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Toxicity and Genotoxicity of five pyrolytic Bio-Oils produced from Wood, measured as Root Growth Inhibition and DNA Double-Strand Breaks in *Allium cepa*

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ABSTRACT

Pyrolytic bio-oil is considered as a potential renewable energy resource that might replace fossil fuels in the future. Chemically, bio-oils are highly complex mixtures consisting of several hundred different compounds, formed during anaerobic thermal degradation of biomass. Several of the compounds are known to exert both toxic and genotoxic effects on living organisms. Genotoxic compounds include polycyclic aromatic hydrocarbons (PAHs), furan and catechol, while various acids, alcohols, aldehydes, ketones and phenols are associated with the toxic properties. So far, only a handful of previous studies have assessed the toxicological effects of bio-oils in living organisms, and contradictory results point of the need to perform more studies to elucidate the harmfulness of bio-oils.

The aim of this master's project was thus to evaluate the toxic properties and the genotoxic potential of five bio-oils made from different feedstock species of wood (beech, pine, poplar, spruce, unspecified wood). Toxicity and genotoxicity were respectively measured as root growth inhibition and DNA double-strand breaks (DSBs), in the *in vivo Allium cepa* test system. The hypotheses were: 1. Small differences in chemical composition or concentration of bio-oils may cause highly different toxicological responses in *A. cepa*, 2. It might be possible to identify differences in chemical composition influencing the toxicological effect of bio-oils by comparing the results with provided chemical data.

The results for toxicity showed that the five bio-oils caused a significant reduction in onion root growth with increasing concentrations. The toxic effect varied between the different bio-oils, and was ranked in the following order, according to the obtained EC_{50}-values: *Pine* > *Beech* > *Wood* > *Poplar* > *Spruce.* The obtained results for measurement of DNA DSBs indicated that bio-oils produced from fast pyrolysis of wood can cause severe DNA damage at concentrations of about 0.0004 ml bio-oil/ml solution and higher. The different feedstock species of wood applied in the pyrolysis process, may also affect the genotoxic potential of the bio-oils. The provided chemical data did not explain the observed toxic or genotoxic effect very well, but differences in specific chemical composition were observed, which might have caused the difference in toxicological response. Considering that further upgrade of bio-oils is needed to obtain applicable bio-oils, the toxicological effects of the finished upgraded products is likely to be altered compared to the effects seen for the crude bio-oils. Further studies should thus focus on the upgraded bio-oil products, as these are most likely to be of environmental concern in the future.

OPPSUMMERING

Pyrolyseolje er betraktet som en potensiell fornybar energiressurs som kanskje kan erstatte fossilt brensel i fremtiden. Kjemisk sett så er pyrolyseolje en svært kompleks blanding bestående av flere hundre ulike forbindelser, dannet under anaerobisk varmenedbrytning av biomasse. Flere av forbindelsene er kjent for å utøve både toksiske og genotoksiske effekter på levende organismer. Genotoksiske forbindelser inkluderer blant annet polysykliske aromatiske hydrokarboner (PAH), furan og catechol, mens ulike syrer, alkoholer, aldehyder, ketoner og fenoler er forbundet med de toksiske egenskapene. Kun et fåtall tidligere studier har evaluert toksikologiske effekter av pyrolyseoljer i levende organismer, og motstridende resultater viser behovet for å utføre flere studier som belyser skadeligheten av pyrolyseoljer.

Hensikten med dette masterprosjektet var derfor å evaluere de toksiske egenskapene og det genotoksiske potensialet til fem pyrolyseoljer laget av ulike arter trematerialer (bøk, furu, poppel, gran og uspesifisert tre). Toksisitet og genotoksisitet ble respektivt målt som rotvekst-reduksjon og DNA dobbelttrådbrudd (DSB), i det *in vivo* test systemet *Allium cepa*. Hypotesene var: 1. Små forskjeller i kjemisk sammensetning eller konsentrasjon av pyrolyseoljer, kan føre til svært forskjellige toksikologiske responser i *A. cepa*, 2. Det vil kanskje være mulig å identifisere forskjeller i kjemisk sammensetning som påvirker den toksikologiske effekten til pyrolyseoljene ved å sammenligne resultatene med mottatte kjemiske data.

Resultatene for toksisitet viste at de fem pyrolyseoljene førte til en signifikant reduksjon i rotvekst med økende konsentrasjoner. Toksisiteten varierte mellom de ulike pyrolyseoljene, og ble rangert i følgene rekkefølge i henhold til oppnådde EC_{50}-verdier: *Furu > Bøk > uspesifisert tre > Poppel > Gran.* De oppnådde resultatene for DNA DSB indikterte at pyrolyseoljer produsert ved rask pyrolyse av trematerialer can føre til alvorlig DNA-skade ved en konsentrasjon av 0.0004 ml pyrolyseolje/ml løsning eller høyere. De ulike artene av trematerialer anvendt i pyrolyseprosessen, kan også ha innvirkning på det genotoksiske potentialet til pyrolyseoljene. De mottatte kjemiske data kunne ikke forklare den observerte toksiske eller genotoksiske effekten , men forskjeller i spesifikk kjemisk sammensetning var observert, og kan ha ført til forskjellen i toksokologisk respons. Med tanke på at vidre oppgradering av pyrolyseoljer er nødvendig for å oppnå anvendbare oljer, er det sannynlig at det ferdig oppgraderte produktet vil ha andre toksikologiske egenskaper enn den primære pyrolyseoljen. Videre studier bør dermed fokuserere på de oppgraderte produktene, siden disse mest sannsynlig vil være av miljøbekymring i fremtiden.

ABBREVIATIONS

A. cepa	*Allium cepa*
C. elegans	*Caenorhabditis elegans*
CA	Chromosomal aberration
CFGE	Constant-field gel electrophoresis
CYP450	Cytochrome P450 mixed-function oxidase system
D. magna	*Daphnia magna*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double-strand break
DNA-FTM	DNA fraction, of total DNA, that migrated
EC_{50}	Half-maximal effective concentration
EC_{75}	75 % effective concentration
EDTA	Ethylenediaminetetraacetic acid
ESI-MS	Electrospray ionization mass spectrometry
EU	European Union
FAME	Fatty acid methyl ester
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography – mass spectrometry
GPC	Gel permeation chromatography
HHV	Higher heating value
HMW	High molecular weight
HPLC	High-pressure liquid chromatography
HRR	Homologous recombination repair

HS	Hazelnut shell
IARC	International Agency for Research on Cancer
IR	Infrared
Kbp	Kilo base pair
L. multiflorum	*Lolium multiflorum*
LMPA	Low melting-point preparative agarose
LMW	Low molecular weight
MML	Median molecular length
MMS	Methyl methanesulfonate
NHEJR	Non-homologous end-joining repair
NMR	Nuclear magnetic resonance
P. radiata	*Pinus radiata*
PAH	Polycyclic aromatic hydrocarbon
PCA	Principal component analysis
PFGE	Pulsed-field gel electrophoresis
PFI	Paper and Fibre Research Institute
RF	Relative front
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SSB	Single-strand break
US EPA	United States Environmental Protection Agency
UV	Ultraviolet radiation
WSF	Water-soluble fraction

1 INTRODUCTION

1.1 General background

The need for energy resources has highly increased during the last century and will continue to increase proportionally with population growth and industrialization in the future (Armaroli and Balzani, 2007). With the major energy resource today being non-renewable fossil fuel, new ways to generate energy are necessary to overcome the increasing demand. The application of plant biomass as a more environment-friendly and CO_2 neutral energy resource has gained increased interest in the latest decades, and may be a good alternative to fossil fuel (Mohan et al., 2006). Several different processing methods have been applied to obtain the most favourable biomass fuel (bio-fuel), including trans-esterification of plant oils into fatty acid methyl esters (FAME) (Andrade et al., 2011), ethanol production through hydrolysis and fermentation of polysaccharides or lignocelluloses (Hahn-Hagerdal et al., 2006) and pyrolysis of various biomass into liquid oil (Mohan et al., 2006).

Fast pyrolysis of biomass is one of the most promising methods, and research on wood-based pyrolytic bio-oil has already been established in Norway (Kleinert and Barth, 2008). With increasing production and eventually application of bio-oils, it will be important to assess the risks related to environmental contamination. Bio-oils are highly complex mixtures consisting of hundreds to thousands of different compounds, which might interact with each other and cause unknown adverse effects on biological systems (Lu et al., 2012). The shortage of toxicological data related to bio-oil exposure, makes it thus urgent to study possible harmful effects prior to large-scale commercial release of these bio-oils.

1.2 Pyrolytic bio-oil

1.2.1 Definition

Pyrolytic bio-oil, also called bio-oil/bio crude/pyrolysis oil (henceforth bio-oil), is the primary product created by heating biomass in the absence of oxygen, a process called pyrolysis. The resulting product is a dark brown oxygenated oil with a smoky odour and high heating value (Mohan et al., 2006). Potential biomass feedstock includes different wood species (Mohan et al., 2006), grasses (Mohanty et al., 2011, Greenhalf et al., 2013) and algae (Miao et al., 2004), in addition to residues from forest industry (bark, needles, branches) (Oasmaa et al., 2010), agriculture (straw, corn stover) (Oasmaa et al., 2010) and municipal biological waste (Muhammad Saiful Islam, 2010).Wood is an abundant resource in the Nordic countries, leading this master's project to focus on bio-oils made from different species of wood. Some main physical properties for wood-based bio-oils are listed in table 1.

Table 1: Typical properties of wood-based bio-oils

Physical property	**Typical values**
Moisture content (H_2O)	15-30 %
pH	2.5
Density	1.2 kg/l
Elemental composition (dry basis)	
Carbon	48-60 wt. %
Hydrogen	6-7 wt. %
Oxygen	34-45 wt. %
Nitrogen	0-0.1 wt. %
Sulphur	60-500 ppm
Ash	0-0.2 wt. %
Higher heating value (HHV)	16-19 MJ/kg
Viscosity (at 50 °C)	40-100 cP
Solids (Char)	0.2-1.0 wt. %

(Czernik and Bridgwater, 2004, Lu et al., 2012)

1.2.2 Production methods

There are two main production methods to produce bio-oils, namely slow and fast pyrolysis. In general the two processes are quite similar, but occur with different physical reaction terms (Bridgwater, 2012a). *Slow pyrolysis*, also known as the conventional method, applies a temperature between 400-500 °C, a heating rate of 10-30 °C/min and a vapour residence time between 5-30 min, which favours the formation of charcoal (Bridgwater et al., 1999). The method is not so favourable with respect to bio-oil outcome (≈ 30 wt. %), but slow pyrolysis bio-oil may still be a valuable by-product from charcoal production.

On the other hand, *fast pyrolysis* has been developed to obtain a high bio-oil yield and to minimize the amount of char and gases formed in the process (Bridgwater, 2012a). To do so, fast pyrolysis applies very high heating rates (10^3-10^5°C/s) and short vapour residence time (< 2 s), which allows rapid quenching of volatiles into liquid bio-oil (Lu et al., 2012). The process reaches a moderate final temperature of 500-600 °C. Fast pyrolysis produces about 60-80 wt. % bio-oil, which makes it the most preferred method for large-scale bio-oil production (Mohan et al., 2006). High bio-oil outcome is also dependent on the pyrolysis technique, and several heating devices have been explored that meet the rapid heat-transfer requirement for fast pyrolysis. This includes bubbling fluidized beds, circulating and transported beds, ablative reactors, rotating cone reactors and vacuum reactors (Mohan et al., 2006).

1.2.3 Chemical composition and characterization

Bio-oils can chemically be described as complex mixtures consisting of various organic acids, alcohols, phenols, ethers, esters, ketones, aromatic compounds, aliphatic compounds, sugars and water (Marsman et al., 2007). In total, several hundred different compounds have been identified (Bridgwater et al., 1999). The versatile composition is the final result from thermal degradation of the three main components of wood, namely cellulose, hemi-cellulose and lignin. Rapid quenching of the volatiles during fast pyrolysis saves many intact functional groups of these three components, and explains why different wood-based bio-oils exhibit similarities in chemical composition (Lu et al., 2012). However, the specific composition of each bio-oil is unique and is determined by the feedstock, pyrolysis technique and the applied physical reaction terms.

A thorough chemical characterization of a bio-oil demands various analytical techniques due to the complex composition of different functional groups. Gas chromatography-mass spectrometry (GC-MS) is the most commonly applied method for detection of volatile compounds, while high-pressure liquid chromatography (HPLC) and electrospray ionization mass spectroscopy (ESI-MS) can be used to detect non-volatile compounds (Lu et al., 2012). Additional techniques can also be applied to determine functional group composition (e.g. Fourier transform infrared spectroscopy, FTIR), molecular weight distribution (e.g. gel permeation chromatography, GPC) and specific bonds between atoms (e.g. nuclear magnetic resonance spectroscopy, NMR). Also, separation of the bio-oil into fractions containing compounds of similar polarities facilitates chemical characterization. However, a complete identification of all compounds is almost impossible due to the complex nature of bio-oil.

1.2.4 Solubility and time-dependent properties

The solubility of bio-oils is a highly important factor that influences application and physical behaviour. The high oxygen content makes bio-oils almost completely miscible with polar solvents, such as methanol, acetone, dimethyl sulfoxide (DMSO) etc., and immiscible with conventional liquid transportation fuels (Bridgwater, 2012b). Compared to fossil fuels, the water solubility of bio-oils is much higher and varies in the range of 60-80 wt. % (Sipila et al., 1998). Also, the addition of water results in phase separation, into a yellow water-soluble fraction (WSF), and a sticky or powdered insoluble bottom phase (Sipila et al., 1998). The WSF contains volatile acids, alcohols and other low molecular weight (LMW) degradation products (aldehydes, carbohydrates, ketones, phenols) (Sipila et al., 1998). The water-insoluble fraction is better known as 'pyrolytic lignin' due to its primary content of high molecular weight (HMW) lignin-derived compounds (Scholze and Meier, 2001), and includes aliphatic and aromatic hydrocarbons, and longer chained aldehydes, ketones and phenols.

A main issue when considering bio-oils as a potential energy resource is the ageing properties of the liquid (Bridgwater, 2012b). The stability of bio-oils has been found to decrease during long-time storage due to the high acidity and the content of various reactive compounds. For instance, some of the compounds tend to self-react at standard temperature and light conditions to form larger molecules and water (Mohan et al., 2006). Phase separation has also been observed, together with other time-dependent changes in behaviour, such as increased viscosity and decreased volatility (Bridgwater, 2012b). As a result, the physical properties of the primary pyrolysis product do not satisfy commercial applications.

1.2.5 Application areas

To produce commercial bio-oil products, further upgrade of the bio-oils is needed to increase the stability and to remove undesirable compounds or properties (Bridgwater, 2012b). The main motive is to develop applicable bio-oils that can be used as bio-fuel for transportation or to generate electricity. For instance, with respect to transportation, it is desirable to adapt bio-oil to diesel engines. To do so, modifications such as catalytic cracking, hydro-treatment, emulsification with diesel/bio-diesel, blending with alcohol fuel or steam reforming is required (Lee et al., 2014). Concerning energy/fuel production, water removal is essential to increase the higher heating value (HHV).

Increased interest has also been observed for particularly valuable compounds in bio-oils, which might be extracted and sold as separate products on the market. Compounds of interest include alcohols, organic acids, phenols, plastics, preservatives, resins, fibres, bio-pesticides and fertilizers (Bridgwater, 2012b, Hossain et al., 2013). However, the area requires more devotion to develop reliable low-cost separation and refining techniques (Zhang et al., 2007).

1.2.6 Advantages and concerns

Biomass exists in a theoretical infinite amount, which makes it a renewable resource with a stable market value. Biomass is also cheaper and easier to acquire compared to fossil resources, and transport-costs can be reduced by utilizing local available plant material. From an environmental perspective, bio-oils might be less harmful compared to fossil fuels due to lower content of heavy metals, and lower atmospheric emissions of NO_X and SO_X when combusted (Zhang et al., 2006). However, the main environmental advantage of bio-oils is the zero net CO_2 release. Plants absorb atmospheric CO_2 when growing, which is released back into the atmosphere when burning the biomass. Replacement of fossil fuels with bio-oils might thus reduce the global warming effect.

Considerations should also be directed toward possible negative impacts on the society and the environment. For instance, bio-oil production demands large areas, implying that areas meant for food production might compete with bio-oil resources. Also, deforestation disrupts natural ecosystems and wildlife, and will further increase the extermination of species (Demirbas, 2009). Replanting of biomass is thus essential to maintain a sustainable production, and to limit the environmental impact within a certain area. Even so, replanting requires economical resources and is highly time-consuming. Notwithstanding the negative perspective, new energy resources are needed in the future, and the main objective should be to find a more suitable alternative compared to fossil fuels considering the well-being of the environment and humanity.

Environmental exposures

Bio-oils are not yet a fully available commercial product and unintended environmental releases are thus limited so far. Eventually, when released on the market, contamination may occur from production sites, transport leakages and accidents. The distribution and retention time of bio-oils in the environment is then likely to depend on both the water-solubility and the bio-degradability of the different compounds. For instance, the water-soluble compounds can readily be transported over long distances by drainage, while the pyrolytic lignin is likely to precipitate and

stick to biota nearby the spill source. Because little is known about the toxicological properties of the complex mixture existing in bio-oils, a precautionary approach is necessary.

1.3 Complex mixture toxicity

Complex mixture toxicity is characterized by the various interactions that can occur between the compounds in the mixture and the biological system. These interactions involve non-additive effects such as synergism, potentiation and antagonism (Cassee et al., 1998), and may occur in the toxicokinetic phase (uptake, distribution, metabolism, excretion) or in the toxicodynamic phase (effect on biological receptors, cellular target or organ) (Cassee et al., 1998). Chemical identification of single hazardous compounds in a complex mixture is thus rarely adequate for toxicological evaluations, and might be misleading. The harmfulness of bio-oils or other highly complex mixtures is thus best studied by assessing the biological effects of the whole mixture and not of individual compounds.

1.4 Bio-oil toxicity

1.4.1 Harmful components

Bio-oils are composed of a wide set of compounds, some of them being recognized as harmful to human beings and ecosystems. This particularly includes polycyclic aromatic hydrocarbons (PAHs), which are organic compounds consisting of fused aromatic carbon rings arranged in a planar structure. Several PAHs have been classified by the International Agency for Research on Cancer (IARC) as group 2A - probable human carcinogens, and by the United States Environmental Protection Agency (US EPA) as priority pollutants (Tsai et al., 2007). Chemical analyzes of bio-oils show that LMW 2-3 ringed PAHs dominate, while the heavier and more lipophilic 4-6 ringed PAHs are found in lower concentrations (Tsai et al., 2007, Cordella et al., 2012). The LMW PAHs are favourable due to lower toxicity and higher bio-degradability.

