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Toxic and Genotoxic Effects of Fast Pyrolysis Oils Generated from Three Different Feedstocks Evaluated in an *Allium cepa* Chromosome Aberration Assay

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Abstract

Non-renewable energy sources cover the majority of today's energy demand. However, due to uncertainty of available petroleum reserves and great environmental impacts associated with the use of petroleum, renewable energy sources are of growing importance. Biomass is one of the main energy sources available, and bio-oils derived from biomasses can be converted to fuels, be used in heat and electricity production, or be used as a source of chemicals. Pyrolysis is regarded as the most efficient process for biomass conversion, and the pyrolysis oil yield may be as high as 80% if fast pyrolysis is used. Pyrolysis oils are complex mixtures of compounds, where content and properties depend on the biomass feedstock, process type and conditions. Even though pyrolysis oils are considered environmental friendly compared with petroleum oils, increasing production and application of these oils hasten the importance to assess their hazard in the environment.

The aim of this master's project was to determine if the pyrolysis oils generated from fast pyrolysis of three different wood feedstocks, namely poplar, beech and spruce, exerted any toxic and/or genotoxic effects in an *Allium cepa* chromosomal aberration assay. It was hypothesized that the toxicity and the genotoxicity of the different pyrolysis oils would vary with the feedstock used for making the oils and would increase with increasing concentrations of the oils tested.

To achieve the aim, an *Allium cepa* test was performed on different concentrations of the three pyrolysis oils. Since the oils were diluted with water to gain the different concentrations, only the water-soluble fraction of the oils was tested in this assay. First, a root inhibition test was conducted, where the roots of *Allium cepa* onions were exposed directly to the different solutions of the oils. The roots were measured after exposure, and dose-response curves were made. Root inhibition can be seen as a measure of general toxicity. Further, roots exposed to three concentrations of the different oils were selected for microscopic examination, consisting of determination of the mitotic index and scoring of chromosomal aberrations. These can be seen as measures of cytotoxicity and genotoxicity, respectively.

The toxicity-curves for the three pyrolysis oils were somewhat different; however, all showed a sigmoid shape: decreasing relative root length with higher concentrations. The mitotic index was significantly lower for onion roots exposed to all the oils and concentrations of oils

compared with negative control. The frequency of damaged dividing cells, in total and within the phases of division, was, in general, higher in onion roots exposed to all the concentrations of the different oils compared with negative control; however, a concentration-dependency was lacking for the ones exposed to the spruce-oil. The poplar-oil, followed by the beech-oil, seemed to have the highest genotoxic potential. The chromosomal aberrations largely contributing to the high frequency of damage, were those that are usually induced by chemicals exerting effects on the spindle apparatus during cell division.

The results indicate that all the three pyrolysis oils exert a toxic, cytotoxic and genotoxic effect. Different "fingerprinting" techniques have revealed strong similarities between the oils; however, some differences are seen in the abundance of different compounds or groups of compounds. The differences in toxicity and genotoxicity between the oils are probably due to these variations in chemical composition, as a result of the different biomass feedstocks used in the pyrolysis oils.

The results from the *Allium cepa* test are presumed to be a sensitive indicator of toxicity and genotoxicity, and may be seen as an early warning to other biological systems. However in this case, further studies should be carried out to evaluate the effects of the whole complement of the oils, and to identify compounds, or interactions between compounds, responsible for the observed effects. In this way, pyrolysis oils may be manufactured to cause less hazard in the environment. Additionally, the pyrolysis oils tested in the present master's project are considered to be crude bio-oils that need further upgrading prior to application. Thus, the toxicity of refined oils, that may have a different composition of compounds compared with crude oils, should also be tested. Furthermore, other test systems may be used to test differences in toxicity between fossil oils and pyrolysis oils, and thus, get a comparable measure of toxic effects exerted by oils derived from non-renewable energy sources and the alternative, oils derived from renewable energy sources.

Sammendrag

Ikke-fornybare energikilder dekker majoriteten av dagens energibehov, men grunnet usikkerhet knyttet til tilgjengelighet av petroleumreserver og store miljøpåvirkninger assosiert med bruken av petroleum, vokser betydeligheten av fornybare energikilder. Biomasse er en av de viktigste tilgjengelige energikildene, og bio-oljer derivert fra biomasser kan bli omdannet til brensel, brukt i varme- og elektrisitetsproduksjon, eller bli brukt som en kilde til kjemikalier. Pyrolyse er sett på som den mest effektive prosessen for biomasse omdanning, og pyrolyseolje-utbyttet kan bli så høyt som 80% hvis rask pyrolyse blir brukt. Pyrolyseoljer er komplekse blandinger av forbindelser, hvor innholdet og egenskapene avhenger av råmaterialet av biomasse, prossesstype og forhold. Selv om pyrolyseoljer er sett på som miljøvennlige sammenlignet med petroleumsoljer, vil økt produksjon og bruk av disse oljene fremskynde viktigheten av å evaluere deres risiko for miljøet.

Hensikten med denne mastergradsoppgaven var å bestemme om pyrolyseoljene laget fra rask pyrolyse av tre forskjellige råmaterialer av tre, poppel, bøk og gran, hadde noen toksiske og/eller genotoksiske effekter i en *Allium cepa* kromosomaberrasjonstest. Det ble utarbeidet hypoteser om at toksisiteten og genotoksisiteten av de forskjellige pyrolyseoljene ville variere med råmaterialet brukt for å lage oljene og ville øke med økte konsentrasjoner av oljene som ble testet.

En *Allium cepa* test ble utført på de forskjellige konsentrasjonene av de tre pyrolyseoljene. Siden oljene ble fortynnet med vann for å oppnå ulike konsentrasjoner, var det kun den vannløselige delen av oljene som ble testet i denne analysen. Først ble en rotinhibisjonstest utført, hvor røttene av *Allium cepa* løk ble eksponert direkte for de forskjellige løsningene av oljer. Røttenes vekst ble målt etter eksponering, og dose-respons kurver ble laget. Inhibering av røttenes vekst kan bli sett på som et mål på generell toksisitet. Videre ble røtter som hadde vært eksponert for tre konsentrasjoner av de forskjellige oljene, valgt for mikroskopisk analyse, som bestod av bestemmelse av mitotisk indeks og deteksjon av kromosomaberrasjoner. Dette kan bli ansett som mål på henholdsvis cytotoksisitet og genotoksisitet.

Toksisitetskurvene for de tre pyrolyseoljene var noe forskjellig, men alle viste en sigmoid form, der relativ rotvekst minket med økte konsentrasjoner. Mitotisk indeks var signifikant lavere for løkrøtter eksponert for alle oljene og konsentrasjoner av oljer sammenlignet med negativ

kontroll. Frekvensen av skadede celler i deling, totalt og innenfor de forskjellige delingsfasene, var generelt sett høyere for alle konsentrasjoner av de forskjellige oljene sammenlignet med negativ kontroll, selv om konsentrasjons-avhengighet manglet for røttene eksponert for gran-oljen. Poppel-oljen, fulgt av bøk-oljen, syntes å ha det høyeste genotoksiske potensialet. De kromosomaberrasjonene som i stor grad bidro til den høye frekvensen av skade, var de som vanligvis blir indusert av kjemikalier som utøver effekter på spindelapparatet under celledeling.

Resultatene indikerer at alle tre pyrolyseoljene utøver en toksisk, cytotoksisk og genotoksisk effekt. Forskjellige «fingeravtrykk»-teknikker har avslørt en sterk likhet mellom oljene, men noen forskjeller kan bli sett i mengden av ulike kjemiske forbindelser eller grupper av forbindelser. Forskjellene i toksisitet og genotoksisitet mellom oljene kan komme av disse variasjonene i kjemisk innhold, som et resultat av de forskjellige biomasse råmaterialene brukt i pyrolyse oljene.

Resultatene fra *Allium cepa* testen er antatt å være en sensitiv indikator på toksisitet og genotoksisitet og kan bli sett på som en tidlig advarsel til andre biologiske systemer. I dette tilfellet bør imidlertid flere studier bli utført for å studere effekter av hele komplementet av oljene, og for å identifisere forbindelser, eller interaksjoner mellom forbindelser, ansvarlig for de observerte effektene. På denne måten kan pyrolyseoljer bli framstilt slik at de fører til mindre fare for miljøet. I tillegg er pyrolyseoljene som ble testet i denne mastergradsoppgaven ansett å være råoljer som trenger oppgradering før de kan anvendes. De raffinerte oljene kan ha ulik komposisjon av forbindelser sammenlignet med råoljer, og derfor bør også toksisiteten av disse bli testet. Videre bør andre testsystem bli brukt for å teste forskjeller i toksisitet mellom fossile oljer og pyrolyseoljer, og dermed få et sammenlignbart mål på toksiske effekter utøvd av oljer fra ikke-fornybare energikilder og alternativet, oljer fra fornybare energikilder.

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Abbreviations

CA	Chromosomal aberrations
CO_2	Carbon dioxide
C-metaphase	Colchicine-metaphase
C-mitosis	Colchicine-mitosis
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EC_{50}	Half maximal effective concentrations
ESI-MS	Electrospray ionization-mass spectrometry
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
GHG	Greenhouse gas
HCl	Hydrochloric acid
HS	Hazelnut shell
MFO	Mixed function oxidase
MI	Mitotic index
MMS	Methyl methanesulfonate
m/z	Mass-to-charge ratio
NO_x	Oxides of nitrogen
PAH	Polycyclic aromatic hydrocarbon
PM	Particulate matter
ROS	Reactive oxygen species
SO_x	Oxides of sulphur

1 Introduction

1.1 Bio-oil – a renewable energy source

Non-renewable energy sources, such as petroleum, coal and natural gas, cover the majority of today's energy demand. However, the uncertainty of available petroleum reserves in a world with rapid growth in energy consumption has led to concern. Additionally, environmental impacts, resulting in increased health risk and the threat of global climate change, are a negative consequence of fossil fuel usage (Omer, 2008, Cherubini, 2010, Ng et al., 2010). Climate change is one of the primary concerns for humanity in the 21st century (Tingem and Rivington, 2009), and in particular global warming caused by emissions of carbon dioxide and other greenhouse gases (GHGs) (Panwar et al., 2011).

Renewable energy is of growing importance in satisfying concerns over the use of fossil fuel. In 2009, renewable energy sources supplied 14% of the total world energy demand, and in future aspects, it is expected that the fossil oil production will decrease, while production and application of renewable energy sources, like bio-oil from biomass, will increase (Demirbas, 2009) (Fig. 1.1). Biomass, or organic material, is one of the main renewable energy sources available, and today biomass supplies approximately 10% of the global annual primary energy consumption (World Energy Council, 2010). The most important benefit of renewable energy sources is the decrease of environmental pollution, particularly reduced greenhouse gas emission (Charters, 2001, Kalogirou, 2004, Dijkman and Benders, 2010, Jagoda et al., 2011). Renewable energy sources have massive energy potential, but they are generally not fully accessible, with distinct regional variability, which give rise to technical and economical challenges. However, by improving the collection and conversion efficiency, the cost may be lowered, and increased reliability and applicability may be obtained (Kalogirou, 2004).

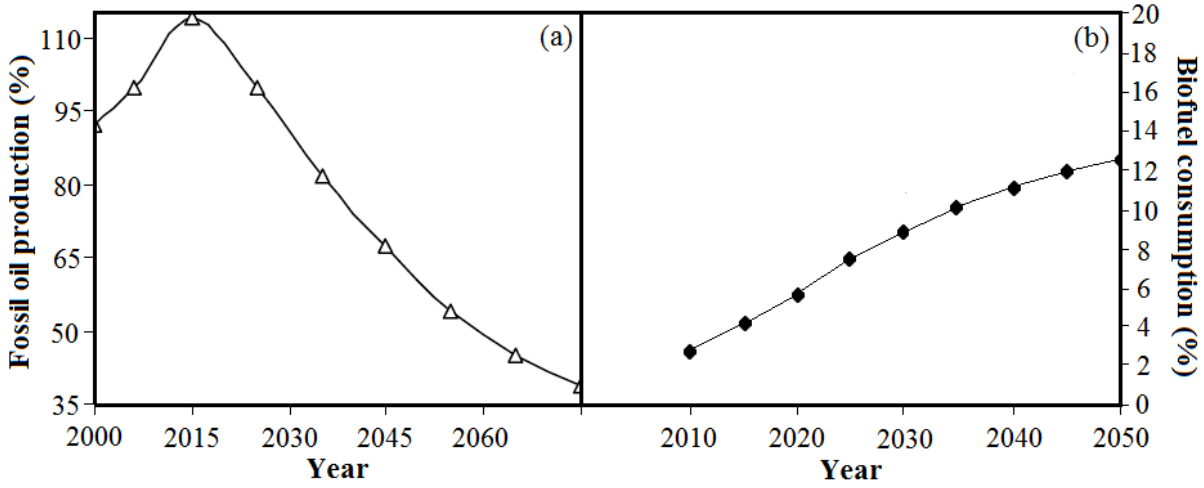


Figure 1.1. Future predictions of fossil oil production and biofuel consumption. Future predictions of global fossil oil production as percentage (%) of today's production (a), and biofuel consumption as percentage of the total automotive fuel consumption (b) in the world (modified from Demirbas (2006a) and Demirbas (2009)**).**

1.1.1 Applications

Bio-oils derived from biomasses can be converted to fuels, be used in heat and electricity production, or be used as a source of chemicals (Bridgwater et al., 1999, Mohan et al., 2006), as illustrated in figure 1.2. Liquid bio-oils are attractive high density energy carriers allowing low cost storage, handling and transport (Celaya et al., 2012). The production of fuels and chemicals from biomass is not a new concept. Biomass has been used as a source of energy all over the world since ancient times (Demirbas, 2006b). Since the 1800's biomass-based chemicals have been used in a variety of different products, like paint and glue. However, in the 1930's and 40's petrochemicals started to displace chemicals and products from biomass (Demirbas, 2006b), and became the preferred energy resources.

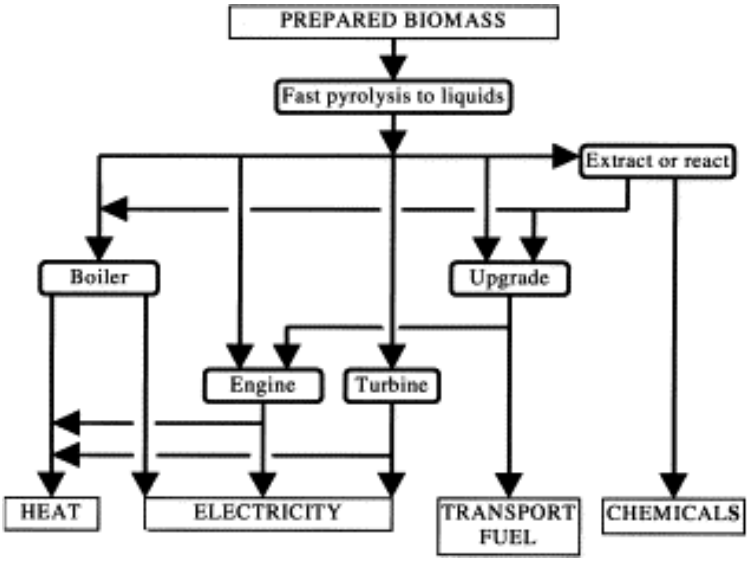


Figure 1.2. Applications of bio-oils (Bridgwater et al., 1999).

The use of biofuel as an alternative for petroleum-derived fuel is environmental beneficial. Biomasses from plants emit approximately the same amount of carbon during conversion as is taken up during plant growth, thus, the use of plant biomass does not contribute to a build-up of CO_2 in the atmosphere (McKendry, 2002). Furthermore, biofuels derived from biomasses have a higher oxygen content for improved combustion efficiency (Demirbas, 2007), and contain lower amounts of aromatic, sulphur, nitrogen and metal compounds compared to fossil fuels (Pütün, 2002, Kalam et al., 2003), causing less emissions of pollutants like oxides of sulphur (SO_x), oxides of nitrogen (NO_x), carbon dioxides (CO_2) and particulate matter (PM) (Kalam et al., 2003, Mohan et al., 2006, Zhang et al., 2007).

Although bio-oils may be promising and environmental beneficial as fuel constituents, most of the oils should be considered as crude bio-oils that need further upgrading (Eide and Neverdal, 2014). Today, bio-fuel prices are high relative to fossil fuel prices due to high production costs, and only tax credits make bio-fuel commercially viable (Ng et al., 2010, Sadeghinezhad et al., 2014). However, with development of techniques, standardization and increased volume of production, these costs can be significantly reduced (Ryan et al., 2006, Ng et al., 2010), making an enhanced commercialization of biofuels possible.

1.1.2 Plant biomass

Plant biomass is the only current renewable source of carbon that can be used directly for liquid fuels and chemicals (Huber et al., 2006). Wood biomass accounts for 87% of the total bio-

energy produced today (World Energy Council, 2010). Wood and other plant biomasses, like bark, nuts and seeds, essentially consist of oxygen-containing organic polymers, and these large amounts of oxygen in plant carbohydrate polymers differ substantially from fossil feeds (Mohan et al., 2006). Generally, the order of abundance of the major organic content in terrestrial whole-plant biomass is cellulose, hemicellulose and lignin (McKendry, 2002, Klass, 2004, Huber et al., 2006, Mohan et al., 2006). The cellulose, hemicellulose and lignin polymers make up 40-80, 15-30 and 10-25 wt.% of terrestrial biomass, respectively (Huber et al., 2006). The lipid and protein fractions are normally much less abundant than the carbohydrate components (Klass, 2004).

Lignin can bind cellulose and hemicellulose, making up lignocellulose compounds (Huber et al., 2006). These create more tightly bound fibres in the plant biomass. The amounts of lignin, cellulose and hemicellulose differ between different types of lignocellulosic materials. Woody plants, composed of tightly bound fibres, contain a higher proportion of lignin than herbaceous plants, composed of more loosely bound fibres (McKendry, 2002). The relative amount of cellulosic and lignin content also varies between softwood (gymnosperms) and hardwood (angiosperms); the lignin to cellulose and hemicellulose ratio is found to be higher in softwoods, as compared to the hardwoods (Pandey, 1999). Cellulose and hemicellulose are polysaccharides made up of sugar monomers, while lignin is an aromatic biopolymer, a polyphenol with a complex, random structure. Cellulose and hemicellulose may be broken down (hydrolyzed) into sugars. The lignin fraction, on the other hand, is nonfermentable (Wyman et al., 2005, Huber et al., 2006, Mohan et al., 2006). Hence, the biodegradability of cellulose is greater than that of lignin, and the overall conversion of carbon-containing plant biomass present as cellulose is greater than for plants with a higher proportion of lignin. This makes some plant species more suitable for processing as energy crops than others (McKendry, 2002).

1.1.3 Pyrolysis processes

Pyrolysis is regarded as the most efficient process for biomass conversion and the most promising technology to compete with, and maybe even replace, non-renewable fossil fuel resources (Demirbas, 2001). Biomass pyrolysis is the chemical decomposition, depolymerization and fragmentation of organic material, usually wood (lignocellulose) or just lignin. This happens through the application of heat (375-525°C) in absence of oxygen or when significantly less oxygen is present than required for complete combustion (Huber et al., 2006,

Mohan et al., 2006, Cordella et al., 2012), as illustrated in figure 1.3. During the process, biomass is converted to liquid products (pyrolysis oils), a solid residue (char), and several light gaseous compounds (e.g. carbon dioxide, carbon monoxide, hydrogen, light hydrocarbons) (Cordella et al., 2012).

Conventional slow pyrolysis is not a new invention, but dates back to ancient Egyptian times. Since then, this pyrolysis process has been improved and is widely used in charcoal and coke production (Mullaney et al., 2002). In the 1980's, researchers found that the pyrolysis liquid yield could be increased using fast pyrolysis to replace traditional slow pyrolysis processes, which have a much lower yield and where char is the main product (Mullaney et al., 2002). In fast pyrolysis the biomass is heated at moderate temperatures (around 500°C) at a rapid rate, and the vapors produced are cooled and condensed rapidly, achieving yields of liquid products as high as 80% (Bridgwater and Peacocke, 2000). Process conditions that favor liquid products are moderate temperatures, fast heating rates, and short hot vapor residence times to minimize secondary reactions (Huber et al., 2006, Celaya et al., 2012).

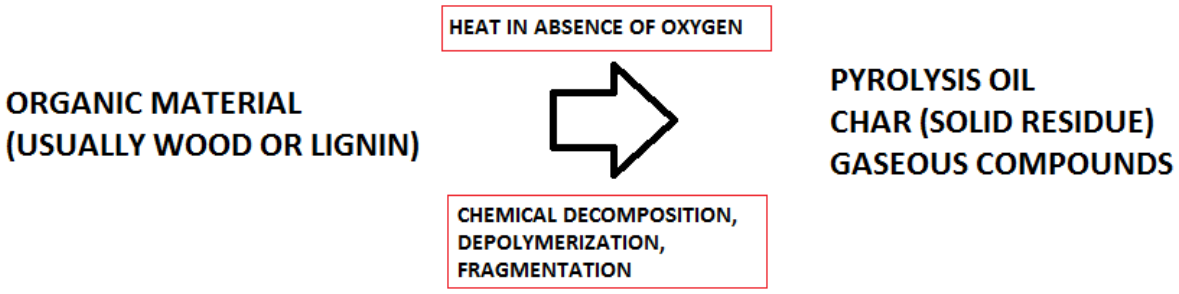


Figure 1.3. Overview of conditions and products from a pyrolysis process.

1.1.4 Pyrolysis oils

Pyrolysis oils are usually dark brown, free-flowing liquids with a distinctive smoky odor, and are particular attractive because they present a much better opportunity for high-efficiency energy production compared to other biomass fuels (Czernik and Bridgwater, 2004, Mohan et al., 2006). The yield and properties of the oils depend on the biomass feedstock, the process type and conditions, and the product collection efficiency (Czernik and Bridgwater, 2004).

The pyrolytic breakdown, depolymerization and fragmentation of wood produce a large number of chemical substances (Mohan et al., 2006), and consequently pyrolysis oils are composed of

a wide mixture of compounds, containing carbon, hydrogen and oxygen, like acids, esters, alcohols, ketones, aldehydes, phenols, sugars, furans, miscellaneous oxygenates, alkenes and aromatics (Rue and Breton, 2006). Pyrolysis oils contain 45-50 wt.% oxygen, and oxygen is present in most of the identified compounds in the oils (Oasmaa and Czernik, 1999). This is the main reason for differences in properties and behavior between fossil oils and biomass pyrolysis oils. The most abundant compound in the pyrolysis oils is water. Other major groups of compounds are hydroxyaldehydes, hydroxyketones, sugars, carboxylic acids and phenolic compounds, mostly present as oligomers (Oasmaa and Czernik, 1999, Mohan et al., 2006).

Pyrolysis oils are not yet commercial products due to several challenges. The high oxygen content of pyrolysis oils makes them poorly miscible with petroleum-based liquids, and there are problems using them as a fuel in standard equipment constructed for combustion of petroleum-derived fuels, mainly due to poor volatility, high viscosity, chemical instability, coking, and corrosiveness (Czernik and Bridgwater, 2004). Fast pyrolysis oils contain a significant amount of aldehydes, ketones and carboxylic groups, which are unstable and undergo a number of reactions during storage. To improve storage stability, crude fast pyrolysis liquids therefore need mild upgrading to reduce the amount of these compounds (Toven et al., 2013, Eide and Neverdal, 2014). A significant effort is being spent on research and development directed to the application of pyrolysis oils for heat and power, and for the use as a transport fuel (Czernik and Bridgwater, 2004).

1.2 Toxicity of pyrolysis oils

Even though pyrolysis oils are considered environmental friendly compared with petroleum oils, increasing production and eventually application and commercialization of pyrolysis oils hasten the importance to assess their hazard in the environment. Pyrolysis oils can reach the environment through accidental releases and/or routine losses associated with use or transport (Leme et al., 2012), and may produce adverse effects on the environment, animals and humans. However, it should be noted that, compared with petroleum oils, biomass pyrolysis oils are less persistent in the aquatic and soil environment, due to a higher biodegradability (Blin et al., 2007, Oasmaa et al., 2012).

1.2.1 Toxicity studies of pyrolysis oils

Pyrolysis oils have been shown to exert eco-, cyto- and genotoxic effects on living organisms and cells, as shown in the studies below. The studies resulted in different toxicity results. This is probably due to the use of different biomass feedstocks and different pyrolysis conditions, leading to different composition of compounds in the pyrolysis oils, as well as the use of different toxicity tests and endpoints.

1.2.1.1 Toxicity studies – eco- and cytotoxicity

Ecotoxicological effects may among others be detected through survival or inhibition of growth of living organisms. Chatterjee et al. (2013) observed a dose-dependent decrease in survival of the nematode *Caenorhabditis elegans* after exposure to slow pyrolysis oils generated from two different biomass feedstocks (rice straw and sawdust of oak tree). Pimenta et al. (2000) and Park et al. (2008) detected immobilization of the freshwater crustacean *Daphnia magna* after exposure to a slow pyrolysis oil produced from *Eucalyptus grandis* wood and a fast pyrolysis oil produced from radiata pine, respectively. Moreover, Pekol et al. (2012) observed a significant concentration-dependent inhibition in root growth of the onion *Allium cepa* when exposed to a fast pyrolysis oil from hazelnut shell. However, within the Biotox project, 21 pyrolysis oil samples (19 obtained from fast pyrolysis and 2 from slow pyrolysis) from different feedstocks showed no ecotoxicological effects, apart from inhibition of algal growth at high concentrations (Girard et al., 2005). Additionally, Oasmaa et al. (2012) selected one of the oils from the Biotox project for further analysis, namely a fast pyrolysis bio-oil from spruce, and did not detect inhibition of algal growth or immobilization of *D. magna* after exposure.

