (n = 3) together with NPD, Na-azid and BaP controls (n = 3), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| | TA98 | | | | | | TA100 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Experiment 7 | | | Experiment 11 | | | Experiment 6 | | |
| Exposure | 48 h | 72 h | BTL | 48 h | 72 h | BTL | Exposure | 48 h | 72 h | BTL |
| Spontane | 15 | 21 | 0 | 18 | 27 | 0 | Spontane | 116 | 125 | 0 |
| | 23 | 31 | | 28 | 32 | | | 101 | 117 | |
| | 27 | 33 | | 20 | 29 | | | 103 | 119 | |
| | 22 | 27 | | 20 | 29 | | | 108 | 121 | |
| | 17 | 22 | | 28 | 38 | | | 110 | 124 | |
| Mean | 21 | 27 | | 23 | 31 | | Mean | 108 | 121 | |
| ± SD | 5 | 5 | | 5 | 4 | | ± SD | 6 | 3 | |
| DMSO | 20 | 31 | 0 | 27 | 40 | 0 | DMSO | 114 | 126 | 0 |
| | 33 | 46 | | 25 | 41 | | | 109 | 115 | |
| | 18 | 34 | | 20 | 31 | | | 111 | 128 | |
| | 32 | 44 | | 26 | 30 | | | 102 | 118 | |
| | 20 | 25 | | 28 | 38 | | | 110 | 121 | |
| Mean | 25 | 36 | | 25 | 36 | | Mean | 109 | 122 | |
| ± SD | 7 | 9 | | 3 | 5 | | ± SD | 4 | 5 | |
| 0.1 µL | 29 | 39 | 0 | | | | 0.1 µL | 86 | 103 | 0 |
| | 26 | 36 | | | | | | 86 | 96 | |
| | 37 | 46 | | | | | | 78 | 91 | |
| Mean | 31 | 40 | | | | | Mean | 83 | 97 | |
| ± SD | 6 | 5 | | | | | ± SD | 5 | 6 | |
| 0.5 µL | 45 | 53 | 0 | | | | 0.5 µL | 107 | 118 | 0 |
| | 41 | 51 | | | | | | 94 | 110 | |
| | 28 | 44 | | | | | | 101 | 115 | |
| Mean | 38 | 49 | | | | | Mean | 101 | 114 | |
| ± SD | 9 | 5 | | | | | ± SD | 7 | 4 | |
| 1 µL | 38 | 42 | 0 | | | | 1 µL | 93 | 105 | 0 |
| | 45 | 65 | | | | | | 77 | 87 | |
| | 33 | 43 | | | | | | 86 | 96 | |
| Mean | 39 | 50 | | | | | Mean | 85 | 96 | |
| ± SD | 6 | 13 | | | | | ± SD | 8 | 9 | |
| 2 µL | 40 | 50 | 0 | | | | 2 µL | 85 | 96 | 0 |
| | 51 | 60 | | | | | | 64 | 79 | |
| | 37 | 48 | | | | | | 84 | 97 | |
| Mean | 43 | 53 | | | | | Mean | 78 | 91 | |
| ± SD | 7 | 6 | | | | | ± SD | 12 | 10 | |
| 5 µL | 47 | 55 | 0 | 34 | 45 | 0 | 5 µL | 97 | 112 | 0 |
| | 47 | 57 | | 29 | 42 | | | 73 | 82 | |
| | 53 | 60 | | 23 | 37 | | | 88 | 102 | |
| Mean | 49 | 57 | | 29 | 41 | | Mean | 86 | 99 | |
| ± SD | 3 | 3 | | 6 | 4 | | ± SD | 12 | 15 | |
| 6 µL | | | | 33 | 46 | 0 | Na-azid | 676 | | 0 |
| | | | | 37 | 48 | | | 604 | | |
| | | | | 22 | 35 | | | 766 | | |
| Mean | | | | 31 | 43 | | Mean | 682 | | |
| ± SD | | | | 8 | 7 | | ± SD | 81 | | |
| 8 µL | | | | 33 | 41 | 0 | Bap | 466 | | 0 |
| | | | | 26 | 33 | | | 398 | | |
| | | | | 31 | 38 | | | 366 | | |
| Mean | | | | 30 | 37 | | Mean | 410 | | |
| ± SD | | | | 4 | 4 | | ± SD | 51 | | |
| 10 µL | | | | 29 | 40 | 0 | | | | |
| | | | | 26 | 38 | | | | | |
| | | | | 20 | 37 | | | | | |
| Mean | | | | 25 | 38 | | | | | |
| ± SD | | | | 5 | 2 | | | | | |