The key factor related to the carcinogenic effect of PAHs is the formation of reactive epoxide metabolites by the cytochrome P450 mixed-function oxidase system (CYP450) (Shimada and Fujii-Kuriyama, 2004). These metabolites are known to affect DNA replication through DNA-adduct formation (Baird et al., 2005). Further, carcinogenesis can be induced when adducts form at a site critical to regulation of cell differentiation or growth (Gehle, 2011). Another known effect of PAHs is the formation of reactive oxygen species (ROS), which may cause oxidative stress and lipid peroxidation (Niki et al., 2005). Reactions between ROS and DNA or proteins may also indirectly lead to mutations and altered protein function.

Another harmful compound found in bio-oils, is the heterocyclic organic compound named furan, which consist of a five-membered aromatic ring with one oxygen atom. Furan was scored as a potentially hazardous compound found in bio-oils (Cordella et al., 2012) and has been related to tumour incidences in rodents (Kellert et al., 2008). As for PAHs, the furan compound itself is relatively non-toxic, but toxicity is initiated by CYP450 oxidation into cis-2-butene-1, 4-dial (Peterson, 2006). This highly electrophilic metabolite can react with proteins and DNA, and has showed similar genotoxic potency as methyl methanesulfonate (MMS) (Kellert et al., 2008).

Catechol has also been detected in bio-oils (Cordella et al., 2012), and is a phenol compound known to exert mutagenic responses through oxidative stress. The compound has also been found to directly inactivate protein function and disrupt electron transportation in cellular membranes (Schweigert et al., 2001). Other phenols and substituted phenol compounds are not known to exert genotoxic effects (Moridani et al., 2003), but many are known to cause acute toxicity in living organisms (Cordella et al., 2012). Also, several other compounds in bio-oils are known to be toxic, e.g. acetic acid, furfuryl alcohol, furfural, hydroxyacetaldehyde (Cordella et al., 2012).

1.4.2 Previous toxicological studies

Considering that bio-oils have been investigated for about 25 years, there is surprisingly little to be found on their toxicity in the literature. However, this may be due to the fact that bio-oils have been considered as an unfinished product. A Material Safety Data Sheet for bio-oils have been proposed, but is based on a limited set of hazardous properties (CIRAD-Forêt, 2006). The information is based on a study funded by the European Union (EU), named the BIOTOX project, which assessed 21 different bio-oils in relation to toxicity, ecotoxicity, mutagenicity and bio-degradability using standard screening methods (Girard et al., 2005). Besides this, only a handful of separate studies have assessed toxicological responses of bio-oils in biological systems (Pimenta et al., 2000, Park et al., 2008, Pekol et al., 2012, Chatterjee et al., 2013).

The previous studies have shown that bio-oils are capable of inducing negative effects, at both the cellular and the genetic level. Cellular responses include reduced cell viability and increased apoptosis in human and rodent cell lines, with increasing bio-oil concentrations (Chatterjee et al., 2013, Park et al., 2008). Reduced cell viability with increasing concentrations was also found using the bacterial trypan blue exclusion assay (Park et al., 2008, Chatterjee et al., 2013).

Several of the studies were found to assess the potential ecotoxicological effects of bio-oils in aquatic environments by applying the *Daphnia magna* immobility test (Pimenta et al., 2000, Girard et al., 2005, Park et al., 2008). Here, *D. magna* mobility was reduced to 50 % at concentrations between 23-55 mg/l for *Pinus radiata* bio-oil (Park et al., 2008) and at 170 mg/l for *Eucaluptus grandis* bio-oil (Pimenta et al., 2000). In the BIOTOX project, none of the bio-oils caused 50 % reduction in swimming capacity when a maximum concentration of 100 mg/l was applied (Girard et al., 2005).

Caenorhabditis elegans, a common nematode found in temperate soil environments, showed dose-dependent survival after exposure to slow pyrolyzed oak, with a sharp decrease in vitality at a concentration of 0.5 % and 100 % death at a 1.0 % concentration (Chatterjee et al., 2013). Exposure to different mutant strains of *C. elegans* indicated that oxidative stress and impairment of the immune system were possible underlying mechanisms of toxicity. This finding is supported by the known toxicological effects of PAHs, furan and catechol (c.f. 1.4.1).

Bio-oil obtained from fast pyrolysis of hazelnut shells was found to increase the level of chromosomal aberrations in the *Allium cepa* test system (Pekol et al., 2012). This finding is consistent with other studies that observed increased DNA fragmentation after bio-oil exposure (Park et al., 2008, Chatterjee et al., 2013), as chromosomal aberrations are assumed to be caused by DNA strand breaks (c.f. 1.5). Mutagenic activity has been observed in the Ames test, where a positive response was seen with and without metabolic activation (Girard et al., 2005).

On the other hand, absence of genotoxic responses have been registered, including lack of reversions in the mutant luminescent bacteria *Vibrio fisheri* (Pimenta et al., 2000), non-significant results in micronuclei assays in lymphoma cells (Girard et al., 2005) and non-significant results in Ames test (Pimenta et al., 2000). In brief, the lack of extensive toxicological data and contradictory results point out the need to perform more studies to acquire knowledge of which bio-oil is more toxic and which is not.

1.5 DNA double-strand breaks as a genotoxic endpoint

Structural damage to the DNA is often an early-warning signal of possible adverse effects appearing at higher levels of biological complexity. Analyses of structural DNA damage caused by exposures to exogenous substances are useful preliminary examination tools to determine if further toxicological evaluation of the substance is needed. Of the various forms of damage, DNA double-strand break (DSB) probably is the most dangerous (Jackson, 2002). Formation of

DNA DSBs occurs when the two complementary DNA strands break simultaneously at sites that are sufficiently close to each other to cause complete rupture of the double helix (Jackson, 2002). As a consequence, the two DNA ends may become physically dissociated from each other, making repair difficult to perform and provides opportunity for inappropriate recombination with other sites in the genome (Jackson, 2002). Compared to single-strand break (SSB), DNA DSB is much more severe due to the lack of an intact template to restore the original sequence.

Ionizing radiation, certain chemicals (e.g. bleomycin) and free radicals are known to directly induce DNA DSBs (Gent et al., 2001). However, DNA DSBs are most frequently caused indirectly during replication when DNA polymerase encounters a DNA SSB or another type of DNA lesion that has escaped the cellular repair-mechanisms (Jackson, 2002). These lesions can occur from spontaneous endogenous events (e.g. abasic sites, 8-oxoguanine, thymine glycol, 3-methyladenine) (Vilenchik and Knudson, 2003) or from exposure to various exogenous DNA damaging agents (adducts, intercalates, base-modificators) (Jackson, 2002). Also, DNA DSBs are naturally occurring intermediates in several essential cellular processes, such as meiotic recombination and somatic recombination in developing lymphocytes (Gent et al., 2001, Jackson, 2002).

Formation of DNA DSBs is believed to be a critical primary step in the formation of chromosomal aberrations (e.g. fragmentation, translocation and deletion) (Jackson, 2002). Chromosomal aberrations are potent inducers of apoptosis and have been related to elevated risk of cancer development (Pfeiffer et al., 2000). Chromosomal aberrations are likely to occur through DNA DSB repair mechanisms due to the high rate of miss-repair. Correct repair is especially difficult if the two DNA ends become physically dissociated from each other or need individual processing before DNA ligation can occur (Jackson, 2002). The two main repair mechanisms also differ in their accuracy. In non-homologous end-joining repair (NHEJR) the two DNA ends are directly ligated, but the mechanism is highly error-prone due to small unavoidable deletions in the ligation area (Pfeiffer et al., 2000). These deletions can have tumour-generic effects if occurring in tumour suppressor genes. Homologous recombination repair (HRR) will most likely restore the original sequence, but frequently causes genome-rearrangement aberrations, such as translocations with possibility to activate proto-oncogens (Pfeiffer et al., 2000).

1.5.1 Measurement of DNA double-strand breaks by agarose gel electrophoresis

Determination of DNA double-strand breaks can be performed relatively rapid by applying neutral agarose gel electrophoresis according to Theodorakis et al. (1994). Here, cellular material is embedded into agarose plugs, which are enzymatically digested and sealed into the wells of an agarose gel. Constant field gel electrophoresis (CFGE) is then applied, causing DNA fragments to migrate out of the wells and be separated by molecular size in the gel. Increased frequency of DNA strand breaks in a sample will cause the super-coiled 'ball' of DNA in the well to relax, and release DNA fragments to a higher extent (Collins et al., 2008). The relative amount of DNA strand breaks can thus be estimated by calculating the percentage of DNA migrating out of the well relative to the total DNA amount loaded into the gel (Theodorakis et al., 1994). Also, the median molecular length of the migrated DNA fragments will reflect genetic damage, as the size of the DNA fragments is inversely proportional to the number of DNA strand breaks in the sample. By applying either neutral or alkaline conditions, the amount of DNA DSBs or the total amount of DNA strand breaks (DSBs and SSBs) can respectively be measured (Theodorakis et al., 1994).

Theodorakis et al. (1994) originally applied the method to fish blood cells, but later studies have applied the method to several other species as well, such as birds (Krøkje et al., 2006, Fenstad et al., 2014), amphipods (Costa et al., 2002) and mussels (Siu et al., 2008), in addition to rat hepatoma cell lines (Haldsrud and Krøkje, 2009). Adaption of the method to plant material (i.e. *Allium cepa* and *Lolium multiflorum)* has also been successfully tested at the Department of Biology at NTNU (unpublished work).

Standard methods for measurement of genotoxic insult in living organisms, such as microscopic analysis of chromosomal aberrations, have a highly subjective element. In contrast, the method developed by Theodorakis and co-workers (1994) is more objective due to damage-quantification by instruments and mathematical computations. The method also has the advantage of minimizing DNA-damage during sample processing, implying that the majority of damage detected is caused by the genotoxic agent, and not the preparation procedure.

1.6 Allium cepa as test species

1.6.1 Test reliability

Plant systems have long been employed as genetic models for toxicological screening and monitoring of environmental pollutants (Grant, 1982, Fiskesjø, 1985, Rank and Nielsen, 1993, Rank, 2003). The *Allium cepa* is a well accepted *in vivo* test system for assessment of genotoxicity of pollutants. The onions can either be exposed in the laboratory or placed directly in the contaminated environment of interest. Evaluation of chromosomal aberrations and micronuclei formation in meristem root cells are the most investigated endpoints, but newer studies have also applied the comet assay for detection of DNA fragmentation (Leme and Marin-Morales, 2009). The *A. cepa* test system is commonly applied due to the advantage of high sensitivity, high percentage of dividing cells, uniform chromosome size and easy handling in laboratory and *in situ* conditions (Seth et al., 2008).

1.6.2 Applicable test samples

The *A. cepa* test system has been applied to pollutants such as heavy metals (Seth et al., 2008), radioactive compounds (Kovalchuk et al., 1998), different water contaminations (SmakaKincl et al., 1996, Leme and Marin-Morales, 2008), soil contamination (Cotelle et al., 1999) and fly ash (Chakraborty et al., 2009). *A. cepa* also possesses an oxidase enzyme system, which makes it applicable for evaluations of secondary mutagens without the addition of an exogenous metabolic system (Fiskesjø, 1985). For instance, positive response have been observed for PAHs such as benzo(a)pyrene and benzene. (Fiskesjø, 1985, Rank and Nielsen, 1994). However, some authors have also pointed out some restriction towards the detection of secondary mutagens, as plants presents lower enzyme concentration and limited substrate specification compared to mammalian CYP450 (Leme and Marin-Morales, 2008, Rodrigues et al., 2010).

1.6.3 Extrapolation to mammals and environmental exposure

Besides the advantages mentioned above, the *A. cepa* test system has shown good correlation for genotoxicity, when compared with other test systems, e.g. mammals (Fiskesjø, 1985, Rank and Nielsen, 1994, Leme and Marin-Morales, 2009). Due to the higher metabolic capacity of mammals, even small positive results for secondary mutagens in *A. cepa* should be taken into considerations, since it might imply an even higher risk to mammals. In general, detection DNA damage as a result of contaminant exposure in any species may on one level be extrapolated to all species since the genomic material is highly conserved between species (Leme and Marin-Morales, 2009).

Aim and hypotheses

The aim of this master's project was to evaluate the toxic properties and the genotoxic potential of five bio-oils made from different species of wood. Toxicity and genotoxicity was respectively measured as root growth inhibition and DNA double-strand breaks, in the *in vivo Allium cepa* test system.

The hypotheses are:

- Small differences in bio-oil composition or concentration may cause highly different toxicological responses in *A. cepa*, as numerous non-additive interactions will likely occur between the various bio-oil components and the test system.
- By comparing the obtained results for bio-oil toxicity with chemical data, it might be possible to identify differences in chemical composition influencing the toxicity of the bio-oils.
- By comparing the obtained results for bio-oil genotoxicity with chemical data, it might be possible to identify differences in chemical composition influencing the genotoxicity of the bio-oils.

2 MATERIALS AND METHODS

2.1 Test species

Allium cepa was used as test species in this master's project. The onion bulbs were provided as a gift by Johan A. Hveem, Lena. All onions were between 30-40 mm in diameter and were harvested September/October 2013. The experiments were performed 2-4 months after harvest to assure sufficient onion root growth. The onions had not been treated with any growth inhibitors, which was essential for the project. After receiving the onions, they were stored in a cardboard box placed in a dark closet at room temperature until use.

2.2 Bio-oils

Five different bio-oils produced from fast pyrolysis of whole wood (100 %) were applied in the master's project. The bio-oils were provided by the Paper and Fibre Research Institute (PFI). All bio-oils have previously been analyzed by Centre for Research, Development and Innovation, Statoil ASA, using electrospray ionization-mass spectrometry (ESI-MS), Fourier transform infrared spectroscopy (FTIR) and gas chromatography-mass spectrometry (GC-MS) (Eide and Neverdal, 2014). The bio-oils derived from beech and spruce, were produced at the University of Aston, UK. The three other bio-oils were produced by different foreign companies. Brief information about the bio-oils, e.g. notation used, is listed in table 2.

Table 2: Overview of the five different bio-oils applied in the project

Feedstock/notation	***Beech***	***Pine***	***Poplar***	***Spruce (100-0-0)***	***Wood (unspecified)***
Producer	University of Aston, UK	Company I	Company II	University of Aston, UK	Company III
Year of production	2012	2011	2009	2011	2010 or older
Storage	The bio-oils were stored in the dark at room temperature at PFI before they were received. After receiving the bio-oils, they were stored dark in the fridge (4°C).				

Bio-oil made from spruce is referred to as 100-0-0 in Celaya et al. (2012) and Toven et al. (2013). The notation is also used in the chemical data obtained from Statoil (Eide and Neverdal (2014)).

2.3 Chemical characterization of bio-oils

Chemical data for the five bio-oils were provided by Ingvar Eide, Centre for Research, Development and Innovation, Statoil ASA, and have been published earlier elsewhere (Eide and Neverdal, 2014). Several different fingerprinting techniques were applied, including positive and negative ESI-MS, FTIR and GC-MS. Qualitative identification of single components was not performed. To better illustrate the differences in chemical composition between the bio-oils, Ingvar Eide kindly performed principal component analysis (PCA) on the obtained mass spectra

and chromatograms. Individual spectra/chromatograms are presented for three bio-oils that showed large variation for all analyses, i.e. beech, poplar and wood.

2.3.1 ESI-MS

Electrospray ionization mass spectrometry detects volatile and non-volatile polar compounds in bio-oils. By applying either positive or negative ionization, the presence of certain classes of compounds can be indicated (Eide and Zahlsen, 2012). However, the technique cannot be applied to identify single components, but is used for comparison of mass distribution of the resulting ions. Positive ESI-MS detects compounds that readily form positive ions, especially compounds that contain nitrogen, oxygen and sulphur groups (Eide and Neverdal, 2014). Negative ESI-MS detects compounds that form negative ions, such as organic acids, alcohols and phenols (Eide and Zahlsen, 2012). In contrast, non-polar compounds cannot be ionized and are thus not detected by standard ESI-MS.

The provided PCA score plots for positive and negative ESI-MS of the bio-oils applied in this project, demonstrated that the bio-oils were very similar in their ion mass distribution, but still not equal (figure 1).

For positive ionization, the PCA score plot showed that bio-oil made from beech differed from the other bio-oils. The four other bio-oils appeared highly similar, but the bio-oil made from unspecified wood was slightly different. According to the mass spectra, beech bio-oil showed a higher amount of low m/z (mass-to-charge-ratio) positive ions relative to the two other bio-oils (figure 1). Wood bio-oil showed the highest intensities in the dominant mass region, but seemed to lack the tail of heavier positive ions observed for the mass spectra of beech and poplar bio-oil.

The PCA score plot for the negative ionization showed that the two softwood species, spruce and pine, were highly similar in negative ion mass distribution. Poplar bio-oil displayed high similarity to the softwood species, but some variation was seen by the second principal component. In contrast to the positive ion analysis, beech bio-oil showed high similarity to the other bio-oils in the negative ion analysis. The largest difference in negative ion composition was seen between wood bio-oil and the softwood species. The individual mass spectra of beech, poplar and wood bio-oil highly overlapped (figure 1), and it was clear that specific peaks in the range of 100-200 m/z largely contributed to the bio-oils' composition. No apparent difference was seen between the mass distribution of poplar and beech bio-oil, while wood bio-oil showed higher intensities in the dominant mass region and did not contain ions above 800 m/z.