Park et al. (2008) and Chatterjee et al. (2013) detected cytotoxic effects linked to the pyrolysis oils tested. A dose-dependent increase in cytotoxicity and apoptosis in cultured cell systems was observed when testing toxicity of the slow pyrolysis oils produced from rice straw and sawdust of oak three (Chatterjee et al., 2013), and a decrease in cell viability was observed with higher concentrations of the fast pyrolysis oil derived from radiata pine (Park et al., 2008). Cytotoxicity may damage cell function, which results in inability of cells to proliferate. These disturbances may appear before genotoxic effects, or even in the absence of the latter, hence, the cytotoxic effect may be considered an earlier indication of cellular damage (Park et al., 2008).

1.2.1.2 Genotoxicity studies

Genotoxicity of environmental contaminants is of great concern, due to the capability of genetic damage to cause health problems and affect future generations, since these damages may be inheritable (Bickham et al., 2000). Genotoxicity studies have been conducted for both whole pyrolysis oil samples and fractions of the oils. Pimenta et al. (2000) found no observable mutagenic activity of liquid products from slow pyrolysis of *Eucalyptus grandis* wood in Microtox™ bioassays; however, when taking the polycyclic aromatic hydrocarbon (PAH) fraction of the pyrolysis oil, genotoxic effects were shown. Cordella et al. (2012) also suggested a carcinogenic potential of oil fractions generated from slow pyrolysis of different biomass samples (corn stalks, poplar and switchgrass). In this case, carcinogenicity was associated with 11 compounds (i.e. catechol and PAHs) found in the oils.

Whole sample studies have revealed genotoxic potential of different types of pyrolysis oils. Pekol et al. (2012) showed mutagenicity associated with a pyrolysis oil obtained from fast pyrolysis of hazelnut shell (HS) in an *Allium cepa* test, in the form of chromosomal aberrations, and Park et al. (2008) detected DNA damage, particularly DNA strand breaks, after exposure to a bio-oil from the fast pyrolysis of radiata pine, using a single-cell gel electrophoresis (comet) assay. Chatterjee et al. (2013) found a dose-dependent increase in genotoxicity of cultured cell systems in a comet assay linked to pyrolysis oils from slow pyrolysis of rice straw and sawdust of oak tree.

Within the Biotox project, the 21 selected pyrolysis oils were tested for mutagenicity using a bacterial reverse mutation assay (Ames test) and a micronucleus test *in vivo* and *in vitro* on mice cells (Girard et al., 2005). In the Ames test, all the fast pyrolysis oils showed a positive indication of mutagenicity, and the two slow pyrolysis oils tested were too toxic for the bacteria to give a result. The micronucleus test also indicated a potential for slight mutagenicity; however, the authors concluded that further testing was needed. Oasmaa et al. (2012) detected mutagenicity in a bacterial reverse mutation assay with and without metabolic activation of the fast pyrolysis oil derived from spruce feedstock, which indicated that the oil contained both direct acting mutagens and compounds that were mutagenic active after metabolic transformation. Micronucleus testing on mice cells, however, indicated a lack in ability of the oil to induce chromosome changes or damage to the mitotic apparatus (Oasmaa et al., 2012).

1.2.2 Toxicity of single pyrolysis oil components

Hazard assessment of single pyrolysis oil components has been conducted (Diebold, 1997, Zhou et al., 2010, Cordella et al., 2012); however, this information may be misleading. It is important to keep in mind that pyrolysis oils are complex mixtures composed of a wide variety of compounds, as mentioned earlier, making it difficult to identify single toxic components and their behavior. The chemicals may undergo synergistic or antagonistic interactions by altering metabolism or transport characteristics, and this may have a significant effect on toxicity (Donnelly et al., 1995).

The possible presence of PAHs in pyrolysis oils, however, should be given attention due to their known genotoxic effects. PAHs need to be metabolically activated to cause damage. Biotransformation of PAHs is usually catalyzed by cytochrome P450 enzymes and creates PAH-epoxides that can form adducts with DNA (Hall and Grover, 1990, Boström et al., 2002, Parkinson and Ogilvie, 2007). Incomplete repair of these adducts may give rise to mutations (Boström et al., 2002). Additionally, some types of PAH metabolites may generate reactive oxygen species (ROS) and indirectly cause DNA damage (Bolton et al., 2000). Fortunately, pyrolysis oils generated with temperatures at around 500^{o}C contain small amounts of PAHs (Williams and Horne, 1994, Williams and Horne, 1995, Diebold, 1997); however, the qualitative and quantitative amounts of PAHs may vary between various pyrolysis oils, due to different pyrolysis conditions and feedstocks (Tsai et al., 2007, Lu et al., 2009). Furthermore, it is evident that a high quantity of PAHs may be formed during possible upgrading of the oils by zeolite catalysts to produce refined bio-fuels (Williams and Horne, 1994, Williams and Horne, 1995, Vitolo et al., 2001).

1.2.3 Chromosomal aberrations

Sensitive genetic endpoints for exposure of mutagens can range from point mutations to chromosomal aberrations (CA) in cells of different organs and tissues (Preston and Hoffmann, 2007). Chromosomal aberrations are defined as any departure from the normal in chromosomal structure or number (Preston and Hoffmann, 2007). These aberrations may be detected in both mitotic and meiotic cell divisions (Grant, 1978). In the normal process of mitotic division, chromosomes in metaphase should be organized in the metaphasic plate in the middle of the cell; in anaphase, the centromeres connect with the spindle apparatus which causes a pull of the chromatids of each chromosome to the opposite pole of the cell; in telophase, the daughter

chromosomes arrive and assemble at each pole, and in cytokinesis, the cell divides into two identical daughter cells (Fig. 1.4).

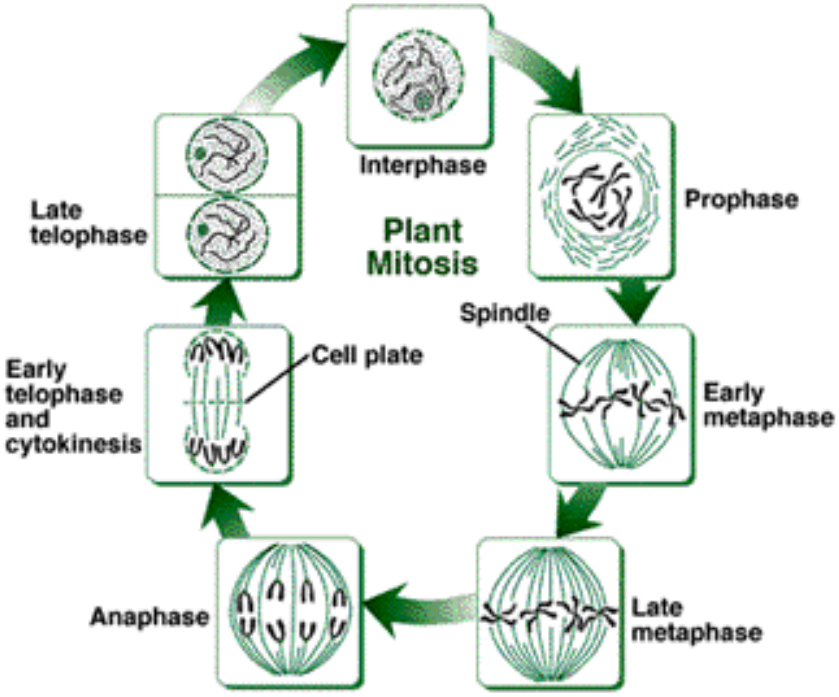


Figure 1.4. Mitosis in a plant cell (Stern, 2000).

Mutagenic chemicals may induce many different types of chromosomal aberrations. The chromosomal aberrations may arise directly from clastogens, mutagens that create DNA strand breaks with subsequently survival or misrepair of the damage, or indirectly from breaks created from unravelling, synthesis and repair processes (Bignold, 2009). The breaks can occur anytime (pre-synthesis, synthesis or post-synthesis) in the cell cycle (Bignold, 2009). The majority of breaks induced in interphase, are repaired by rejoining to the original configuration. However, repair-failure of single breaks may give rise to chromosomes from which a portion has been broken off, called fragments. Two or more repair-failed breaks in the same cell may occur, either in the same or in different chromosomes. This may lead to multiple fragment appearance, or the multiple breaks may interact, if they are in close spatial and temporal proximity, and sometimes form bizarre chromosomes, called rearrangements or exchanges (Savage, 1976, Bender et al., 1988). One of the most observed rearrangements in microscopic analyses is bridges, and this aberration is often seen together with fragments (Rank, 2003). Figure 1.5 shows how chromatid breakage and rejoining can result in these aberrations.

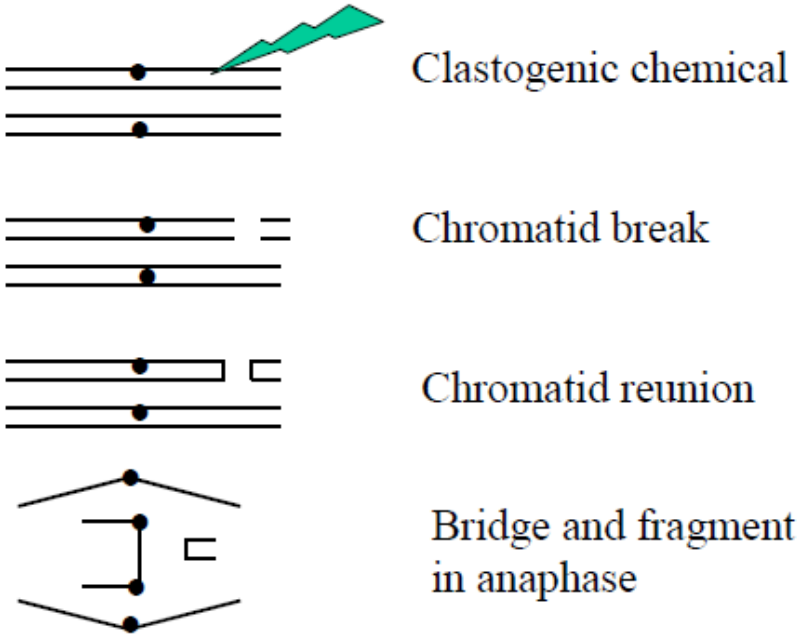


Figure 1.5. Formation of a bridge and a fragment. Chromatid breakage and rejoining during mitosis resulting in the formation of a bridge and a fragment (Rank, 2003).

Mutagenic chemicals may also induce chromosomal aberrations by affecting the spindle apparatus or the centromere-regions during cell division, and this may affect the organization in metaphase and the ability to segregate the sister chromatids to separate daughter cells (Preston and Hoffmann, 2007). Colchicine-mitosis (c-mitosis) was first described by Levan (1938) as random scattering of condensed chromosomes due to an inactivation of the spindle apparatus in colchicine-treated cells. Colchicine, and other c-mitotic chemicals, may prevent formation of the mitotic spindle by inducing microtubule depolymerization (Caperta et al., 2006). If some sign of assembling of the chromosomes outside the metaphase plate is detected in the cell, it is defined as a disturbed metaphase cell. Disturbed metaphases, laggard chromosomes, a lag observed in the chromosomal migration to the poles (Akintonwa et al., 2009), and vagrant chromosomes, chromosomes that are not organized to a specific stage of the mitotic division (Akintonwa et al., 2009), may be caused by weak c-mitotic disturbances, in which the spindle is made somewhat ineffective but is not totally inhibited (Levan, 1947). The induction of c-mitotic effects may lead to the separation of an unequal number of chromosomes in the daughter nuclei, which can result in cells that contain multiples of the normal complement of chromosomes (polyploidy) or cells that contain up to a few more or a few less chromosomes than the normal complement (aneuploidy) (Albertini et al., 2000). Disturbances of the spindle apparatus may also result in chromosome configurations like ‘star anaphase’, in which

chromosomes are drawn into an area at the poles in a star-like aggregation (Vaughn and Lehnen, 1991).

Another type of chromosomal aberration is stickiness. Sticky chromosomes lose their normal appearance, and they are seen as agglomerations of chromosomes adhered to each other. This may happen if chromosome fibers fail to condense properly in preparation of mitosis. Then inter-chromosomal chromatin fibres may be trapped in and tangled with fibers of other chromosomes, resulting in stickiness, sub-chromatid connections between chromosomes (McGill et al., 1974). Sticky chromosomes indicate that the agent is affecting the organization of the chromatin, which is related to a disturbed balance in the quantity of histones or other proteins responsible for controlling the proper structure of nuclear chromatin (Radić et al., 2010). Stickiness may be associated with chromatin breaks (McGill et al., 1974) and with the formation of bridges (Radić et al., 2010), which may occur when sticky chromosomes separate during anaphase and telophase. During cell division, sticky chromosomes may also produce aneuploid or polyploid cells (Onwuamah et al., 2014).

Examples of different kinds of chromosomal aberrations in meta-, ana- and telophase are shown in figures 1.6, 1.7 and 1.8, respectively.

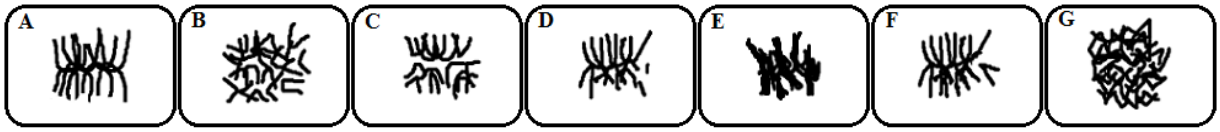


Figure 1.6. Chromosomal aberrations in metaphase. Illustrations of different kinds of chromosomal aberrations that may be observed in meristematic cells in metaphase of *Allium cepa* roots. A: Normal metaphase. B: C-metaphase. C: Disturbed metaphase. D: Fragments. E: Sticky metaphase. F: Vagrant. G: Polyploid cell.

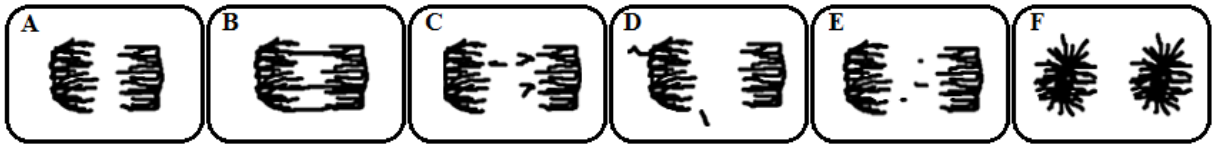


Figure 1.7. Chromosomal aberrations in anaphase. Illustrations of different kinds of chromosomal aberrations that may be observed in meristematic cells in anaphase of *Allium cepa* roots. A: Normal anaphase. B: Bridges. C: Laggards. D: Vagrants. E: Fragments. F: Star anaphase.

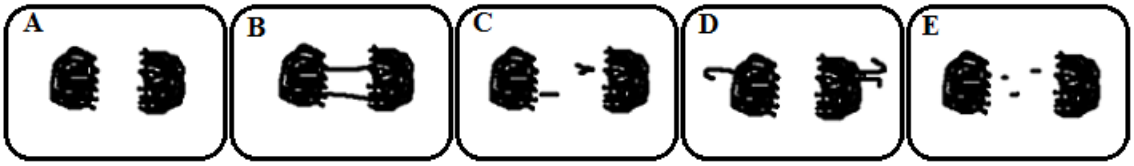


Figure 1.8. Chromosomal aberrations in telophase. Illustrations of different kinds of chromosomal aberrations that may be observed in meristematic cells in telophase of *Allium cepa* roots. A: Normal telophase. B: Bridges. C: Laggards. D: Vagrants. E: Fragments.

It is also important to recognize that chromosomal aberrations may arise spontaneously, and consequently, it will always be a background level of "naturally" occurring aberrations (Bender et al., 1988).

1.2.4 The *Allium cepa* test

The *Allium cepa* test is a plant test system used to examine both toxicity and genotoxicity. The basic steps of the *A. cepa* test are measurement of root length, determination of the mitotic index (the proliferation status of a cell population, MI) and observation of chromosomal aberrations of the common onion, *Allium cepa*, after exposure to a test solution. The root appearance and root length can be used as measures of toxicity (Fiskesjö, 1985), the mitotic index can be used to evaluate cytotoxicity, and chromosomal aberrations can be used to verify genotoxicity of different chemicals (Leme and Marin-Morales, 2009). The *Allium cepa* test was introduced by Levan in 1938 (Levan, 1938), demonstrating disturbances in the mitotic spindle due to colchicine exposure, and later Levan (1945) showed that inorganic salt solutions of different concentrations induced chromosomal aberrations in root cells of *Allium cepa* (Leme and Marin-Morales, 2009). Since then, technical modifications of the test have been made to create a more comprehensive assessment of chemicals in the complex mixtures of environmental samples (Grant, 1982, Fiskesjö, 1985, Rank et al., 1993, Ma et al., 1995, Rank and Nielsen, 1997, Rank, 2003, Matsumoto et al., 2006, Fernandes et al., 2007, Leme et al., 2008).

Many researchers have used the test, mainly as a bioindicator of environmental pollution. Single components considered hazardous environmental pollutants, like metals (Liu et al., 1995, Seth et al., 2008, Yıldız et al., 2009), pesticides (Ateeq et al., 2002, Bolle et al., 2004) and aromatic hydrocarbons (Fiskesjö, 1981), as well as waste effluents from pollution sources (Odeigah et al., 1997, Rank and Nielsen, 1998, Grover and Kaur, 1999, Chandra et al., 2005, Bagatini et al., 2009) and environmental samples, as water and soil samples, from polluted areas (Smaka-Kincl

et al., 1996, Kovalchuk et al., 1998, Cabrera and Rodriguez, 1999, Leme and Marin-Morales, 2008, Souza et al., 2009) have been evaluated in the *Allium cepa* test. As earlier mentioned, PAHs need to be metabolically activated by cellular enzymes (cytochrome P450-dependent mixed function oxidases (MFO) system) before exerting their biological effect (Parkinson and Ogilvie, 2007), and the *Allium cepa* onion has mechanisms involving these enzymes (Fatima and Ahmad, 2006, Tabrez and Ahmad, 2013).

Even though the *Salmonella* mutagenicity assay (Ames test) is the most commonly applied genotoxicity test for complex mixtures (Claxton et al., 1998), the *Allium cepa* test is sensitive to heavy metals (Fiskesjö, 1988), and there are many benefits using *Allium cepa* onions as test organisms; they are easy to store and handle, they have many and equivalent roots that do not branch, they mostly have good chromosome conditions, such as large and few chromosomes (2n=16), and they can be exposed directly to the test samples in the laboratory. In this short-term *in vivo* model, the roots grow in direct contact with the substance of interest, and since the cells in the root tips are in constant mitotic division, the toxic effects and alterations occurring over a cell cycle can be identified (Tedesco and Laughinghouse IV, 2012). The results may serve as a warning to other biological systems, since the target is DNA, common to all organisms (Leme and Marin-Morales, 2009), and have shown to be comparable with results from other tests performed in mammalian systems (Grant, 1978, Fiskesjö, 1985, Rank and Nielsen, 1994, Chauhan et al., 1999). The test has been recognized as a sensitive, reliable, simple, cheap and rapid bioassay (Fiskesjö, 1985, Rank and Nielsen, 1993, Rank and Nielsen, 1994, Rank, 2003).

1.3 Complex mixtures

A complex mixture is defined as a mixture that consists of tens, hundreds or thousands of chemicals, and of which the composition is qualitatively and quantitatively not fully known (Feron et al., 1998).

1.3.1 Combined actions

The combined action in complex mixtures is any outcome of exposure for two or more chemicals, and may be ‘no interaction’ (additivity), ‘more than additivity’ (synergism, potentiation), or ‘less than additivity’ (antagonism) (Könemann and Pieters, 1996).

If no interaction between components in a mixture occurs, the terms dose addition and response addition are used. Response additivity describes a situation where the chemicals in a mixture are functionally independent, and the grades of effect are adding up. This will only occur if the individual compounds exceed their own thresholds of tolerance (Könemann and Pieters, 1996). With dose additivity the chemicals all act on the same biological site, by the same mechanisms, and differ only in their potencies. The toxicity of these mixtures can be estimated by summing up the doses of the components after adjustment for their potency (Könemann and Pieters, 1996, Groten, 2000). No threshold exists for dose additivity (Könemann and Pieters, 1996).

Interaction between chemicals may result in a stronger effect (synergism, potentiation) or weaker effect (antagonism) than expected on the basis of additivity (Groten, 2000, Feron and Groten, 2002). A synergistic effect occurs when the combined effect of two chemicals is larger than the sum of the effect of each individual chemical (Eaton and Gilbert, 2007). Potentiation, a form of synergism, occurs when a compound that is not toxic itself, increases the toxicity of another chemical on a particular organ or system (Eaton and Gilbert, 2007). Antagonism occurs when two chemicals inhibit the effect of each other, or when one chemical inhibits the effect of the other (Eaton and Gilbert, 2007). The interaction may be of physiochemical and/or biological nature, and might occur in the toxicokinetic phase (i.e. processes of uptake, distribution, metabolism and excretion) or/and in the toxicodynamic phase (i.e. effects of the chemicals on the receptor, cellular target or organ) (Groten, 2000, Groten et al., 2001). This makes it difficult to identify toxic compounds and their behavior in complex mixtures.

1.3.2 Strategies for evaluations: experimental design

It can be distinguished between whole-mixture analysis, also known as top-down approach, and component joint action or interaction analysis, also known as bottom-up approach (Feron and Groten, 2002). In the case of top-down approaches, test systems are exposed to the whole mixture and exposure-response studies are conducted to evaluate the nature and magnitude of the hazard associated with the mixture (Mauderly, 1993). The results give no information about the individual compounds or interactions between them (Eide, 1996, Groten et al., 2001).

The bottom-up approach, however, makes it possible to analyze the effect of individual components or groups of components. By fractionation, the mixture is separated into individual

constituents or families of constituents, and further tested for biological activity (Mauderly, 1993). Interactions may also be detected in a bottom-up approach by the use of synthetic mixtures. In these mixtures it will be necessary to vary the composition of the mixture and the relative amounts of the different compounds, and compare the effects of the mixture to the effects of the individual constituents. These studies usually begin with two agents and stepwise use increasingly complex combinations of agents to get an enhanced understanding of the causative agents, or mechanisms, of the effects of the complete mixture (Mauderly, 1993).

In practice a bottom-up approach for studying the toxicology of complex mixtures is nearly impossible (Groten, 2000); however, combinations of top-down and bottom-up approaches are often used to study the toxicology of mixtures (Feron and Groten, 2002).

1.4 Aims and hypotheses

The aim of this master's project is to study if the pyrolysis oils, generated from fast pyrolysis of three different wood feedstocks, exert any toxic and/or genotoxic effects in an *Allium cepa* chromosomal aberration assay. The following hypotheses are made:

- The toxicity and the genotoxicity of the different oils will vary with the feedstock used for making the oils.
- The toxicity and the genotoxicity of the different oils will increase with increasing concentrations of the oils.

2 Materials and methods

2.1 The test organism *Allium cepa*

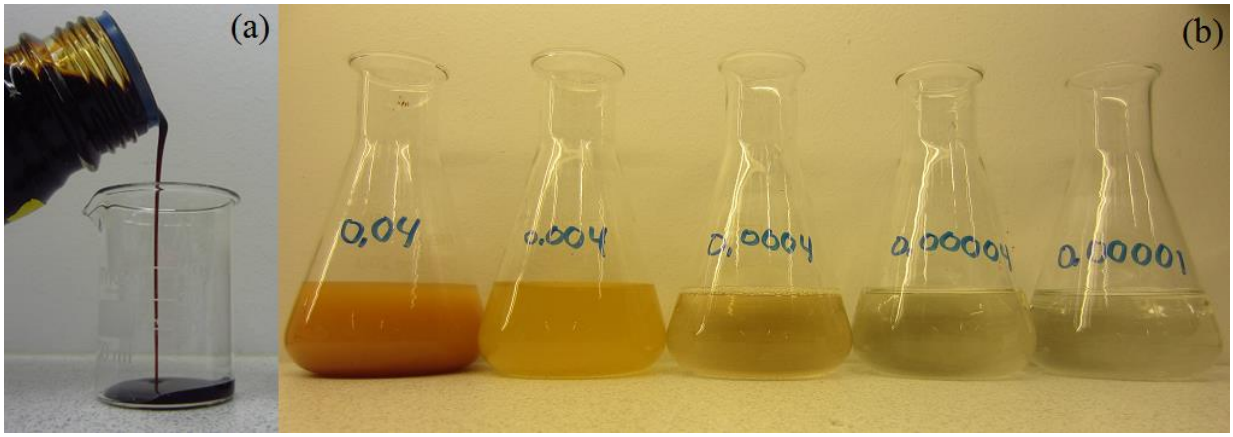
Small, non-treated bulbs (2.0-4.0 cm in diameter) of the common onion, *Allium cepa* (2n=16) were provided from the farm "Hveem Nord Østre", Toten, Norway. The onions were harvested in the end of August/beginning of September, and needed a couple of months to gain ability to grow roots. Prior to initiating the test, the yellow shallows of the bulbs, and the dry, brownish bottom plate, were removed without destroying the root primordia. The peeled bulbs were put into fresh tap water during the cleaning procedure to protect the primordia from drying. Dried and mould-attached, as well as poorly growing, onions were discharged. The onions should not have started shooting of green leaves (Fiskesjö, 1985), since the energy used for this growth may compete with the downward root growth. During the experiment, the onions were kept in a dry, dark closet at room temperature.