| NPD | 1528 | | | 1212 | | | | | | |
| | 1628 | | | 1164 | | | | | | |
| | 2084 | | | 1300 | | | | | | |
| Mean | 1747 | | | 1225 | | | | | | |
| ± SD | 296 | | | 69 | | | | | | |
| Bap | 121 | | | 106 | | | | | | |
| | 107 | | | 140 | | | | | | |
| | 99 | | | 111 | | | | | | |
| Mean | 109 | | | 119 | | | | | | |
| ± SD | 11 | | | 18 | | | | | | |

**Table D 2.4: Results on secondary mutagenicity observed in TA98 and TA100 induced by 80-15-5 in Experiment 6-7 and 10-11 of the Ames assay.** The preincubation version of the Ames assay was applied with S9 exposing TA98 and TA100 to a concentration range (0.1-5 µL/plate) of the spruce-derived fast pyrolysis oil 80-15-5 (n = 3) together with NPD, Na-azid and BaP controls (n = 3), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| TA98 | | | | | | | TA100 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Experiment 7 | | | Experiment 11 | | | | Experiment 6 | | | Experiment 10 | | |
| Exposure | 48 h | 72 h | BTL | 48 h | 72 h | BTL | Exposure | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | 15 | 21 | 0 | 18 | 27 | 0 | Spontane | 116 | 125 | 0 | 111 | 117 | 0 |
| | 23 | 31 | | 28 | 32 | | | 101 | 117 | | 105 | 113 | |
| | 27 | 33 | | 20 | 29 | | | 103 | 119 | | 124 | 130 | |
| | 22 | 27 | | 20 | 29 | | | 108 | 121 | | 122 | 124 | |
| | 17 | 22 | | 28 | 38 | | | 110 | 124 | | 122 | 126 | |
| Mean | 21 | 27 | | 23 | 31 | | Mean | 108 | 121 | | 117 | 122 | |
| ± SD | 5 | 5 | | 5 | 4 | | ± SD | 6 | 3 | | 8 | 7 | |
| DMSO | 20 | 31 | 0 | 27 | 40 | 0 | DMSO | 114 | 126 | 0 | 102 | 113 | 0 |
| | 33 | 46 | | 25 | 41 | | | 109 | 115 | | 107 | 118 | |
| | 18 | 34 | | 20 | 31 | | | 111 | 128 | | 110 | 118 | |
| | 32 | 44 | | 26 | 30 | | | 102 | 118 | | 105 | 116 | |
| | 20 | 25 | | 28 | 38 | | | 110 | 121 | | 116 | 126 | |
| Mean | 25 | 36 | | 25 | 36 | | Mean | 109 | 122 | | 108 | 118 | |
| ± SD | 7 | 9 | | 3 | 5 | | ± SD | 4 | 5 | | 5 | 5 | |
| 0.1 µL | 28 | 41 | 0 | | | | 0.1 µL | 121 | 132 | 0 | | | |
| | 37 | 44 | | | | | | 133 | 146 | | | | |
| | 35 | 46 | | | | | | 105 | 115 | | | | |
| Mean | 33 | 44 | | | | | Mean | 120 | 131 | | | | |
| ± SD | 5 | 3 | | | | | ± SD | 14 | 16 | | | | |
| 0.5 µL | 40 | 51 | 0 | | | | 0.5 µL | 167 | 168 | 0 | | | |
| | 31 | 42 | | | | | | 181 | 183 | | | | |
| | 38 | 59 | | | | | | 152 | 154 | | | | |
| Mean | 36 | 51 | | | | | Mean | 167 | 168 | | | | |
| ± SD | 5 | 9 | | | | | ± SD | 15 | 15 | | | | |
| 1 µL | 40 | 50 | 0 | | | | 1 µL | 340 | 342 | 0 | | | |
| | 30 | 42 | | | | | | 328 | 331 | | | | |
| | 20 | 33 | | | | | | 329 | 333 | | | | |
| Mean | 30 | 42 | | | | | Mean | 332 | 335 | | | | |
| ± SD | 10 | 9 | | | | | ± SD | 7 | 6 | | | | |