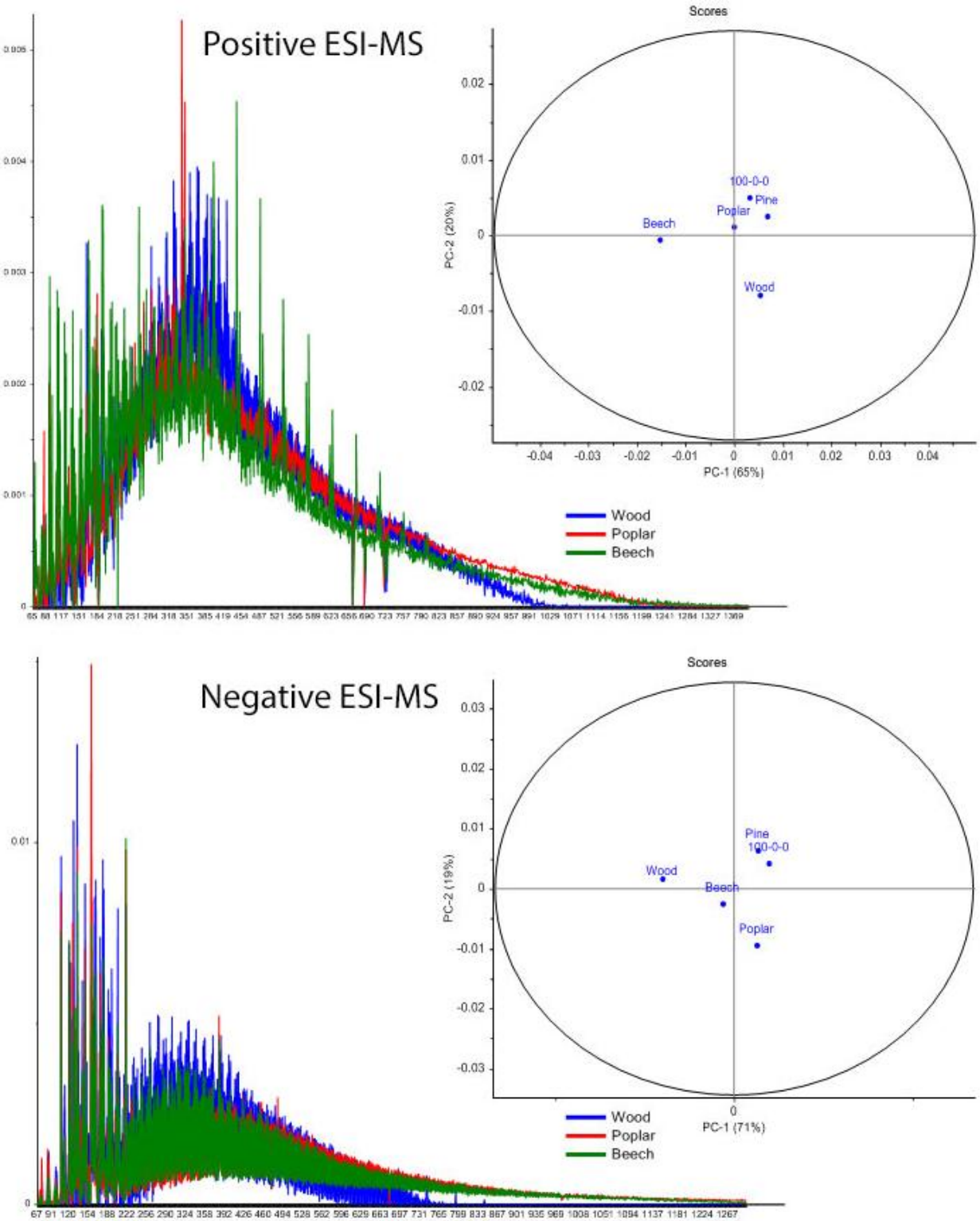

Figure 1: Positive and negative ESI-MS spectra of beech, poplar and wood bio-oil are presented to the left, while the PCA score plots showing the variation between all five bio-oils (beech, pine, poplar, spruce (100-0-0), wood) for the two analyses are presented to the right.

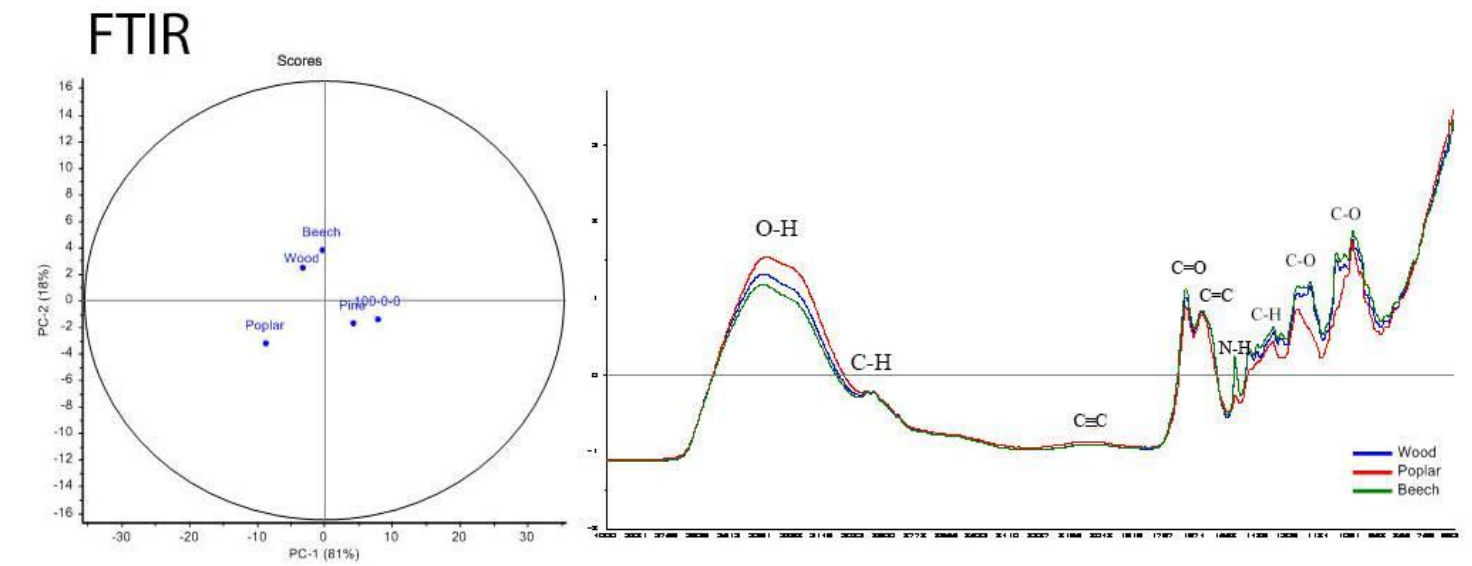

Figure 2: The PCA score plot of tabulated IR spectra of the five bio-oils (beech, pine, poplar, spruce, wood) obtained by FTIR is presented to the left, while the individual IR spectra of beech, poplar and wood bio-oil is presented to the right.

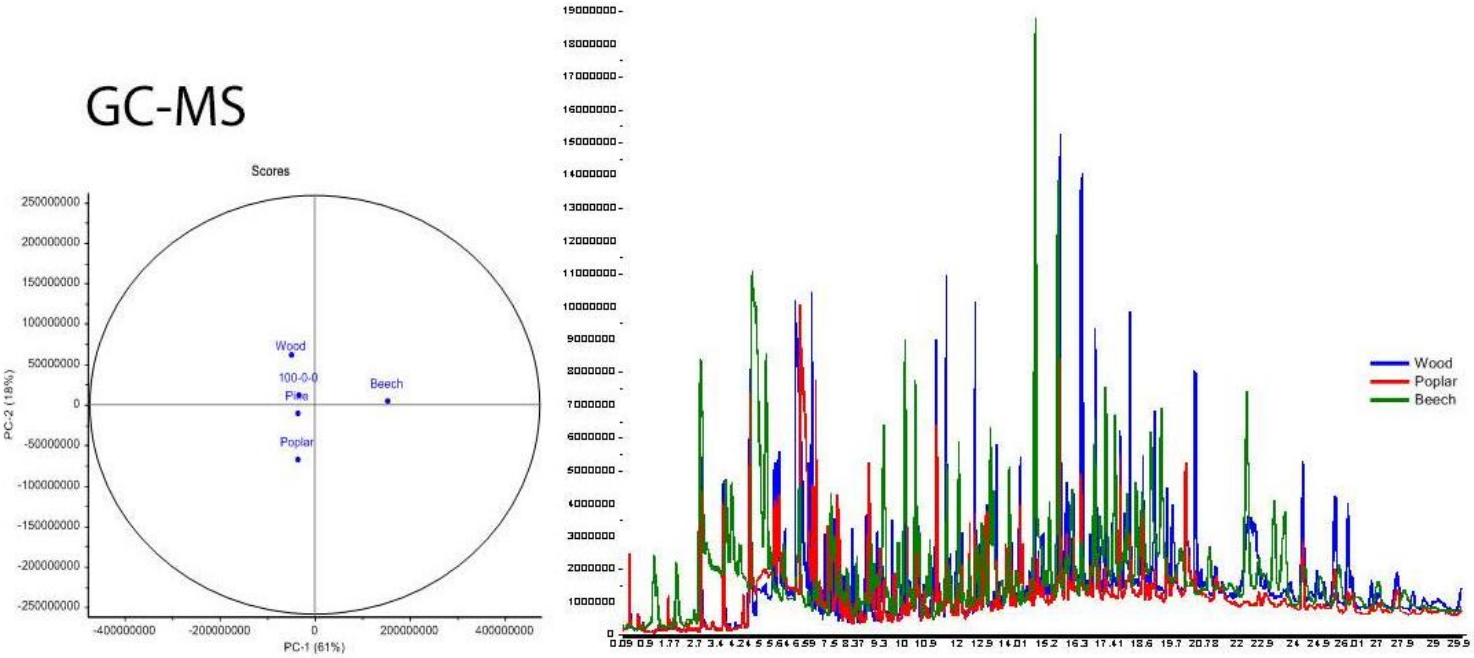

Figure 3: The PCA score plot of the chromatograms of the five bio-oils (beech, pine, poplar, spruce, wood) obtained by GC-MS is presented to the left, while the individual chromatograms of beech, poplar and wood bio-oil is presented to the right.

FTIR

Fourier transform infrared spectroscopy can characterize all functional groups present in bio-oils (Eide and Neverdal, 2014). It has previously been observed that the proportions of the functional groups correlate well with the content of cellulose, hemi-cellulose and lignin found in the feedstock (Stas et al., 2014). For instance, the two bio-oils made from softwood species (spruce and pine) showed the highest similarity in functional group composition in the obtained PCA score plot (figure 2). Poplar and beech bio-oil differed from the softwood species, and also differed from each other. The bio-oil made from unspecified wood showed high similarity to beech bio-oil in functional group composition.

Table 3: Functional group composition of the five bio-oils, obtained by FTIR. The observed intensity peaks in the IR spectra were assigned to their respective functional groups according to values found in the literature (Qiang et al., 2008, Stas et al., 2014). Classes of compounds that contain the specific functional groups are given.

Wave number (cm-1)	**Functional groups**	**Compound class**
3600-3200	O-H	Phenols, alcohols, water, carboxylic acids
2980-2870	C-H	Alkanes
2350-2000	C≡C	Alkynes
1850-1650	C=O	Aldehydes, ketones, carboxylic acids, esters
1680-1580	C=C	Alkenes
1550-1490	N-H	Nitrogenous compounds
1470-1350	C-H	Alkanes
1300-950	C-O	Alcohols, phenols, ethers

The main difference in functional group composition between the bio-oils made from poplar, wood and beech, can be seen from their IR spectra (figure 2). The wavelength peaks in the spectra have been assigned to their respective functional groups, together with classes of compounds containing these groups (table 3). Poplar bio-oil showed the highest content of O-H, which is associated with water, alcohols, phenols and carboxylic acids. The two other bio-oils contained a higher amount of all other functional groups, especially C-H and C-O, but beech bio-oil was the one that showed the highest intensities between the two bio-oils.

2.3.2 GC-MS

Gas chromatography-mass spectrometry detects volatile compounds in bio-oils and is often used to identify single components. Compounds in bio-oils typically identified by GC-MS include acids, aldehydes, ketones, alcohols, phenols, esters, monosaccharide and nitrides. However, some volatile compounds of low concentrations cannot be perfectly determined, due to the complex peaks displayed on the chromatogram (Qiang et al., 2008). Also, only partial information of the bio-oil is obtained by GC-MS, since non-volatile compounds such as lignin oligomers (long-chained phenols) cannot be detected (Stas et al., 2014).

Due to the lack of identification of single compounds in the bio-oils used in this master's project, only visible differences in the chromatograms, including the PCA score plot, could be applied to evaluate the chemical differences between the bio-oils. According to the obtained PCA scores, it was evident that beech bio-oil has a chemical composition different from the other bio-oils (figure 3). The other four bio-oils appeared very similar according to the first principal component, but some variation was seen by the second principal component. Wood and poplar showed the largest variation, while pine and spruce showed very high similarity with each other. The chromatograms of beech, poplar and wood bio-oil showed several overlapping peaks with varying intensities, implying that the bio-oils contained many of the same compounds, but in different concentrations. Wood and poplar bio-oil showed many of the same peaks, but wood showed generally higher intensities. In contrast, the chromatogram of beech bio-oil contained some high peaks that were not observed for any of the other two bio-oils.

2.4 Experimental setup

2.4.1 Chemicals, equipment and consumption material

The chemicals, equipment and consumption material used during bio-oil preparation, exposure to onions and for fixation and storage of the onion root tips are listed below.

Equipment and consumption material	***Prod. Nr***	***Producer***
Chemical thermometer, 100 °C	3201	Assistent, Karl Hecht
Pasteur pipette, 150 mm	612-1701	VWR International
Pipettes (100 µl, 1000 µl, 2500 µl,5000 µl)		Eppendorf
Pipette tips		
200 µl	70.760.502	Sarstedt
1000 µl	70.762.100	Sarstedt
2500 µl	0030 000.951	Eppendorf
5000 µl	0030 000.978	Eppendorf
Plastic ruler, 15 cm	560 01-15	Staedtler
Surgical blade, carbon steel (size 11)	0203	Swann-Morton
Surgical blade handles (no. 3)		Swann-Morton
Test tubes, glass (50 ml)		
Thermostatic water bath	D3165	Köttermann
Vortex vibrofix VF1 Electronic		Janke & Kunkel, Ika labortechnik
Watchmaker tweezers		

Chemicals	***Prod. Nr***	***Producer***
8-hydroxyquinoline (C_9H_7NO), saturated solution	1.07098.0250	Merck
Acetic acid (CH_3COOH), 100 % glacial	1.00063.1000	Merck
Ethanol (CH_3CH_2OH), 96 %	20821.31	VWR International
Dimethyl sulfoxide ($(CH_3)_2SO$), $\geq$ 99.8 %	1.02950.0500	Merck
Hydrochloric acid (HCl), 37 % fuming	1.00317.1000	Merck

Methane methylsulfonate ($C_2H_6O_3S$)	M4016-25G	Sigma
Sodium hydroxide (NaOH)	S5881	Sigma

2.4.2 Preparation of bio-oil solutions

A stock solution of bio-oil, sufficient for a whole experiment, was prepared the day before the exposure of onions started. The stock solution had a concentration of 0.04 ml bio-oil/ml solution (ml/ml). Tap water was used as the main solvent to facilitate root growth conditions, but 0.1 % dimethyl sulfoxide (DMSO) was also applied to enhance solubility of organic compounds. Before making the stock solution, the bio-oils were heated in a water bath for 1 hour (50 °C) (Neverdal, pers. comm.) and homogenized by a vortex mixer. The stock solution was then made by first dissolving the bio-oils in DMSO followed by dilution with tap water. The soluble fraction was replaced in a new container, and the bottom phase was discarded. Between each day of exposure, the stock solution was stored in the dark at 4 °C.

Seven exposure solutions were prepared from the stock solution at each day of bio-oil exposure, and were of the following concentrations (ml/ml): 0.04 (stock solution), 0.004, 0.001, 0.0004, 0.0001, 0.00004 and 0.00001. Due to limited volume of beech bio-oil, a dilution of 0.01 ml/ml was prepared as the highest concentration instead of 0.04 ml/ml. The actual exposure concentrations, including the stock solution, were relative to the stated concentrations based on the amount of bio-oil soluble in tap water and 0.1 % DMSO. Before preparation of the exposure solutions, the stock solution was heated for 1 hour in a water bath (50 °C), and the seven solutions were then made by diluting with tap water (50 °C). DMSO was added to the dilutions to maintain the 0.1 % concentration and the pH was adjusted to about 7.00 for all solutions to eliminate the pH effect on onion root growth. The solutions were cooled down to room temperature before exposing the onions.

2.4.3 Bio-oil exposure

Exposure of the onions for the five bio-oils was carried out in five separate experimental runs. The experimental setup was adapted from the *Allium cepa* test developed by Fiskesjø (1985) and further described by Rank and Nielsen (1993). The lower half of the onions was peeled and the dry bottom plate was carefully removed. Onions were then placed in glass beakers filled with tap water to induce growth for 48 h. The onions were kept in a dark closet during growth and the water was changed after 24 h. Up to five extra onions were used for each experiment, so that onions with poorer growth could be discarded from the exposures. Root growth induction was followed by a 72 h exposure to the seven bio-oil concentrations (about 50 ml solution), using

three parallels for each concentration. The bio-oil solutions were replaced every 24 h with newly made solutions.

Tap water was used as a negative control and methyl methanesulfonate (MMS), 10 mg/l, was used as a positive control for genotoxicity. Exposure to the two controls was performed in a separate experiment instead of repeating the same exposure five times. Onions were also exposed to tap water at the beginning and at the end of the two-month experimental period to verify that the rate of root growth remained unchanged. An additional experiment was also run to test for any bias according to the method applied for preparation of the exposure solutions. This experiment was referred to as the *blind* experiment. Here, onions were handled equally as in the bio-oil experiments and were exposed to dilutions of a stock solution containing only the solvent (0.1 % DMSO in tap water). The DMSO concentration and the pH were adjusted in the same way as in the bio-oil experiments. The *blind* stock solution was also used as a solvent control for genotoxicity.

2.5 Measurement of bio-oil toxicity

2.5.1 Root growth inhibition

Bio-oil toxicity was examined as inhibition of *A. cepa* root growth by measuring the root growth during the 72 h exposure period. Root length was first measured after the 48 h growth induction period and for every following 24 h during the exposure. The onion root length was calculated as the average length of all roots on one individual.

Root growth inhibition was expressed as percentage root growth relative to the average root growth of individuals exposed to the lowest bio-oil concentration (0.00001 ml/ml) of the respective bio-oil tested, henceforth referred to as *relative root growth*. The average relative root growth of onions exposed to the lowest concentration was thus equal to 100 %.

$$\textit{Relative root growth} = \frac{\textit{Root length}_{72h\ exposure} - \textit{Root length}_{48h\ tap\ water}}{(\textit{Root length}_{72h\ exposure} - \textit{Root length}_{48h\ tap\ water})\ \textit{of the 0.00001 ml/ml exposure}} \times 100\ \%$$

The half-maximal effective concentration (EC_{50}) was calculated for comparison of toxicity between the different bio-oils, and was the concentration corresponding to 50 % relative root growth.