2.2 The pyrolysis oils

The samples of pyrolysis oils used in this experiment were received from the Paper and Fibre Research Institute (PFI), and were generated from fast pyrolysis of different whole wood feedstocks (100% wood), spruce (*Picea abies*), beech (*Fagus sylvatica*) and poplar (*Populus tremula*). The samples from beech and spruce were made at the University of Aston (Celaya et al., 2012, Toven et al., 2013, Eide and Neverdal, 2014). The spruce-, beech- and poplar-oil were made in 2011, 2012 and 2009, respectively. The wood materials were collected from different countries; the spruce was from Norway, the poplar was from Canada and the beech was collected from the United Kingdom. The oils were kept in dark at 4°C.

The oils were heated for one hour at 50°C to homogenize them, as advised by Gunhild Neverdal, Statoil (pers. comm.). Further, the oils were added 0.1% dimethyl sulfoxide (DMSO) and diluted with tap water prior to the *A. cepa* test, to gain seven different concentrations, namely: 0.00001, 0.00004, 0.0001, 0.0004, 0.001, 0.004 and 0.04 (or 0.01) ml pyrolysis oil/ml of solution (tap water+DMSO). Picture 2.1 illustrates the appearance of a pyrolysis oil generated from poplar feedstock (Pic. 2.1a), and five poplar solutions of different concentrations (0.04, 0.004, 0.0004, 0.00004 and 0.00001 ml oil/ml solution) (Pic. 2.1b). The DMSO of 0.1% was chosen as a dominance of literature demonstrate that DMSO concentrations <0.1% cause no

toxic effects in neither *in vitro* nor *in vivo* studies (Stratton, 1987, Blancaflor et al., 1998, Vijayan et al., 2004, Pagan et al., 2006, Iakimova et al., 2008, Quinn et al., 2008, Yuan et al., 2012), and a quantity of <0.25% DMSO has been suggested to be appropriate in an earlier conducted *Allium cepa* study (Chauhan et al., 1999). Since the possible toxic and genotoxic effects, that may influence the interpretation of the results, are tested in the present master's project using 'blind' solutions (tap water+DMSO; 0.1%), reliably observations of the effects of exposure are conducted. Water was gradually added to the oils, and the solutions were shaken thoroughly between each adding on a vortex mixer. None of the oils were phase separated. Only the water-soluble part of the oils was used for further exposure. The pH of all concentrations of the different oils was measured, and adjusted to approximately pH=7.5 every 24 hours.



Picture 2.1. Pyrolysis oil generated from poplar feedstock and poplar-oil solutions Appearance of a pyrolysis oil generated from poplar feedstock (a), and five concentrations of the poplar-oil (0.04, 0.004, 0.0004, 0.00004 and 0.00001 ml oil/ml solution (tap water+dimethyl sulfoxide, DMSO)) (b).

2.2.1 Composition of the oils

The oil samples obtained from poplar, beech and spruce feedstocks have been characterized at Statoil's Center for Research, Development and Innovation, using different chemical "fingerprinting" techniques: positive and negative electrospray ionization-mass spectrometry (ESI-MS), Fourier transform infrared spectroscopy (FTIR) and gas chromatography-mass spectrometry (GC-MS) (Eide and Neverdal, 2014). The oils were analyzed after they were produced. The chemical information in the analyses is associated with molecular size and functional groups. Positive ESI-MS detects mostly polar compounds containing oxygen, nitrogen or sulfur, negative ESI-MS primarily detects organic acids, FTIR detects functional groups, and GC-MS detects hydrocarbons and other organic compounds sufficiently volatile for the GC (Eide and Neverdal, 2014). Chemical "fingerprinting" is used as an initial step to

provide a compositional overview, without identifying and quantifying every individual compound in the sample (Eide and Zahlsen, 2005, Eide et al., 2011).

In the ESI-MS analysis, there is one distinct line per integer mass number (m/z). The lines represent those parent compounds (unfragmented) that have become ionized. Electrospray ionization typically occurs by the addition or loss of a proton, but sometimes positive ionization occurs by adducts. Non-polar hydrocarbons, such as paraffins and aromatics, are not ionizable and as a consequence not detectable in the ESI-MS analysis (Gellerstedt et al., 2008, Kleinert et al., 2011, Eide and Neverdal, 2014), and non-volatile, less-polar compounds may not be as efficiently ionized as others (Smith et al., 2012, Olcese et al., 2013). Thus, ESI-MS spectra represent detection of mostly polar, water-soluble components, as are present in the test solutions in this master's project. Figure 2.1a shows a positive ESI-MS of the three pyrolysis oils generated from poplar, beech and spruce, and illustrates a quite similar pattern, with dominant masses in the range 100-900 m/z. The pyrolysis oils also show a somewhat similar pattern in the negative ESI-MS (Fig. 2.1b), with dominant masses in the range 100-700 m/z, especially at lower mass numbers. Even though the oils follow the same trend throughout both spectra, some differences are seen between the oils for the lines at different mass numbers.

Fourier transform infrared spectroscopy allows analysis of groups of compounds, and do not allow identification of individual pyrolysis oil components (Eide and Zahlsen, 2005, Staš et al., 2013). Characterization of the entire pyrolysis oil sample is conducted, regardless of the volatility of the compounds (Staš et al., 2013). The absorption bands that can be found in FTIR spectra of pyrolysis oils are presented in Table 2.1. Fourier transform infrared spectroscopy analysis of the three pyrolysis oils revealed strong similarities between the samples; however, some differences are seen at the different signal clusters (Fig. 2.1c). The first peak in the spectra contain the wavenumber interval 3600-3200 cm^{-1}, and partly the interval 3100-3000 cm^{-1}, and is corresponding to groups of compounds like phenols, alcohols, water, carboxylic acids, amides, amines and aromatics. Within this peak, the pyrolysis oil generated from poplar shows the highest absorbance, followed by beech and spruce, respectively. Within the wavenumber interval 1850-1580 cm^{-1}, some higher absorbance is observed for the poplar- and beech-oil compared with the spruce-oil, and this interval corresponds to chemical compounds like aldehydes, ketones, carboxylic acids, esters and alkenes. The beech- and spruce-oil show the highest tendency of absorbance within the wavenumber interval 1550-950 cm^{-1} compared with the poplar-oil. This interval corresponds to classes of compounds like nitrogenous compounds,

aromatics, alkanes, alcohols and ethers, where the spruce-oil is partly dominating in the interval corresponding to alcohols and ethers. For the wavenumber interval 915-700 cm^{-1}, corresponding to aromatics, the three oils have a somewhat similar absorbance. However, the spruce-oil is showing a weakly higher absorbance with wavenumbers close to 915 cm^{-1}, and the poplar- and beech-oil are showing a weakly higher absorbance with numbers approaching 700 cm^{-1}.

GC-MS can be used for the analysis of the chemical composition of the pyrolysis oils (Staš et al., 2013); however, chromatographic separation and curve deconvolution may become complicated with the complex oils. Additionally, large molecules (with boiling points of > 400-450 °C) are difficult to analyze (Eide and Zahlsen, 2005). Gas chromatography is also unable to characterize the non-volatile compounds present, like sugar and lignin oligomers (Staš et al., 2013). The GC-MS spectra of the three different pyrolysis oils used in this project (Fig. 2.1d) illustrate some similarity of pattern; however, the oils show differences in abundance at the different retention times.

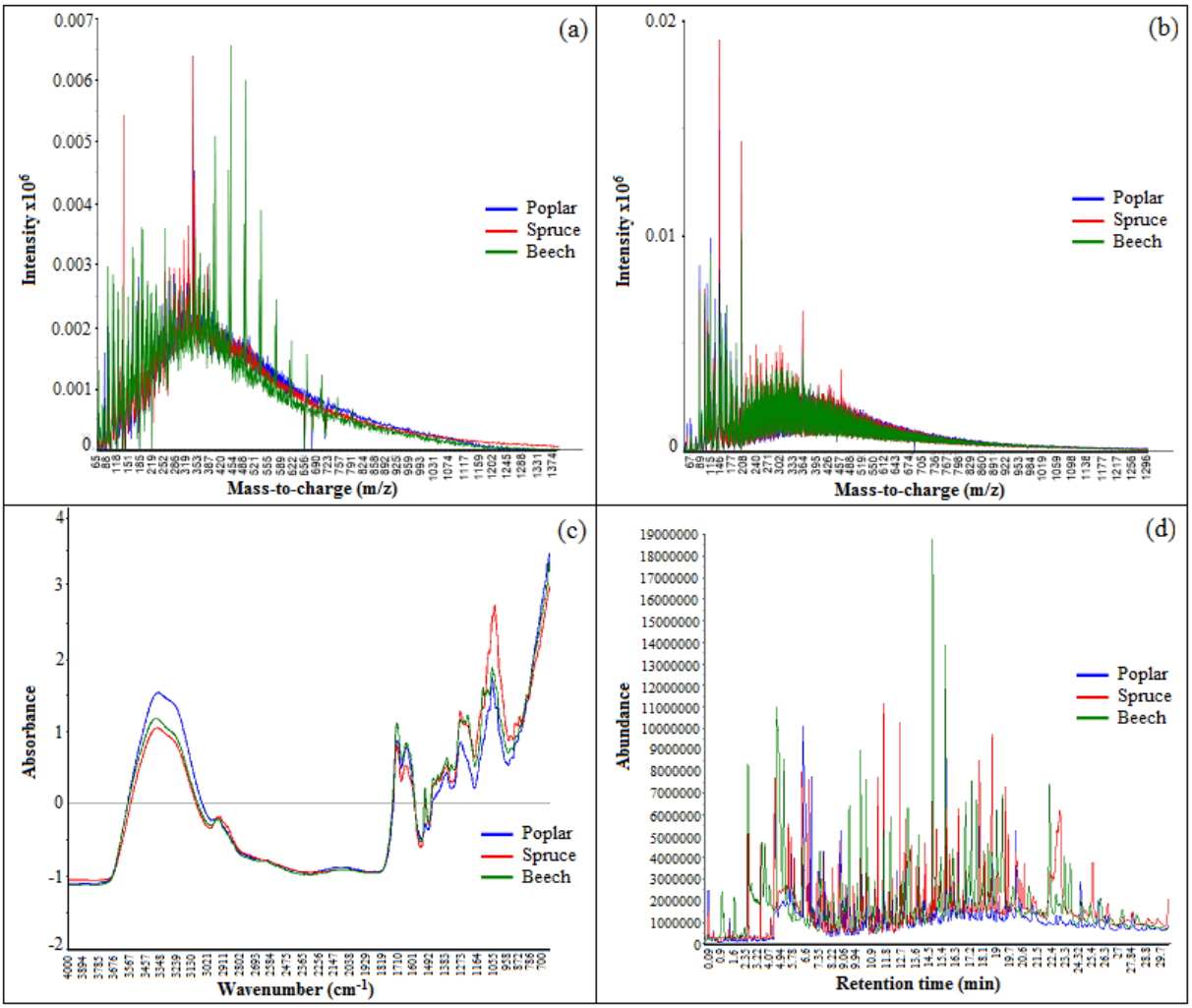


Figure 2.1. Fingerprinting spectra. Positive electrospray ionization-mass spectrometry (ESI-MS) spectra (a), negative ESI-MS spectra (b), Fourier transform infrared spectroscopy (FTIR) spectra (c) and gas chromatography-mass spectrometry (GC-MS) chromatogram (d) of pyrolysis oils made from poplar (blue), spruce (red) and beech (green) feedstocks. New spectra were obtained from Eide and Neverdal (2014).

Table 2.1: Absorption bands in FTIR spectra. Absorption bands in fourier transform infrared spectroscopy (FTIR) spectra of pyrolysis oils (Pütün et al., 1999, Lu et al., 2008, Staš et al., 2013).

Wavenumber (cm^{-1})	Type of vibration	Classes of compounds
3600-3200	O-H, N-H stretching	Phenols, alcohols, water, carboxylic acids, amides, amines
3100-3000	C-H stretching	Aromatics
2980-2870	C-H stretching	Alkanes
2350-2000	C≡C stretching	Alkynes, cyanides
1850-1650	C=O stretching	Aldehydes, ketones, carboxylic acids, esters
1650-1580	C=C stretching	Alkenes
1550-1490	NO_2 stretching, N-H bending, aromatic C=C stretching	Nitrogenous compounds, aromatics
1470-1350	C-H bending	Alkanes
1300-950	C-O stretching, O-H bending	Alcohols, ethers
915-650	C-H in-plane bending	Aromatics

2.3 The *Allium cepa* test

2.3.1 Root inhibition test

The root inhibition test was carried out in collaboration with the MSc student Serina Beate Engebretsen (Engebretsen, 2014).

2.3.1.1 Chemicals, equipment and consumption materials

Chemicals	Product number	Producer
Dimethyl sulfoxide (DMSO, ≥99.8%)	1.02950.0500	Merck
Methyl methanesulfonate (MMS, 99%)	M4016-25G	Sigma

Equipment and consumption material	Product number	Producer
Blade handles, No. 3		Swann-Morton
Carbon steel surgical blades, No. 11	0203	Swann-Morton
Chemical thermometer, 100 °C	3201	Assistent
Erlenmeyer flask, 250 ml	4980-250	Pyrex
Glass beakers, 150 ml		
Glass bottle with top, 500 ml		Schott & Gen, Mainz
Glass test tubes, 50 ml		
Measuring cylinder, 250 ml		Brand Blaubrand Eterna
Pipette tips		
100µl	70.760.502	Sarstedt
1000µl	70.762.100	Sarstedt
2500µl		
5000µl		
Pipettes; 100µl, 1000µl, 2500µl, 5000µl		Eppendorf
Plastic ruler, 15 cm	560 01-15	Staedtler
Termostatic water bath	D3165	Köttermann
Vortex Vibrofix VF1 Electronic		Janke & Kunkel
		Ika Labortechnik

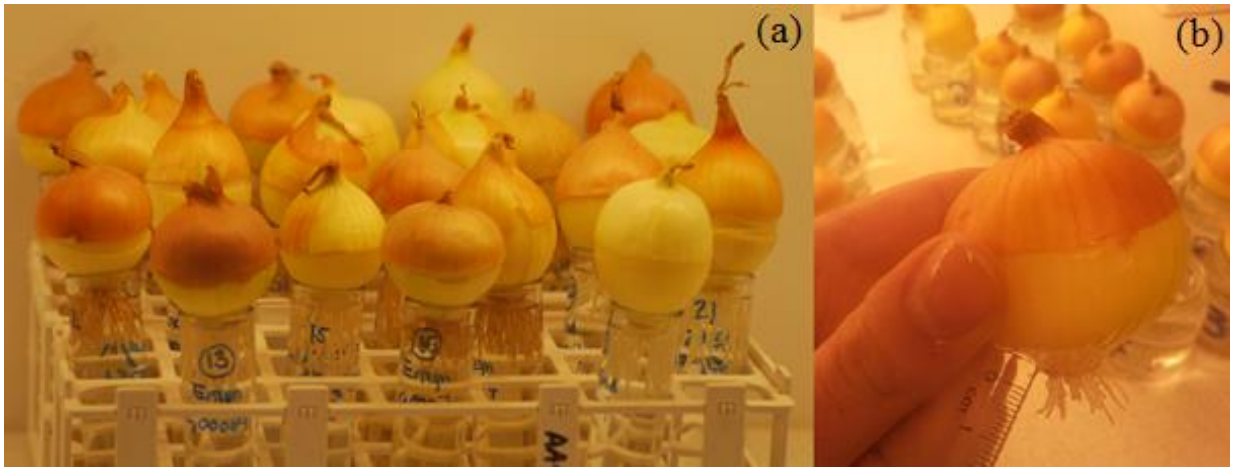
2.3.1.2 Method

Prior to exposure, peeled bulbs of *Allium cepa* onions were placed in 150 ml glass beakers filled with tap water for 48 hours. Further, three replicates of the onions were placed in 50 ml glass test tubes with their respective treatment-solutions (Photo 2.2a); seven different pyrolysis oil concentrations, seven 'blind' solutions (tap water+DMSO), one for each concentration, a negative control (tap water of good quality) and a positive control (methyl methanesulfonate, MMS, 10 mg/l), for 72 hours. The 'blind' solutions were made in the same way as the oil-solutions; however, the oils were replaced by tap water. Every 24 hours the test solutions were

replaced by fresh solutions and all the roots of each onion were measured with a plastic ruler (Photo 2.2b). The average root length was calculated for each onion. At the end of each experiment the appearance of the roots was observed and noted. The experiments were conducted separately for each treatment-group.

For further discussion, all the different solutions tested (negative control, positive control, seven different concentrations of pyrolysis oils and 'blind' solutions) are referred to as "treatment-groups", while the oil-solutions alone are referred to as "exposure-groups".

Toxicity curves for each oil were generated and displayed as oil concentration against root length inhibition. Root length inhibition was measured as mean root length gained during the 72 hours exposure period as a percentage of the root length of onions exposed to the lowest concentration of the respective oil. From the regression curve half maximal effective concentrations (EC_{50}s), concentrations of the solutions that reduce the growth of the roots by half, were calculated using Sigma Plot (SYSTAT Software Inc.).



Picture 2.2. Onion setup and root measurement. Setup (a) and measure of root lengths (b) of the onion *Allium cepa*.

2.3.2 Pre-treatment and fixation

2.3.2.1 Chemicals, equipment and consumption materials

Chemicals	Product number	Producer
8-hydroxyquinoline (C_9H_7NO)	1.07098.0250	Merck
Acetic acid (CH_3COOH, 100%)	1.00063.1000	Merck
Ethanol absolute (CH_3CH_2OH, 96%)	20821.310	VWR International

Equipment and consumption material	Product number	Producer
Glass pasteur pipettes, 150 mm	612-1701	VWR International
Tweezers		Comaco
Snap-cap vials		

2.3.2.2 Method

After 72 hours of exposure, root tips (~ 1.0 cm in length) were pinched off and collected in snap-cap vials containing distilled water. The water was subsequently replaced by 8-hydroxyquinoline (saturated solution in distilled water), and the vials were kept cold (4°C) in a refrigerator for five hours. After five hours, the root tips were washed three times with distilled water, before fixated in Carnoy's solution (3:1 ethanol/acetic acid solution), and the vials were kept cold for 70 minutes. The ethanol/acetic acid solution was subsequently replaced by 70% ethanol and the vials were stored cold, not longer than two months, until microscope slide preparation was carried out.

2.3.3 Microscope slide preparation

2.3.3.1 Chemicals, equipment and consumption materials

Chemicals	Product number	Producer
Acetic acid (CH_3COOH, 100%)	1.00063.1000	Merck
Ethanol absolute (CH_3CH_2OH, 100%)	20821.310	VWR International
Eukitt ($C_{13}H_{22}O_4$)		O. Kindler GmbH
Hydrochloric acid (HCl, 37%)	1.00317.1000	Merck
Liquid nitrogen (N_2, -196 °C)		AGA
Orcein, Synthetic ($C_{28}H_{24}N_2O_7$)	07380	Sigma-Aldrich

Equipment and consumption material	Product number	Producer
Aluminium blocks		
Balance, type AE 260 S	2524 GK	Mettler
Blade handles, No. 3		Swann-Morton
Blade handles, No. 4		Swann-Morton
Carbon steel surgical blades, No. 11	0203	Swann-Morton
Carbon steel surgical blades, No. 22	0208	Swann-Morton
Cover slips, 18 x 18 mm	VD1 1818 Y100A	Knittel Gläser
Cover slips, 24 x 32 mm	BB024032A1	Menzel-Gläser
Ethanol burner		
Filter paper circles, 70 mm	10300008	Schleicher & Schuell
Glass pasteur pipettes, 150 mm	612-1701	VWR International

Glass trough with cover, 9 x 7 x 6.5 cm	1205/1	Assistent
Incubator		Julabo
Measuring cylinder, 10 ml		Assistent
Measuring cylinder, 100 ml		Hirschmann
Microscope slides	631-1554	VWR International
Pencil	120-2	Staedtler
Snap-cap vials		
Tray of stainless steel, for 10 slides	1205/5	Assistent
Tweezers		

2.3.3.2 Method

For every onion, 3 mm of a minimum of 3 root tips were cut off and placed in a snap-cap vial with 2 ml acetic acid (45%)/hydrochloric acid (HCl; 1 M) (9:1) and heated for 5 minutes at 50°C. Thus, the root cells became fixed and macerated. Further, the acetic acid/HCl solution was immediately replaced by 2 ml 45% acetic acid in order to stop the reaction. The root tips were then placed on pre-labelled microscope slides. Two drops of filtrated 2% orcein solution were added to the root tips on each slide. The orcein solution was made from 2 g orcein solved in 100 ml acetic acid (60%), which was shaken and filtrated prior to use. Further, cover slips were placed on the root tips and the slides were placed above an ethanol burner for a couple of seconds. The slides were allowed to absorb stain for 5-10 minutes. Afterwards, the cells were spread evenly in a monolayer by gently pressing the cover slips down with the thumb and tapping with a pencil. Prepared slides were subsequently placed upon flat aluminium blocks precooled in liquid nitrogen (N_2), and the cover slips were removed with a sharp scalpel. Once the cover slips were removed, the slides were immediately, whilst still frozen solid, placed into glass troughs with 70% ethanol for 5 seconds, 96% ethanol for 8 seconds, 100% ethanol for one minute and a second glass trough with 100% ethanol for further 5 minutes. The slides were then allowed to air dry for at least 30 minutes, before gluing on the final cover slips with eukitt.

2.3.4 Microscopic examination

2.3.4.1 Equipment and consumption material

Equipment and consumption material	Product number	Producer
Digital camera	Coolpix 990	Nicon
Hand tally counter	FM40047	Tamaco
Microscope	ECLIPSE E400	Nicon

2.3.4.2 Method

The microscopic analysis includes calculation of the mitotic index (MI) and scoring of chromosome aberrations in meta-, ana- and telophase cells. The initial screening analysis was examined in a microscope at 400x magnification. The mitotic index is a measure of the proliferation status of a cell population, and was found by counting all stages of mitotic cells (pro-, meta-, ana- and telophase) out of 1000 cells (Rank, 2003). MI is calculated as:

$$MI = \frac{\text{total number of dividing cells}}{\text{total cell number}} x100$$

At least 100 cells in meta-, ana- and telophase were scored for chromosomal aberrations for each individual onion, within each treatment-group. As many slides as needed to get over hundred cells in division for each individual, were made. One type of chromosomal aberration was only detected once for each aberrant cell. Where damage was observed, photos of the cell were taken for documentation (600x magnification), and the coordinates were noted. The cells uncertain to have a damage, were analyzed by two persons (Bingham, pers. comm.) before the final decision was made. Cells of bad quality, where it was difficult to judge whether a damage was present or not, were excluded from the results. Bad quality may, among others, be due to poorly staining of the cells, overlapping cells, or mechanical damages. The microscopic slides were coded and examined blindly, due to expectations that may affect the results.

Identification character of a cell in metaphase was condensed chromosomes that laid in, or were heading to, the metaphase plate. Cells with chromosomes segregating to each pole, with a clearly "finger"-form, were detected as cells in anaphase. Cells assembled to each pole, without any chromosome "fingers", were detected as cells in telophase. For the counting of the mitotic index, cells were classified as a cell in interphase if single chromosomes could not be detected. The other cells were classified as dividing cells.

2.4 Statistics

2.4.1 Evaluation of toxicity

Statistical differences among treatment-groups were tested using a two-sample Student t-test. The SPSS 21.0 statistical package was used for this analysis. For all the tests the level of significance was set at $p<0.05$.

To conduct a statistical comparison between two different sets of toxicity data (T_1 and T_2), a null-hypothesis was assumed: H_0: $T_1=T_2$. These evaluations were made under the assumptions that the experimental single value was normally distributed around the estimates, T_1 and T_2, and that a Student t-test could be used. If the probability was less than 5% ($P(T_1=T_2)<0.05$), the H_0 was rejected, and the anti-hypothesis H_1: $T_1 \neq T_2$ was assumed; it is a statistical significant difference between T_1 and T_2.

The conclusion of the statistical comparisons of T_1 against T_2 vary in strength after which degree of probability it can be claimed that H_0 is correct. Dependent on the level of P, the conclusion can be graded as in Table 2.2, and will in the results be presented as the implicated symbols.

Table 2.2: Two-tailed t-test of a null-hypothesis in the form H_0: $T_1=T_2$. The conclusions are made by the combination of column 'Δ', the difference T_1-T_2 (bigger (+) or smaller (-) than zero), and 'P', the interval the levels of the probability P fall within (dependent on the t-value sizes and the number of degrees of freedom). P is the probability of H_0 being fulfilled (5% significant level, two-tailed test). The symbols are the ones used for presentation of statistical conclusions.