| 2 µL | 39 | 46 | 0 | 38 | 55 | 0 | 1,5 µL | | | | 447 | 449 | 0 |
| | 41 | 49 | | 30 | 47 | | | | | | 405 | 408 | |
| | 40 | 47 | | 28 | 38 | | | | | | 409 | 410 | |
| Mean | 40 | 47 | | 32 | 47 | | Mean | | | | 420 | 422 | |
| ± SD | 1 | 2 | | 5 | 9 | | ± SD | | | | 23 | 23 | |
| 3 µL | | | | 18 | 30 | 1 | 2 µL | 721 | 722 | 0 | 564 | 567 | 0 |
| | | | | 24 | 32 | | | 833 | 835 | | 581 | 582 | |
| | | | | 18 | 27 | | | 903 | 908 | | 591 | 591 | |
| Mean | | | | 20 | 30 | | Mean | 819 | 822 | | 579 | 580 | |
| ± SD | | | | 3 | 3 | | ± SD | 92 | 94 | | 14 | 12 | |
| 4 µL | | | | 1 | 9 | 2 | 3 µL | | | | 127 | 141 | 1 |
| | | | | 1 | 13 | | | | | | 165 | 176 | |
| | | | | 0 | 15 | | | | | | 183 | 190 | |
| Mean | | | | 1 | 12 | | Mean | | | | 158 | 169 | |
| ± SD | | | | 1 | 3 | | ± SD | | | | 29 | 25 | |
| 5 µL | 0 | 8 | 2 | | | | 4 µL | | | | 0 | 0 | 2 |
| | 0 | 2 | | | | | | | | | 0 | 1 | |
| | 0 | 0 | | | | | | | | | 0 | 5 | |
| Mean | 0 | 3 | | | | | Mean | | | | 0 | 2 | |
| ± SD | 0 | 4 | | | | | ± SD | | | | 0 | 3 | |
| NPD | 1528 | | | 1212 | | | 5 µL | 0 | 14 | 2 | | | |
| | 1628 | | | 1164 | | | | 0 | 11 | | | | |
| | 2084 | | | 1300 | | | | 0 | 16 | | | | |
| Mean | 1747 | | | 1225 | | | Mean | 0 | 14 | | | | |
| ± SD | 296 | | | 69 | | | ± SD | 0 | 3 | | | | |
| Bap | 121 | | | 106 | | | Na-azid | 676 | | 0 | | 936 | |
| | 107 | | | 140 | | | | 604 | | | | 876 | |
| | 99 | | | 111 | | | | 766 | | | | 740 | |
| Mean | 109 | | | 119 | | | Mean | 682 | | | | 851 | |
| ± SD | 11 | | | 18 | | | ± SD | 81 | | | | 100 | |
| | | | | | | | Bap | 466 | | 0 | | 376 | |
| | | | | | | | | 398 | | | | 340 | |
| | | | | | | | | 366 | | | | 324 | |
| | | | | | | | Mean | 410 | | | | 347 | |
| | | | | | | | ± SD | 51 | | | | 27 | |

**Table D 2.5: Results on secondary mutagenicity observed in TA98 and TA100 induced by 60-40-0 in Experiment 6-7 and 10-11 of the Ames assay.** The preincubation version of the Ames assay was applied with S9 exposing TA98 and TA100 to a concentration range (0.1-5 µL/plate) of the spruce-derived fast pyrolysis oil 60-40-0 (n = 3) together with NPD, Na-azid and BaP controls (n = 3), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| TA98 | | | | | | |
|---|---|---|---|---|---|---|
| | Experiment 7 | | | Experiment 11 | | |
| Exposure | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | 15 | 21 | 0 | 18 | 27 | 0 |
| | 23 | 31 | | 28 | 32 | |
| | 27 | 33 | | 20 | 29 | |
| | 22 | 27 | | 20 | 29 | |
| | 17 | 22 | | 28 | 38 | |
| Mean | 21 | 27 | | 23 | 31 | |
| ± SD | 5 | 5 | | 5 | 4 | |
| DMSO | 20 | 31 | 0 | 27 | 40 | 0 |
| | 33 | 46 | | 25 | 41 | |
| | 18 | 34 | | 20 | 31 | |
| | 32 | 44 | | 26 | 30 | |
| | 20 | 25 | | 28 | 38 | |