2.6 Preservation of *A. cepa* root tips

Straight after the final measurement of root growth, the onion roots were preserved for later analysis of genotoxicity. All root tips were cut off, about 5 – 7 mm in size, using tweezers and were treated with 8-hydroxyquinoline (saturated solution) for 5 hours (4 °C). The root tips were then washed three times with distilled water before fixation in 3:1 ethanol-acetic acid solution for 70 min. After fixation, the solution was changed to 70 % ethanol for storage at 4 °C. The root tips belonging to one of the onions exposed to 0.0004 ml/ml beech bio-oil were not fixated, due to a manual slip in the process.

2.7 Detection of DNA double-strand breaks by agarose gel electrophoresis

2.7.1 Chemicals, equipment and consumption material

The chemicals, equipment and consumption material used during measurement of DNA DSBs are listed below.

Equipment and consumption material	***Prod. Nr***	***Producer***
50 - well plug moulds, CHEF Mapper XA System	1703713	Bio-Rad
Biofuge fresco	75005510	Heraeus
Centrifuge tube w/screw cap, 10 ml		Nunc
Comb, 15 wells		BioRad
Dri-block heater, DB-2D		Techne
Electrophoresis power supply- EPS200	56117302	Heraeus
Filter paper, any kind		
Gel Doc 2000	755/00715	BioRad
Gel mould, 10 cm gels		BioRad
Metal spatulas		
Microtubes, 1.5 ml	72.690.001	Sarstedt
Microwave oven, max 900 W		Electrolux
Pipette (100 µl, 1000 µl)		Eppendorf
Pipette (2 µl, 10 µl, 20µl, 200µl)		Gilson Pipetman
Pipette tips		
10 µl	3512	Molecular BioProducts
200 µl	70.760.502	Sarstedt
1000 µl	70.762.100	Sarstedt
Porcelain mortar and pestle (x 6)		Haldenwahger
Rotamax 120 orbital mixer		Heidolph
Scale, Metler AE260-S	2524GK	Metler
Surgical blades, carbon steel (size 22)	0208	Swann-Morton
Vortex vibrofix VF1 Electronic		Janke&Kunkel, Ika labortechnik
White plastic tray		
Wide mini-sub cell GT	63S 28031	BioRad

Chemicals	***Prod. Nr***	***Producer***
Activated carbon	C3014	Sigma-Aldrich
Agarose for routine use	A9539	Sigma
Boric acid (H_3BO_3), for electrophoresis	B7901	Sigma
Ethidium Bromide ($C_{21}H_{20}BrN_3$), 10 mg/ml	161-0433	BioRad
Ethylenediaminetatraacetic acid ($C_{10}H_{16}N_2O_8$), EDTA	161-0729	BioRad
Hydrochloric acid (HCl), 37 % fuming	1.00317.1000	Merck
Lambda DNA	#SD0011	Fermentas
Lambda/HindIII marker 2	#SD0102	Fermentas
Liquid nitrogen (N_2), -196 °C		AGA
Loading dye x6	#R0611	Fermentas
Low melt preparative grade agarose, LMPA	162-0019	BioRad
Protein kinase K (from *Tritirachium album*)	P2308	Sigma-Aldrich
Sodium dodecylsulfate ($NaC_{12}H_{25}SO_4$), SDS	L-3771	Sigma
Sodium hydroxide (NaOH)	S5881	Sigma
Trizma base ($C_4H_{11}NO_3$), Tris	T6066	Sigma

2.7.2 Bio-oil concentrations for genotoxic assessment

Three bio-oil concentrations were selected for evaluation of genotoxicity based on the obtained results for root growth inhibition. The two highest concentrations were avoided as the toxic effect might alter the measured genotoxic endpoint (DNA DSBs) by e.g. causing DNA degradation. Also, highly toxic concentrations would inhibit cell cycle progression, and therefore, also the formation of DNA DSBs. The lowest bio-oil concentration was not selected as it might be too low to have any influence on the test organism. The selected concentrations where thus the following three concentrations (ml/ml): 0.00004, 0.0004 and 0.001. The highest concentration was around EC_{75}, the middle concentration around EC_{50} and the lowest concentration did not show any apparent effect on root growth for most of the bio-oils.

2.7.3 Agarose plug preparation

The preparation method for the agarose plugs was adapted from Theodorakis et al. (1994) together with own adjustments for application of plant material. Fixated root tips (30 tips) from single individuals were ground in a porcelain mortar with liquid nitrogen and mixed with 0.3 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). The diluted root material was heated to 37 °C and mixed with an equal volume of pre-melted 1 % low melting-point preparative agarose (LPMA) (37 °C). The solution was homogenized using a vortex mixer followed by centrifugation for a few seconds to sort out un-ground material. Only the upper suspension layer was used to cast the agarose plugs by loading 35 µl into each plug mould and chilling the plugs

at 4 °C for 15 min. The plugs were then released from the mould, placed in digestion buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.5 % SDS, pH 7, 1mg/ml proteinase K) and incubated for 6 h at 55 °C.

2.7.4 Constand field agarose gel electrophoresis

After digestion, the agarose plugs were chilled, loaded onto a 0.6 % agarose gel and sealed into place with 1 % LMPA (37 °C). The plugs were run in triplicates for each individual onion. TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8) was used both for the gel and as running buffer. The electrophoresis was run at 2.3 V/cm for 14 h. After electrophoresis, the gel was placed in an ethidium bromide staining solution (1 drop of 10 mg/ml per 0.5L TBE) and stained for 2 h before measurement of UV-intensity with BioRad Gel Doc 2000. The DNA fragment size distribution resulting from the electrophoresis was scaled with respect to DNA size-standards run on the same gel (mixture of whole lambda DNA and HindIII digested lambda DNA fragments).

The experiment was designed to minimize variation between the parallels due to the low number of individuals applied (n=3). All individuals from the same exposure were thus run on the same gel (figure 4). Due to some inhomogeneous distribution of DNA in the obtained DNA bands, three internal measurement lanes were applied in Gel Doc 2000 for each lane on the gel, and the average value of the internal measurements was used.

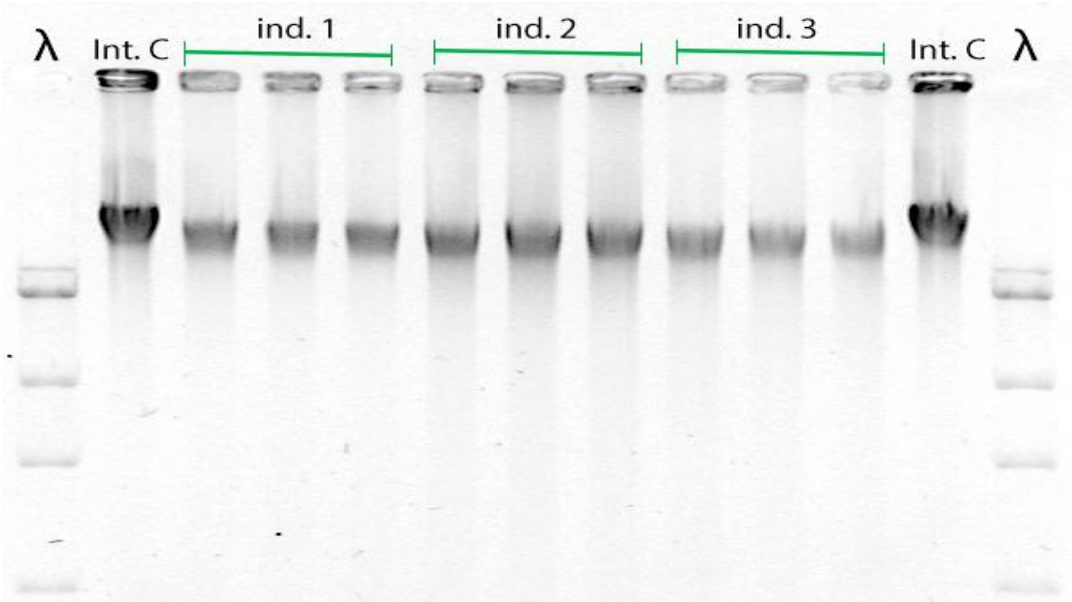

Figure 4: Demonstration of the gel-loading design. The gel contained all parallels (ind.1, ind.2, ind.3) exposed to the same bio-oil concentration using triplicate agarose plugs (marked with green line). The lambda DNA size-marker (λ) was run on both sides of the gel together with an internal constant control (Int. C). The internal constant control was applied to check for variation in migration distance between the different runs (c.f. 2.7.7).

2.7.5 Fraction of total DNA that migrated

The DNA-fraction, of total DNA, that migrated into the gel (DNA-FTM) was used as a relative measure of DNA DSBs (Fenstad et al., 2014). The DNA-FTM was determined by calculating the area under the two absorbance peaks (figure 5).

Total DNA = Area [DNA left in well] + Area [Migrated DNA fragments]

DNA-FTM = Area [Migrated DNA fragments] /Total DNA x 100 %

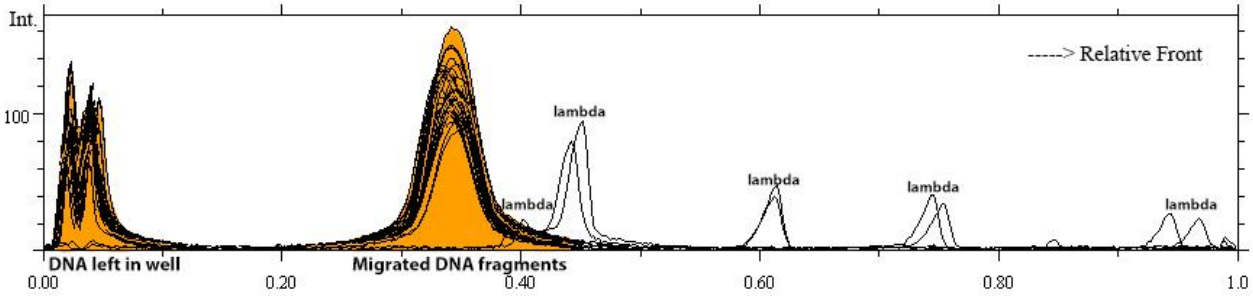

Figure 5: Fluorescence intensity is plotted against the electrophoretic migration distance (relative front). The DNA peaks (orange coloured) represent DNA left in the well (to the left) and DNA fragments that have migrated into the gel (to the right). The lambda DNA-ladder is also seen with decreasing DNA fragment size from left to right.

2.7.6 Median molecular length

The relative number of DNA DSBs was analyzed by calculating the median molecular length (MML) of the migrated DNA fragments. This was done by creating a plot in Gel Doc 2000, which showed the UV-absorbance vs. the electrophoretic migration distance (relative front, RF) (figure 5). The area under the intensity curve of the migrated DNA fragments was then divided into equal halves. The corresponding migration distance for this median value was compared to the migration distances of the lambda DNA-ladder, and could thus be converted into a MML-value given in kilo base pairs (kbp). Due to the lower range of the DNA size-marker, the MML had to be calculated from an extrapolated curve of the lambda DNA-ladder (appendix A.2.2).

2.7.7 Internal constant control of agarose gels

A tap water control sample was chosen as an internal constant control of variation in median molecular length between different electrophoresis runs (figure 4). All plugs of the internal control were made at the first day of gel electrophoresis and were stored at 4 °C between each run. The internal control was found to have a standard deviation of ± 28 kbp in MML between the different runs (appendix A.2.5). However, the variation was likely lower for the bio-oil exposed samples, as the agarose plugs of the internal constant control were highly overloaded (figure 4).

2.7.8 DNA fragment distribution

An additional measurement of genotoxicity, not previously applied with this method, was performed by calculating the skewness of the DNA fragment distribution curve. The skewness was calculated using Pearson's second skewness coefficient:

$Sk_p = 3 \times (mean - median/standard\ deviation)$

The formula gives the skewness as a value between -3 and +3. A more positive-skewed distribution seen for a sample relative to the negative control implies a higher ratio of smaller DNA fragments, which is associated with increased occurrence of DNA DSBs.

2.8 Data calculations/analysis

Calculation of the EC_{50} was performed by plotting the relative root growth values against the respective bio-oil concentrations in a semi-log plot. A four-parameter sigmoid regression line was then created by applying the built-in function in SigmaPlot 12.5, and the equation for the regression line was used to estimate the EC_{50}-values. All parameters, together with the fitness of the regression curves can be found in the appendix (A.1.4)

The DNA-FTM and MML was calculated in Excel 2007. The relative amount of DNA in the well and in the migrated DNA bands was calculated by respectively summing the intensity peaks within the two areas. Extrapolation of the lambda ladder was performed in SigmaPlot 12.5, by adding a fake point for a higher molecular size, and applying a power regression to obtain a standard curve (c.f. appendix A.2.2)

A two-sided Student's t-test (equal variance) was applied to indicate exposures that differed significantly from the negative tap water control in DNA-FTM and in MML. The calculations were performed in Excel 2007, and the significance level was set to $p < 0.01$. All p-values are listed in the appendix (A.2.1 and A.2.3).

3 RESULTS

The toxicological effects of the five bio-oils on *Allium cepa* were assessed by different endpoints. First, a description of macroscopic changes in *A. cepa* induced by exposure to bio-oil is presented to give an overall indication of how severely the test species was affected by the exposure. The result from the quantitative measurement of bio-oil toxicity, analyzed as reduction in *A. cepa* root growth, is then presented. Genotoxic insult to the onions is presented last and was assessed by examining the relative amount of DNA double-strand breaks (DSBs) in *A. cepa* root meristem cells.

3.1 Macroscopic changes induced by bio-oil exposure

Exposure of *A. cepa* to different concentrations of bio-oil (beech, pine, poplar spruce and wood) induced macroscopic changes in appearance that were already seen after 24 h. The onion bulb itself was not affected, only differences in root appearance were observed, and the effects had increased after every 24 h observation. The final appearance of the onion roots after 72 h bio-oil exposure is shown in figure 6. Increased bio-oil exposure caused overall browning of the *A. cepa* roots, starting with a light brown colour for onions exposed to a concentration of 0.0004 ml bio-oil ml/ml solution (ml/ml) and a dark brown colour for onions exposed to the highest concentration (0.04 ml/ml). Additionally, browning at the outermost of the root tips was observed at all bio-oil concentrations. The change in colour was not observed for onions grown in tap water, which had roots that remained white during the whole exposure period (not shown).

The consistence of the onion roots was also altered by bio-oil exposure. Increased bio-oil concentrations caused softening of the roots, and the roots became smooth and slimy for the two highest concentrations. Roots of onions exposed to tap water were hard and crisp, and the same consistence was observed for the two lowest bio-oil concentrations (0.00001 ml/ml and 0.00004 ml/ml). All five bio-oils showed the same effect on colour and consistence of onion roots, with no apparent difference at the same concentrations.

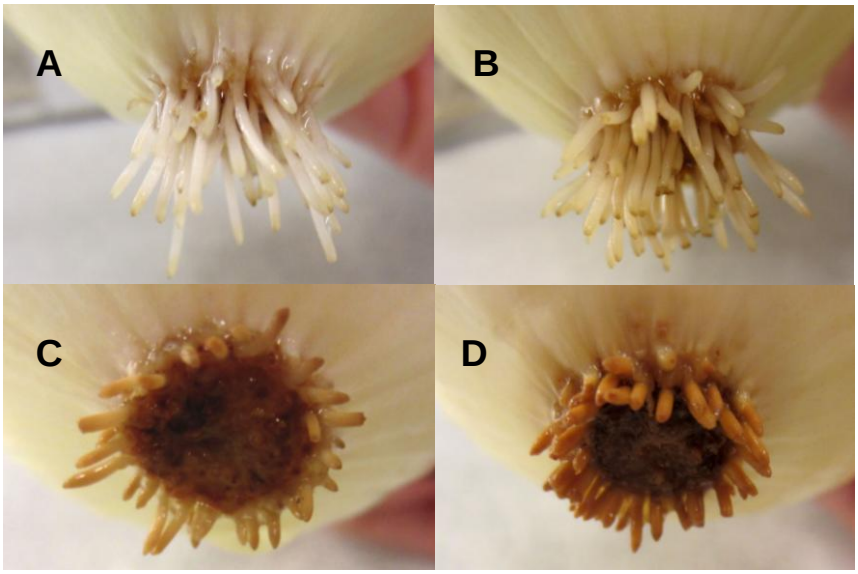

Figure 6: The appearance of *A. cepa* roots after 72 h bio-oil exposure, shown for exposure to four different concentrations (ml bio-oil/ml solution). A = 0.00004, B=0.0004, C = 0.004 and D=0.04.

3.2 Root growth inhibition

Bio-oil toxicity was evaluated based on the obtained root length for *A. cepa* after 72 h exposure to seven different bio-oil concentrations. The toxic effect on the individual onion was quantitatively expressed as percentage root growth relative to the average root growth for onions exposed to the lowest bio-oil concentration (relative root growth). The results are illustrated in figure 7, where relative root growth is presented as a function of bio-oil concentration. The individual relative root growth values and the average values for the three parallels can be found in the appendix (A.1.1). Toxicity curves for all five bio-oils were obtained by a four-parametric sigmoid regression using SigmaPlot 12.5. The curves showed good fit for all bio-oils ($R^2 \geq 0.7$) and were used to estimate the EC_{50}-values. The values are given in table 4.