Δ	P	Conclusion	Symbols
-	P < 0.001	T1 is strongly significant less than T2	---
-	0.001 < P < 0.01	T1 is clearly significant less than T2	--
-	0.01 < P < 0.05	T1 is weakly significant less than T2	-
+ and -	0.05 < P	T1 and T2 is not significantly different	is
+	0.01 < P < 0.05	T1 is strongly significant larger than T2	+
+	0.001 < P < 0.01	T1 is clearly significant larger than T2	++
+	P < 0.001	T1 is weakly significant larger than T2	+++

3 Results

In this chapter, the results are presented in the same order as they were performed in the laboratory (Chapter 2). Firstly, the results from the root inhibition test are presented, showing dose-response curves and the associated half maximal effective concentrations. Results from the microscopic examination include scores of mitotic index, percentage of damaged dividing cells (displayed both in total and within the different phases of division), distribution of dividing phases and the specific chromosomal aberrations observed in the dividing cells.

3.1 Root inhibition test

The average length of roots was measured for three parallels of onions, exposed to seven different concentrations of pyrolysis oils (0.00001, 0.00004, 0.0001, 0.0004, 0.001, 0.004, and 0.01 or 0.04 ml oil/ml solution), generated from fast pyrolysis of poplar (Fig. 3.1a), spruce (Fig. 3.1b) and beech (Fig. 3.1c) feedstocks, as a percentage of the lowest concentration (0.00001 ml oil/ml solution). All the three dose-response curves had sigmoid shapes, with decreasing percentage of root length with increasing concentrations of the oils.

The four lowest concentrations of the poplar-derived oil contribute to the plateau of its curve (~53-134%). Then the curve has a significant decrease (Appendix D) down to the 0.001-concentration (~12-30%). The five lowest concentrations contribute to the plateau of the dose-response curve of the spruce-derived oil (~44-167%), and then the curve decreases significantly (Appendix D) down to the 0.004-concentration (~3-13%). The dose-response curve of the beech-derived oil decreases gradually from the 0.00001-concentration (69-127%) to the 0.01-concentration (9-17%). For all the three dose-response curves, the percentage of root length reaches the stationary phase at the 0.004-concentration.

A dose-response curve for percentage of root length as a function of concentration is also generated for the roots exposed to the ‘blind’ test solutions (tap water+DMSO) (Fig. 3.1d). The curve does not have a significant decrease or increase with increasing “concentrations” (Appendix D). The root growth of onions exposed to MMS was not lower compared with negative control (Appendix A).

Dose-response curves obtained between the concentrations of the pyrolytic oils and relative *Allium cepa* root growth determined the EC_{50}-values as 0.000605, 0.000364 and 0.001816 ml oil/ml solution for pyrolysis oils derived from poplar, beech and spruce, respectively (Table 3.1).

Raw data for the root lengths after exposure to different treatments are presented in Appendix A.

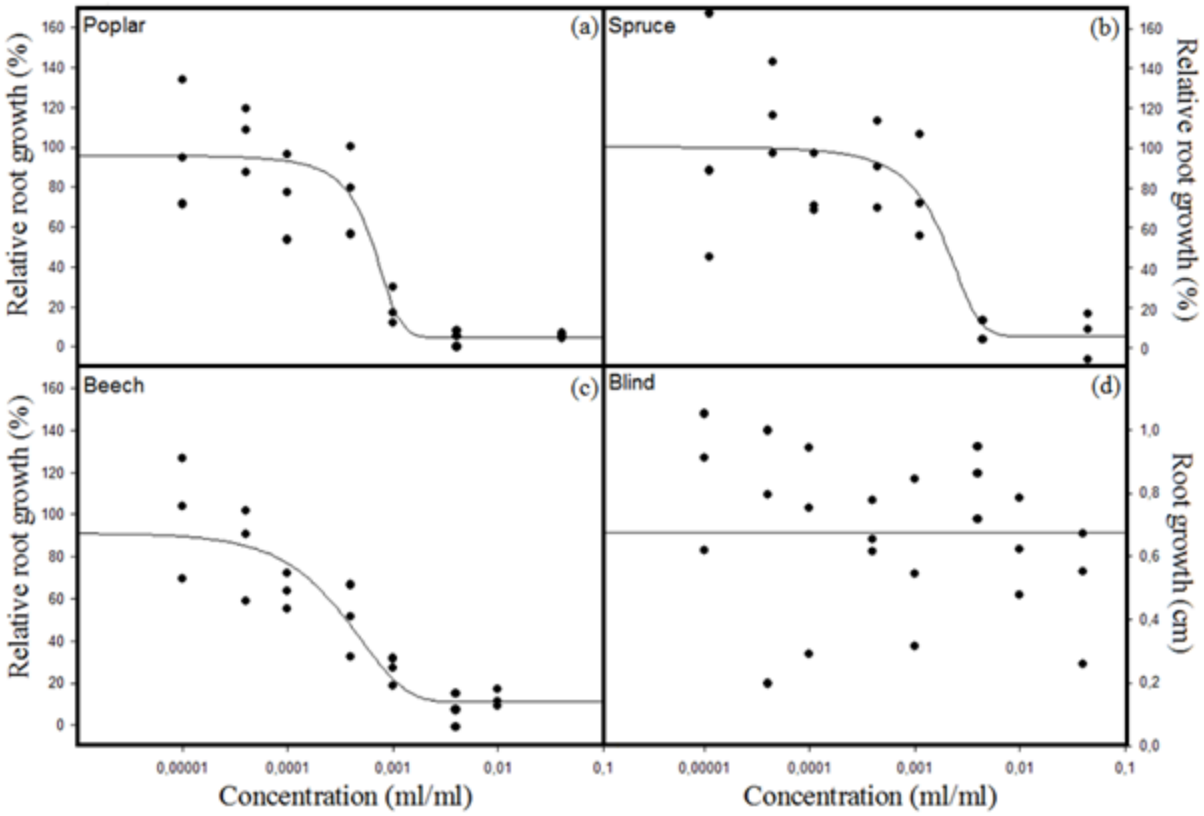
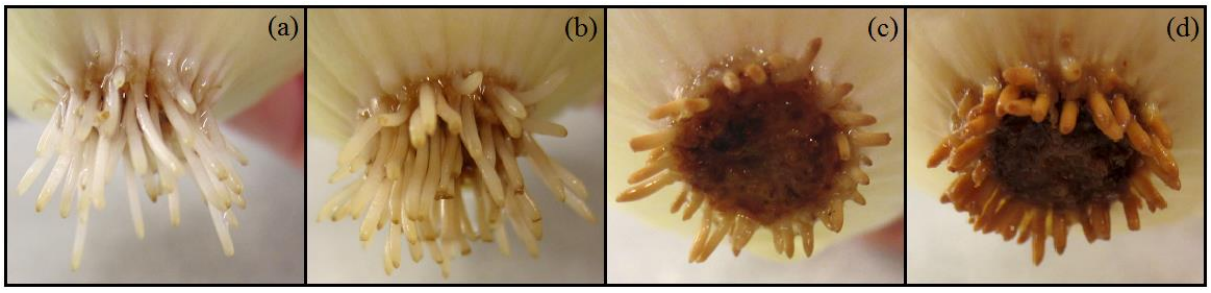


Figure 3.1. Dose-response curves. Mean values for root length of onions (*Allium cepa*) as a function of concentrations of the tested pyrolysis oils (ml pyrolysis oil/ml solution (tap water+dimethyl sulfoxide, DMSO)) made from fast pyrolysis of poplar (a), beech (b), and spruce (c) feedstocks, and a 'blind' control (tap water+DMSO) (d) after 72 hours of exposure. The data are given as percentage (%) of the lowest concentration of the pyrolysis oils, and as root growth (cm) for the 'blind' solutions. Three parallels of onions and a regression line are included for each treatment concentration. Each dot represent one individual, and the lines are sigmoid four parametric curves, R^2=0.85 (a), 0.83 (b), 0.70 (c) and $-6.61*10^{-13}$ (d).

Table 3.1: EC_{50}-values. Toxicity of the tested pyrolysis oils, made from poplar, beech or spruce feedstocks, expressed as the half maximal effective concentration (EC_{50}), which is the effect concentration for 50% inhibition of the root length.

Pyrolysis oil feedstock	EC_{50} (ml oil/ml solution)
Poplar	0.000605
Beech	0.000364
Spruce	0.001816

The roots of the onions exposed to different pyrolysis oils for 72 hours showed no apparent difference in quality when exposed to the same concentrations; however, between different concentrations the appearance differed. The roots became gradually more brown, viscous and soft with increasing concentrations (Picture 3.1). The colour change was more apparent at the root tips, except at the highest concentrations (0.004-0.04 ml oil/ml solution), where the colour was evenly brown throughout the whole root. Additionally, the roots exposed to the highest concentrations were vulnerable, fragile and more easily damaged. Roots exposed to negative control, positive control, 'blind' solutions and the two lowest concentrations of the oils (0.00001-0.00004 ml oil/ml solution) for 72 hours, on the other hand, were fresh, stiff, light, and easy to handle.



Picture 3.1. Appearance of roots of the onion *Allium cepa* after a 72 hour exposure to four different concentrations (0.00004 (a), 0.0004 (b), 0.004 (c) and 0.04 (d) ml oil/ml solution) of a pyrolysis oil generated from poplar feedstock.

3.2 Mitotic index (MI)

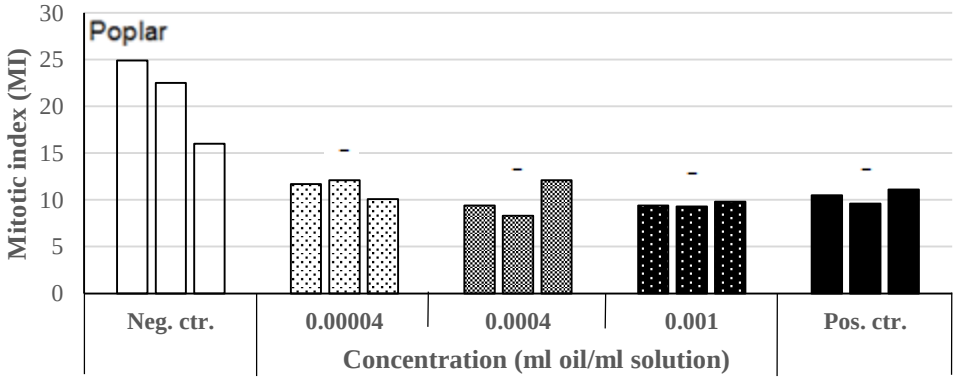
The mitotic index was determined by examination on microscopic slides prepared from roots of triplicates of onions exposed 72 hours to three different concentrations (0.00004, 0.0004 and 0.001 ml oil/ml solution) of pyrolysis oils produced from poplar (Fig. 3.2a), beech (Fig. 3.2b) and spruce (Fig. 3.2c) feedstocks. The mitotic index of a 'blind' solution was included for each concentration (Fig. 3.2d), as well as for triplicates of onions exposed to tap water (negative

control) and methyl methanesulfonate (positive control) to compare with the MI of the exposure-groups.

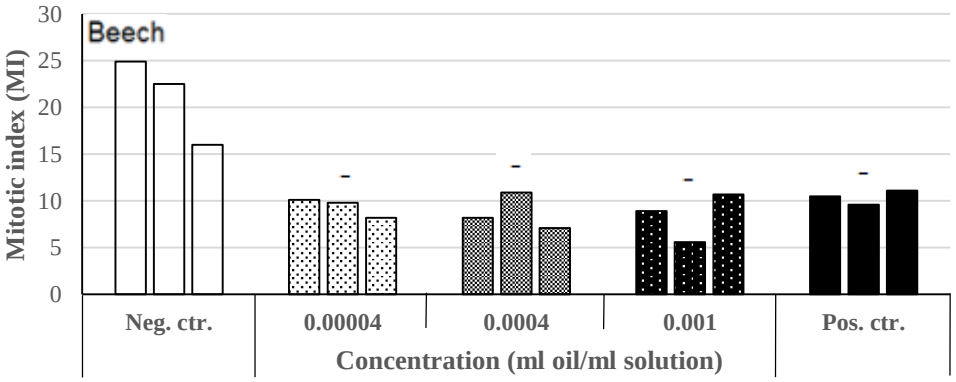
The MI of the different concentrations of pyrolysis oils, as well as the MI of MMS, were weakly significant lower than the MI of the negative control. There are no clear decrease in MI with increasing concentrations of the different pyrolysis oils; however, the mitotic index seem to be decreasing slightly with higher concentrations. The MI of the 'blind' solutions was not significantly different from the MI of the negative control.

Values of the mitotic index are presented in Appendix B.

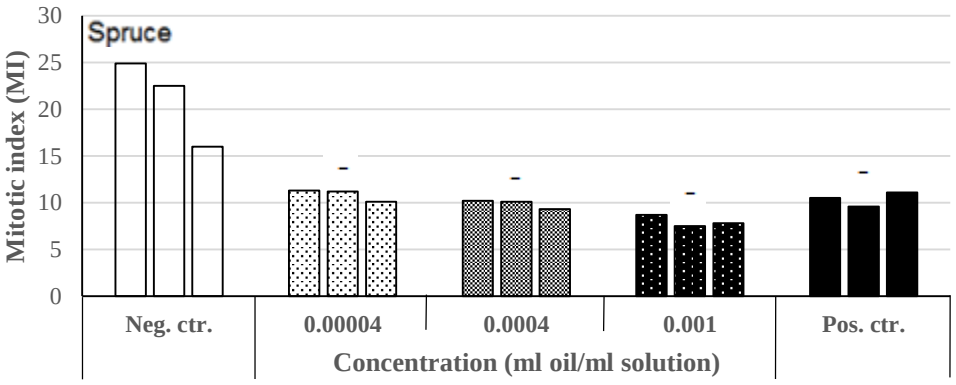
a)



b)



c)



d)

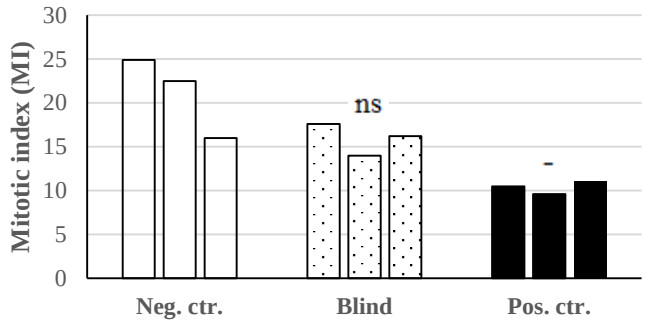


Figure 3.2. Mitotic index (MI). MI values of root tip meristems of *Allium cepa* onions exposed 72 hours to pyrolysis oils generated from fast pyrolysis of poplar (a), beech (b) or spruce (c) feedstocks at different concentrations (0.00004, 0.0004 and 0.001 ml oil/ml solution (tap water+dimethyl sulfoxide, DMSO)). MI values for onion roots exposed to a 'blind' solution (tap water+DMSO) for each concentration (0.00004, 0.0004 and 0.001 ml/ml, respectively) were inluded, as well as values for negative control (tap water)- and positive control (methyl methanesulfonate, MMS)-exposed onion roots for comparison. Three parallels of onions are included for each treatment, one column representing one individual. Different symbols denote the means of the different treatment-groups that are not significantly different (ns) or significantly different (weakly (-/+), clearly (--/++) or strongly (---/+++)) from the mean of negative control using a two-sample (independent group) t-test ($p<0.05$).

3.3 Damaged dividing cells

3.3.1 Frequency of damaged dividing cells

Damaged *Allium cepa* root cells in division (meta-, ana- or telophase) as percentage of total dividing cells were determined from the microscopic slides of each treatment-group. Onions exposed to pyrolysis oil from poplar feedstock had a clearly significant higher percentage of damaged cells compared with those exposed to negative control, and had apparent increases of damage with increasing concentrations (Fig. 3.3a).

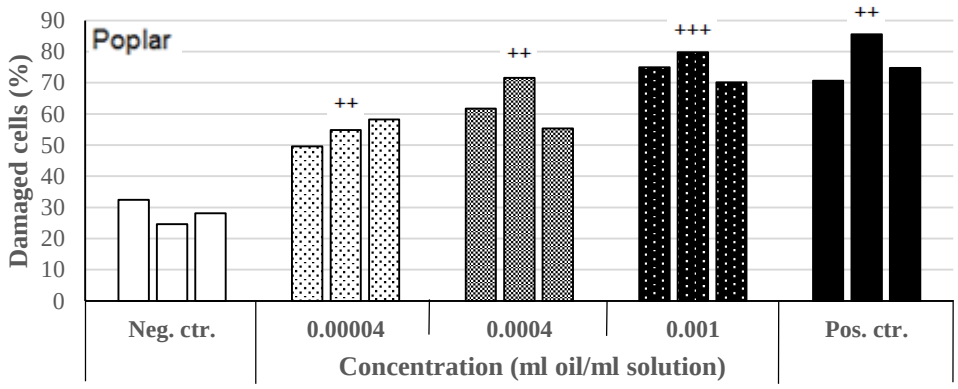
Onions exposed to pyrolysis oil from beech feedstock had a similar trend as for the ones exposed to the poplar-oil, with clearly significant and concentration-dependent increases in frequency of damaged cells compared with negative control (Fig. 3.3b). However, the percentage of damaged cells was slightly lower for the ones exposed to the beech-oil within all the three concentrations. The percentage of damage for onions exposed to the two highest concentrations of the poplar- and beech-oil was not significantly different from positive control (Appendix D).

The onions exposed to the pyrolysis oil from spruce feedstock showed a significantly higher frequency of damage for all the concentrations compared with negative control (Fig. 3.3c). However, no apparent differences in percentage of damaged cells with increasing concentrations were observed, and the percentage for all the concentrations was clearly significant different from positive control (Appendix D). Moreover, the onions exposed to the lowest concentration of this oil had a similar percentage of damage as for the two other oils, while the onions exposed to the two highest concentrations had a percentage of damage that was clearly lower compared with the other oils.

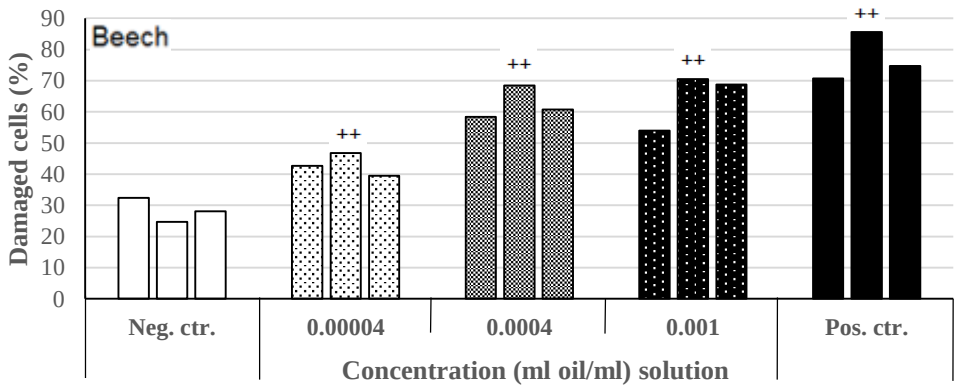
The percentage of damage in onions exposed to the 'blind' solutions was not significantly different from the once exposed to negative control (Fig. 3.3d), and positive control was clearly significant different from negative control.

Raw data for the total number of damaged and normal dividing cells for each of the treatment-groups are presented in Appendix B.

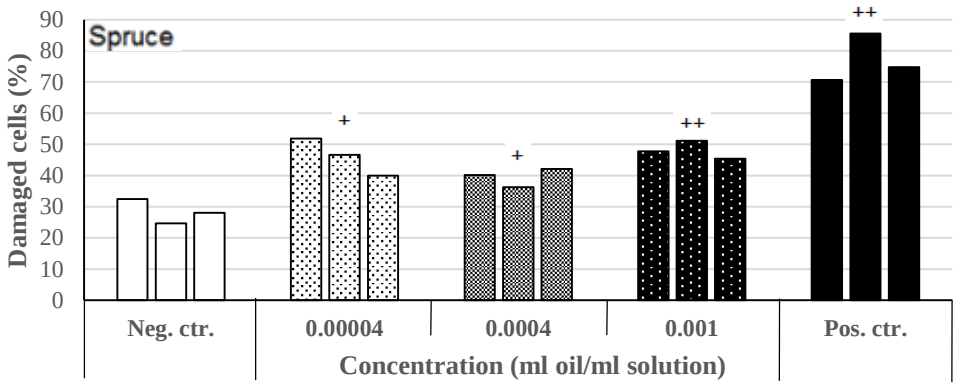
a)



b)



c)



d)

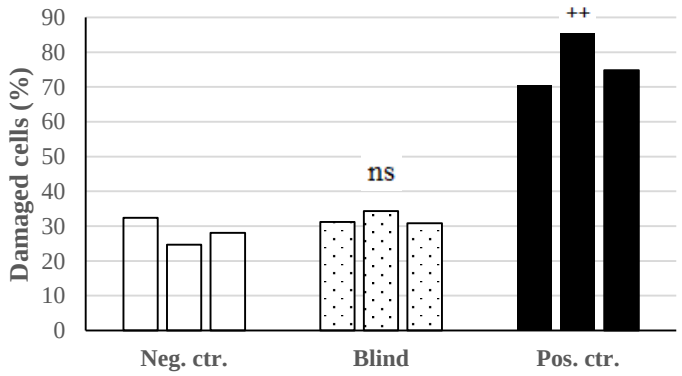


Figure 3.3. Damaged dividing cells. Damaged dividing cells observed in meta-, ana- and telophase of root meristems of *Allium cepa* onions after 72 hours exposure to pyrolysis oils generated from fast pyrolysis of poplar (a), beech (b) or spruce (c) feedstocks at three different concentrations (0.00004, 0.0004 and 0.001 ml oil/ml solution (tap water+dimethyl sulfoxide, DMSO)). The data are given as percentage (%) of total dividing cells. The percentage of damaged cells for onions exposed to a 'blind' solution (tap water+DMSO) for each concentration (0.00004, 0.0004 and 0.001 ml/ml, respectively) was also included, as well as the percentage for negative control (tap water)- and positive control (methyl methanesulfonate, MMS)-exposed onion roots for comparison. Three parallels of onions are included for each treatment, one column representing one individual. Different symbols denote the means of the different treatment-groups that are not significantly different (ns) or significantly different (weakly (-/+), clearly (--/++) or strongly (---/+++)) from the mean of negative control using a two-sample (independent group) t-test ($p<0.05$).

3.3.2 Distribution of dividing phases

The distribution of metaphase, anaphase and telophase was examined on the microscopic slides made from *Allium cepa* roots exposed to the different treatment solutions (negative control, different concentrations of pyrolysis oils generated from poplar, beech and spruce feedstocks, a 'blind' control for each concentration and a positive control) (Fig. 3.4). Three onions were pooled for each treatment-group, except for the 'blind' solutions (one onion per concentration). The distribution of phases from onions exposed to all the different treatments showed a similar pattern, with metaphase, and partially telophase, being the dominant phases (~42-59 and 30-44%, respectively), while the anaphase contributed to a small part of the total phases (~7-16%).

Raw data for the number of cells in the different division stages are presented in Appendix B.

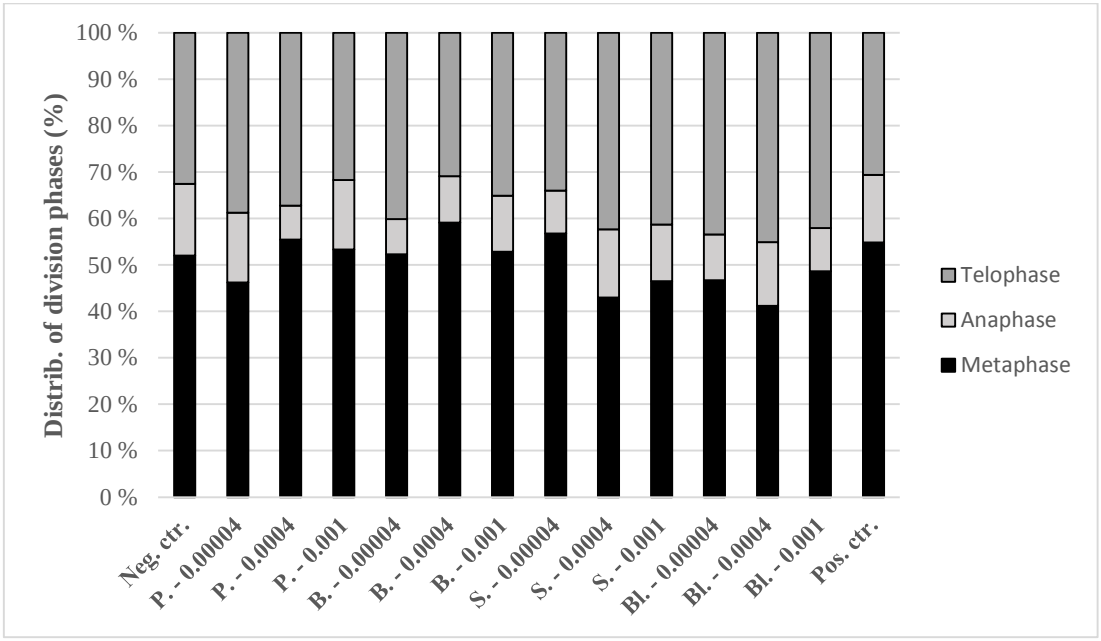


Figure 3.4. Distribution of dividing phases. The distribution of dividing phases (meta-, ana- and telophase) observed in root tip meristems of *Allium cepa* onions after 72 hours exposure to different treatment-groups; a negative control (tap water), pyrolysis oils generated from fast pyrolysis of poplar, beech and spruce feedstocks at different concentrations (0.00004, 0.0004, 0.001 ml oil/ml solution (tap water+dimethyl sulfoxide, DMSO)), a 'blind' solution (tap water+DMSO) for each concentration, and a positive control (methyl methanesulfonate, MMS), respectively. Results from three parallels of onions are pooled for each treatment-group, except for the 'blind' treatments (one onion per concentration).