| Mean | 25 | 36 | | 25 | 36 | |
| ± SD | 7 | 9 | | 3 | 5 | |
| 0.1 µL | 34 | 46 | 0 | | | |
| | 41 | 50 | | | | |
| | 54 | 64 | | | | |
| Mean | 43 | 53 | | | | |
| ± SD | 10 | 9 | | | | |
| 0.5 µL | 31 | 42 | 0 | | | |
| | 31 | 42 | | | | |
| | 40 | 48 | | | | |
| Mean | 34 | 44 | | | | |
| ± SD | 5 | 3 | | | | |
| 1 µL | 21 | 26 | 0 | | | |
| | 24 | 34 | | | | |
| | 28 | 43 | | | | |
| Mean | 24 | 34 | | | | |
| ± SD | 4 | 9 | | | | |
| 2 µL | 42 | 50 | 0 | 28 | 36 | 0 |
| | 29 | 39 | | 38 | 50 | |
| | 30 | 40 | | 31 | 41 | |
| Mean | 34 | 43 | | 32 | 42 | |
| ± SD | 7 | 6 | | 5 | 7 | |
| 3 µL | | | | 30 | 41 | 0 |
| | | | | 37 | 50 | |
| | | | | 32 | 40 | |
| Mean | | | | 33 | 44 | |
| ± SD | | | | 4 | 6 | |
| 4 µL | | | | 29 | 41 | 0 |
| | | | | 20 | 28 | |
| | | | | 26 | 39 | |
| Mean | | | | 25 | 36 | |
| ± SD | | | | 5 | 7 | |
| 5 µL | 8 | 18 | 2 | 11 | 30 | 1 |
| | 8 | 21 | | 19 | 30 | |
| | 10 | 20 | | 19 | 47 | |
| Mean | 9 | 20 | | 16 | 36 | |
| ± SD | 1 | 2 | | 5 | 10 | |
| NPD | 1528 | | | 1212 | | |
| | 1628 | | | 1164 | | |
| | 2084 | | | 1300 | | |
| Mean | 1747 | | | 1225 | | |
| ± SD | 296 | | | 69 | | |
| Bap | 121 | | | 106 | | |
| | 107 | | | 140 | | |
| | 99 | | | 111 | | |
| Mean | 109 | | | 119 | | |
| ± SD | 11 | | | 18 | | |

| TA100 | | | | | | |
|---|---|---|---|---|---|---|
| | Experiment 6 | | | Experiment 10 | | |
| Exposure | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | 116 | 125 | 0 | 111 | 117 | 0 |
| | 101 | 117 | | 105 | 113 | |
| | 103 | 119 | | 124 | 130 | |
| | 108 | 121 | | 122 | 124 | |
| | 110 | 124 | | 122 | 126 | |
| Mean | 108 | 121 | | 117 | 122 | |
| ± SD | 6 | 3 | | 8 | 7 | |
| DMSO | 114 | 126 | 0 | 102 | 113 | 0 |
| | 109 | 115 | | 107 | 118 | |
| | 111 | 128 | | 110 | 118 | |
| | 102 | 118 | | 105 | 116 | |
| | 110 | 121 | | 116 | 126 | |
| Mean | 109 | 122 | | 108 | 118 | |
| ± SD | 4 | 5 | | 5 | 5 | |
| 0.1 µL | 128 | 136 | 0 | | | |
| | 160 | 167 | | | | |
| | 124 | 130 | | | | |
| Mean | 137 | 144 | | | | |
| ± SD | 20 | 20 | | | | |
| 0.5 µL | 148 | 155 | 0 | | | |
| | 175 | 181 | | | | |
| | 168 | 177 | | | | |
| Mean | 164 | 171 | | | | |
| ± SD | 14 | 14 | | | | |
| 1 µL | 251 | 253 | 0 | | | |
| | 249 | 254 | | | | |
| | 256 | 261 | | | | |
| Mean | 252 | 256 | | | | |
| ± SD | 4 | 4 | | | | |
| 1,5 µL | | | | 291 | 291 | 0 |
| | | | | 287 | 289 | |
| | | | | 331 | 331 | |
| Mean | | | | 303 | 304 | |
| ± SD | | | | 24 | 24 | |
| 2 µL | 365 | 368 | 0 | 366 | 368 | 0 |
| | 364 | 364 | | 378 | 382 | |
| | 377 | 377 | | 367 | 369 | |
| Mean | 369 | 370 | | 370 | 373 | |
| ± SD | 7 | 7 | | 7 | 8 | |
| 3 µL | | | | 399 | 401 | 0 |
| | | | | 415 | 417 | |