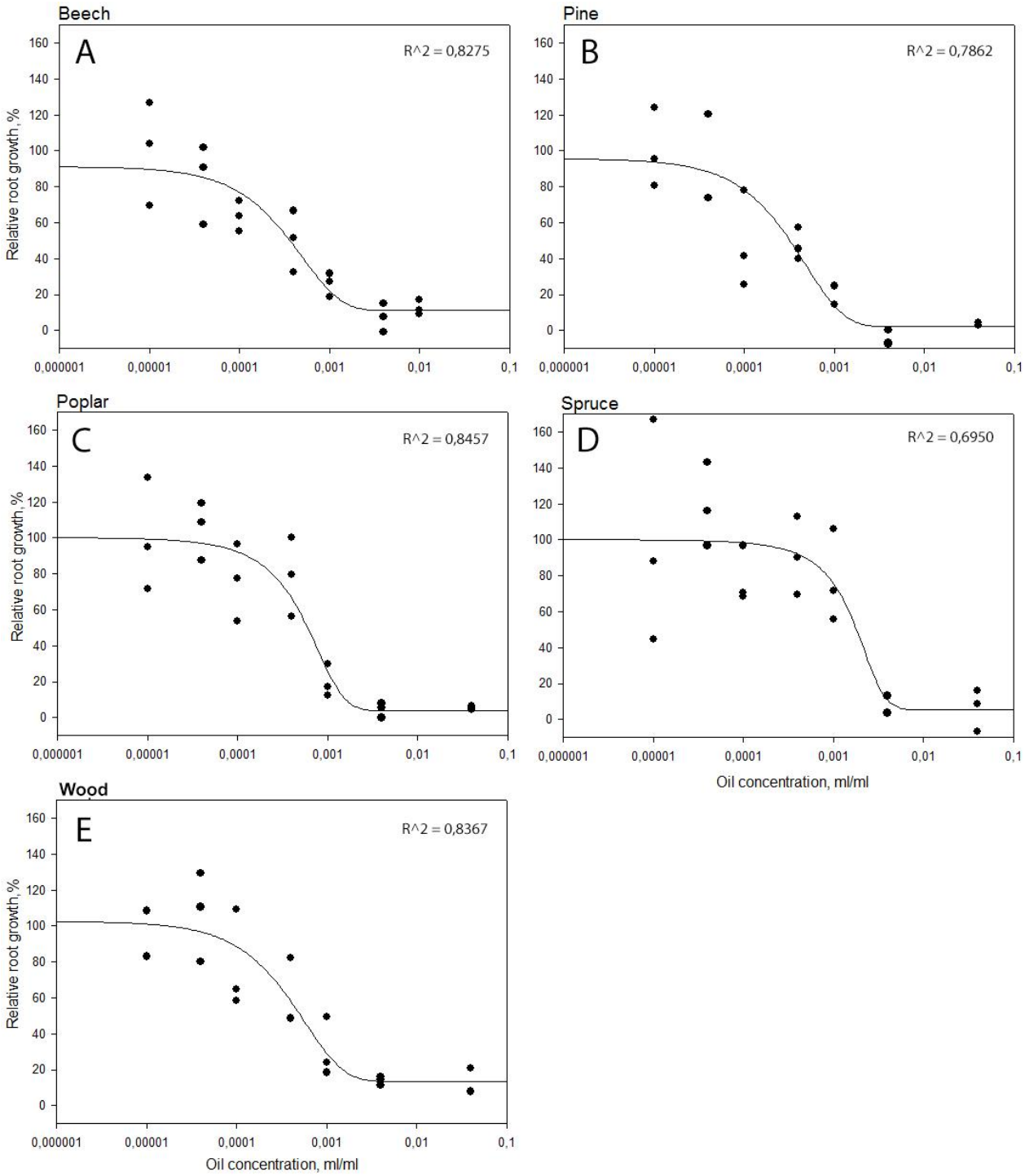

Figure 7: Relative root growth of onions (*Allium cepa*) as a function of bio-oil concentration (ml bio-oil/ml solution). The relative root growth was calculated as percentage root growth relative to the average root growth of individuals exposed to the lowest bio-oil concentation. Three paralells of onions (black dots) and the estimated sigmoid regression curve are shown for bio-oil made from beech (A), pine (B), poplar (C), spruce (D) and wood (E). The fitness of the regression curves to the data points are shown by the coefficient of determination (R^2).

Table 4: Growth inhibition of *A cepa* roots after bio-oil exposure expressed as EC_{50}

Bio-oil feedstock	**EC_{50}** (ml bio-oil/ml solution)
Beech	0.000364
Pine	0.000322
Poplar	0.000605
Spruce	0.001816
Wood	0.000516

According to the EC_{50}-values, the toxicity of the tested bio-oils can be ranked in the following order:

Pine > Beech > Wood > Poplar > Spruce

All five bio-oils caused a concentration-dependent reduction in relative root growth with increasing concentrations (figure 7). In general, the lowest bio-oil concentrations showed no or little effect on the relative root growth, while the two highest bio-oil concentrations caused almost complete inhibition.

Increasing concentrations of beech bio-oil caused a steady linear decrease in relative root growth in the concentration range of 0.00004 – 0.004 ml/ml (figure 7A). A notable difference in relative root growth was seen between the two lowest concentrations, and was not observed for any of the other bio-oils. The root growth of onions exposed to the two highest concentrations of beech bio-oil was not completely inhibited, but showed about 90 % reduction. Relatively small variation was observed between the parallels, but the variation increased with lower concentrations.

Pine bio-oil was the most toxic bio-oil according to the EC_{50}-values and showed a very similar toxicity curve (figure 7B) to the second most toxic bio-oil (beech). Reduction in relative root growth was first seen at the 0.0001 ml/ml exposure. Here, the measured relative root growth also showed some deviation from the regression curve. The two highest concentrations of pine bio-oil caused about 100 % inhibition of *A.* cepa root growth. As for beech bio-oil, a higher variation among the parallels was observed with decreasing concentrations, i.e. the difference in relative root growth between parallels was between 40-50 % for the three lowest concentrations.

The toxicity curve for poplar bio-oil (figure 7C) showed the best fit to its respective data points, indicated by the highest R^2-value (0.85). However, there was some variation between the parallels, which was particularly high for concentrations below 0.001 ml/ml. No difference in root growth was seen between the two lowest concentrations, while the two next concentrations

caused a similar slight reduction in relative root growth. The largest increase in toxicity for poplar bio-oil was observed between the 0.0004 ml/ml exposure and the 0.001 ml/ml exposure. Here, the average relative root growth decreased with about 60 %.

Spruce bio-oil had the most notable toxicity curve (figure 7D), and also the highest EC_{50}-value. The regression curve for spruce bio-oil had the poorest fit to its respective data points ($R^2 \approx 0.7$), but still explained the observed trend. No evident reduction in relative root growth was observed in the concentration range of 0.00001 – 0.001 ml/ml, but a sudden change in toxicity was seen between the 0.001 ml/ml and the 0.004 ml/ml concentration. Here, the relative root growth dropped by 68 %, reaching a value of 9 %. The variation between parallels was especially high for onions exposed to the lowest concentration and varied in the range of 45-167 %, which means that one onion had roots that on average were over two times the length of roots from another onion.

The toxicity curve for wood bio-oil (figure 7E) had a good fit to the data points ($R^2=0.84$). The variation between the parallels increased with lower concentrations, but was smaller than observed for many of the other bio-oils. Onions exposed to the lowest concentration showed especially close values. The linear region was seen in the concentration range of 0.00004 ml/ml-0.001 ml/ml, where the 0.0001 ml/ml concentration was the first concentration to cause growth inhibition. Onions exposed to the two highest concentrations of wood bio-oil reached a higher relative root growth (12-14 %) than observed for onions exposed to the same concentrations of any of the other bio-oils.

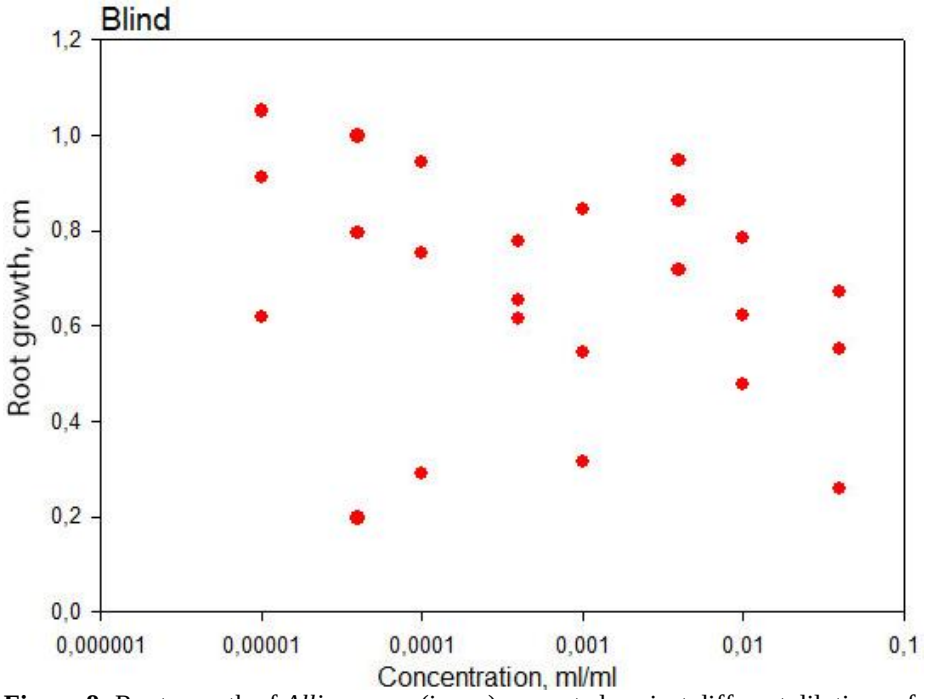

Figure 8: Root growth of *Allium cepa* (in cm) presented against different dilutions of a solution containing tap water and 0.1 % DMSO (called *blind*). The blind solution was diluted in the same way as the bio-oil stock solutions into a total of eight solutions. Onions exposed to the undiluted blind solution can be seen at the 0.04 ml/ml concentration. Three parallels of onions (red dots) are shown in the figure.

An additional experiment was conducted to check for any bias according to the experimental method used and was referred to as the *blind* experiment. Here, onions were exposed to different dilutions of a stock solution containing only tap water and 0.1 % DMSO. The stock solution was handled and diluted in the same way as the bio-oil stock solutions. Results from the blind experiment can be seen in figure 8, and are presented as root growth of onions in centimetres versus the different dilutions. A weak increase in root growth was observed with increasing dilutions of the blind stock solution. On the other hand, large variation in root growth was seen between the parallels and between all individuals in general, which caused a very random distribution pattern. Accordingly, no significant relationship was found when a linear regression was performed (not shown). It should also be mentioned that the average root growth of all individuals (≈ 0. 67 cm, ±) was about 30 % lower than the average root growth obtained for onions grown in only tap water (≈ 1.00 cm) (appendix A.1.2 and A.1.3)

3.3 Genotoxic responses in *A. cepa* root meristem

The relative amount of DNA DSBs occurring in *A. cepa* root meristem cells after exposure to the five bio-oils was analyzed for evaluation of genotoxicity. For this approach, onion root tips were embedded into agarose plugs, which were enzymatically digested, loaded onto an agarose gel and run under neutral electrophoresis. Three different measurements of DNA DSBs were then applied. First, the DNA-fraction, of total DNA, that migrated into the gel (DNA-FTM) was calculated. Then, the median molecular length (MML) of the migrated DNA fragments was determined, and the skewness of the DNA fragment distribution was calculated as an additional measurement of DSBs.

The following three bio-oil concentrations were tested for genotoxicity (ml/ml): 0.00004, 0.0004 and 0.001 (c.f. 2.7.2). A forth concentration (0.004 ml/ml) was also considered to check if even higher bio-oil concentrations could further increase the genotoxic response. However, the obtained fluorescence intensity was very vague at this concentration for all five bio-oils, and the results could thus not be applied in the analyses (picture shown in appendix A.2.4).

3.3.1 Analysis of the DNA-fraction of total DNA that migrated

The DNA-FTM for onions exposed to different bio-oil concentrations was compared to the negative and positive control for evaluation of genotoxicity (figure 9). A two-tailed Student's T-test was performed to identify exposures that were significantly different ($p < 0.01$) from the negative control. However, the statistical difference was less emphasized due to the lack of statistical power (n=3), and was only applied to illustrate trends. The p-values along with the estimated DNA-FTM for each individual can be found in the appendix (A.2.1).

Surprisingly, onions exposed to the positive control, methyl methanesulfonate (MMS), showed no average increase in the DNA-FTM compared to onions exposed to tap water. The solvent control (0.1 % DMSO in tap water) did not show any elevation of DNA-FTM compared to the negative control, but had a lower average value (c.f. appendix A.2.1).

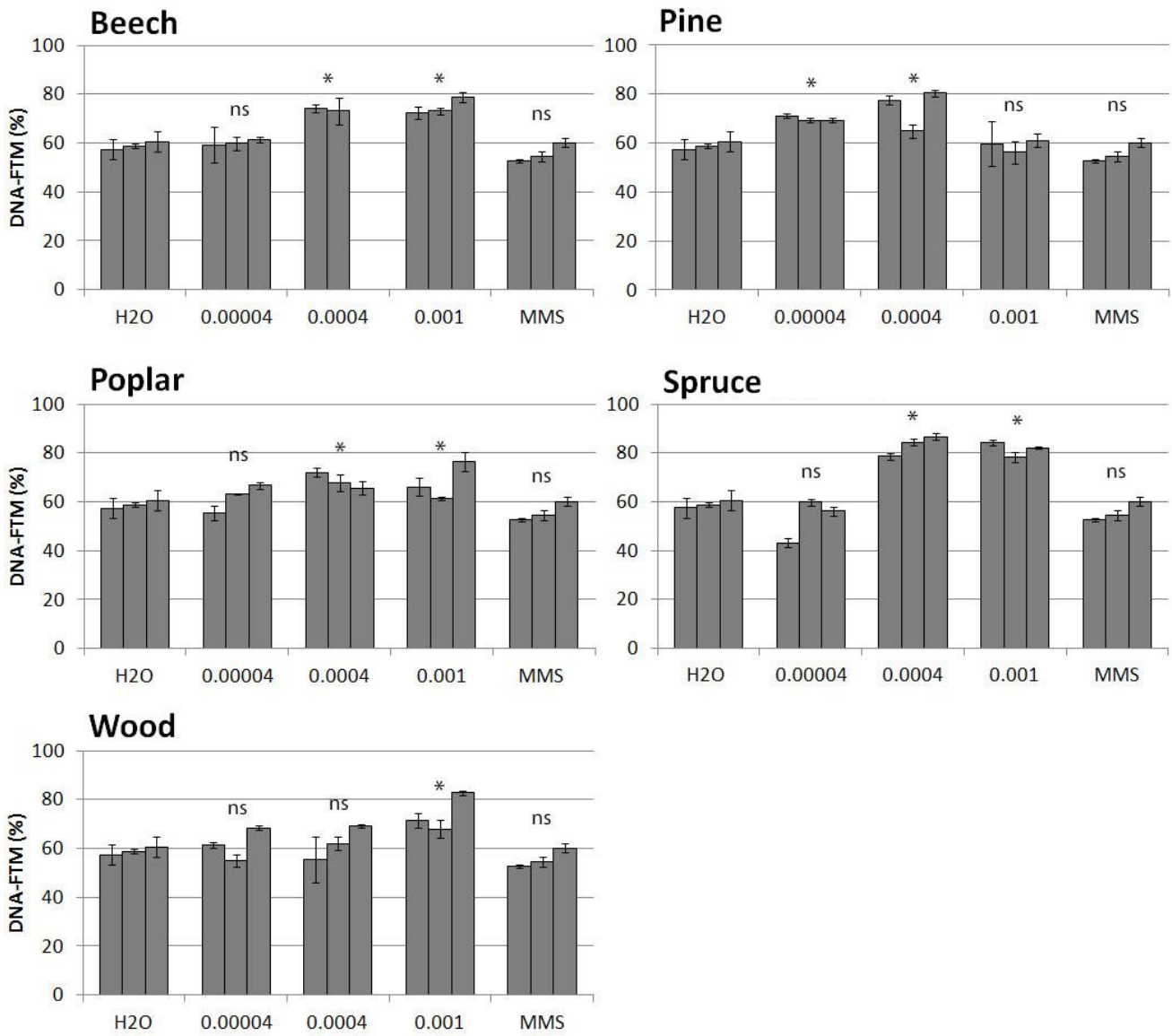

Figure 9: The DNA-fraction, of total DNA, that migrated into the gel (DNA-FTM), as a relative measure of DNA double-strand breaks (DSBs) in *A. cepa*, after exposure to tap water (H2O), three different bio-oil concentrations (ml bio-oil/ml solution) and methyl methanesulfonate (MMS). Results are shown for five bio-oils (beech, pine, poplar, spruce and wood). Each column represents one individual and shows the standard deviation of three replicates. The asterisks indicate exposures that were significantly different from the tap water control ($p < 0.01$, two-tailed Student's T-test, in Microsoft Excel 2007).

Onions exposed to bio-oil made from beech showed a concentration-dependent increase in DNA-FTM with increasing concentrations. The lowest concentration (0.00004 ml/ml) did not cause any alteration in DNA-FTM compared to tap water, while both of the two higher concentrations caused a similar increase in DNA-FTM.

Pine bio-oil was the only bio-oil that showed a decrease in the average DNA-FTM from the lowest (0.00004 ml/ml) to the highest (0.001 ml/ml) concentration. An elevation in DNA-FTM was seen for the two lower concentrations, with a slightly higher average value observed for the

middle concentration (0.0004 ml/ml). It was also noted that onions exposed to the middle concentration showed high variation in DNA-FTM. The highest exposure (0.001 ml/ml) of pine bio-oil caused no apparent genotoxic effect, and thus deviated in genotoxic response compared to the highest exposure of the other bio-oils. Also, one of the individuals exposed to the highest concentration showed a relatively high standard deviation for the three replicates.

Poplar bio-oil caused a weak increase in DNA-FTM with increasing concentrations. The lowest concentration showed no difference from the negative control, while the two higher concentrations caused a small similar increase in DNA-FTM. Some variation in response was observed between the parallels for all three concentrations.

Exposure to spruce bio-oil caused the highest increase in DNA-FTM seen for *A. cepa* in the experiments. Genotoxicity was not observed for the lowest exposure (0.00004 ml/ml), but the two higher concentrations (0.0004 ml/ml and 0.001 ml/ml) clearly showed a high and similar genotoxic response. Also, it was noted that one of the individuals exposed to the lowest concentration showed a much lower DNA-FTM than the tap water control.

Increasing concentrations of bio-oil made from unspecified wood caused a gradually increase in average DNA-FTM. The genotoxic response showed a shift towards a higher concentration compared to the other bio-oils, as only the 0.001 ml/ml exposure caused a higher response relative to tap water. All three exposures showed some variation between the parallels, and one individual from the 0.0004 ml/ml exposure showed a relatively high standard deviation for the replicates.

3.3.2 Median molecular length

The MML of the DNA fragments that migrated into the gel was determined for all onions exposed to bio-oil and compared to the negative and the positive control (figure 10). A two-tailed Student's T-test was performed to identify exposures that were significantly different ($p < 0.01$) from the negative control. However, the statistical difference was less emphasized due to the lack of statistical power (n=3), and was only applied to illustrate trends. The obtained p-values, estimated MML for each individual and an example of one of the standard curves used to calculate the MML can be found in the appendix (A.2.2 and A.2.3).