3.3.3 Frequency of damaged dividing cells within the dividing phases

The percentage of damaged dividing cells in meta-, ana- and telophase was analyzed for triplicates of onions exposed to pyrolysis oils from poplar, beech and spruce feedstock of different concentrations, namely 0.00004, 0.0004 and 0.001 ml oil/ml solution. A 'blind' solution for each concentration was included (one onion for each concentration), as well as triplicates of onions exposed to a negative control (tap water) and a positive control (MMS) for comparison.

3.3.3.1 Metaphase

The frequency of damaged cells in metaphase of onions exposed to all the three types of pyrolysis oils was apparently higher than negative control and lower than positive control, and the positive control had a clearly significant higher frequency compared with negative control.

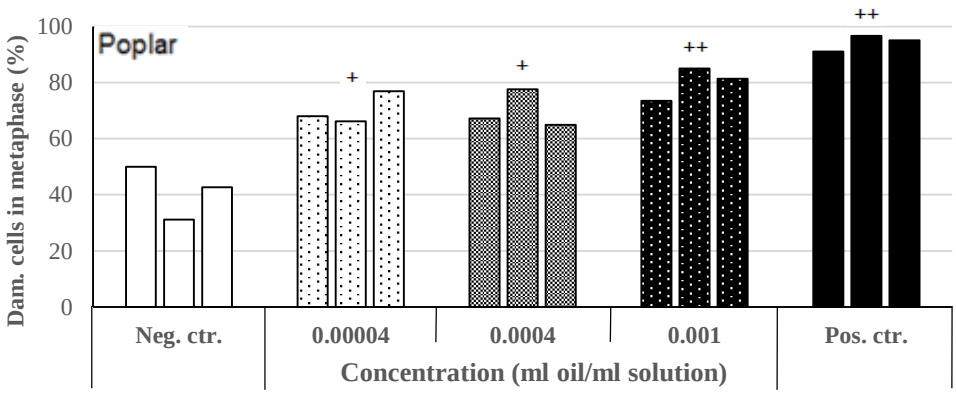
The frequency of damaged cells in metaphase for the onions exposed to pyrolysis oil from poplar feedstock was approximately similar for the two lowest concentrations (weakly significant higher compared with negative control), and then had a slight increase to the highest concentration (clearly significant higher compared with the negative control and almost as high as for the positive control) (Fig. 3.5a).

The percentage of damaged cells in metaphase for the onions exposed to the pyrolysis oil from beech feedstock seemed to be higher for the lowest concentration compared with negative control, although not significantly (Fig. 3.5b). The percentage of damage for onions exposed to the two highest concentrations was clearly significant higher than negative control and almost as high as positive control.

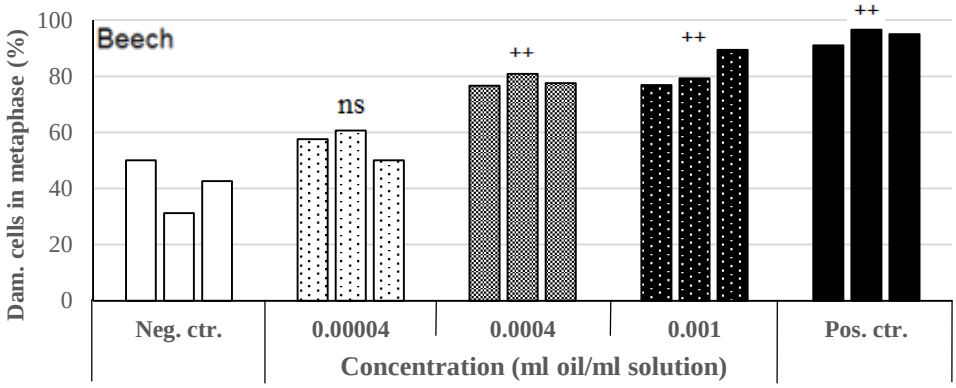
The onions exposed to the pyrolysis oil made from spruce feedstock showed a somewhat different pattern (Fig. 3.5c). The ones exposed to the lowest and highest concentrations showed a weakly significant higher frequency of damaged cells in metaphase compared with negative control, while the ones exposed to the 0.0004-concentration, although appearing to be higher, showed no significantly differences compared with negative control.

The percentage of damaged cells in metaphase for the onions exposed to the different concentrations of 'blind' solutions was not significantly higher compared with negative control (Fig. 3.5d).

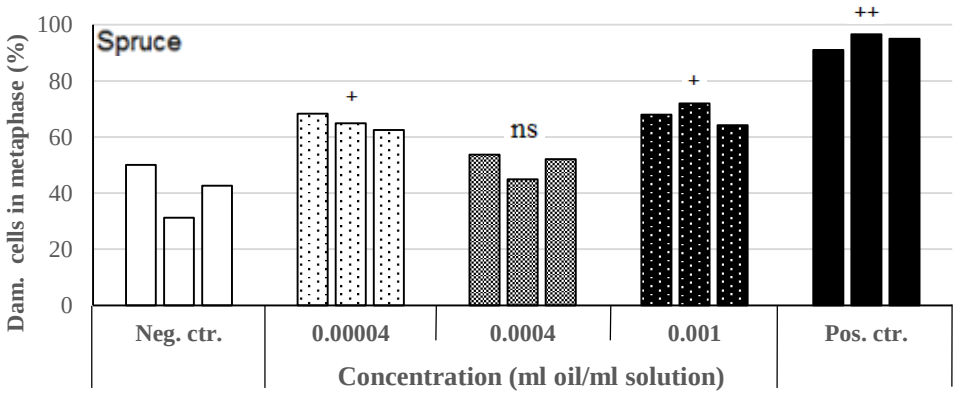
a)



b)



c)



d)

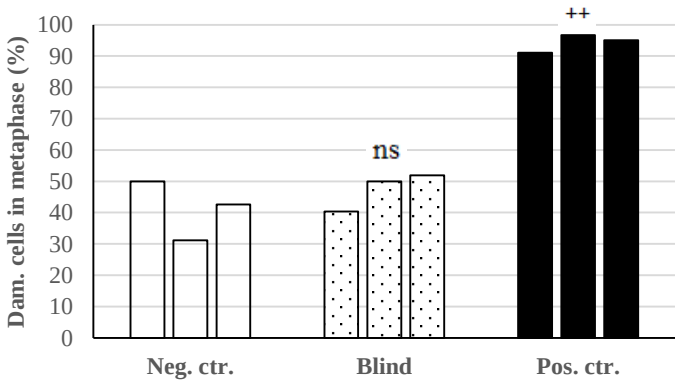


Figure 3.5. Damaged dividing cells in metaphase. Damaged dividing cells observed in metaphase of root meristems of *Allium cepa* onions after 72 hours exposure to pyrolysis oils generated from fast pyrolysis of poplar (a), beech (b) or spruce (c) feedstocks at three different concentrations (0.00004, 0.0004 and 0.001 ml oil/ml solution (tap water+dimethyl sulfoxide, DMSO)). The data are given as percentage (%) of total dividing cells in metaphase. The percentage of damaged cells for onions exposed to a 'blind' solution (tap water+DMSO) for each concentration (0.00004, 0.0004 and 0.001 ml/ml, respectively) was also included, as well as the percentage for negative control (tap water)- and positive control (methyl methanesulfonate, MMS)-exposed onion roots for comparison. Three parallels of onions are included for each treatment, one column representing one individual. Different symbols denote the means of the different treatment-groups that are not significantly different (ns) or significantly different (weakly (-/+), clearly (--/++) or strongly (---/+++)) from the mean of negative control using a two-sample (independent group) t-test ($p<0.05$).

3.3.3.2 Anaphase

All the onions exposed to different pyrolysis oils and concentrations of oils had an apparently higher percentage of damaged cells in anaphase compared with negative control. However, for all the oils, no pattern of increase in damage was found with increasing concentrations. A relatively high variation in percentage of damaged cells in anaphase was found between the onions within each treatment-group compared with the other cell-division phases. The positive control had a clearly significant higher frequency compared with negative control.

The percentage of damaged cells in anaphase was clearly significant higher for the onions exposed to the lowest and the highest concentrations of poplar-oil compared with negative control (Fig. 3.6a). The onions exposed to the 0.0004-concentration seemed to have a higher frequency of damaged cells in anaphase compared with negative control, although not significantly due to a high variation between the onions.

For the onions exposed to the pyrolysis oil from beech feedstock, the lowest and highest concentrations had a clearly significant higher percentage of damaged cells in anaphase, and the ones exposed to 0.0004-concentration had a weakly significant higher percentage, compared with negative control (Fig. 3.6b).

For the onions exposed to the spruce-oil, the percentage of damaged cells in anaphase for the lowest concentration was strongly significant higher than for the negative control-group (Fig. 3.6c). The onions exposed to the two highest concentrations, however, had a clearly significant higher percentage compared with negative control.

The onions exposed to a ‘blind’ solution of each concentration showed a weakly significant higher percentage of damaged cells in anaphase compared with negative control, with a decreasing trend of damage with increasing “concentrations” (Fig. 3.6d).

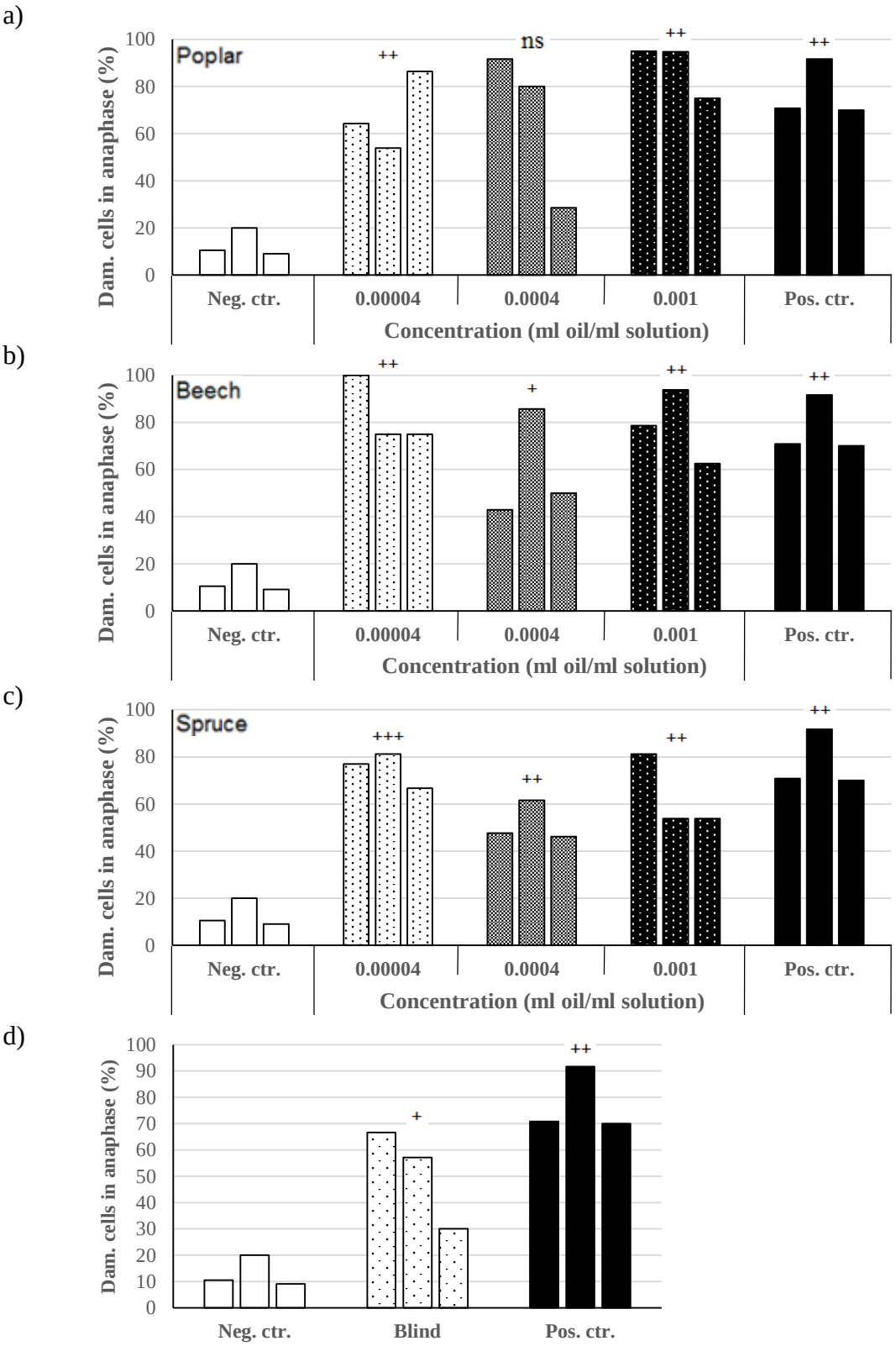


Figure 3.6. Damaged dividing cells in anaphase. Damaged dividing cells observed in anaphase of root meristems of *Allium cepa* onions after 72 hours exposure to pyrolysis oils generated from fast pyrolysis of poplar (a), beech (b) or spruce (c) feedstocks at three different concentrations (0.00004, 0.0004 and 0.001 ml oil/ml solution (tap water+dimethyl sulfoxide, DMSO)). The data are given as percentage (%) of total dividing cells in anaphase. The percentage of damaged cells for onions exposed to a 'blind' solution (tap water+DMSO) for each concentration (0.00004, 0.0004 and 0.001 ml/ml, respectively) was also included, as well as the percentage for negative control (tap water)- and positive control (methyl

methanesulfonate, MMS)-exposed onion roots for comparison. Three parallels of onions are included for each treatment, one column representing one individual. Different symbols denote the means of the different treatment-groups that are not significantly different (ns) or significantly different (weakly (-/+), clearly (--/++) or strongly (---/+++)) from the mean of negative control using a two-sample (independent group) t-test ($p<0.05$).

3.3.3.3 Telophase

The percentage of damaged cells in telophase was relatively low compared with the other cell-division phases. The percentage of damage for the positive control showed a high variation between the three onions, and was weakly significant higher compared with negative control.

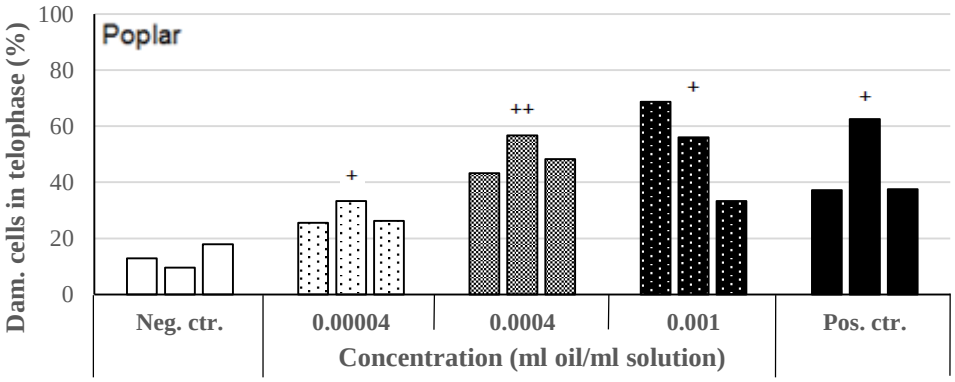
The percentage of damaged cells in telophase of onions exposed to pyrolysis oil from poplar feedstock was weakly significant higher for the lowest and the highest concentration, and was clearly significant higher for the 0.0004-concentration, compared with negative control (Fig. 3.7a). The variation in percentage of damaged cells was relatively high for onions exposed to the highest concentration.

For the onions exposed to the pyrolysis oil from beech feedstock, the percentage of damaged cells in telophase was not significant higher, clearly significant higher and weakly significant higher for the lowest, middle and highest concentrations, respectively, compared with negative control (Fig. 3.7b).

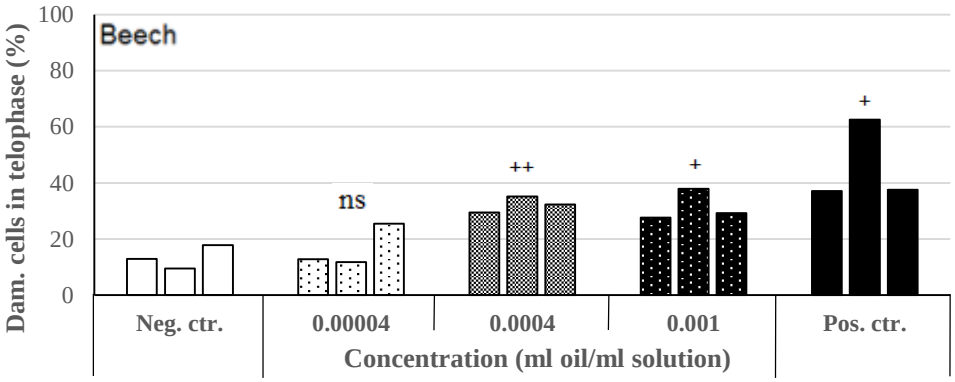
For all the concentrations, the percentage of damaged cells in telophase for onions exposed to spruce-oil was not significantly higher compared with negative control, although it appeared higher for the ones exposed to the two highest concentrations compared with the lowest concentration (Fig. 3.7c).

For the onions exposed to ‘blind’ solutions of different concentrations, the percentage of damaged cells in telophase was not significantly higher compared with negative control (Fig. 3.7d).

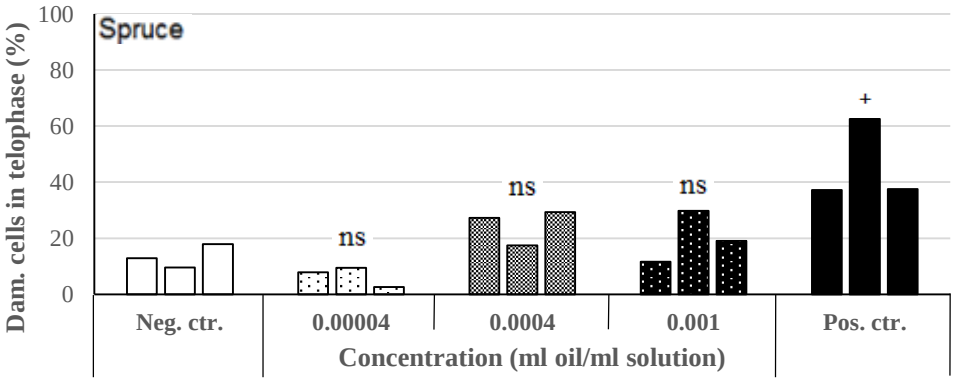
a)



b)



c)



d)

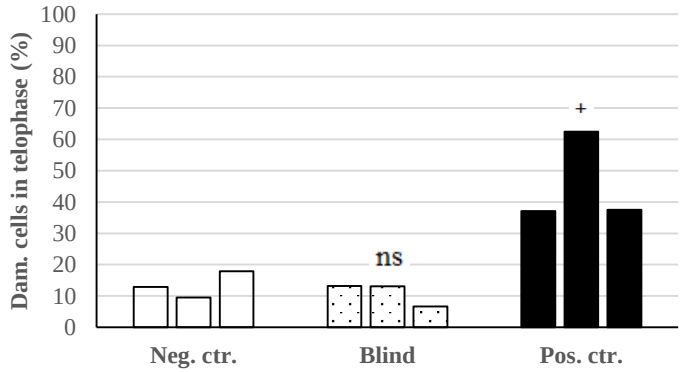


Figure 3.7. Damaged dividing cells in telophase. Damaged dividing cells observed in telophase of root meristems of *Allium cepa* onions after 72 hours exposure to pyrolysis oils generated from fast pyrolysis of poplar (a), beech (b) or spruce (c) feedstocks at three different concentrations (0.00004, 0.0004 and 0.001 ml oil/ml solution (tap water+dimethyl sulfoxide, DMSO)). The data are given as percentage (%) of total dividing cells in telophase. The percentage of damaged cells for onions exposed to a 'blind' solution (tap water+DMSO) for each concentration (0.00004, 0.0004 and 0.001 ml/ml, respectively) was also included, as well as the percentage for negative control (tap water)- and positive

control (methyl methanesulfonate, MMS)-exposed onion roots for comparison. Three parallels of onions are included for each treatment, one column representing one individual. Different symbols denote the means of the different treatment-groups that are not significantly different (ns) or significantly different (weakly (-/+), clearly (--/++) or strongly (---/+++)) from the mean of negative control using a two-sample (independent group) t-test ($p<0.05$).

3.3.4 Specific chromosomal aberrations observed in dividing cells

The mean number of different chromosomal aberrations (bridges, c-metaphases, disturbed metaphases, fragments, laggards, polyploids, star anaphases stickiness and vagrants), as percentage of total dividing cells, was measured from triplicates of onions exposed to pyrolysis oils generated from fast pyrolysis of poplar, beech or spruce feedstocks at three different concentrations (0.00004, 0.0004 and 0.001 ml oil/ml solution) (Table 3.2). The percentage of aberrations for onions exposed to a 'blind' solution of each concentration (one onion per concentration) was also included, as well as the mean percentage of triplicates of onions exposed to negative control (tap water) and to positive control (MMS) for comparison.

The most dominant aberration in all treatment-groups was disturbed metaphases. Other dominant damages were vagrants, laggards and c-metaphases. All these types of aberrations had a clearly higher frequency for the onions exposed to their positive control compared with their respective negative control. Most of the frequency-values of these aberrations for the exposure-groups of onions lay within the values of their respective negative- and positive control-group. Fragments, polyploidy and star anaphases contributed to a very low frequency of the total number of dividing cells for all of the treatment-groups.

For onions exposed to the pyrolysis oil generated from poplar feedstock, the frequency of disturbed metaphases, fragments, laggards, vagrants and polyploidy was increased with higher concentrations. The poplar-oil did not induce a significantly increase in frequency of fragments or c-metaphases compared with negative control (Appendix D). The frequency of bridges was only significantly higher for onions exposed to the lowest concentration of poplar-oil compared with negative control (Appendix D). Within the disturbed metaphase-, laggard-, and vagrant-types of aberrations, the poplar-oil induced an apparent higher frequency compared with negative control, and the frequency of vagrants was apparently higher for the onions exposed to the poplar-oil compared with the frequency for the onions exposed to the two other oils. The frequency of stickiness was significantly higher for onions exposed to all the concentrations of the poplar-oil compared with negative control (Appendix D). A few polyploid cells and cells

with star anaphases were found for onions exposed to the highest and lowest concentration of poplar-oil.

The frequency of fragments, laggards, vagrants, polyploidy and stickiness was increased with increasing concentrations of the pyrolysis oil generated from beech feedstock. For the bridge- and fragment-types of aberrations, the beech-oil did not induce a significantly higher frequency compared with negative control (Appendix D). The onions exposed to the beech-oil appeared to have a higher frequency of c-metaphases, disturbed metaphases and laggards compared with negative control, although not significantly for most of the concentrations (Appendix D). The frequency of vagrants was significantly higher for the onions exposed to all the concentrations of beech-oil compared with negative control (Appendix D). A few polyploid cells were found in onions exposed to the two highest concentrations of the beech-oil. No star-anaphases were detected. Stickiness was found at a higher frequency for onions exposed to the two highest concentrations of beech-oil compared with negative control, although only significantly for the ones exposed to the 0.0004-concentration (Appendix D).

Only the frequency of polyploidy and star anaphases was increased with increasing concentrations for the onions exposed to the pyrolysis oil generated from spruce feedstock. The frequency of bridges and fragments was not higher for onions exposed to spruce-oil compared with the ones exposed to negative control. The frequency of c-metaphases was apparently higher only for onions exposed to the lowest concentration of the spruce-oil compared with negative control. Disturbed metaphases seemed to exhibit a higher frequency in onions exposed to the lowest and highest concentration of the spruce-oil compared with negative control; however, the onions exposed to the two highest concentrations had a lower frequency compared with the respective ones from the poplar- and beech-oil. The frequency of laggards was significantly higher for the onions exposed to the two highest concentrations of the spruce-oil compared with negative control (Appendix D); however, it was apparently lower compared with the respective ones from the two other oils. Within the vagrant-type of aberration, the onions exposed to the two highest concentrations of spruce-oil had a significantly higher frequency compared with negative control (Appendix D). Some polyploidy and star anaphases were found for onions exposed to the two highest concentrations of the spruce-oil. No apparent increases were found in frequency of stickiness for onions exposed to the spruce-oil compared with negative control, except for a weakly significant increase for the 0.0004-concentration (Appendix D).

No clear pattern of increasing frequency within the types of chromosomal aberrations with increasing “concentrations” of the ‘blind’ solutions was observed, except for the c-metaphase. The onion roots exposed to ‘blind’ solutions had no significant higher frequency of chromosomal aberrations compared with those exposed to negative control, except for a weakly significant larger frequency for the vagrant aberration type (Appendix D).

The different types and numbers of aberrations detected within each treatment-group, and pictures of different types of aberrations observed during the microscopic examination period of this master’s project are presented in Appendix C and Appendix E, respectively.