| | | | | 419 | 420 | |
| Mean | | | | 411 | 413 | |
| ± SD | | | | 11 | 10 | |
| 4 µL | | | | 105 | 119 | 1 |
| | | | | 97 | 105 | |
| | | | | 120 | 141 | |
| Mean | | | | 107 | 122 | |
| ± SD | | | | 12 | 18 | |
| 5 µL | 27 | 36 | 2 | | | |
| | 23 | 38 | | | | |
| | 45 | 60 | | | | |
| Mean | 32 | 45 | | | | |
| ± SD | 12 | 13 | | | | |
| Na-azid | 676 | | 0 | | 936 | |
| | 604 | | | | 876 | |
| | 766 | | | | 740 | |
| Mean | 682 | | | | 851 | |
| ± SD | 81 | | | | 100 | |
| Bap | 466 | | 0 | | 376 | |
| | 398 | | | | 340 | |
| | 366 | | | | 324 | |
| Mean | 410 | | | | 347 | |
| ± SD | 51 | | | | 27 | |

**Table D 2.6: Results on secondary mutagenicity observed in TA98 and TA100 induced by 60-30-10 in Experiment 6-7 and 10-11 of the Ames assay.** The preincubation version of the Ames assay was applied with S9 exposing TA98 and TA100 to a concentration range (0.1-5 µL/plate) of the spruce-derived fast pyrolysis oil 60-30-10 (n = 3) together with NPD, Na-azid and BaP controls (n = 3), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| TA98 | | | | | | |
|---|---|---|---|---|---|---|
| | Experiment 7 | | | Experiment 11 | | |
| Exposure | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | 15 | 21 | 0 | 18 | 27 | 0 |
| | 23 | 31 | | 28 | 32 | |
| | 27 | 33 | | 20 | 29 | |
| | 22 | 27 | | 20 | 29 | |
| | 17 | 22 | | 28 | 38 | |
| Mean | 21 | 27 | | 23 | 31 | |
| ± SD | 5 | 5 | | 5 | 4 | |
| DMSO | 20 | 31 | 0 | 27 | 40 | 0 |
| | 33 | 46 | | 25 | 41 | |
| | 18 | 34 | | 20 | 31 | |
| | 32 | 44 | | 26 | 30 | |
| | 20 | 25 | | 28 | 38 | |
| Mean | 25 | 36 | | 25 | 36 | |
| ± SD | 7 | 9 | | 3 | 5 | |
| 0.1 µL | 39 | 45 | 0 | | | |
| | 35 | 45 | | | | |
| | 28 | 45 | | | | |
| Mean | 34 | 45 | | | | |
| ± SD | 6 | 0 | | | | |
| 0.5 µL | 37 | 51 | 0 | | | |
| | 33 | 40 | | | | |
| | 35 | 36 | | | | |
| Mean | 35 | 42 | | | | |
| ± SD | 2 | 8 | | | | |
| 1 µL | 27 | 33 | 0 | | | |
| | 29 | 35 | | | | |
| | 31 | 50 | | | | |
| Mean | 29 | 39 | | | | |
| ± SD | 2 | 9 | | | | |
| 2 µL | 35 | 45 | 0 | 25 | 40 | 0 |
| | 33 | 40 | | 27 | 35 | |
| | 28 | 36 | | 24 | 36 | |
| Mean | 32 | 40 | | 25 | 37 | |
| ± SD | 4 | 5 | | 2 | 3 | |
| 3 µL | | | | 35 | 46 | 0 |
| | | | | 34 | 45 | |
| | | | | 28 | 46 | |
| Mean | | | | 32 | 46 | |
| ± SD | | | | 4 | 1 | |
| 4 µL | | | | 21 | 28 | 0 |
| | | | | 26 | 34 | |
| | | | | 36 | 47 | |
| Mean | | | | 28 | 36 | |
| ± SD | | | | 8 | 10 | |
| 5 µL | 17 | 27 | 1 | | | |
| | 11 | 24 | | | | |
| | 14 | 19 | | | | |
| Mean | 14 | 23 | | | | |
| ± SD | 3 | 4 | | | | |
| NPD | 1528 | | | 1212 | | |
| | 1628 | | | 1164 | | |
| | 2084 | | | 1300 | | |
| Mean | 1747 | | | 1225 | | |
| ± SD | 296 | | | 69 | | |
| Bap | 121 | | | 106 | | |