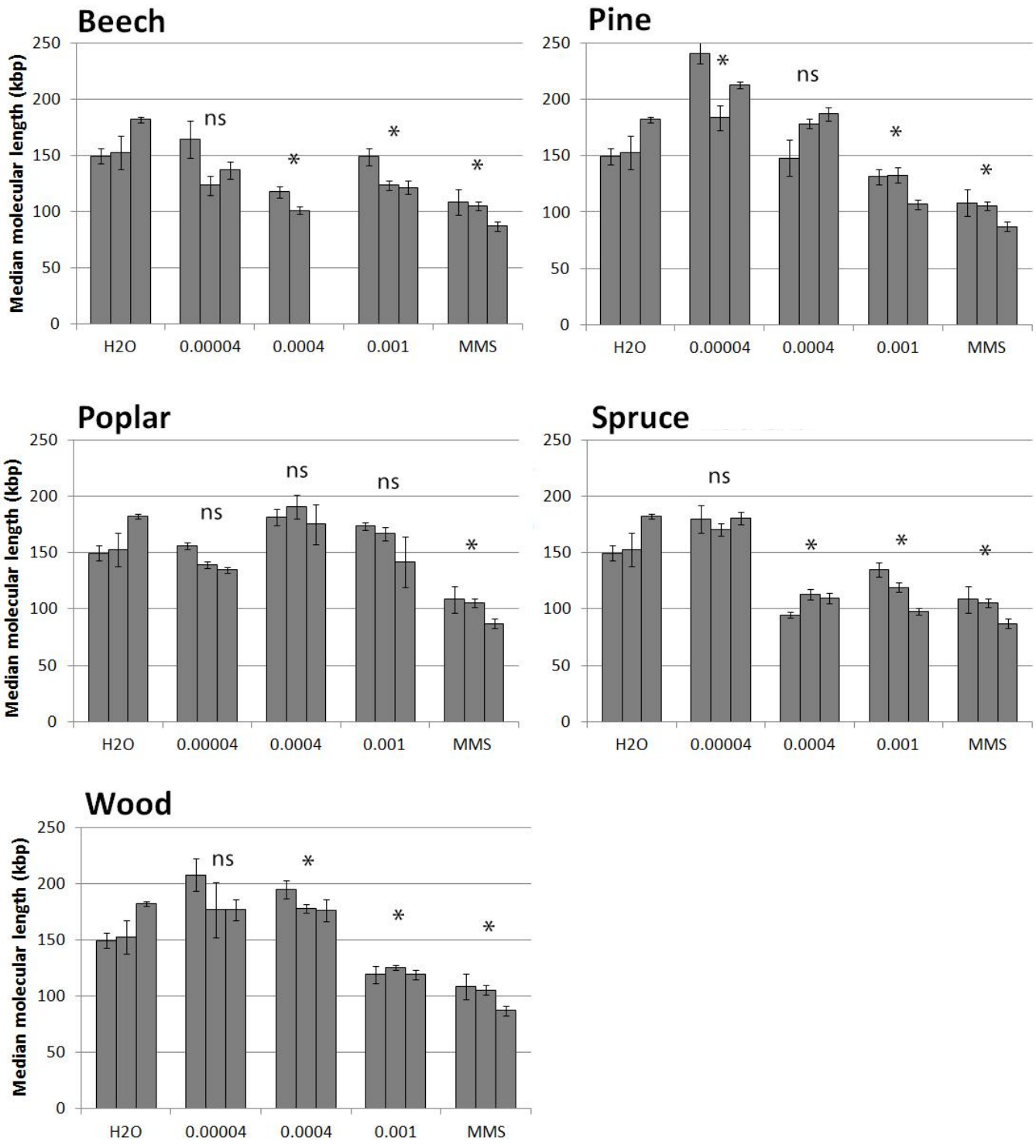

Figure 10: The median molecular length (MML) of the DNA fragments that migrated into the gel during electrophoresis of *A. cepa* DNA, after exposure to tap water (H2O), three different bio-oil concentrations (ml bio-oil/ml solution) and methyl methanesulfonate (MMS). Results are shown for five bio-oils (beech, pine, poplar, spruce and wood). Each column represents one individual and shows the standard deviation of three replicates. The asterisks indicate exposures that were significantly different from the tap water control ($p < 0.01$, two-tailed Student's T-test, in Microsoft Excel 2007).

Onions exposed to the negative control had an average MML of 162 kilo base pairs (kbp) for the migrated DNA fragments. It was noted that one individual showed much higher MML than the other two onions, and thereby increased the average value. The positive MMS control showed an evident reduction in MML compared to tap water and had an average MML of 100 kbp. Onions exposed to the solvent control (0.1 % DMSO in tap water) showed an average MML of 114 kbp (c.f. appendix A.2.3) which was unexpectedly low compared to tap water. The average MML for onions exposed to bio-oil varied between 95 to 241 kbp.

Beech bio-oil caused no clear concentration-dependent trend in MML of the migrated DNA fragments, but all concentrations caused a reduction in average MML compared to tap water. The MML decreased from the lowest concentration (0.00004 ml/ml) to the middle concentration (0.0004 ml/ml), which caused a similar reduction in MML as the MMS exposure. Further, the MML increased from the middle to the highest (0.001 ml/ml) exposure. High variation between the parallels was seen at all concentrations.

Pine bio-oil caused a clear reduction in MML with increasing bio-oil concentrations. However, only the highest concentration (0.001 ml/ml) caused an increase in fragmentation compared to the negative control. The middle concentration showed a slight increase in average MML, while the lowest concentration clearly showed less fragmentation than the negative control. Onions exposed to the lowest concentration (0.00004 ml/ml) also showed large variation in response, with one individual having a particularly high MML (241 kbp).

None of the three concentrations of poplar bio-oil caused a clear reduction in MML of the migrated DNA fragments. A slight decrease in MML was seen for onions exposed to the lowest concentration (0.00004 ml/ml), but the genotoxic response was not further increased with higher concentrations. Instead, onions exposed to the two higher concentrations showed higher MML than the tap water control. It was noted that one of the individuals exposed to the highest concentration (0.001 ml/ml) had a lower MML than the two other onions, and also showed high standard deviation between the replicates.

A clear reduction in MML was seen for onions exposed to the two highest concentrations (0.0004 ml/ml and 0.001 ml/ml) of spruce bio-oil. The MML obtained at these concentrations was similar to the MML for onions exposed to the positive MMS control. On the other hand, onions exposed to the lowest concentration of spruce bio-oil had a higher average MML than onions exposed to tap water.

Bio-oil made from unspecified wood caused a reduction in MML with increasing concentrations. However, only the highest exposure (0.001 ml/ml) increased DNA fragmentation relative to the negative control. The obtained MML for this concentration was not very far from the value obtained for the positive control. Both of the two lower concentrations showed a higher MML than onions exposed to tap water, and the observed MML for middle concentration (0.0004 ml/ml) was slightly reduced compared to the lowest concentration.

3.4 Skewness of the DNA fragment distribution

The molecular size distribution of the DNA fragments that migrated into the gel was analysed by calculating the skewness of the obtained DNA intensity distribution curve. Pearson's second coefficient of skewness was applied for the measurement (c.f. 2.7.8). A more positive skew compared to the negative control would indicate a higher amount of smaller DNA fragments, and thus a higher genotoxic insult.

Unexpectedly, the skewness showed poor correlation with expected observations for the control exposures. Two of the onions exposed to tap water showed some of the highest obtained positive skewness values for *A. cepa*, while only one onion showed a negative skew. Onions exposed to the positive MMS control showed very variable values, with one onion having a large negative skew, another having a positive skew and one showing about no skew at all. According to the observed results for the control exposures, the skewness of the DNA fragment distribution could not be used as a reliable measurement of DNA fragmentation. The obtained results were thus not evaluated in the project, but are included in the appendix (A.2.6.).

4 DISCUSSION

4.1 Bio-oil toxicity

4.1.1 Relative root growth as quantitative measure of toxicity

Analysis of root growth inhibition is commonly applied as a quantitative measurement of toxicity in higher plants, and is particularly known for being included as the first part of the *Allium cepa* chromosomal aberration assay (Fiskesjø, 1985). Root elongation is highly sensitive to stress since the root cells are in direct contact with the environment. Reduction in root growth is thus readily seen when roots are exposed to toxic substances, wrong pH or substances that might prevent nutrition uptake (Fiskesjø, 1993).

Usually, root growth inhibition is expressed as reduction in percentage root growth relative to onions grown in tap water, according to Fiskesjø (1985). However, the separate water exposures carried out in the master's project were not comparable with the bio-oil experiments. For instance, the root growth in two of the bio-oil experiments seemed to converge toward 60 %, and not 100 %, of the tap water exposed onions with decreasing bio-oil concentrations. The ability to grow roots thus seemed to have varied between experiments according to differences in experimental conditions. Variables that may have influenced the rate of root growth, include light conditions, temperature, onion storage time/conditions and measurement/fixation of roots at varying time of the day.

According to the lack of a comparable water control, it was decided that root growth inhibition was best expressed as percentage root growth relative to the average root growth of individuals exposed to the lowest (0.00001 ml/ml) bio-oil concentration (referred to as relative root growth). With the exception of beech bio-oil, no further increase in root growth was observed between the second lowest and the lowest bio-oil exposure, indicating that these concentrations did not exert a toxic effect. Therefore, it was assumed that onions exposed to the lowest bio-oil concentration had reached a root growth comparable to a potential negative control. However, this was not certain for beech bio-oil, but the lowest exposure was still assumed to be very close to a potential control exposure. The obtained EC_{50}-values, based on the relative root growth, were thus considered to be reliable for comparison of bio-oil toxicity. It was also considered more accurate to examine the reduction in root growth caused by bio-oil exposure by comparing with onions exposed to the same solvent (0.1 % DMSO), and not only tap water. For illustration, the average

root growth of onions exposed to the solvent control was found to be about 30 % lower than the root growth of tap water exposed onions.

4.1.2 Influence of feedstock species on bio-oil toxicity

Bio-oils produced from wood have previously shown higher toxicity than other biomass feedstock applied in the pyrolysis process (Chatterjee et al., 2013, Holteberg, 2014). The toxicity of wood-based bio-oils is likely dependent on the ratio of cellulose, hemi-cellulose and lignin in the feedstock species, as pyrolysis of each component results in many of the same compounds in different bio-oils (Mohan et al., 2006). A study estimating the pesticidic properties of pyrolytic bio-oils, found that toxicity decreased in order for lignin > hemi-cellulose > cellulose (Hossain et al., 2013). However, it is difficult to predict the toxicity based on the composition of the feedstock pyrolyzed, because of the complex interactions occurring between the compounds in the mixture. The ratio of the three components may also vary for one species depending on how old the trees were when chopped. Also, the exact species utilized for production of the five bio-oils applied in this project are mostly unknown, meaning that the amount of different wood components cannot be looked up in the literature. The most reliable method to determine the toxicity of a bio-oil is thus considered to be through experimental exposure studies.

The obtained results in the *A. cepa* root growth inhibition test applied in this master's project indicated that all five bio-oils exerted a very similar toxic effect. For instance, the induced macroscopic changes in *A. cepa* appearance after exposure were identical for all five bio-oils. Also, the toxic effect on root growth was exerted in about the same concentration range and an almost complete inhibition of root growth was seen at the two highest concentrations in all bio-oil experiments. However, some differences in toxicity were still seen, as the bio-oils caused dissimilar reduction of relative root growth at the same concentrations.

The complete inhibition of root growth seen for concentrations above 0.001 ml/ml implied that root growth already was affected during the first hours of exposure. According to the estimated EC_{50}-values, bio-oil made from pine wood was the most toxic bio-oil with an EC_{50} of 0.000322 ml bio-oil/ml solution (ml/ml). On the other hand, beech bio-oil showed nearly the same EC_{50} (0.000364 ml/ml) and could not for certain be separated from pine in toxicity, due to the regression uncertainty caused by the high variation between the parallels. The bio-oil produced from wood of unknown species (EC_{50}, 0.000516 ml/ml) differed with about 0.00015 ml in EC_{50} from beech bio-oil, and showed very similar toxicity to poplar bio-oil (EC50, 0.000605ml/ml).

Fast pyrolysis of spruce resulted in the least toxic bio-oil, as the EC_{50} (0.001816 ml/ml) was found to be six times higher than for pine bio-oil, and three times higher than for poplar bio-oil. The obtained results thus indicated that different feedstock species most likely affect the toxic properties of pyrolytic bio-oils.

4.1.3 Comparison with other toxicity studies

This master's project is the second study to apply the *A. cepa* root growth inhibition test for evaluation of toxicity of pyrolytic bio-oils. Pekol et al. (2012) examined root growth inhibition after exposure to bio-oil made from hazelnut shells (HS), and also found that onion root growth significantly deceased with increasing bio-oil concentrations. The estimated EC_{50} for the HS bio-oil was found to be 0.0002 ml/ml, which was not far from the lowest EC_{50} obtained in this master's project. The previous study also observed browning of the roots, but in contrast, the roots seemed to harden and not soften with higher exposure concentrations.

Among the previous studies on bio-oil toxicity, three were found to assess the WSF of wood-based bio-oils by applying the *D. magna* immobility bioassay. For comparison, the stated concentrations in the studies (given in mg/l) were converted to ml/ml, assuming a bio-oil density of 1.2 kg/l (Bridgwater, 2012b). The obtained EC_{50} for *D. magna* swimming capacity was found to be 0.000046 ml/ml for *P. radiata* bio-oil (upper bio-oil phase) (Park et al., 2008) and 0.000142 ml/ml for *E. grandis* bio-oil (whole bio-oil in water) (Pimenta et al., 2000). These concentrations were in the range of onion root growth inhibition, even though the inhibition was small in this concentration area. However, it was not unexpected that the toxicity was higher in the *D. magna* immobility bioassay since the bioassay is known to be more sensitive than the *A. cepa* root inhibition test (Arkhipchuk et al., 2000). In the third study, the EC_{50} was not reached for any of the tested bio-oils (Girard et al., 2005), because the maximum concentration applied (0.000083 ml/ml) was too low.

One of the studies also performed a preliminary screening of cytotoxicity of the WSF of bio-oil (upper bio-oil phase) derived from *P. radiata* wood (Park et al., 2008). Here, the EC_{50} for viability of mouse lymphoma cells was found to be 0.0001125 ml/ml, which was about one third of the lowest obtained EC_{50} values for root growth inhibition. However, *in vitro* assays are generally more sensitive compared to *in vivo* assays, because the whole surface area of the cell is exposed, and the toxic insult cannot be dealt with in the same way as in an intact functional organism.

Additional studies assessing the toxic potential of whole bio-oils (dissolved in DMSO), were considered not to be comparable with the results obtained in this project, because the insoluble pyrolytic lignin fraction was included. In conclusion, previous results from studies assessing toxicity of the WSF of bio-oils showed high correspondence with the concentration range for toxicity obtained in this project.

4.1.4 Environmental relevance of bio-oil exposure studies

In an eventual spill scenario in the natural environment the bio-oil concentration will be higher close to the spill source and decrease with increasing distance, making it important to test a wide concentration range. Also, the polar fraction and the pyrolytic lignin fraction will most likely behave different in the environment due to their difference in water-solubility. The water-soluble compounds are highly bio-available and assumed to be readily distributed in the environment by drainage. On the other hand, the non-polar pyrolytic lignin is less bio-available and likely to stick to the surface of biota in the environment near the spill source. Studies assessing bio-oil toxicity by dissolving whole bio-oils in DMSO are thus considered to be less comparable to environmental spill scenarios, compared to studies dissolving the bio-oils in water.

The retention time of the various bio-oil compounds in the environment is also likely to vary, due to differences in bio-degradability. The bio-degradability during 28 days was estimated to be about 40-50 % for whole bio-oil, 30 % for the pyrolytic lignin fraction and over 70 % for the aqueous fraction (Girard et al., 2005). The WSF thus have a much shorter residence time in the environment before it is degraded, implying that the water-soluble compounds are most harmful only in the nearest time period after the exposure event. The pyrolytic fraction, containing HMW phenols and hydrocarbons, will remain in the environment for a longer time period. Even though they are much less bio-available, the compounds may eventually be incorporated into organisms through feeding on contaminated biota. Further approaches should thus consider testing the toxicity of both the WSF and the water-insoluble fraction separately to imitate the physical behaviour in the environment.

4.2 Genotoxic effects of bio-oils

4.2.1 Influence of feedstock species on the relative amount of DNA DSBs

Evaluation of the relative amount of DNA DSBs in *A. cepa* roots after exposure to bio-oils was performed by comparing the obtained endpoint levels with the levels of the negative and the positive control. The negative tap water control was assumed to reflect the basal level of fragmentation occurring in healthy onions, while exposure to MMS was applied to indicate harmful levels of DNA DSBs. Unexpectedly, the MMS exposure did not cause any elevation in DNA-FTM relative to the tap water exposure. The obtained results for DNA-FTM in this project was thus considered to be less reliable compared to the calculated MML, since MMS clearly decreased the MML of the migrated DNA fragments. On the other hand, the two measurements showed relatively good correlation for many of the bio-oil exposures.

The obtained results for DNA-FTM and MML implied that all bio-oils, except poplar, clearly induced the formation of DNA DSBs in *A. cepa* root meristem cells. Several of the bio-oils also caused a higher genotoxic response with increasing bio-oil concentrations.

Poplar bio-oil was considered the least genotoxic bio-oil, as no reduction in MML of the DNA fragments was observed compared to the negative control. A very slight increase in DNA-FTM was seen for the two higher concentrations of poplar bio-oil, which might imply that the fragment size do not decrease before a significant increase in DNA-FTM is observed. The bio-oils made from pine and unspecified wood were considered to be the second least genotoxic bio-oils. For both bio-oils, a decrease in MML was only observed for the highest concentration, but was very close to the MML of the positive control. The DNA-FTM obtained for wood bio-oil correlated well with the MML by showing an increase only for the highest exposed onions. In contrast, the DNA-FTM did not correlate with the MML for the pine bio-oil exposure. A higher DNA-FTM was seen for the two lowest exposures, while no reduction in MML was observed. For the highest exposure, no increase in DNA-FTM was seen, even if the MML was significantly reduced. The disagreement might be due to the lower reliability of the DNA-FTM measurements.