Table 3.2: Specific chromosomal aberrations. Mean (±SEM) number of specific chromosomal aberrations (CAs) as a percentage (%) of total dividing cells found in root meristems of *Allium cepa* onions after 72 hours exposure to pyrolysis oils generated from fast pyrolysis of poplar, beech and spruce feedstocks at different concentrations (0.00004, 0.0004, 0.001 ml oil/ml solution (tap water+dimethyl sulfoxide, DMSO)). The percentage in onions exposed to a 'blind' solution (tap water+DMSO) for each concentration (0.00004, 0.0004 and 0.001 ml/ml, respectively) was also included, as well as the percentage for negative control (tap water)- and positive control (methyl methanesulfonate, MMS)-exposed inion roots for comparison. Results from three parallels of onions are pooled for each treatment-group, except for the 'blind' treatment (one onion per treatment).

Treatment	Conc.	Specific aberrations as % of total dividing cells								
		B.	C-M.	D.M.	F.	L.	P.	Star	S.	V.
Poplar	0.00004	9.61 (±0.11)	4.09 (±1.57)	19.87 (±3.22)	2.17 (±0.90)	8.47 (±1.30)	0.00 (±0.00)	0.32 (±0.32)	8.11 (±0.98)	15.88 (±4.50)
	0.0004	2.69 (±0.39)	3.40 (±0.76)	25.22 (±6.07)	2.53 (±1.22)	12.67 (±0.82)	0.00 (±0.00)	0.00 (±0.00)	10.37 (±1.83)	16.20 (±2.73)
	0.001	6.69 (±2.44)	4.40 (±2.71)	28.14 (±8.58)	2.88 (±1.12)	15.64 (±6.36)	0.62 (±0.62)	0.00 (±0.00)	9.56 (±2.05)	23.30 (±6.60)
Beech	0.00004	6.69 (±1.36)	8.02 (±1.55)	21.84 (±2.95)	1.83 (±0.08)	6.33 (±0.62)	0.00 (±0.00)	0.00 (±0.00)	0.28 (±0.28)	9.89 (±1.69)
	0.0004	4.00 (±1.57)	15.04 (±3.32)	27.18 (±4.55)	2.96 (±0.57)	11.49 (±4.11)	0.52 (±0.52)	0.00 (±0.00)	3.74 (±0.74)	11.88 (±0.92)
	0.001	5.22 (±2.15)	9.47 (±2.88)	26.40 (±5.43)	3.63 (±0.57)	15.82 (±3.27)	1.91 (±1.05)	0.00 (±0.00)	5.17 (±1.93)	16.67 (±3.67)
Spruce	0.00004	4.58 (±2.08)	16.13 (±2.58)	20.07 (±1.14)	4.00 (±0.73)	6.37 (±2.26)	0.00 (±0.00)	0.00 (±0.00)	0.92 (±0.58)	7.77 (±1.92)
	0.0004	4.57 (±2.28)	5.39 (±1.02)	12.44 (±0.87)	1.59 (±0.37)	10.16 (±1.45)	0.33 (±0.33)	0.28 (±0.28)	4.89 (±1.49)	15.79 (±1.22)
	0.001	1.75 (±0.09)	6.62 (±0.69)	19.25 (±1.02)	2.24 (±1.17)	6.66 (±1.07)	3.16 (±0.39)	0.57 (±0.29)	2.32 (±1.32)	12.76 (±0.39)
Blind	0.00004	7.38	1.64	13.11	0.82	4.10	2.46	1.64	1.64	9.84
	0.0004	8.82	6.86	13.73	1.96	5.88	0.00	0.00	0.00	10.78
	0.001	4.67	12.15	9.35	0.93	2.80	0.00	0.00	2.80	5.61
Neg. ctr.		4.32 (±0.40)	5.68 (±2.84)	15.08 (±3.78)	2.70 (±0.98)	2.54 (±0.22)	0.31 (±0.31)	0.00 (±0.00)	0.54 (±0.28)	3.00 (±0.59)
Pos. ctr.		11.63 (±3.40)	13.45 (±2.96)	36.73 (±3.57)	2.40 (±1.27)	15.94 (±2.37)	0.29 (±0.29)	1.29 (±0.64)	1.54 (±0.29)	20.50 (±3.29)

B., bridge; C-M., c-metaphase; D.M., disturbed metaphase; F., fragment; L., laggard; V., vagrant; P., polyploidy; Star, star anaphase; S., stickiness

4 Discussion

In this chapter the results will be discussed in the same order as presented in Chapter 3. Firstly, evaluation and possible explanations of the different results will be conducted, followed by relating the trends of (geno)toxic damage to the composition of the pyrolysis oils. Further, optimalization and evaluation of the method will be conducted in the same order as presented in Chapter 2. Finally, the relevance of testing pyrolysis oils in an *Allium cepa* test will be evaluated.

4.1 Discussion of results

4.1.1 Root inhibition test

As expected, all the dose-response curves for the onions exposed to the three different pyrolysis oils, generated from poplar, beech and spruce feedstocks, showed a sigmoid shape, with a decreasing percentage of root length with increasing concentrations, indicating the presence of toxic substances in the oils having sub lethal effects on plants. Sustained root growth is regulated by cell division in the division zone of the apical meristem and by cell elongation in the elongation zone (Obroucheva, 2008, Shishkova et al., 2008). These events occur simultaneously in the root tips of *Allium cepa* onions. Thus, chemical agents affecting any part of these processes, like metabolism, protein/DNA/RNA synthesis, spindle functionality etc., may inhibit root growth. Since the pathways leading to root growth inhibition are many, it is difficult to discover which mechanisms the oils exhibit that are causing the toxic effects on the roots.

Supporting our results, Pekol et al. (2012) also detected reduction in growth of *A. cepa* roots with increasing concentrations of liquid products obtained from the pyrolysis of hazelnut shell. Other studies have also indicated toxic effects of different pyrolysis oils, like decreased survival (Chatterjee et al., 2013) and immobilization of living organisms (Pimenta et al., 2000, Park et al., 2008). It should be noted, however, that there are conflicting reports on the toxicity of pyrolysis oils. Within the Biotox project, ecotoxic effects, like inhibition of growth and immobilization, were tested in algal cultures and in *Daphnia magna*, respectively, after exposure of different pyrolysis oils (Girard et al., 2005). Twenty-one pyrolysis oils were tested, 19 from fast pyrolysis and two from slow pyrolysis. Twelve pyrolysis oils showed no ecotoxic

effects, eight pyrolysis oils showed very weak ecotoxic effects, and one pyrolysis oil showed ecotoxic effects (slow pyrolysis sample). The variable results from different studies are probably due to the use of different pyrolysis oils derived from different pyrolysis processes and biomass feedstock, resulting in different chemical compositions, as well as the use of different toxicity tests and concentrations.

The sigmoid shapes of the dose-response curves for the three oils were different from each other, which indicates a somewhat different toxicity of the oils. Both the poplar- and the spruce-oil showed steep decreases in percentage of root length between two concentrations (0.0004 to 0.001, and 0.001 to 0.004 ml oil/ml solution, respectively), indicating that the threshold for toxic effect of these oils lay in-between these concentrations. The dose-response curve of the beech-oil, however, showed a gradually decreasing pattern, which corresponds to a gradually increase in toxicity between all the concentrations. This indicates that the beech-oil already has reached its threshold for toxicity at the lowest concentration (0.00001 ml oil/ml solution), or that the threshold is absent. For all the oils, a threshold for reaching the maximum response is obtained at the 0.004-concentration, and from here, no additional decrease in percentage of root length (increase in toxicity) is observed with increasing concentrations.

The root growth of onions exposed to the 'blind' solutions showed no significant difference with increased "concentrations", which suggests that the DMSO present in the oil-solutions does not affect the interpretation of the comparisons between oils and concentrations of oils, even if they induced a slight root growth inhibition. The root growth of onions exposed to MMS was not inhibited, as might be expected since MMS clearly disturbs proliferation in the root meristem (Section 4.1.2). This may be due to the possible continuing of roots to grow due to elongation of pre-existing cells, and emphasize that MMS can not be used as a positive control when detecting toxic effects in a root inhibition test.

Toxicity expressed as the half maximal effective concentration, calculated from the dose-response curves obtained for each of the pyrolytic oils, seemed to be lowest for the spruce derived oil, with a concentration of 0.001816 ml oil/ml solution needed to reduce the relative root growth by 50%. The poplar- and beech-oil seem to exert more toxicity, with concentrations of 0.000605 and 0.000364 ml oil/ml solution, respectively, needed to reduce the relative root growth by half. Caution should be taken, however, when comparing the EC_{50} values between the oils. The curves for the poplar- and spruce-oil have a steep decrease only between two of

the measured concentrations, and thus, increasing concentrations in-between these two concentrations cause a relatively great increase in inhibition of the root growth compared with the beech-oil that exerts a gradually increase between all the concentrations.

The appearance of the roots did vary between different concentrations of the oils, with changes from fresh, stiff and light roots to a gradually higher degree of brown and soft roots with higher concentrations. At treatments causing high toxicity, the roots will be softer, slacken and die, and the root tips may turn brownish due to toxic effects causing cell death (Fiskesjö, 1985). This, along with the root inhibition results, support a higher toxicity with increasing concentrations of the oils. The evenly brown colour throughout the whole root at the highest concentrations of the oils (0.004-0.04 ml oil/ml solution) may indicate that all the cells in these roots were dead, and this assumption corresponds well with the root inhibition results, showing no relative root growth at concentrations above 0.004 ml oil/ml solution.

4.1.2 Mitotic index (MI)

As expected, all of the different types and concentrations of pyrolytic liquid exposures resulted in a reduction of the mitotic index in the *Allium cepa* root tips compared with negative control. Inhibition of mitotic activity can be used as a measure of cytotoxicity (Linnainmaa et al., 1978, Smaka-Kincl et al., 1996, Leme and Marin-Morales, 2009), due to the cytotoxic effects on cell function resulting in an inability of cells to proliferate (Park et al., 2008). Hence, these results illustrate a cytotoxic potential of fast pyrolysis oils derived from poplar, beech and spruce feedstocks. Cytotoxic effects may be considered as an early indication of cellular damage, because these disturbances frequently appear long before genotoxic effects manifest, or even in the absence of the latter (Park et al., 2008).

A mitotic index significantly lower than negative control may indicate alterations, deriving from the chemical action, in the growth and development of exposed organisms (Leme and Marin-Morales, 2009). Reduction in the mitotic index could be due to inhibition of DNA synthesis at S-phase (Sudhakar et al., 2001), or inactivation or repression of control sites (G1- or G2-stage), by direct or indirect actions of chemicals, may prolong interphase and prevent the cell from entering mitosis (Van't Hof, 1968, Webster and Davidson, 1969). Such mito-depressive effects may also be due to the inhibition of RNA- (González-Fernández et al., 1974, Chauhan et al., 1998) and protein synthesis (Rost, 1984), or deficiency of DNA and RNA

contents in the nucleus of dividing cells (El-Ghamery et al., 2000). In addition, the rate of mitosis has been shown to be related to the ATP level (Epel, 1963), and in this sense, compounds in the pyrolytic oils may function as inhibitors of energy metabolism, and thus, disturb mitosis.

Similar effects on mitotic index were described by Pekol et al. (2012) studying the effect of liquid products obtained from the pyrolysis of hazelnut shell on an *Allium cepa* test system. Morever, Park et al. (2008) observed a decrease in cell viability in a mammalian cell line exposed to a fast pyrolysis oil from radiata pine compared with control cells, and Chatterjee et al. (2013) showed an increase in cytotoxicity in cultured cell systems when testing toxicity of a slow pyrolysis oil produced from biomasses of rice straw and sawdust of oak three compared with an untreated control. However, Park et al. (2008), Pekol et al. (2012) and Chatterjee et al. (2013) observed dose-dependent cytotoxicity. This is in conflict with the results in this study, which showed no clear pattern of decrease of mitotic index with increasing concentrations, with quite similar index values between different oils and concentrations of oils. The absence of a concentration-dependent cytotoxicity might be due to the concentrations being too close to each other, making it difficult to detect any clear difference in mitotic index. It should be noted that even though a clear pattern of concentration-dependent increase was not detected, the mitotic index seemed to be decreasing with higher concentrations. Pekol et al. (2012) observed a much clearer concentration-dependency after exposure to the hazelnut-pyrolysis oil for 24 hours, compared with the dependency after 48 hours of exposure, which is the normal exposure period in an *Allium cepa* test. This indicates that the differences are diminishing with increasing exposure times, and that the concentration-dependent decrease of the mitotic index might have been higher in the present master's project at exposure times lower than 72 hours.

As expected, the mitotic index for the 'blind' solutions was not significantly different from negative control, indicating that DMSO did not show any significant cytotoxic effects on the *Allium cepa* test system. The MI for the positive control, however, was weakly significant lower compared with negative control, and quite similar to the MI for the onions exposed to different concentrations of the three different pyrolysis oils. Other studies have shown contradictory results when comparing the MI for MMS (10 mg/l) and negative control in an *Allium cepa* test, where some authors have found a decreased MI (Rank and Nielsen, 1997, Rank et al., 2002, Rank, 2003, Bolle et al., 2004, Grisolia et al., 2004, Barbosa et al., 2010, Tabrez and Ahmad, 2011, Tabrez and Ahmad, 2012), some have found an increased MI (Evandri et al., 2003,

Siddiqui et al., 2011) and others have found both decreased and increased MI (Rank and Nielsen, 1994, Rank and Nielsen, 1998, Promkaew et al., 2010) compared with negative control. This implies that the MMS can not with certainty be used as a positive control when testing for cytotoxic effects, as is also the case when testing the toxic effects in the root inhibition test.

4.1.3 Damaged dividing cells

4.1.3.1 Frequency of damaged dividing cells

The onions exposed to all the concentrations of the different pyrolysis oils had a significant higher percentage of damaged dividing cells compared with negative control. These results correspond well with the cytotoxicity results, showing significant decreases in mitotic index for all the concentrations of poplar, beech and spruce pyrolysis oils, and were expected, as whole sample studies of different types of pyrolysis oils have revealed genotoxic potential. Pekol et al. (2012), who to my knowledge are the only ones who have studied the genotoxicity of a pyrolysis oil in an *Allium cepa* test, showed a concentration-dependent increase in the percentage of chromosomal aberrations in onions after exposure to fast pyrolysis oil from hazelnut shell. Park et al. (2008) showed that the liquid fraction of a fast pyrolysis oil from radiata pine seemed to have a genotoxic property in a comet assay; however, no genotoxic effect was observed for the tar fraction at any of the concentrations studied. This may indicate that the liquid fraction of the oil, as examined in this study, has the highest genotoxic potential. Additionally, Chatterjee et al. (2013) found a dose-dependent increase in genotoxicity of cultured cell systems in a comet assay after exposure to pyrolysis oils from slow pyrolysis of rice straw and sawdust of oak tree.

The onions exposed to the different pyrolysis oils exhibited a different pattern in their percentage of damage. The ones exposed to poplar- and beech-oil showed a concentration-dependent increase in damage, as did Pekol et al. (2012) and Chatterjee et al. (2013), while the spruce-oil showed no apparent differences in percentage of damage with increasing concentrations. This resembles the root inhibition results, which show somewhat similar percentage of root growth for all of the three concentrations (0.00004, 0.0004 and 0.001 ml oil/ml solution) of onions exposed to spruce-oil, while much clearer differences is observed between the three concentrations for the onions exposed to the poplar- and beech-oil. This might suggest that concentrations resulting in a steep increase for both toxicity and genotoxicity not

yet have been reached at the highest concentration (0.001 ml oil/ml solution) for the onions exposed to the spruce-oil.

While the percentage of damage was fairly similar between onions exposed to the three different oils at the lowest concentration, the onions exposed to the two highest concentrations of the poplar- and beech-oil had a percentage of damage that was not significantly different from positive control. The onions exposed to the two highest concentrations of the spruce-oil, on the other hand, had a percentage of damage that was clearly lower than for the other oils, and had a significantly lower percentage of damage compared with positive control. This indicates a high genotoxic potential of the poplar- and beech-oil at these concentrations. Additionally, exposure of the poplar-oil caused a higher percentage of damage at these concentrations compared with exposure to the beech-oil, indicating that the poplar-oil exerted the highest percentage of damage.

The percentage of damage in onions exposed to the 'blind' solutions was not significantly different from the once exposed to negative control. This was expected as, with few exceptions, a large battery of *in vitro* and *in vivo* studies have confirmed a lack of genotoxic potential of DMSO (McCann et al., 1975, Latt et al., 1981, Leifer et al., 1981, Heidelberger et al., 1983, Lee et al., 1983, Brockman et al., 1984, Valencia et al., 1984). The positive control induced a clearly significant higher frequency of damaged cells compared with negative control, as expected since MMS often is used as a positive control in mutagenicity testing, and is a potent inducer of chromosomal aberrations (Rank and Nielsen, 1997).

4.1.3.2 Distribution of dividing phases

The distribution of phases from onions exposed to different treatments showed a similar pattern, with metaphase dominating (~42-59%), followed by telophase (~30-44%), while anaphase contributed to a small part of the dividing phases (~7-16%). Cell division takes approximately the same time in cells of the same cell type, provided that the cells are from individuals of the same species, and that the cell cycle is not affected by genotoxic compounds. The similar pattern of the different oils and the negative control indicates that components in the oils do not affect the distribution of the cell phases. The duration of different dividing phases has been determined for *A. cepa* roots, and has here been modified to gain percentage of duration time for the meta-, ana- and telophase in the DNA-division cycle. The metaphase contributed to

24%, the anaphase to 18% and the telophase to 59% of total dividing cells (in meta-, ana- and telophase) (Utrilla et al., 1993). Comparing with these numbers, too many cells are present in metaphase, and too few are present in telophase. The underestimated number of cells in telophase may be due to difficulty of detecting these cells. Cells in telophase are the ones that are most similar to cells in interphase, especially when other cells are partially covering them, or when cells are indistinct.

Pekol et al. (2012) found a more similar pattern of distribution of phases as observed in this study. For normal cells, the negative control value was 35-38%, 23-24% and 38-42% for meta-, ana- and telophase, respectively, and the distribution showed no apparent trend of changes after onions were exposed to different concentrations of hazelnut-pyrolysis oil. This may indicate that the process of preparing microscopic slides, and/or difficulties of detecting some of the phases during slide examination, cause differences in the distribution of phases compared with the original time duration of the different phases.

4.1.3.3 Frequency of damaged dividing cells within the dividing phases

The frequency of damaged dividing cells in metaphase had a more similar pattern with the frequency in total compared with the two other phases. This is due to the distribution of dividing phases in the different treatment-groups, in which metaphase are clearly dominating, and thus, contributes to a larger part of the total. Most of the damage types are easiest to detect in metaphase, due to the chromosomes being more tightly assembled in anaphase and telophase, which may result in covering of the area of damage. This makes it difficult to detect specific damages within these phases. Thus, damage in metaphase probably gives the most correct picture of the relatively different genotoxic potential of each pyrolysis oil.

The frequency of damaged cells in metaphase of onions exposed to all the three concentrations of the different types of pyrolysis oils was apparently higher compared with negative control, indicating a toxic effect on the metaphase of cell division. The onions exposed to the lowest concentration of the oils showed quite similar percentage of damaged cells in metaphase; however, not significant higher for those exposed to the beech-oil compared with negative control. At the two highest concentrations, the spruce-oil seemed to exert the lowest percentage of damaged cells in metaphase compared with the two other oils. Additionally, a concentration-dependent increase in percentage was lacking, as discussed for the frequency of total damaged

dividing cells (Chapter 4.1.3.1). The two highest concentrations of the beech-oil and the highest concentration of the poplar-oil exerted a clearly significant higher frequency of damage compared with negative control, with frequency almost as high as for positive control. This indicates a high disturbance of cells in metaphase at these concentrations. As expected, the positive control had a clearly significant higher percentage of damaged cells in metaphase, and the 'blind' solutions showed no significant difference in frequency compared with negative control.

Looking at the frequency of damaged cells in anaphase, the variation of the three replicates of onions in each treatment-group was high. This is due to the low contribution of anaphase to the total mitotic phases (meta-, ana- and telophase), which resembles the low number of anaphases detected for each treatment-group (Appendix B). The results from the frequency of damaged cells in anaphase are, thus, not as reliable as those for the cells in metaphase and telophase. The high variation makes it difficult to make comparisons, and to clearly state a conclusion about differences between the oils and the different concentrations of the oils. However, it is a clear pattern of higher frequency of damaged cells in anaphase of onions exposed to different concentrations of pyrolysis oils, as well as for the positive control, compared with negative control. This suggests that the pyrolysis oils can affect the anaphase-stage of cell division. The frequency of damaged cells for the onions exposed to the 'blind' solutions is weakly significant higher than negative control, but due to a high variation of frequency between the onions exposed to these solutions, and a low number of anaphases detected in these onions, this will not be further discussed.

The frequency of damaged cells in telophase was in general clearly lower than the frequency of damaged cells in metaphase and anaphase. It may be speculated in that spindle effects generated in metaphase may proceed into anaphase; however, that the cell fails to segregate the chromosomes into two poles, thereby the aberration is detected in anaphase. Due to the relatively short time duration of the anaphase stage of mitosis (Utrilla et al., 1993), healthy cells in metaphase are quite rapidly converted to cells in telophase, and thus, a higher frequency of normal cells in telophase is observed relatively to the two other phases. The onions exposed to the poplar-oil appeared to have the highest frequency of damaged cells in telophase compared with the ones exposed to the other oils, with a significantly higher frequency of damage at all the concentrations, and an apparent increase in frequency of damage from the lowest to the two highest concentrations. The onions exposed to the beech-oil had a significantly higher

frequency of damage at the two highest concentrations. The ones exposed to the spruce-oil, however, did not have a significantly higher frequency of damage at any concentrations, although the two highest concentrations appared to exert a higher frequency compared with the lowest concentration. This indicates that the spruce-oil, at these concentrations, may lack an effect or have a weak effect on damage of cells in telophase in the mitotic cell division. As expected, the positive control had a significantly higher percentage of damaged cells in telophase, and the 'blind'-solutions were not significantly different compared with negative control.

4.1.4 Specific chromosomal aberrations observed in dividing cells

The most dominant aberration in all treatment-groups was disturbed metaphases, followed by vagrants, laggards and c-metaphases. All of these aberrations indicate complete or partly inhibited spindle mechanisms. Since these types of aberrations also were dominating in the negative control-group, this indicates that the spindle apparatus in the *Allium cepa* system may be particularly sensitive to spontaneously occurring damage. Fragments, polyploid cells and star anaphases contributed to a very low frequency of the total number of dividing cells for all the treatment-groups, indicating that these aberrations are relatively rarely induced, both spontaneously and by chemicals, in *Allium cepa* cells. Due to the low number of cells possessing these types of aberrations, the results within these groups will not be emphasized further in the discussion.

The tendency of increasing frequency of different types of chromosomal aberrations with increasing concentrations for the onions exposed to the poplar- and beech-pyrolysis oil was higher compared with the ones exposed to the spruce-oil. This resembles the results above indicating that the onions exposed to spruce-oil were lacking a dose-depending increase in frequency of damaged dividing cells. Only the frequency of polyploidy and star anaphases was increasing with increasing concentrations of the spruce-oil. However, these results are not valid due to a very low number of cells detected within these types of aberrations. A clear lack of a dose-response relationship is observed in the bridge-group, which may be due to a low effect on this group of all the tested concentrations of pyrolysis oils. The lack of a relationship between concentrations and percentage of c-metaphases for all the different oils may be due to misinterpretation. The detection of effect of spindle inhibiting substances may appear at other types of aberrations, like disturbed metaphases. Thus, it might be beneficial to look at

chromosomal aberrations induced by spindle inhibitors, like c-metaphases, disturbed metaphases, laggards and vagrants, as one group, and for the same reason, chromosomal aberrations induced by clastogens, like bridges and fragments, as another.

No clear pattern of increasing frequency with increasing "concentrations" of 'blind' solutions was observed within the types of chromosomal aberrations. Additionally, no significant increases in frequency of different types of chromosomal aberrations were detected in roots of onions exposed to the 'blind' solutions compared with negative control, except for a weakly significant increase for the vagrant aberration type. These findings suggest that the DMSO added in the pyrolysis oils did not contribute to an enhanced frequency of chromosomal aberrations, and that the results from the onions exposed to the different pyrolysis oils may be directly compared with the ones from the onions exposed to negative control.

Clastogenic compounds can cause breakage in a DNA-strand, which may create fragments or interact with breakages on other chromosomes or chromatids and create bridges (Savage, 1976). For the onions exposed to the spruce- and beech-oil, no apparent higher frequency of fragments or bridges was observed compared with negative control, which indicates a lack of clastogenic compounds present in these oils. The onions exposed to the poplar-oil did not show increases in frequency of fragments compared with negative control. However, a strongly significant higher frequency of bridges was observed for the lowest concentration, implying a clastogenic potential for this oil. It should be mentioned that the frequency of fragments may have been underestimated. Fragments in both meta- and anaphase may have been present without detection, due to covering of other chromosomes.