| | 107 | | | 140 | | |
| | 99 | | | 111 | | |
| Mean | 109 | | | 119 | | |
| ± SD | 11 | | | 18 | | |

| TA100 | | | | | | |
|---|---|---|---|---|---|---|
| | Experiment 6 | | | Experiment 10 | | |
| Exposure | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | 116 | 125 | 0 | 111 | 117 | 0 |
| | 101 | 117 | | 105 | 113 | |
| | 103 | 119 | | 124 | 130 | |
| | 108 | 121 | | 122 | 124 | |
| | 110 | 124 | | 122 | 126 | |
| Mean | 108 | 121 | | 117 | 122 | |
| ± SD | 6 | 3 | | 8 | 7 | |
| DMSO | 114 | 126 | 0 | 102 | 113 | 0 |
| | 109 | 115 | | 107 | 118 | |
| | 111 | 128 | | 110 | 118 | |
| | 102 | 118 | | 105 | 116 | |
| | 110 | 121 | | 116 | 126 | |
| Mean | 109 | 122 | | 108 | 118 | |
| ± SD | 4 | 5 | | 5 | 5 | |
| 0.1 µL | 116 | 119 | 0 | | | |
| | 95 | 100 | | | | |
| | 100 | 111 | | | | |
| Mean | 104 | 110 | | | | |
| ± SD | 11 | 10 | | | | |
| 0.5 µL | 145 | 152 | 0 | | | |
| | 146 | 157 | | | | |
| | 143 | 150 | | | | |
| Mean | 145 | 153 | | | | |
| ± SD | 2 | 4 | | | | |
| 1 µL | 189 | 192 | 0 | | | |
| | 204 | 207 | | | | |
| | 191 | 195 | | | | |
| Mean | 195 | 198 | | | | |
| ± SD | 8 | 8 | | | | |
| 1,5 µL | | | | 233 | 235 | 0 |
| | | | | 209 | 211 | |
| | | | | 229 | 234 | |
| Mean | | | | 224 | 227 | |
| ± SD | | | | 13 | 14 | |
| 2 µL | 360 | 363 | 0 | 281 | 284 | 0 |
| | 378 | 382 | | 310 | 311 | |
| | 422 | 426 | | 286 | 290 | |
| Mean | 387 | 390 | | 292 | 295 | |
| ± SD | 32 | 32 | | 16 | 14 | |
| 3 µL | | | | 389 | 391 | 0 |
| | | | | 360 | 361 | |
| | | | | 382 | 382 | |
| Mean | | | | 377 | 378 | |
| ± SD | | | | 15 | 15 | |
| 4 µL | | | | 281 | 282 | 1 |
| | | | | 299 | 302 | |
| | | | | 297 | 303 | |
| Mean | | | | 292 | 296 | |
| ± SD | | | | 10 | 12 | |
| 5 µL | 95 | 108 | 1 | | | |
| | 129 | 148 | | | | |
| | 152 | 164 | | | | |
| Mean | 125 | 140 | | | | |
| ± SD | 29 | 29 | | | | |
| Na-azid | 676 | | 0 | | 936 | |
| | 604 | | | | 876 | |
| | 766 | | | | 740 | |
| Mean | 682 | | | | 851 | |
| ± SD | 81 | | | | 100 | |
| Bap | 466 | | 0 | | 376 | |
| | 398 | | | | 340 | |
| | 366 | | | | 324 | |
| Mean | 410 | | | | 347 | |
| ± SD | 51 | | | | 27 | |

**Table D 2.7: Results on secondary mutagenicity observed in TA98 and TA100 induced by 43-22-35 in Experiment 6-7 and 10-11 of the Ames assay.** The preincubation version of the Ames assay was applied with S9 exposing TA98 and TA100 to a concentration range (0.1-10 µL/plate) of the spruce-derived fast pyrolysis oil 43-22-35 (n = 3) together with NPD, Na-azid and BaP controls (n = 3), DMSO controls and registration of spontaneous reversions (n = 5). Individual revertants, mean values ± SD as well as background toxicity levels (BTLs) from 0-3 are listed.