Bio-oil produced from beech wood was evaluated as the second most genotoxic bio-oil. The two higher concentrations significantly reduced the MML of the migrated DNA fragments and also increased the DNA-FTM compared to the negative control. The same trend was observed for the two highest concentrations of spruce bio-oil, but the measurements were induced to a higher

degree. Therefore, spruce bio-oil was considered the most genotoxic bio-oil in relation to DNA DSBs.

A forth concentration of bio-oil (0.004 ml/ml) causing complete inhibition of root growth, was assessed to check if even higher genotoxic responses could be obtained. However, no clear DNA fragment bands were seen in the agarose gel and very low intensities were seen in the well area. Ida Holan (2014), who assessed chromosomal aberrations in the same onions, did not observe any dividing cells for this concentration through microscopic examination. The high bio-oil concentration thus seemed to have caused DNA degradation, probably due to apoptosis.

4.2.2 Comparison with other studies assessing bio-oil gentoxicity

The already mentioned studies that assessed bio-oil toxicity were also found to assess the genotoxic potential of bio-oils. One of the studies assessed DNA fragmentation by applying the comet assay to mammalian cells exposed to *P. radiata* bio-oil (Park et al., 2008). Here, the highest concentration, about 0.0000175 ml/ml, was found to significantly increase DNA fragmentation. This concentration was lower than those applied in this project for assessment of genotoxicity, which might indicate that *P. radiata* wood results in a more genotoxic bio-oil than the feedstock species in this project. On the other hand, mammalian cells might be more sensitive, especially to secondary mutagens, due to their higher metabolic capacity (Leme and Marin-Morales, 2009).

Bio-oil made from three of the applied species; beech, pine and spruce, were assessed in the BIOTOX project by applying the Ames test (Girard et al., 2005). Mutagenic responses were seen for all three bio-oils, but the difference in genotoxic response between the bio-oils could not be determined because most of the applied concentrations were too toxic to obtain representative results.

Pekol et al. (2012) assessed chromosomal aberrations (CA) in *A. cepa* exposed to HS bio-oil, and one concentration (0.0004 ml/ml) was equal to one of the concentrations applied in this project. Here, about 80 % of the dividing cells showed various types of CAs after 48h exposure to HS bio-oil. A high ratio of the DNA DSBs induced by bio-oil exposure is thus likely converted into CAs in onion root cells. Ida Holan (2014), who applied the *A. cepa* chromosomal aberration test to the onions exposed to beech, spruce and poplar bio-oil in this project, also found high levels of CAs for all three bio-oils.

In summary, pyrolytic bio-oils might have a potential genotoxic effect on living organisms, but due to the limited studies performed the severity cannot yet be evaluated. It is also difficult to compare the obtained results for genotoxicity with studies applying a variety of different bio-assays. Further studies thus need to focus on developing standard protocols for testing genotoxicity of different bio-oils.

4.2.3 Increased DNA fragment length at low exposures to bio-oils

The notable increase in MML of DNA fragments observed for exposure to some bio-oil concentrations might be an indication of hormesis. Hormesis is an adaptive response to damage seen for low-dose exposures to various toxic and genotoxic agents (Calabrese and Baldwin, 2002). The most common hypothesis proposes the formation of reactive oxygen species as a triggering pathway, which leads to induction of DNA repair systems and/or radical detoxification (Dimova et al., 2008). For instance, adaptive responses to DNA DSBs have been observed after exposure to low doses of bleomycin, which is a strong inducer of DNA DSBs (Schlade-Bartusiak et al., 2002). Further, DNA repair-deficient mutants were found to exhibit no induced radio-resistance compared to normal cells, suggesting the involvement of DSB rejoining (Skov et al., 1994). Other studies have also found that hormesis can be interpreted in terms of increased non-homologous end-joining repair or increased homologous recombination repair of DNA DSBs (Raaphorst et al., 2006, Chankova et al., 2007). Evidence of radical detoxification/ROS protection, i.e. up-regulation of superoxide dismutase, catalase and glutathione peroxidise genes, have also been demonstrated (Lanza et al., 2005).

Adaptive responses have previously been observed in *A. cepa* to aluminium - and chromium - induced DNA-damage, mediated through the formation of ROS (Achary and Panda, 2010, Patnaik et al., 2013). It is thus not unlikely that bio-oils might cause hormesis in onions at low-concentration exposures, since bio-oils contain several ROS-inducing chemicals (e.g. PAHs, phenols, furans).

A potential hormesis effect is proposed for pine and wood bio-oil tested in this master's project due the observed increase in MML for the two lowest exposures. According to Calabrese and Baldwin (2002), the adaptive effect is not valid when stimulation is observed for the low doses, but no inhibitory/damaging effect is demonstrated at higher doses. Hormesis could thus not be confirmed for exposure to the other bio-oils. However, bio-oils are very similar in chemical composition which implies that hormesis might occur at low exposures to bio-oils in general, but

might not be seen in the applied concentration range due to differences in genotoxic response. For instance, all concentrations of beech bio-oil reduced the average MML compared to tap water, which means that the applied concentrations were too high to observe any potential adaptive response in *A. cepa.*

The DNA-FTM did not show a decrease for any of the exposures that caused an increase in MML. This might imply that the smaller fragments were more likely to undergo ligation/rejoining into larger fragments, but these resulting fragments were still small enough to migrate through the gel. Also, it should be empathized that the DNA-FTM was considered a less accurate measure of DNA DSBs in this master's project, and might thus not represent the actual level of fragmentation.

4.2.4 Constant-field gel electrophoresis for assesment of DNA strand breaks

The method applied for assessment of genotoxity in this master's project was first introduced by Theodorakis et al. (1994), who examined DNA strand breaks in fish blood cells. The method demands very small amounts of DNA to obtain usable results, which makes it especially applicable for evaluation of DNA damage in species inhabiting nucleated blood cells (Krøkje et al., 2006). Damage to the DNA can thus be evaluated in blood cells of species such as fishes, birds and reptiles without sacrificing the animal. Later studies have also found the method highly suitable for genotoxic field monitoring (Krøkje et al., 2006, Theodorakis et al., 2012, Fenstad et al., 2014), and sequential samples can also be collected from the same individuals during time course studies (Theodorakis et al., 1994, Fenstad et al., 2014). As previously mentioned, the method has not been found to be adapted to plant material in literature, but have been applied to both *L. multiflorum* and *A. cepa* previously at the genotoxicity lab, Department of Biology, NTNU. Constant field gel electrophoresis (CFGE) was found to be a good method for evaluation of DNA DSBs in this project, but some considerations should be evaluated concerning application of plant material.

For instance, it is more difficult to determine the approximate amount of DNA in root tips of *A. cepa*, than in volumes of blood. If a low DNA concentration is applied to the agarose plugs, the lower end of the fragment tail might not be detected in the DNA fragment bands (Theodorakis et al., 1994). In contrast, DNA overloading might trap smaller DNA fragments together with larger fragments, and thus inhibit the migration ability in general.

In this project, several pre-experiments were run to determine the optimal number of root tips to apply in each plug, and about 30 root tips were found to give reliable results. However, the porcelain mortars first used for crushing the material were later replaced by smaller mortars having a smoother surface. A higher amount of plant material was then obtained when scraping the mortars, but the number of roots was not changed when the actual experiments were performed. DNA overloading was thus observed, but did not significantly affect the MML of the migrated fragments. However, it most likely affected the skewness of the DNA distribution curve, as the difference between the mean and the median becomes less apparent with increasing brightness of the DNA bands in the gel. Further experiments should thus try to optimize the number of root tips and also eventually weighing the material to apply about the same amount of DNA in each agarose plug.

Improper digestion of cellular material will also be a problem when applying tissue and not fluids. Undigested plant material might appear as lumps of DNA in the well, inhibiting migration of DNA fragments, and thus affect the calculation of DNA-FTM. Centrifugation of the diluted material is thus highly important to remove less ground tissue. Three internal measurements of each lane were thus performed in this project to generally obtain more reliable results.

The amount of DNA migrating into the gel (DNA-FTM) has previously been found to be qualitatively similar to the obtained MML values (Theodorakis et al., 1994). The measurement has also been applied in several later studies as a reliable measure of DNA DSBs (Chankova et al., 2007, Neijenhuis et al., 2009, Fenstad et al., 2014). However, in this project, a non-genotoxic response was obtained for the MMS exposure for repeated trial gel runs. This might have been caused by DNA overloading or improper digestion, but it might also imply that MMS do not function as a suitable positive control for this measurement. Further studies should thus clarify the non-genotoxic response, or evaluate alternative positive controls, giving a positive response for both the MML and the DNA-FTM.

Compared to similar methods for assessing DNA fragmentation, such as single cell gel electrophoresis (comet assay) and pulse field gel electrophoresis (PFGE), the applied method was preferred for assessment of DNA DSBs in *A. cepa* root cells. The comet assay might be the most commonly applied, but demands complex and expensive image analysis equipment to obtain data (Theodorakis et al., 1994). Direct quantification of strand breaks is thus not possible in the comet assay as several calculations using the obtained image data must be performed.

While in CFGE, differences between control samples and exposed samples can directly be measured from the obtained fluorescence intensity graph. Additionally, the comet assay do not measure the amount of DNA fragments, as the comet tail consists of DNA loops connected to the comet head, and not separate fragments (Collins et al., 2008). Compared to PFGE, the CFGE method was found to be equally sensitive, but more rapid and economical beneficial for measurement of DNA DSBs (Wlodek et al., 1991). The applied method is thus recommended, especially when a high number of DNA samples are being analyzed for DNA fragmentation. The digested agarose plugs can also be stored at 4°C up to two weeks without significant DNA degradation (Theodorakis et al., 1994).

4.3 Correlation between chemical data and obtained results

No clear relationship was observed when comparing the order of bio-oil toxicity or genotoxicity with the PCA score plots obtained from each fingerprinting analysis. On the other hand, the toxicological response of the single bio-oils might be related to some of the variation seen in chemical composition. For instance, bio-oil made from poplar was found to cause little or no genotoxicity, and also showed generally lower intensities in the chromatogram and the ESI-MS spectra relative to the other bio-oils. The IR spectra showed that poplar bio-oil had lower amounts of C-H and C-O, which is associated with alkanes, alcohols and phenols. Especially, phenols are known to exert genotoxic effects, and a lower content of C-O might thus explain the lower genotoxicity observed.

Beech bio-oil showed a clear deviation from the other bio-oils in PCA score plots for positive ESI-MS and GC-MS. A higher amount of some low molecular mass positive ions was observed, and high intensity peaks in the GC-MS chromatogram that did not match the chromatogram of the other bio-oils, indicated a difference in chemical composition. Also the IR spectra showed that beech bio-oil contained a higher amount of C=O, N-H, C-H and C-O functional groups than poplar and wood bio-oil. However, further analyses are needed to examine whether the corresponding compounds contributed to the high toxicity and genotoxicity observed for beech bio-oil.

Pine bio-oil did not show any close similarity in chemical composition with beech bio-oil, although it was the most toxic bio-oil and also caused a high genotoxic response. This could indicate that the damaging compounds found in beech bio-oil were different from the damaging compounds found in bio-oil made from pine.

The two bio-oils made from softwood (spruce and pine) showed very high similarities in all chemical analyses, and a similar toxic and genotoxic response would thus be expected. In contrast, spruce was found to be the least toxic bio-oil, and caused a genotoxic effect at lower concentrations than pine bio-oil. Still, the small differences in chemical composition might have caused the observed difference in toxicological response, and it would be interesting to assess these differences in further studies.

The bio-oil made from unspecified wood showed low similarity with spruce and pine relative to the high similarity observed between the two softwood species, indicating that it might be a hardwood species. In general, wood showed higher intensities in all analyses, but since wood bio-oil caused an average toxic and genotoxic response, it was not possible to assign specific differences in the chemical data to the observed toxicological effects.

4.4 Reliability of the obtained results

4.4.1 Other variables affecting the chemical composition of bio-oils

Except from the applied feedstock species, there are other variables that may affect the chemical composition of bio-oils and have an influence on the toxic and genotoxic potential. The observed toxicity and genotoxicity might thus only be partially, or not at all, caused by the applied feedstock species. Very limited information has been received for each bio-oil, but it is known that they originate from several different companies, except for beech and spruce bio-oil, which was produced at the University of Aston, UK. Most of the bio-oils are thus likely produced by different pyrolysis techniques and with slightly different physical reaction terms, which is likely to have an influence of the resulting bio-oil composition. For instance, the formation of PAHs increases with increasing temperature and increasing vapour time applied in the pyrolysis process (Girard et al., 2005).

The year of production might also influence the harmfulness of the bio-oils, since bio-oils contain compounds that spontaneously react at ambient conditions during long-time storage and may form heavier and more stable compounds (Mohan et al., 2006). Water is also known to be produced in the process, causing the water content of bio-oils to increase with time. These reactions are assumed to result in overall lower toxicity, since the amount of reactive compounds is decreased. For instance, poplar bio-oil was the oldest bio-oil applied in this project, and also showed no apparent genotoxicity and relatively low toxicity, which might thus be age-related.

4.4.2 *In vivo* variations in onion response

High variation in root growth between the parallels was observed for some concentrations and was most likely caused by natural biological variation between the individuals. For instance, some individuals might be more resistant to bio-oil toxicity than others or grow roots at a faster rate. Differences in previous growth conditions on the field could also have caused some variation in health conditions between onions. The variation in root growth decreased with higher exposures, which is logical since the toxic impact limited the growth potential. The obtained results for the higher concentrations might thus also be more reliable. According to the results obtained for the blind exposure, the normal root growth of *A. cepa* during 72 h varied within a wide range. The use of only three parallel individuals per exposure is thus a small number, but might still give a good indication of the concentration-effect relationship.

Individual variation in the rate of cellular repair might also influence the ability to cope with genotoxic insults. Individuals having a slow rate of DNA DSB repair would show a decreased MML and increased DNA-FTM compared to parallel exposed individuals. In this project, we did not have the capacity to use more than three parallels, but this should be considered in further experiments to obtain more reliable results.

4.4.3 Differences in water-solubility of the bio-oils

The water solubility of the bio-oils was unfortunately not measured due to no apparent visual difference between the bio-oils. However, a previous study has shown that bio-oils vary in their water solubility (Sipila et al., 1998). Owing to the fact that the five oils were produced from species most likely having unequal proportions of cellulose, hemicelluloses and lignin, their water solubility is also expected to be different. Especially, higher lignin content results in a larger amount of insoluble pyrolytic lignin produced during pyrolysis. The amount of soluble compounds in each bio-oil was thus most likely different, implying that the relative concentrations varied between the different bio-oil exposures, and might have caused the observed difference in genotoxicity.

4.5 Extrapolation of the results to mammalian species

Higher plant systems have proven to be excellent indicators of cytogenetic and mutagenic effects of environmental chemicals, which might cause a threat to human and environmental health (Grant, 1999). For instance, the *A. cepa* chromosomal aberration assay has shown high sensitivity for various compounds that induces mutagenic and carcinogenic responses in rodents (Rank and Nielsen, 1994). The obtained results for genotoxicity in this project may thus also be relevant for other species as well, including humans. However, differences in cellular pathways between species may influence the genotoxic response. Depending on which compounds in the bio-oil exerting the genotoxic effect, the obtained results in the *A. cepa* test system might cause false positives/negatives when extrapolating to other species.

For instance, mammalian species have a higher metabolic capacity than plant systems (Sandermann, 1999, Leme and Marin-Morales, 2009), which implies that the genotoxic effect of bio-oils would be more severe in mammals than in *A. cepa*, if the DNA insult is caused by secondary mutagens in the mixture, e.g. PAHs. On the other hand, eventual primary mutagens in bio-oils are likely to be degraded and excreted by a faster rate in mammals, and thus cause a lower genotoxic response than observed in *A. cepa*.

Because the genotoxic compounds in the bio-oils were not identified, the molecular mechanisms behind the observed genotoxic effect are unknown. However, since a genotoxic response was observed in *A. cepa*, the bio-oils should be considered as potential genotoxic mixtures in relation to mammalian exposures as well.

4.6 Bio-oil as a future energy resource

Pyrolytic bio-oils have proven to be a potential future energy resource, and have many advantages compared to fossil fuel. The main advantage, is the neutral CO_2 release, as plants absorb CO_2 when growing, and releases it back to the atmosphere when the bio-oil is combusted. Increased application of bio-oils relative to fossil fuels might thus reduce the increasing global warming effect seen today. Additionally, combustion of bio-oils shows lower atmospheric missions of harmful metals, NO_X and SO_X relative to fossil fuels. Transport emissions and costs are also most likely reduced, since bio-oils can be locally produced from any abundant feedstock species.

Several studies, including this one, have shown that bio-oils might be of environmental concern, as positive results have been obtained for both toxicity and genotoxicity. On the other hand, bio-oils are only the primary product of fast pyrolysis, and further upgrade is needed to form commercial products. So far, no such product have been released on the market, and it is thus unknown if the toxic and genotoxic potential is increased or reduced according to the upgrading process. In literature, the pyrolytic lignin fraction is regarded as the undesirable product, together with water. The pyrolytic lignin is also considered the most hazardous fraction, as it contains both toxic and genotoxic phenol compounds. If removed, the obtained product is likely to become less hazardous. However, pyrolytic lignin is considered to be water-insoluble, and genotoxic effects were still seen for the WSF of bio-oils in this study. Removal of water might thus only increase the concentration of harmful components, but this cannot be concluded for certain. Further toxicological evaluations of both bio-oils and its upgraded products are of high importance to better understand which factors influences the toxic and genotoxic properties.

5 Conclusions

The obtained results in this study indicated that pyrolytic bio-oils produced from fast pyrolysis of whole wood, can exert highly toxic effects in living organisms. All tested bio-oils caused a concentration-dependent inhibition of *A. cepa* root growth at environmental relevant concentrations. The results also indicated that the different feedstock species of wood applied in the pyrolysis process might influence the toxic properties of the different bio-oils. According to the obtained EC_{50}-values, the toxicity of the five bio-oils was ranked in the following order: *Pine > Beech > Wood > Poplar > Spruce.*

The obtained results also indicated that bio-oils can cause severe DNA damage at concentrations of about 0.0004 ml bio-oil/ml solution and higher. Different feedstock species of wood is also likely to affect the genotoxic potential of bio-oils. For instance, bio-oil produced from poplar caused no apparent genotoxicity, while beech and spruce bio-oil showed larger for both measurements of DNA double-strand breaks.