A higher frequency of chromosomal aberrations that indicate effects on the spindle apparatus was observed for onions exposed to the different pyrolysis oils compared with negative control. A higher frequency of disturbed metaphases, laggards and vagrants indicates that substances in the oils have interfered with the spindle apparatus of the dividing *Allium cepa* cells and caused a weak c-mitotic effect, affecting the organization in metaphase and the ability to segregate the chromosomes to the poles (Preston and Hoffmann, 2007). A higher frequency of c-metaphases indicates a complete inhibition of the spindle apparatus, resulting in a random scattering of chromosomes in the cell. This was only observed for the beech-oil and the lowest concentration of the spruce-oil compared with negative control. The lack of this higher frequency for the onions exposed to poplar-oil is, as explained above, probably not due to less substances in this

oil interacting completely with the spindle apparatus, but rather a distribution of detection of effect at other types of aberrations. The detection of polyploid cells at the highest concentrations of the different oils may reflect the c-mitotic effect, which can result in cells that contain multiples of the normal complement of chromosomes (Fiskesjö, 1981, Wierzbicka, 1994, Albertini et al., 2000, Gadeva and Dimitrov, 2008). Some star-anaphases were also seen for onions exposed to the poplar- and spruce-oil, further indicating interactions with the spindle apparatus. Substances present in the different pyrolysis oils may prevent formation of the mitotic spindle by inducing microtubule depolymerization (Caperta et al., 2006).

Of the three oils, the poplar- and the beech-oil showed the highest induction of different types of spindle-affecting chromosomal aberrations. C-metaphases were most frequently observed in onions exposed to the beech-derived oil, except for the lowest concentration of spruce-oil, while vagrants were most frequently observed in onions exposed to the poplar-oil. Additionally, both within the disturbed metaphase- and the laggard-type of aberration, the highest concentrations for the pyrolysis oils from poplar and beech feedstocks showed the highest frequency. These findings indicate that all the three oils exert a spindle effect on dividing *Allium cepa* cells; however, the potency of spindle-toxicity was highest for the poplar- and beech-oil.

The onions exposed to poplar-oil showed a significantly higher frequency of stickiness, while the ones exposed to the beech- and spruce-oil only showed a slight increase in frequency of stickiness for the two highest concentrations compared with negative control. Observation of sticky chromosomes suggest an entanglement effect of inter-chromosomal chromatin fibers (McGill et al., 1974), revealing a failure of condensation of the chromosome fibers as a result of exposure to substances in the pyrolysis oil derived from poplar feedstock. This is probably due to an effect on the organization of the chromatin (Radić et al., 2010), and reflects the present of highly toxic substances, which probably will cause cell death (Fiskesjö, 1985, Liu et al., 1992). Stickiness between chromosomes may cause the formation of bridges during the separation in anaphase (Radić et al., 2010). This may have contributed, together with a clastogenic effect, to a higher frequency of observed bridges for the onions exposed to the lowest concentration of poplar-oil compared with the frequency of the two other pyrolysis oils. Kong and Ma (1999) actually suggest that bridge formations without accompanying fragments indicate that they are formed from adherence. Additionally, stickiness may contribute to the observed polyploid cells, since sticky chromosomes may not separate properly during cell division (Onwuamah et al., 2014).

Pekol et al. (2012), who also detected specific types of aberrations after exposure to a pyrolysis oil from hazelnut feedstock in an *Allium cepa* test, found c-metaphases and stickiness to be major aberrations at all concentrations. Since they lack a group called disturbed metaphases, it may be assumed that they have been scored as c-metaphases. Taking these two aberrations together, this group is also the dominating aberration detected in the present study. This implies that compounds exerting colchicine-like effects are common in fast pyrolysis oils derived from different kinds of feedstocks. Stickiness, however, was only found to be a dominating group in the onions exposed to poplar-oil, which indicates that the poplar-oil may share, to a higher degree than the beech- and spruce-oil, some mutual, highly toxic compounds with the hazelnut-oil that induces stickiness in *Allium cepa* cells. Pekol et al. (2012) did not detect any increase in bridges and fragments in onions exposed to different concentrations of the hazelnut-oil compared with negative control. This further indicates a lack of clastogenic chemicals present in fast pyrolysis oils from different feedstocks. Contradictory to the results in this thesis, Pekol et al. (2012) did not detect any increase in vagrants and laggards compared with negative control after exposure. However, since a high amount of c-metaphases were found, and laggards and vagrants indicate a weak c-mitotic effect, the potential of the hazelnut-oil to induce these types of aberrations is present.

4.1.5 (Geno)toxic damage related to composition of the pyrolysis oils

The pyrolysis oils used in this master's project have been characterized by the use of different chemical "fingerprinting" techniques (Section 2.2.1). The different oils show essentially the same major trend in all the spectra, although there are minor differences between the oils in abundance of compounds or classes of compounds. This is, however, not surprising because the oils are expected to have some diversity in composition due to their generation of different feedstocks. Additionally, they are from different geographic locations, with some differences in climate and soil conditions, which may cause differences in chemical composition (Pettersen, 1984). Since the oils were analyzed after they were produced, and they were produced at different times (Section 2.2), storage of the oils may have altered their composition. Thus, the spectra may have shown a even larger variety if analyzed prior to utilization in this master's project.

From the positive and negative ESI-MS spectra, compound identification of the lines detected at different mass number is not provided, thus, a detailed discussion on chemical differences between the oils is impossible to execute. Nevertheless, a various intensity in some of the mass numbers are detected between the different oils, indicating a somewhat difference in the content of polar compounds containing oxygen, nitrogen or sulfur and organic acids. All the lines detected in the spectra are most likely to be water-soluble, due to a low ionization of non- or less-polar hydrocarbons (Smith et al., 2012). Thus, the water-soluble part of the various pyrolysis oils tested in this project probably contains different amounts of some compounds, and this may explain some of the differences observed in the toxic and genotoxic effects tested. As earlier mentioned, Park et al. (2008) found that the liquid fraction of a fast pyrolysis oil generated from radiata pine wood seemed to be more cyto- and genotoxic compared with the tar fraction. Main components in the liquid fraction of the pine-oil, which may have contributed to these effects, were acetic acids and phenols. These compounds may represent some of the lines in the present ESI-MS spectra that exhibited differences between the oils.

The findings of the liquid fraction of a pyrolysis oil being the most genotoxic (Park et al., 2008) might not be expected, since pyrolysis oils generated with temperatures around 500°C, which is the temperature used in fast pyrolysis (Bridgwater and Peacocke, 2000), are expected to contain a small amount of water-insoluble PAHs (Williams and Horne, 1994, Williams and Horne, 1995, Diebold, 1997). Polycyclic aromatic hydrocarbons are known to exert genotoxic effects through their biotransformation to reactive epoxide-species (Hall and Grover, 1990, Boström et al., 2002, Parkinson and Ogilvie, 2007). Pimenta et al. (2000) found genotoxic effects of the PAH fraction of a slow pyrolysis oil from *Eucalyptus grandis* wood; however, the whole oil showed no genotoxic potential. Cordella et al. (2012) also found a genotoxic potential associated with, among others, the PAH fraction of slow pyrolysis oils from different biomass feedstocks. Even though the amount of PAHs may vary between different pyrolysis oils, due to the use of different pyrolysis conditions and feedstocks (Tsai et al., 2007, Lu et al., 2009), it might be speculated in that fast pyrolysis oils in general exhibit amounts of PAHs that are under the detection limit of genotoxicity. However, possible upgrading of the pyrolysis oils by zeolite catalysts to produce refined bio-fuels may produce a high quantity of PAHs (Williams and Horne, 1994, Williams and Horne, 1995), and thus, increase the genotoxic potential of the oils.

In contrast to the ESI-MS spectra, FTIR allows analysis of the entire pyrolysis oil sample and detects functional groups of compounds (Eide and Zahlsen, 2005, Staš et al., 2013). Thus, some of the groups of compounds detected may not be representing the compounds dissolved in the water-fraction of the pyrolysis oils tested in this master's project. Furthermore, it is difficult to anticipate if small amounts of less water-soluble groups of compounds have dissolved in the 0.1% DMSO added in the oil-solutions. Although the FTIR analysis revealed strong similarities between the pyrolysis oil samples, some differences were detected at the different signal clusters. The poplar- and spruce-oil show the highest and lowest absorbance, respectively, within the spectral ranges representing O-H and N-H stretching (phenols, alcohols, water, carboxylic acids, amides and amines). Additionally, within the spectral ranges representing C=O and C=C stretching (aldehydes, ketones, carboxylic acids and esters), a higher absorbance was observed for the poplar- and beech-oil compared with the spruce-oil. Since the spruce-oil seems to exhibit a lower and less concentration-dependent genotoxicity compared with the two other oils, the compounds detected at a higher rate for the poplar- and beech-oil within the spectra, especially the more soluble ones, may contribute to the difference in genotoxicity observed. The spectral ranges dominated or partly dominated by the spruce-oil, masking the presence of nitrogenous compounds, aromatics, alkanes, alcohols and ethers, might represent less important substances contributing to genotoxicity of the oil-solutions generated in this master's project.

As for the ESI-MS spectra, compound identification for the peaks detected at different retention times is not provided in the GC-MS chromatogram, thus, a detailed description of the difference in chemical composition detected between the oils is not performed. The whole composition of the oils can be examined, except some large molecules and non-volatile compounds, which may be difficult to analyze (Eide and Zahlsen, 2005, Staš et al., 2013). The GC-MS is the one of the four spectra showing the highest variability between the oils, with quite clear differences in abundance of compounds at a dominant of the retention times. This further emphasize that even though the pyrolysis oils, which are all made of whole wood and with fast pyrolysis, naturally contain a lot of the same compounds, some differences in abundance of compounds are present, which may cause a difference in toxicity and genotoxicity between the oils.

Nina Holteberg, a MSc student at the Environmental Toxicology program, tested the genotoxicity of the same whole wood spruce-oil as tested in this master's project, using a Ames *Salmonella* assay (Holteberg, 2014). She found that the test solutions induced a positive

mutagenic concentration-dependent response in the bacterial strain TA98 with S9 as well as the bacterial strain TA100 with and without S9; however, not in the TA98 without S9. The most prominent responses were observed in TA100 with S9, and in both strains an increased toxicity was observed in the presence of S9. This indicates that the spruce-oil particularly exert its mutagenic effects after metabolic activation, and that the effect is primarily related to base-pair substitution, and to a lesser degree, frame-shift mutation. *Allium cepa* onions also contain metabolic systems that may create secondary toxicants (Fatima and Ahmad, 2006, Tabrez and Ahmad, 2013). Nevertheless, a dose-response relationship in genotoxicity of the spruce-derived oil evaluated in the present *Allium cepa* test was not shown. This might be due to different endpoints tested in the Ames test and the *Allium cepa* test. Even within the Ames test, a difference in mutagenic endpoints (base-pair substitution and frame-shift mutation) was observed. Additionally, Holteberg (2014) tested the whole complement of the oils, and the secondary toxicants may have been non-soluble compounds, like PAHs, that were not present in the oil-solutions tested in the *Allium cepa* test performed in this master's project. Moreover, different concentrations were used within the different tests. Higher concentrations, or a larger interval between the concentrations, of the spruce-oil might have caused a dose-dependent genotoxicity. However, this was not possible in the present *Allium cepa* test, due to a lack of root growth at higher concentrations.

It has been shown that softwood contains a higher lignin to cellulosic ratio compared with hardwood (Pandey, 1999). Since the water-insoluble fraction of pyrolysis oils is originally composed of lignin-derived material (Oasmaa and Czernik, 1999), this indicates that the spruce-oil, which is produced from softwood, contains a higher amount of water-insoluble compounds compared with the two other oils, which are produced from hardwood. Thus, the amount of compounds solved in the water-soluble fraction of the oils tested in the present *Allium cepa* assay is probably higher for the poplar- and beech-oil compared with the spruce-oil, and this might be the reason for the lower genotoxicity and the lacking concentration-dependency observed for the spruce-oil. This does not imply, however, that the whole complement of the spruce-oil is less toxic than the whole complement of the two other oils. Since a concentration-dependent increase in mutagenicity was seen in the Ames test (Holteberg, 2014), and the spruce-oil probably contains a higher amount of water-insoluble compounds, it might be speculated in that the water-insoluble fraction of the spruce-oil may exert higher genotoxicity compared with the two other oils.

Even though characterization of specific compounds or groups of compounds was not conducted through this thesis, it is important to emphasize that caution should be taken during hazard assessment of single pyrolysis oil components. Attempts to identify single toxic compounds and their behavior may be difficult and/or misleading. Pyrolysis oils are complex mixtures composed of a wide variety of compounds, and interactions between the different chemicals in the oils can affect the response. The chemicals may undergo synergistic or antagonistic interactions, which may have a significant effect on the toxicity (Donnelly et al., 1995) (Section 1.3.1).

4.2 Optimalization and evaluation of the method

4.2.1 The test organism *Allium cepa*

In the beginning of the experimental period of this master's project, onion (*Allium cepa*) bulbs were purchased from different grocery stores; however, these onions were quite large in diameter (7.0-10.0 cm), and the root growth was limited and highly variable between onions. The large diameter made the experimental setup and storage difficult, and the variable and limited root growth made both statistical comparison and accomplishment of the experiment difficult or impossible. Later, we came in contact with a farmer from "Hveem Nord Østre", Toten, Norway, who provided us with small bulbs (2.0-4.0 cm in diameter) of *Allium cepa* onions. The onions had a great and uniform root growth, with little variation between the onions. These findings emphasize the importance to be aware of individual variations when biological assays are carried out. It also states that the conditions and storing of the onions are crucial factors for obtaining adequate root growth to go through with an *Allium cepa* assay.

For the onions purchased from the grocery stores, chemical agents were probably employed, inhibiting the root growth of the onions. This was not the case for the onions purchased from Toten, where ecological agriculture was emphasized. In general, Norwegian onion production does not use chemical spraying during cultivation. However, since the onions were not cultivated under controlled conditions in our laboratory, they may have been exposed to contaminants or radiation sources in the soil prior to being used in the present experiment. The harvesting of onions in Norway happens late August/early September, and the onions need a couple of months to create roots. This restricts the time period for running an *Allium cepa* test, when using onions harvested in Norway. Storing of onions is an additional factor that may

affect the root growth. The onions should be stored in the dark under dry conditions at +10-20°C (Fiskesjö, 1985). In this experiment, the onions were kept in a dry, dark closet at room temperature, and the duration of storage between the first and last exposure of different treatments, was approximately three months. This duration time may have resulted in a different root growth of onions exposed to the different treatments. However, since the root growth in this thesis was measured as relative root growth compared with the lowest concentration of the respective oil, this should not affect the results.

4.2.2 The pyrolysis oils

Prior to the experiment, the pyrolysis oils were diluted with water and 0.1% DMSO to gain different concentrations of the oils. This dilution-process was quite hard to conduct, due to difficulties with solving the oils in water. Heating the oils (50°C) and adding DMSO improved the solving process. However, a mass of undissolved matter assembled in the bottom of the bottle when water was added, and thoroughly mixing and grinding were needed to solve as much as possible. Even though the three pyrolysis oils were attempted treated the same way, some differences in the solving-processes may have occurred, resulting in more or less water-soluble compounds present in the different oils. This may explain some of the differences observed in (geno)toxicity between the oils; however, to a small degree since the oils were mixed and grinded for several minutes until the matter in the bottom was virtually impossible to dissolve any further.

Attempts were made to dissolve most part of the oils by extraction with dichloromethane (DCM), which makes the oils more soluble. The mixture of oil and DCM was placed in an ultrasound bath, centrifuged, and added additional DCM. This was repeated twice, and the DCM-extract was evaporated to dryness in an atmosphere of pure nitrogen. When drying was complete, DMSO was added to dissolve the DCM residue. The oils were subsequently added water to gain the different concentrations of oil-solutions, and after mixing and heating, they were quite well dissolved. However, when comparing the root growth inhibition after exposure from the two different methods explained above, using only water and 0.1% DMSO gave a much higher root-inhibition after oil-exposure compared with the “same” concentration of oil extracted with dichloromethane. Thus, a decision to use the prior method was made, resulting in a higher concentration of toxic compounds for the same amount of oil.

The pH of all concentrations of the different oils was adjusted to approximately pH=7.5. High concentrations of pyrolysis oils may be very acidic, and the highest concentration tested in the present master's project measured a pH of below 3.0. Fiskesjö (1985) showed that a pH below 3.5 caused growth restrictions of *Allium cepa* onion roots. The pH should, thus, be adjusted to the same level to be able to compare toxicity of different concentrations of oils that is due to toxic chemical compounds present, and not due to the differences in pH.

The spruce-, beech- and poplar-oil were made in 2011, 2012 and 2009, respectively. The pyrolysis oils contain compounds that during storage can react with themselves to form larger molecules. These reactions result in an increase in average molecular weight, and thus, viscosity. With the increase in growth of molecular weight, an increase in the amount of the water-insoluble fraction, originally composed of lignin-derived material, has been observed (Oasmaa and Czernik, 1999). The ageing rate depends on the oil composition, and thus, feedstock and pyrolysis conditions (Oasmaa and Czernik, 1999). Since the different pyrolysis oils are all made from fast pyrolysis of wood feedstocks, and seem to be of quite similar composition, the ageing of the oils should be fairly similar. Since the ageing seems to increase the water-insoluble fraction of the oils, less of the water-soluble fraction should be present in the oldest poplar-oil compared with the two other oils, and thus, a lower toxicity might be expected for this oil. However, age did not seem to affect the toxicity in this experiment, since the poplar-oil showed the highest tendency of genotoxicity. An even higher genotoxicity might have been observed in the *Allium cepa* test for the poplar-oil, as well as for the two other oils, if they were synthesized more recently.

4.2.3 The *Allium cepa* test

4.2.3.1 Root inhibition test

Due to the small variation in root growth of the onions used in this experiment, only three parallels of onions were used for each treatment-group. To further decrease the variation, three additional onions for each exposure experiment were added. This made it possible to discharge onions with damages, like the ones damaged during removal of the bottom plate with a scalpel, or dried or mould-attached onions, and to choose the onions with the greatest and most even root growth prior to exposure (after 48 hours growth in tap water).

To decide whether onions have been exposed to toxic compounds in the tested oils, it is important to compare the damage in the exposed groups with a control-group. Background-values from other studies should not be used, due to differences in storage, size and conditions of the onions, and differences in the *Allium cepa* test procedures. Additionally, chromosomal aberrations may appear spontaneously, thus, an aberration frequency of zero should not be used as a reference value.

Root length inhibition for the different oils was presented as percentage of root length of onions exposed to the lowest concentration of the respective oil, and not as a percentage of negative control, as is more commonly used in literature (Fiskesjö, 1985, Rank and Nielsen, 1998, Ateeq et al., 2002, Radić et al., 2010, Onwuamah et al., 2014). In this experiment, the negative control-exposure and the different pyrolysis oil-exposures were conducted separately, due to an expectation of the onion root growth not to be affected with time of storage. A negative control-exposure was carried out at the end of the experiment period, to test for differences with the prior control-exposure (performed at the beginning of the experiment period), and no apparent difference in root length was found. This indicated that it was reasonable to measure the root length of onions exposed to different pyrolysis oils as percentage of negative control. However, the curves obtained had a different percentage of root length prior to reaching the threshold of effect. Since this area is consistent over several concentrations (at least for the poplar- and spruce-oil), it indicates that these concentrations exert no effects on the root growth, and should be approximately equal to the tap water control, which was not the case for the oils tested. A negative control for each oil-exposure experiment probably should have been used, and thus, a decision was made to compare toxicity against the lowest concentration of the respective oils instead. For the same reason, it was decided to interpret the root growth of onions exposed to 'blind' solutions as length (cm), and not as percentage of negative control.

From the toxicity curves, onion roots exposed to three concentrations (0.00004, 0.0004 and 0.001 ml oil/ml solution) of pyrolysis oils were picked to be used further in the microscopic preparation and examination. The 0.001-concentration was the highest of the seven concentrations used in the root inhibition test that contained a sufficient amount of dividing cells for the cyto- and genotoxic evaluations after exposure for all three oils. Additionally, roots exposed to concentrations above 0.001 ml oil/ml solution were very vulnerable, fragile and easy to damage, making the microscopic slide preparation harder to conduct. For the onions exposed to the poplar- and beech-derived oil, the 0.001-concentration resulted in a low relative root

growth. The 0.00004-concentration represented a low concentration, where no or a small toxic effect were observed for the oils. The 0.0004-concentration represented an intermediate concentration, that was in the middle of (for onions exposed to the beech-oil) or just above (for onions exposed to the poplar- and spruce-oil) the steep region of the toxicity curve.

4.2.3.2 Pre-treatment and fixation

Similar to colchicine, pre-treatment with 8-hydroxyquinoline may cause inactivation of the spindle apparatus. Still, 8-hydroxyquinoline should keep the chromosomes at the same place during squashing by increasing the viscosity of the cytoplasm (Tjio and Lavan, 1950). Nevertheless, the viscosity may not have been equal through all of the cytoplasm, or in all of the cells, which may have resulted in some of the specific spindle effect damages observed. However, since all the exposure- and control-groups were treated with the same agent, this will only contribute to the background-level of aberrations, and will not interfere with the interpretation of the results. Additionally, exceeding of the critical temperature of 15-18°C may cause stickiness (Tjio and Lavan, 1950, Östergren and Heneen, 1962). In this study the roots were exposed for 5 hours to the 8-hydroxyquinoline, at a temperature of approximately 4°C, hence it should not have any effect on the frequency of damage within this type of chromosomal aberration.

4.2.3.3 Microscope slide preparation

Some of the slides in this study were difficult to analyze. When the cover slips were removed with a scalpel, sometimes the root cells followed the cover slips, resulting in damaged areas on the slides. Some slides contained cells with chromosomes that were difficult to examine, probably due to poorly spreading of the cells. This was especially the case for hard roots that probably were not sufficiently softened during the macerating process. The difficulty of detecting chromosomes may also have been due to the dye-quality, which may have been affected by a too short (weakly stained) or too long (strongly stained) treatment-time with orcein. Additionally, some slides could not be analyzed due to mechanical damage caused by too hard tapping and squashing. A last error in the microscope slide preparation was the gluing. Too much glue could cause a blurry appearance when observing the cells in the microscope, and insufficient amounts of glue caused bad observation conditions at the border and edges of the cover slip. However, slides or area of slides with bad scoring conditions did not affect the

results in this study, since they were excluded, and new slides of good quality were made until 100 cells in division were detected at each individual of the treatment-groups.

4.2.3.4 Microscopic examination

The microscopic evaluation is a subjective analysis, and efforts were made to avoid incorrect judgment of damages. Prior to microscopic slide preparation and examination, slides prepared from *Allium cepa* onions in another project were screened for chromosomal aberrations. This was to get familiar with the different dividing phases and types of aberrations that can be observed in *Allium cepa* cells. In the beginning of an experiment, phases of division, damaged and normal cells, and different types of aberrations may be difficult to separate from each other. Thus, this minimized the possible difference in interpretation early and late in the microscopic examination period. Brøgger et al. (1984) showed that one of the greatest variations between different laboratories in the evaluation of human lymphocyte chromosomal aberrations were related to the existence of an aberration. Thus, in this experiment, two persons (Bingham, pers. comm.) analyzed cells that were uncertain to have a damage, before the final decision was made. This increased the probability of damages being evaluated equally throughout the working period, and at the same time, the probability of misjudgment was reduced. Additionally, the slides were examined blindly, which minimized expectations that might have affected the results.

Due to difficulties of detecting possible abnormalities within different cell division phases, Rank and Nielsen (1993) proposed an analysis of abnormalities only in anaphase and telophase to obtain a more sensitive method of scoring, and later, Rank (2003) published another study using only anaphases and telophases to conduct chromosomal aberration analysis in an *Allium cepa* test. However, the analysis within all phases of the cell cycle may give a more comprehensive and accurate evaluation, due to the greater analysis material, and promotes a better investigation of the action mechanisms of the chemicals in the oils, and the resulting effects of these actions (Leme and Marin-Morales, 2009). Even so, detection of damage in prophase was not included in this study. Few studies include damages in prophases, and the ones that have included them, found a very low percentage of abnormalities within this stage (Badr, 1981, El-Shahaby et al., 2003, Fisun and Rasgele, 2009). This might be due to difficulties of separating normal and damaged prophases with certainty, since the chromosomes are not yet condensed and are distributed over the whole cell, or difficulties with separating early prophases

from interphases in some cases. Some underestimation of damaged cells is probably found within all the dividing phases when conducting an *Allium cepa* test; however, this might be greater for the prophase. Thus, inclusion of prophase may result in an incorrect estimation of the frequency of damage.

The number of slides needed to detect over 100 dividing cells was increasing with slides prepared from onion roots exposed to increasing concentrations of pyrolysis oils. This indicates that the proliferation of cells in the onion roots was decreasing with exposure to higher concentrations of the different pyrolysis oils. This was not reflected by the mitotic index, a measure of the proliferation status of a cell population, which showed no clear pattern of decrease with increasing concentrations. When scoring the mitotic index, only areas where cell division was detected were used, to exclude cells that were not a part of the meristematic region undergoing mitosis. Areas of limited cell division may have been in the mitotic region; however, inhibited by toxic compounds present in the oil. This may have resulted in an overestimation of mitotic index for the onion roots exposed to the highest concentrations of the pyrolysis oils.

The number of dividing cells registered for chromosomal aberrations has varied between different scientists, even within the last few years. Among others, Radić et al. (2010) and Pekol et al. (2012) recorded chromosomal aberrations in approximately 300 dividing cells, Herrero et al. (2012) and Musanovic et al. (2013) in 100 dividing cells, and Ozakca and Silah (2013) analyzed chromosomal aberrations in 400-500 dividing cells for each test group. In the present study, at least 100 dividing cells were screened for chromosomal aberrations per onion bulb (Appendix B). A higher number of mitotic cells for each treatment would have given a more certain result; however, the analyses were time-consuming and to analyze an additional amount of cells was not possible within the timeframe of this master's project.