| TA98 | | | | | | |
|---|---|---|---|---|---|---|
| | Experiment 7 | | | Experiment 11 | | |
| Exposure | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | 15 | 21 | 0 | 18 | 27 | 0 |
| | 23 | 31 | | 28 | 32 | |
| | 27 | 33 | | 20 | 29 | |
| | 22 | 27 | | 20 | 29 | |
| | 17 | 22 | | 28 | 38 | |
| Mean | 21 | 27 | | 23 | 31 | |
| ± SD | 5 | 5 | | 5 | 4 | |
| DMSO | 20 | 31 | 0 | 27 | 40 | 0 |
| | 33 | 46 | | 25 | 41 | |
| | 18 | 34 | | 20 | 31 | |
| | 32 | 44 | | 26 | 30 | |
| | 20 | 25 | | 28 | 38 | |
| Mean | 25 | 36 | | 25 | 36 | |
| ± SD | 7 | 9 | | 3 | 5 | |
| 0.1 µL | 30 | 38 | 0 | | | |
| | 42 | 51 | | | | |
| | 31 | 44 | | | | |
| Mean | 34 | 44 | | | | |
| ± SD | 7 | 7 | | | | |
| 0.5 µL | 35 | 51 | 0 | | | |
| | 30 | 41 | | | | |
| | 33 | 50 | | | | |
| Mean | 33 | 47 | | | | |
| ± SD | 3 | 6 | | | | |
| 1 µL | 22 | 36 | 0 | | | |
| | 36 | 52 | | | | |
| | 26 | 37 | | | | |
| Mean | 28 | 42 | | | | |
| ± SD | 7 | 9 | | | | |
| 2 µL | 25 | 32 | 0 | 33 | 51 | 0 |
| | 34 | 40 | | 31 | 37 | |
| | 22 | 31 | | 30 | 47 | |
| Mean | 27 | 34 | | 31 | 45 | |
| ± SD | 6 | 5 | | 2 | 7 | |
| 3 µL | | | | 34 | 44 | 0 |
| | | | | 28 | 43 | |
| | | | | 26 | 39 | |
| Mean | | | | 29 | 42 | |
| ± SD | | | | 4 | 3 | |
| 4 µL | | | | 24 | 41 | 0 |
| | | | | 29 | 32 | |
| | | | | 41 | 60 | |
| Mean | | | | 31 | 44 | |
| ± SD | | | | 9 | 14 | |
| 5 µL | 20 | 33 | 0 | 37 | 45 | 0 |
| | 20 | 29 | | 29 | 39 | |
| | 20 | 24 | | 29 | 41 | |
| Mean | 20 | 29 | | 32 | 42 | |
| ± SD | 0 | 5 | | 5 | 3 | |
| NPD | 1528 | | | 1212 | | |
| | 1628 | | | 1164 | | |
| | 2084 | | | 1300 | | |
| Mean | 1747 | | | 1225 | | |
| ± SD | 296 | | | 69 | | |
| Bap | 121 | | | 106 | | |
| | 107 | | | 140 | | |
| | 99 | | | 111 | | |
| Mean | 109 | | | 119 | | |
| ± SD | 11 | | | 18 | | |

| TA100 | | | | | | |
|---|---|---|---|---|---|---|
| | Experiment 6 | | | Experiment 10 | | |
| Exposure | 48 h | 72 h | BTL | 48 h | 72 h | BTL |
| Spontane | 116 | 125 | 0 | 111 | 117 | 0 |
| | 101 | 117 | | 105 | 113 | |
| | 103 | 119 | | 124 | 130 | |
| | 108 | 121 | | 122 | 124 | |
| | 110 | 124 | | 122 | 126 | |
| Mean | 108 | 121 | | 117 | 122 | |
| ± SD | 6 | 3 | | 8 | 7 | |
| DMSO | 114 | 126 | 0 | 102 | 113 | 0 |
| | 109 | 115 | | 107 | 118 | |
| | 111 | 128 | | 110 | 118 | |
| | 102 | 118 | | 105 | 116 | |
| | 110 | 121 | | 116 | 126 | |
| Mean | 109 | 122 | | 108 | 118 | |
| ± SD | 4 | 5 | | 5 | 5 | |
| 0.1 µL | 115 | 129 | 0 | | | |
| | 101 | 117 | | | | |