The provided chemical data on the bio-oils, did not explain the observed toxic or genotoxic effect very well. However, differences in specific chemical composition were observed, which might have caused the difference in toxicological response. Due to limited information of the applied bio-oils, and the fact that the water solubility of the bio-oils was not measured, other variables than the utilized feedstock species might also have influenced the toxic and genotoxic properties. Further studies should thus apply bio-oils made from different feedstock, but by the same pyrolysis technique and same physical reaction terms, to better elucidate the effect of feedstock on bio-oil toxicity and genotoxicity.

Considering that further upgrade of pyrolytic bio-oils is essential to obtain commercial applicable bio-oils, the toxicological effect of the finished upgraded products is likely to be altered compared to the effects seen for the crude bio-oils. However, it is not known if the toxic and genotoxic potential will increase or decrease as a result of the upgrading process. Further toxicological studies should thus focus on the upgraded bio-oil products, as these are most likely to be of environmental concern in the future.

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APPENDIX

A.1 Root growth inhibition

A.1.1 Relative root growth values

The calculated relative root growth of each individual onion exposed to bio-oil is presented in figure A.1.

Figure A.1.

Conc. (ml/ml)	Relative root growth, %														
	Beech			Pine			Poplar			Spruce			Wood		
	Ind. 1	*Ind. 2*	*Ind. 3*	*Ind. 1*	*Ind. 2*	*Ind. 3*	*Ind. 1*	*Ind. 2*	*Ind. 3*	*Ind. 1*	*Ind. 2*	*Ind. 3*	*Ind. 1*	*Ind. 2*	*Ind. 3*
0.00001	126.5	69.5	104.0	95.3	80.5	124.2	94.7	71.5	133.8	167.0	44.7	88.2	108.4	108.6	83.1
0.00004	58.9	102.0	90.9	120.2	73.5	120.3	119.1	108.5	87.6	116.0	96.7	142.9	129.3	110.6	80.1
0.0001	72.2	55.2	63.7	25.6	41.2	78.0	53.6	96.3	77.5	96.8	68.3	70.5	58.4	64.7	109.0
0.0004	32.4	66.6	51.4	39.7	57.2	45.4	79.6	56.4	100.2	90.2	113.1	69.7	82.2	48.4	48.6
0.001	27.2	31.7	18.8	25.2	24.7	14.3	29.6	17.0	12.1	71.9	106.2	55.7	49.3	24.1	18.4
0.004	14.8	-0.9	7.3	-7.6	-7.0	0.2	-0.1	7.9	5.4	13.0	3.6	13.2	14.5	11.4	16.2
0.01	17.0	9.3	11.4												
0.04				4.5	2.7	4.4	5.4	4.4	6.6	16.2	-6.6	8.6	7.9	7.7	21.0

A.1.2 Negative control

The measured root growth for the two tap water exposures can be found in table A.2. Root growth is presented in centimeters, and the two individuals marked with red deviated from the others and was excluded from calculation of the average root growth.

Table A.2.

	Root growth, cm								Average
H_2O (1)	1.07	1.01	1.81	0.89	0.84	1.03	0.85	1.35	1.00
H_2O (2)	0.77	1.32	1.24	0.28	1.41	0.72	0.55		1.00

A.1.3 Solvent control (blind exposure)

Root growth of onions exposed to different dilutions of the stock solution containing 0.1 % DMSO is presented in table A.3. The root growth is presented in centimeters.

Table A.3.

Root growth, cm								
DMSO, 0.1%	*0.04*	*0.01*	*0.004*	*0.001*	*0.0004*	*0.0001*	*0.00004*	*0.00001*
Ind. 1	0.26	0.48	0.95	0.31	0.65	0.75	1.00	0.62
Ind. 2	0.67	0.78	0.86	0.54	0.78	0.94	0.20	0.91
Ind. 3	0.55	0.62	0.72	0.84	0.62	0.29	0.79	1.05
Average	0.49	0.63	0.84	0.57	0.68	0.66	0.66	0.86
Tot. Average	0.67							

A.1.4 Regression data for estimation of EC_{50}

The following sigmoidal four-parameter regression was applied to the obtained relative root growth values for estimation of the EC_{50}: $f = y0+a/ (1+exp(-(x-x0)/b))$

The fitness of the regression curves and estimated parameter values for all five bio-oil exposures is listed below.

BEECH

R	*Rsqr*	*Adj Rsqr*	*Std. Error of Estimate*	
0.9097	0.8275	0.797	16.428	
Parameters	***Coefficient***	***Std. Error***	***t***	***P***
a	626328	9633152897	6.50E-05	0.9999
b	-0.0005038	0.0006	-0.8066	0.431
x0	-0.0045157	7.755	-0.0006	0.9995
y0	11.0837	6.7373	1.6451	0.1183

PINE

R	*Rsqr*	*Adj Rsqr*	*Std. Error of Estimate*	
0.8867	0.7862	0.7485	21.8981	
Parameters	***Coefficient***	***Std. Error***	***t***	***P***
a	919679.924	20297074935	4.53E-05	1
b	-0.00048	0.0007	-0.7085	0.4882
x0	-0.0044129	10.6012	-0.0004	0.9997
y0	2.11212	8.9549	0.2359	0.8164

POPLAR

R	*Rsqr*	*Adj Rsqr*	*Std. Error of Estimate*	
0.9196	0.8457	0.8185	19.0414	
Parameters	***Coefficient***	***Std. Error***	***t***	***P***
a	145.7183	132.4712	1.1	0.29
b	-0.0004206	0.0003	-1.2538	0.2269
x0	0.00028158	0.0008	0.3352	0.7416
y0	3.83233	7.7909	0.4919	0.6291

SPRUCE

R	*Rsqr*	*Adj Rsqr*	*Std. Error of Estimate*	
0.8337	0.695	0.6411	28.8596	
Parameters	***Coefficient***	***Std. Error***	***t***	***P***
a	112.5989	72.1534	1.5605	0.1371
b	-0.0008678	0.0012	-0.7215	0.4804
x0	0.0014537	0.0012	1.2325	0.2345
y0	5.27391	16.5355	0.3189	0.7537

WOOD

R	*Rsqr*	*Adj Rsqr*	*Std. Error of Estimate*	
0.9147	0.8367	0.8079	17.6575	
Parameters	***Coefficient***	***Std. Error***	***t***	***P***
a	319467.513	2236310382	0.0001	0.9999
b	-0.0005776	0.0007	-0.8057	0.4315
x0	-0.0047268	4.0498	-0.0012	0.9991
y0	13.4885	7.3127	1.8445	0.0826

A.2 Measurement of genotoxicity

A.2.1 The DNA fraction, of total DNA, that migrated

The DNA-FTM values for the three replicates of each individual onion exposed in the project is presented in table A.4., together with the total average DNA-FTM of the three parallels. The obtained p-values from the two-tailed Student's t-test, comparing all exposures against the negative control, are shown to the right in the table. Values marked in red indicate deviating measurements caused by in-homogenous distribution of DNA in the lanes. These values were excluded from the average and the statistical tests. Two gels were run for the individuals exposed to 0.001 ml/ml poplar bio-oil, due to several deviating measurements.

Table A.4.

Exposure - conc. (ml/ml)		**INDIVID 1**			**INDIVID 2**			**INDIVID 3**					T-test
		I	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*	**Average**	**± SD**	**p- value**
H2O	-	56.54	54.15	61.80	58.98	59.81	57.75	62.57	55.81	63.27	58.97	3.18	-
Beech	***0.00004***	64.41	62.29	51.05	61.53	61.59	56.84	62.31	39.64	60.73	60.09	4.23	0.5405
	0.0004	72.66	73.79	75.90	76.10	76.49	66.91	Roots were not fixated			73.64	3.62	0.0000
	0.001	73.58	69.72	74.16	74.20	71.51	73.85	77.98	77.18	81.05	74.80	3.44	0.0000
Pine	***0.00004***	70.87	72.01	70.40	70.61	69.00	68.50	69.20	70.10	68.37	69.90	1.21	0.0000
	0.0004	76.38	76.58	79.73	62.30	64.86	67.46	79.12	81.41	80.59	74.27	7.35	0.0000
	0.001	50.44	68.54	60.26	61.58	53.62	53.54	62.47	57.86	62.92	59.03	5.70	0.9788
Poplar	***0.00004***	56.53	57.61	52.19	62.86	63.07	63.18	65.34	67.12	67.91	61.76	5.25	0.1915
	0.0004	74.04	71.50	70.50	67.96	64.36	71.43	63.85	64.56	68.70	68.54	3.66	0.0000
	0.001	54.51	62.99	65.15	48.80	21.23	62.08	74.96	74.55	79.27			
	0.001(2)	62.36	69.76	70.05	49.99	40.30	61.04	69.95	78.77	80.38	70.10	7.01	0.0003
Spruce	***0.00004***	44.40	25.99	42.04	21.61	58.73	60.88	56.75	54.00	57.71	53.50	7.35	0.0634
	0.0004	77.28	78.70	80.01	86.19	83.20	84.04	87.97	86.93	85.40	83.30	3.82	0.0000
	0.001	85.36	84.35	82.89	80.24	76.53	78.17	82.59	82.01	82.04	81.58	2.83	0.0000
Wood	***0.00004***	61.05	60.21	62.64	57.27	55.85	52.40	67.51	68.94	68.87	61.64	5.93	0.2512
	0.0004	66.07	47.64	52.61	58.87	64.33	62.99	68.21	69.21	69.52	62.16	7.70	0.2667
	0.001	72.44	73.71	68.06	71.51	68.08	64.05	82.47	82.19	83.72	74.03	7.17	0.0000
MMS	***10 mg/ml***	53.41	52.21	52.75	56.58	52.60	54.16	58.55	61.95	60.09	55.81	3.62	0.0671
DMSO	***0.1 %***	60.33	58.35	53.78	43.77	17.64	34.43	45.69	50.837	45.68	49.11	8.48	0.0054

A.2.2 Calculation of median molecular length

For calculation of median molecular length (MML) of the migrated DNA fragments, the lambda DNA ladder had to be extrapolated by applying a fake point for a larger molecular size.

For the first gel, the fake point was positioned at 150 kbp at an RF value of 0.3.

To place the fake point of 150 kbp at similar RF for the other gels, a constant value for the following proportions was calculated for the first gel and applied to the other gels:

$$\frac{RF_{\text{whole lambda}} - RF_{\text{fake point}}}{RF_{\text{fragment 4}} - RF_{\text{whole lambda}}} = 1.03 \text{ (value obtained for the first gel)}$$

The following function showed the best fit to the data points:
Power function: F = a(1-x^(-b))*

The RF values for the first gel are presented in table A.5., along with the fake point and the estimated molecular size of the lambda DNA fragments calculated by the power function. The function showed poorer fit for the lower molecular sizes, but this was not a problem when only larger values were of interest. For making the standard curve, the average RF value of the two lambda markers were plotted against the molecular size of the fragments, along with the calculated coordinates of the fake point (figure A.1.).

Table A.5.

Lambda band	RF (lane I)	RF (lane II)	avg. RF	Fragment size (kbp)	Estimated size (kbp)
Fake point			0.3	150	149.600
Whole lambda	0.371	0.342	0.356	48.502	50.009
Fragment 1	0.417	0.386	0.401	23.13	23.435
Fragment 2	0.566	0.540	0.553	9.416	3.002
Fragment 3	0.693	0.684	0.689	6.557	0.688
Fragment 4	0.893	0.912	0.902	4.361	0.065

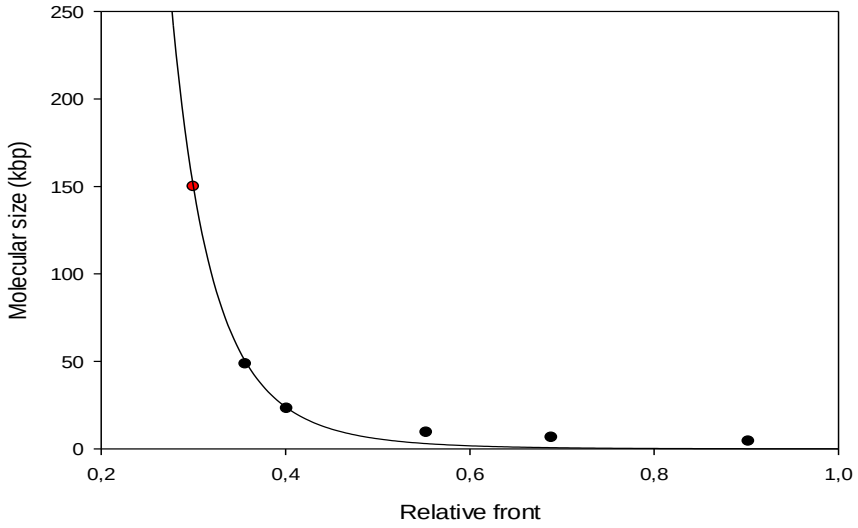

Figure A.1.

A.2.3 Median molecular length

The MML for the three replicates of each individual onion exposed in the project is presented in table A.6., together with the total average MML of the three parallels. The obtained p-values from the two-tailed Student's t-test, comparing all exposures against the negative control, are shown to the right in the table.

Table A.6.

Exposure - conc. (ml/ml)		**INDIVID 1**			**INDIVID 2**			**INDIVID 3**					T-test
		I	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**Average**	**± SD**	**p- value**
H2O	**-**	142	151	156	139	152	168	181	185	181	162	18	-
Beech	***0.00004***	146	175	173	119	119	134	129	144	138	142	22	0.0450
	0.0004	123	114	116	103	97	103	Roots were not fixated			109	10	0.0000
	0.001	149	157	142	120	123	128	116	127	122	131	14	0.0010
Pine	***0.00004***	241	250	231	186	193	172	210	214	215	213	26	0.0002
	0.0004	156	130	158	181	174	181	180	191	191	171	20	0.2866
	0.001	138	132	124	137	137	125	102	110	108	124	14	0.0001
Poplar	***0.00004***	153	158	158	139	142	136	136	137	132	143	10	0.0161
	0.0004	176	179	190	188	181	202	192	178	157	182	13	0.0104
	0.001	172	178	171	165	162	174	167	131	127	161	19	0.9247
Spruce	***0.00004***	192	168	179	135	174	166	177	187	177	173	17	0.1775
	0.0004	95	92	96	108	117	113	105	108	114	106	9	0.0000
	0.001	142	135	128	123	114	120	95	101	98	117	17	0.0001
Wood	***0.00004***	192	211	220	162	164	205	185	166	179	187	22	0.0136
	0.0004	196	187	203	178	182	174	168	174	187	183	11	0.0062
	0.001	122	111	125	127	123	126	122	122	115	121	5	0.0000
MMS	***10 mg/ml***	117	112	95	102	110	105	92	83	86	100	12	0.0000
DMSO		107	106	99	131	123	125	106	113	114	114	11	0.0000

A.2.4 Additional bio-oil concentration

The obtained gel image of onions exposed to a bio-oil concentration of 0.004 ml/ml can be seen in figure A.2. At this bio-oil concentration, the DNA fragment bands in the gel were either invisible or highly diffuse.

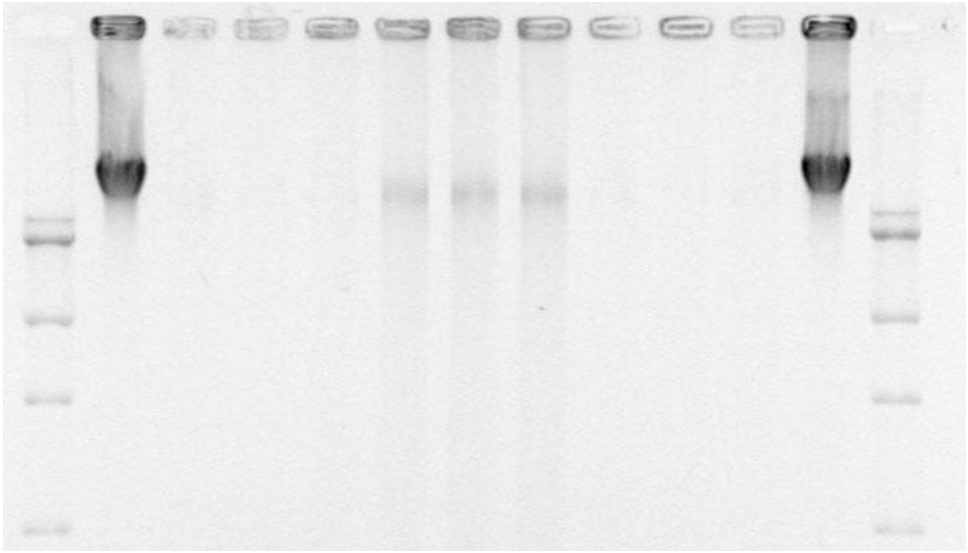

Figure A.2.

A.2.5 Internal constant control for MML

The average MML for the two internal constant control samples applied to each gel can be found in table A.7. The MML between the different gel runs varied with a standard deviation of 28 kbp for the internal control sample.

Table A.7.

Average MML value of the two internal controls pr. gel								
Conc.	***Beech***	***Pine***	***Poplar***	***Spruce***	***Wood***	***H2O***	***MMS***	***DMSO***
0.00004	274	265	290	253	286			
0.0004	283	244	338	258	310			
0.001	331	278	288	285	261			
-						285	233	245
Average	278							
Std. Dev	28							

A.2.6 Skew of the DNA fragment distribution

The skewness of the DNA fragment distribution in the agarose gel for each individual onion exposed to bio-oil, tap water and methyl methanesulfonate is presented in table A.8.

Table A.8.

		Skewness values			
Exposure -	**conc. (ml/ml)**	*Ind. 1*	*Ind. 2*	*Ind. 3*	*Average*
H2O	-	-0.07	0.28	0.49	0.23
Beech	***0.00004***	-0.06	0.03	0.15	0.04
	0.0004	0.00	0.00	-	0.00
	0.001	0.11	0.13	0.18	0.14
Pine	***0.00004***	0.22	0.20	0.13	0.18
	0.0004	0.38	0.23	0.43	0.34
	0.001	-0.18	-0.25	-0.03	-0.15
Poplar	***0.00004***	0.06	-0.13	-0.10	-0.05
	0.0004	0.28	-0.02	0.09	0.12
	0.001	0.10	-0.11	0.33	0.10
Spruce	***0.00004***	-0.32	-0.13	-0.26	-0.23
	0.0004	0.03	0.25	0.08	0.12
	0.001	-0.10	0.03	-0.08	-0.05
Wood	***0.00004***	0.15	-0.14	0.01	0.01
	0.0004	-0.07	0.08	0.19	0.07
	0.001	0.12	0.18	0.38	0.23
MMS	***10 mg/L***	-0.24	-0.01	0.18	-0.02