| | 126 | 149 | | | | |
| Mean | 114 | 132 | | | | |
| ± SD | 13 | 16 | | | | |
| 0.5 µL | 132 | 140 | 0 | | | |
| | 134 | 148 | | | | |
| | 138 | 145 | | | | |
| Mean | 135 | 144 | | | | |
| ± SD | 3 | 4 | | | | |
| 1 µL | 174 | 176 | 0 | | | |
| | 180 | 186 | | | | |
| | 161 | 165 | | | | |
| Mean | 172 | 176 | | | | |
| ± SD | 10 | 11 | | | | |
| 2 µL | 198 | 203 | 0 | | | |
| | 227 | 235 | | | | |
| | 205 | 209 | | | | |
| Mean | 210 | 216 | | | | |
| ± SD | 15 | 17 | | | | |
| 5 µL | 364 | 372 | 0 | 220 | 224 | 0 |
| | 282 | 284 | | 266 | 270 | |
| | 280 | 286 | | 228 | 234 | |
| Mean | 309 | 314 | | 238 | 243 | |
| ± SD | 48 | 50 | | 25 | 24 | |
| 6 µL | | | | 247 | 250 | 0 |
| | | | | 274 | 276 | |
| | | | | 253 | 264 | |
| Mean | | | | 258 | 263 | |
| ± SD | | | | 14 | 13 | |
| 8 µL | | | | 234 | 240 | 0 |
| | | | | 208 | 220 | |
| | | | | 256 | 261 | |
| Mean | | | | 233 | 240 | |
| ± SD | | | | 24 | 21 | |
| 10 µL | | | | 98 | 112 | 1 |
| | | | | 148 | 159 | |
| | | | | 143 | 150 | |
| Mean | | | | 130 | 140 | |
| SD | | | | 28 | 25 | |
| Na-azid | 676 | | 0 | | 936 | |
| | 604 | | | | 876 | |
| | 766 | | | | 740 | |
| Mean | 682 | | | | 851 | |
| ± SD | 81 | | | | 100 | |
| Bap | 466 | | 0 | | 376 | |
| | 398 | | | | 340 | |
| | 366 | | | | 324 | |
| Mean | 410 | | | | 347 | |
| ± SD | 51 | | | | 27 | |

Appendix E-1: Calculated mutagenic potentials of the spruce-derived oils

**Table E 1.1: Values of mutagenic potential in spruce-derived bio-oils calculated from results on the Ames assay.** The results from the preincubation version of the Ames assay exposing TA98 and TA98 with and without S9 to the seven spruce-derived pyrolysis oils, obtained from different relative proportions of wood, bark and needles as feedstock, were used to estimate the oils' mutagenic potentials. Results are shown as slope value of the plotted concentration-effect relationship (number of revertants per μL/plate) where increases in reversion numbers with increasing concentrations were observed.

| | **Pyrolysis oil** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Effect** | **100-0-0** | **0-100-0** | **0-0-100** | **80-15-5** | **60-40-0** | **60-30-10** | **43-22-35** |
| **TA98** | 14.9 | 8.4 | 3.1 | 16.6 | 16.9 | 13.4 | 4.7 |
| **TA98 + S9** | 16.1 | 0 | 9.1 | 22.5 | 0 | 19.7 | 0 |
| **TA100** | 374.8 | 90.2 | 10.1 | 581.1 | 351.9 | 217.1 | 107.9 |
| **TA100 + S9** | 222.2 | 52.9 | 0 | 282.3 | 112.7 | 100.0 | 32.8 |