There are some variation in types of chromosomal damages registered in different studies conducting an *Allium cepa* test. All the chromosomal aberrations registered in this study are common damages that have been detected by several authors (Roychoudhury and Giri, 1989, Kovalchuk et al., 1998, Turkoglu, 2007, Caritá and Marin-Morales, 2008, Udengwu and Chukwujekwu, 2008, Kumari et al., 2009, Patlolla et al., 2012, Pekol et al., 2012); however, some chromosomal aberrations registered in other studies were not detected here, like multipolarity and c-anaphases (Caritá and Marin-Morales, 2008, Leme et al., 2008, Mustafa

and Arikan, 2008). This may be due to the chemical composition of the oils not affecting these types of aberrations; however, the main reason may be that these chromosomal aberrations occur with a low frequency or that there are difficulties of detecting them.

4.2.4 Relevance of testing pyrolysis oils in an *Allium cepa* test

The *Allium cepa* test was used in this study due to its many benefits; onions are easy to store and handle, they have good chromosomal conditions, they can be exposed directly to the test solution and they possess CYP-enzymes that make it possible to detect secondary toxicants. However, the relevance of the exposure to onions may be questioned. Can the results from this study be extrapolated to humans or wildlife?

In a laboratory experiment, exposure doses, pH and other factors that may influence the experiment can be controlled for, which makes it easier to detect toxic actions and responses that are directly caused by the examined exposure. However, since the experiment is performed under controlled conditions, the results cannot immediately be extrapolated to natural conditions. In nature, other pollution sources or compounds found naturally in the environment may interact with the oil chemicals, causing an enhanced or minimized toxicity, and/or affecting bioavailability. Additionally, extrinsic factors, like pH, salinity and temperature, may affect the toxicity of the oils. In this aspect, the present laboratory *in vivo* study to some degree describes the mechanisms and effects of the oils on living organisms; however, *in situ* experiments are needed to further be able to evaluate the effects on plants and animals in their natural habitat.

In this experiment, only the compounds solved in water were tested for (geno)toxic effects, because the onions needed water to grow, and organic solvents are toxic for the root growth. Even though the present experiment did not test the entire complement of the pyrolysis oils, it can be argued that the water-soluble part of the oils makes the greatest environmental threat. If the oils reaches the environment through accidental releases and/or routine losses associated with the use of oils, the compounds that are dissolved and bioavailable in water will due most harm to aquatic plants and animals. However, it should be noted that the compounds with the highest water solubility have a higher biodegradability in the aquatic and soil environment (Blin et al., 2007, Oasmaa et al., 2012), while the more insoluble ones are more persistent. Thus, further testing should be conducted for the whole pyrolysis oils or the water insoluble part of the oils in another test system (e.g. Ames test).

It may be questioned whether the cellular damages observed in the *Allium cepa* test after exposure to different pyrolysis oils and the possible induction of chromosomal aberrations may be extrapolated to mammals and other animals. Leme and Marin-Morales (2009) pointed out that since the target is DNA, common to all organisms, the results may serve as a warning to other biological systems. Besides, the test has shown to be comparable with other tests performed in mammalian systems (Section 1.2.4). However, animals may contain different mechanisms and defense systems, as well as a different organization and types of tissues, compared with plants. Thus, an indication of similar effects may be anticipated; however, the response between different organisms are likely to be somewhat different.

In the current test, chromosomal aberrations were detected as a response of exposure to pyrolysis oils; however, how is this relevant for the cell- and the individual-level? Aberrations may cause cell death by preventing cell division or by losses of vital genetic material. If the cells are chronically affected by toxic chemicals, a great part of the cell population may die or cell division may be blocked, causing reduced supply of new cells contributing to growth of an organism. Earlier studies have shown a correlation between the frequency of chromosomal aberrations, mitotic index and the root growth in an *Allium cepa* test (Fiskesjö, 1988, Liu et al., 1992, Smaka-Kincl et al., 1996). If the aberration is stable, it can give permanent alterations in the genetic material, which can be transferred through several cell generations, becoming a mutation. Mutations can cause a range of effects on living organisms, like cancer, impairments in enzyme function, altered protein turnover, impairment in general metabolism, inhibition of growth and impairments in immune response and reproduction (Kurelec, 1993). The effect mutations are having on the cell and individual may depend on the type of mutation and how important the changed genetic information is for the cell function. Mutations in germ line cells can cause hereditary defects, and this is of concern in ecosystems with respect to adverse effects (Wurgler and Kramers, 1992, Bickham et al., 2000), although these mutations may be selected against and be lost through generations. An increased frequency of chromosomal aberrations in peripheral blood lymphocytes in humans have been linked to an increased frequency of cancer (Hagmar et al., 1994, Bonassi et al., 2008). Thus, the pyrolysis oils tested in this master's project exerted (geno)toxic effects on the *Allium cepa* onions, and may cause sub-lethal effects on plants and animals in wildlife. However, caution should be taken if using the present result to estimate the risk to populations. Appropriate assessment of the genotoxic potential of an

exposure requires use of different assays to evaluate various genetic events in several cell types (Park et al., 2008).

5 Summary and conclusion

The aim of this master's project was to determine the toxic and the genotoxic potentials of pyrolysis oils generated from three different wood feedstocks, namely poplar, beech and spruce, by evaluation in an *Allium cepa* chromosomal aberration assay. It was hypothesized that these potentials would vary with the feedstock used for making the oils and would increase with increasing concentrations of the oils.

The results showed:

- A toxic, cytotoxic and genotoxic potential of all the three pyrolysis oils.
- That the order of genotoxicity of the oils was pyrolysis oils derived from poplar, beech and spruce feedstocks, respectively.
- A concentration-dependency in genotoxicity for the poplar- and beech-oil; however, not for the spruce-oil.
- That the genotoxic potential of the oils was mainly linked to chromosomal aberrations induced by chemicals exerting effects on the spindle apparatus.

The differences in toxicity between the oils are probably due to some variation in the chemical composition of the oils. The results from this master's project could constitute an important knowledge of the aquatic toxicology of pyrolysis products. However, further studies should be carried out to investigate the effects of the whole compliment of the oils and to identify compounds, or interactions of compounds, responsible for the observed toxicity. In this way, pyrolysis oils that exert minor hazard on the environment may be manufactured. Additionally, the pyrosysis oils tested in the present master's project are considered to be crude bio-oils, and many steps of refinement may be needed prior to application. Thus, comprehensive toxicity and genotoxicity studies should also be conducted on upgraded oils. Furthermore, other test systems can be conducted to test differences in toxicity between fossil oils and pyrolysis oils. Thus, a comparable measure of toxic effects exerted by oils derived from non-renewable energy sources and the alternative, oils derived from renewable energy sources, may be achieved.

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Appendix A: Root Lengths

Table A: Average root length (cm) per individual of *Allium cepa* onions was determined after 48 hours exposure to tap water and 72 hours exposure to the respective treatment-group. The treatments were negative control (tap water), pyrolysis oils generated from fast pyrolysis of poplar, beech and spruce feedstocks at seven different concentrations (0.00001, 0.00004, 0.0001, 0.0004, 0.001, 0.004 and 0.01/0.04 ml oil/ml solution (tap water+dimethyl sulfoxide, DMSO)), a 'blind' solution (tap water+DMSO) for each concentration, and a positive control (methyl methanesulfonate, MMS). Six parallels of onions were exposed to negative control, whereas three parallels of onions were included for all the other treatments and concentrations. The difference in average root length after exposure to 72 hours of treatment and 48 hours of tap water was also included for all the treatment-groups. The average of the differences for all the six individuals of onions exposed to negative control was measured, and the root length differences of the onions exposed to the various treatment-groups was calculated as a percentage (%) of this average.

Treatment	Conc.	Ind.	Root length (average per individual)			
			48 h water	72 h water	72h-48h	72h-48h (avg.)
Neg. ctr.		1	0.136	1.207	1.071	0.946
		2	0.263	1.273	1.010	
		3	0.307	1.193	0.886	
		4	0.233	1.071	0.838	
		5	0.288	1.315	1.027	
		6	0.135	0.980	0.845	
Treatment	**Conc.**	**Ind.**	**Root length (average per individual)**			
			48 h water	**72 h treatment**	**72h-48h**	**% of neg. ctr. (avg.)**
Poplar	0.00001	1	0.251	0.300	0.049	5.153
		2	0.639	0.679	0.040	4.209
		3	0.996	1.056	0.060	6.295
	0.00004	1	0.710	0.709	-0.001	-0.058
		2	0.379	0.450	0.071	7.487
		3	0.523	0.572	0.049	5.152
	0.0001	1	0.389	0.655	0.266	28.165
		2	0.590	0.742	0.153	16.152
		3	0.781	0.890	0.109	11.538
	0.0004	1	0.727	1.443	0.716	75.697
		2	0.600	1.107	0.507	53.619
		3	0.705	1.607	0.902	95.352
	0.001	1	0.724	1.206	0.482	50.936
		2	0.610	1.477	0.867	91.592
		3	0.793	1.490	0.698	73.762
	0.004	1	0.152	1.224	1.072	113.283
		2	0.181	1.158	0.977	103.236
		3	0.172	0.960	0.788	83.303
	0.04	1	0.329	1.181	0.853	90.105
		2	0.405	1.048	0.643	68.002
		3	0.935	2.139	1.204	127.241
Beech	0.00001	1	1.494	1.605	0.111	11.744
		2	0.764	0.825	0.061	6.431

		3	1.045	1.120	0.075	7.879
	0.00004	1	0.638	0.734	0.097	10.239
		2	1.152	1.147	-0.006	-0.604
		3	0.928	0.976	0.048	5.065
	0.0001	1	0.563	0.741	0.178	18.808
		2	1.023	1.230	0.207	21.897
		3	0.993	1.116	0.123	12.963
	0.0004	1	0.568	0.780	0.212	22.400
		2	1.138	1.574	0.436	46.041
		3	0.577	0.913	0.336	35.538
	0.001	1	1.076	1.548	0.472	49.903
		2	0.707	1.067	0.361	38.129
		3	0.893	1.310	0.417	44.049
	0.004	1	0.534	0.920	0.385	40.723
		2	0.743	1.410	0.667	70.483
		3	0.747	1.341	0.594	62.825
	0.01	1	0.646	1.473	0.827	87.461
		2	0.446	0.900	0.454	48.015
		3	0.947	1.628	0.680	71.910
Spruce	0.00001	1	0.740	0.830	0.090	9.562
		2	1.017	0.980	-0.037	-3.861
		3	0.600	0.648	0.048	5.045
	0.00004	1	0.800	0.872	0.072	7.633
		2	0.665	0.685	0.020	2.121
		3	0.930	1.004	0.074	7.776
	0.0001	1	0.823	1.224	0.401	42.337
		2	0.832	1.424	0.592	62.560
		3	0.632	0.943	0.310	32.810
	0.0004	1	0.475	0.978	0.503	53.141
		2	0.666	1.296	0.630	66.634
		3	0.641	1.030	0.388	41.046
	0.001	1	0.771	1.311	0.539	57.009
		2	0.446	0.827	0.381	40.218
		3	1.094	1.487	0.393	41.549
	0.004	1	0.640	1.286	0.646	68.316
		2	0.552	1.092	0.539	56.996
		3	0.805	1.602	0.796	84.171
	0.04	1	1.562	2.493	0.931	98.403
		2	0.904	1.153	0.249	26.344
		3	0.408	0.900	0.492	51.990
Blind	0.00001	1	0.429	0.687	0.257	27.209
		2	0.424	1.096	0.672	71.033
		3	0.544	1.094	0.550	58.154
	0.00004	1	0.443	0.921	0.479	50.582
		2	0.552	1.337	0.785	82.953
		3	0.504	1.127	0.622	65.783
	0.0001	1	0.373	1.318	0.945	99.907
		2	0.451	1.313	0.862	91.061

		3	0.349	1.066	0.717	75.743
	0.0004	1	0.308	0.623	0.315	33.273
		2	0.527	1.071	0.544	57.511
		3	0.847	1.690	0.843	89.081
	0.001	1	0.251	0.905	0.654	69.092
		2	0.442	1.219	0.777	82.150
		3	0.360	0.976	0.616	65.066
	0.004	1	0.451	1.205	0.753	79.628
		2	0.667	1.609	0.942	99.586
		3	0.384	0.674	0.291	30.730
	0.01	1	0.809	1.807	0.998	105.443
		2	0.737	0.933	0.196	20.768
		3	0.373	1.167	0.794	83.914
	0.04	1	0.568	1.187	0.620	65.496
		2	1.224	2.137	0.912	96.411
		3	1.484	2.534	1.051	111.064
Pos. ctr.		1	0.559	3.125	2.566	271.166
		2	0.747	3.211	2.464	260.392
		3	0.765	2.521	1.757	185.674

Appendix B: Mitotic Index and Number of Cells in Division

Table B: The mitotic index and number of cells (in total, normal cells and damaged cells) observed in division, and in different stages of division (meta-, ana- and telophase) in root meristems of *Allium cepa* onions after 72 hours exposure to pyrolysis oils generated from fast pyrolysis of polar, beech and spruce feedstocks at three different concentrations (0.00004, 0.0004 and 0.001 ml oil/ml solution (tap water+dimethyl sulfoxide, DMSO)). The values for onions exposed to a 'blind' solution (tap water+DMSO) for each of the concentrations were included, as well as the values in negative control (tap water)- and positive control (methyl methanesulfonate, MMS)-exposed onion roots for comparison. Three individuals were included for each treatment-group.

Treatment	Conc.	Ind.	MI	#cells in division (meta-, ana-, telophase)			#metaphase			#anaphase			#telophase		
				Tot.	N	D	Tot.	N	D	Tot.	N	D	Tot.	N	D
Poplar	0.00004	1	11.7	117	59	58	51	16	35	15	5	10	51	38	13
		2	12.1	113	51	62	64	21	43	13	6	7	36	24	12
		3	10.1	103	43	60	39	9	30	22	3	19	42	31	11
	0.0004	1	9.4	107	41	66	58	19	39	12	1	11	37	21	16
		2	8.3	102	29	73	67	15	52	5	1	4	30	13	17
		3	12.9	121	54	67	58	20	38	7	5	2	56	29	27
	0.001	1	9.4	104	26	78	33	9	24	20	1	19	51	16	35
		2	9.3	104	21	83	60	9	51	19	1	18	25	11	14
		3	9.8	107	32	75	75	14	61	8	2	6	24	16	8
Beech	0.00004	1	10.1	103	59	44	59	25	34	5	0	5	39	34	5
		2	9.8	107	57	50	61	24	37	12	3	9	34	30	4
		3	8.2	119	72	47	52	26	26	8	2	6	59	44	15
	0.0004	1	8.2	101	42	59	60	14	46	7	4	3	34	24	10
		2	10.9	127	40	87	76	14	62	14	2	12	37	24	13
		3	7.1	102	40	62	59	13	46	12	6	6	31	21	10
	0.001	1	8.9	100	46	54	39	9	30	14	3	11	47	34	13
		2	5.6	105	31	74	60	12	48	16	1	15	29	18	11
		3	10.7	128	40	88	77	8	69	10	3	7	41	29	12
Spruce	0.00004	1	11.3	133	64	69	82	26	56	13	3	10	38	35	3
		2	11.2	146	78	68	77	27	50	16	3	13	53	48	5

		3	10.1	100	60	40	56	21	35	6	2	4	38	37	1
	0.0004	1	10.2	117	70	47	41	19	22	21	11	10	55	40	15
		2	10.1	102	65	37	49	27	22	13	5	8	40	33	7
		3	9.3	102	59	43	48	23	25	13	7	6	41	29	12
	0.001	1	8.7	109	57	52	50	16	34	16	3	13	43	38	5
		2	7.5	127	62	65	57	16	41	13	6	7	57	40	17
		3	7.8	108	59	49	53	19	34	13	6	7	42	34	8
Blind	0.00004	1	15.6	122	84	38	57	34	23	12	4	8	53	46	7
	0.0004	1	10.1	102	67	35	42	21	21	14	6	8	46	40	6
	0.001	1	14.2	107	74	33	52	25	27	10	7	3	45	42	3
Neg. ctr.		1	14	108	73	35	58	29	29	19	17	2	31	27	4
		2	22.5	142	107	35	80	53	27	20	16	4	42	38	4
		3	13.8	146	105	41	68	39	29	22	20	2	56	46	10
Pos. ctr.		1	10.5	116	34	82	56	5	51	25	7	18	35	22	13
		2	11.1	104	15	89	60	2	58	12	1	11	32	12	20
		3	9.6	103	26	77	61	3	58	10	3	7	32	20	12

Appendix C: Specific Chromosomal Aberrations

Table C: The number of specific types of chromosomal aberrations observed in meristematic root cells in meta-, ana- and telophase of *Allium cepa* onions after 72 hours exposure to pyrolysis oils generated from fast pyrolysis of poplar, beech and spruce feedstocks at three different concentrations (0.00004, 0.0004 and 0.001 ml oil/ml solution (tap water+dimethyl sulfoxide, DMSO)). The types and numbers for onions exposed to a 'blind' solution (tap water+DMSO) for each of the concentrations were included, as well as for the negative control (tap water)- and positive control (methyl methanesulfonate, MMS)-exposed onion roots for comparison. Three parallels of individuals were included for each of the treatment-groups.

Treatment	Conc.	Ind.	Phase	Classification of damage	#damages
Poplar	0.00004	1	Metaphase	C-metaphase	7
				Disturbed	17
				Fragment	1
				Sticky	11
			Anaphase	Bridge	7
				Laggard	5
				Vagrant	10
			Telophase	Bridge	4
				Laggard	5
				Vagrant	7
		2	Metaphase	C-metaphase	6
				Disturbed	29
				Fragment	2
				Sticky	7
				Vagrant	1
			Anaphase	Bridge	5
				Laggard	2
				Vagrant	5
			Telophase	Bridge	6
				Laggard	5
				Vagrant	4
		3	Metaphase	C-metaphase	1
				Disturbed	20
				Fragment	2
				Sticky	9
			Anaphase	Bridge	8
				Fragment	2
				Laggard	7
				Star	1
				Vagrant	17
			Telophase	Bridge	2
				Laggard	4
				Vagrant	8
	0.0004	1	Metaphase	C-metaphase	3
				Disturbed	19

				Fragment	2
				Sticky	15
				Vagrant	2
			Anaphase	Bridge	3
				Laggard	7
				Vagrant	8
			Telophase	Laggard	5
				Vagrant	13
		2	Metaphase	C-metaphase	5
				Disturbed	38
				Fragment	5
				Sticky	9
			Anaphase	Bridge	2
				Laggard	3
				Vagrant	3
			Telophase	Laggard	10
				Vagrant	12
		3	Metaphase	C-metaphase	3
				Disturbed	25
				Fragment	1
				Sticky	10
			Anaphase	Bridge	1
				Vagrant	1
			Telophase	Bridge	3
				Laggard	17
				Vagrant	14
	0.001	1	Metaphase	Disturbed	12
				Fragment	3
				Sticky	11
				Vagrant	1
			Anaphase	Bridge	5
				Laggard	9
				Vagrant	14
			Telophase	Bridge	7
				Fragment	2
				Laggard	20
				Vagrant	18
		2	Metaphase	C-metaphase	4
				Fragment	2
				Disturbed	34
				Sticky	13
			Anaphase	Bridge	4
				Fragment	1
				Laggard	7
				Vagrant	18
			Telophase	Laggard	6
				Vagrant	11
		3	Metaphase	C-metaphase	10

				Disturbed	43
				Polyploid	2
				Sticky	6
			Anaphase	Bridge	4
				Fragment	1
				Laggard	2
				Vagrant	6
			Telophase	Bridge	1
				Laggard	5
				Vagrant	5
Beech	0.00004	1	Metaphase	C-metaphase	8
				Disturbed	26
				Fragment	1
			Anaphase	Bridge	4
				Fragment	1
				Laggard	2
				Vagrant	2
			Telophase	Bridge	1
				Laggard	4
				Vagrant	5
		2	Metaphase	C-metaphase	11
				Disturbed	26
				Fragment	1
			Anaphase	Bridge	9
				Laggard	3
				Vagrant	9
			Telophase	Bridge	1
				Fragment	1
				Laggard	3
				Vagrant	2
		3	Metaphase	C-metaphase	6
				Disturbed	19
				Fragment	2
				Sticky	1
			Anaphase	Bridge	5
				Laggard	3
				Vagrant	6
			Telophase	Bridge	2
				Laggard	6
				Vagrant	9
	0.0004	1	Metaphase	C-metaphase	20
				Disturbed	22
				Fragment	1
				Sticky	4
			Anaphase	Bridge	2
				Laggard	3
				Vagrant	3
			Telophase	Fragment	2

				Laggard	4
				Vagrant	8
		2	Metaphase	C-metaphase	11
				Fragment	2
				Disturbed	46
				Polyploid	2
				Sticky	3
			Anaphase	Bridge	9
				Fragment	2
				Laggard	12
				Vagrant	11
			Telophase	Fragment	1
				Laggard	13
				Vagrant	3
		3	Metaphase	C-metaphase	17
				Disturbed	24
				Fragment	2
				Sticky	5
			Anaphase	Bridge	3
				Laggard	4
				Vagrant	5
			Telophase	Laggard	4
				Vagrant	9
	0.001	1	Metaphase	C-metaphase	5
				Disturbed	16
				Polyploid	4
				Sticky	5
			Anaphase	Bridge	3
				Fragment	1
				Laggard	10
				Vagrant	9
			Telophase	Fragment	2
				Laggard	10
				Vagrant	8
		2	Metaphase	C-metaphase	9
				Disturbed	36
				Fragment	2
				Polyploid	1
				Sticky	2
			Anaphase	Bridge	9
				Fragment	3
				Laggard	15
				Vagrant	14
			Telophase	Bridge	1
				Laggard	4
				Vagrant	10
		3	Metaphase	C-metaphase	19
				Disturbed	37

				Fragment	3
				Polyploid	1
				Sticky	11
				Vagrant	1
			Anaphase	Bridge	2
				Fragment	1
				Laggard	6
				Vagrant	6
			Telophase	Bridge	2
				Laggard	6
				Vagrant	7
Spruce	0.00004	1	Metaphase	C-metaphase	28
				Disturbed	27
				Fragment	7
				Sticky	1
			Anaphase	Bridge	6
				Laggard	7
				Vagrant	9
			Telophase	Laggard	3
				Vagrant	3
		2	Metaphase	C-metaphase	18
				Disturbed	32
				Fragment	4
			Anaphase	Bridge	12
				Laggard	12
				Vagrant	10
			Telophase	Laggard	2
				Vagrant	5
		3	Metaphase	C-metaphase	15
				Disturbed	18
				Fragment	4
				Sticky	2
			Anaphase	Bridge	1
				Laggard	2
				Vagrant	3
			Telophase	Vagrant	1
	0.0004	1	Metaphase	C-metaphase	4
				Disturbed	15
				Fragment	1
				Sticky	3
			Anaphase	Bridge	8
				Laggard	8
				Star	1
				Vagrant	9
			Telophase	Laggard	7
				Vagrant	12
		2	Metaphase	C-metaphase	6
				Disturbed	14

				Fragment	2
				Sticky	2
			Anaphase	Bridge	5
				Laggard	6
				Vagrant	8
			Telophase	Bridge	2
				Laggard	4
				Vagrant	6
		3	Metaphase	C-metaphase	7
				Disturbed	11
				Fragment	1
				Polyploid	1
				Sticky	6
			Anaphase	Laggard	4
				Vagrant	5
			Telophase	Fragment	1
				Laggard	4
				Vagrant	11
	0.001	1	Metaphase	C-metaphase	6
				Disturbed	20
				Polyploid	3
				Sticky	5
			Anaphase	Bridge	2
				Laggard	7
				Star	1
				Vagrant	11
			Telophase	Laggard	2
				Vagrant	3
		2	Metaphase	C-metaphase	10
				Disturbed	23
				Fragment	3
				Polyploid	5
				Sticky	3
			Anaphase	Bridge	2
				Laggard	2
				Star	1
				Vagrant	5
			Telophase	Fragment	2
				Laggard	7
				Vagrant	12
		3	Metaphase	C-metaphase	7
				Disturbed	23
				Fragment	1
				Polyploid	3
				Vagrant	1
			Anaphase	Bridge	2
				Fragment	1
				Laggard	2

				Vagrant	5
			Telophase	Fragment	1
				Laggard	3
				Vagrant	7
Blind	0.00004		Metaphase	C-metaphase	2
				Disturbed	16
				Polyploid	3
				Sticky	2
			Anaphase	Bridge	6
				Laggard	2
				Star	2
				Vagrant	8
			Telophase	Bridge	3
				Fragment	1
				Laggard	3
				Vagrant	4
	0.0004		Metaphase	C-metaphase	7
				Disturbed	14
				Fragment	1
			Anaphase	Bridge	3
				Laggard	5
				Vagrant	8
			Telophase	Bridge	6
				Fragment	1
				Laggard	1
				Vagrant	3
	0.001		Metaphase	C-metaphase	13
				Disturbed	10
				Sticky	3
				Vagrant	1
			Anaphase	Bridge	2
				Fragment	1
				Laggard	3
				Vagrant	3
			Telophase	Bridge	3
				Vagrant	2
Neg. ctr.		1	Metaphase	C-metaphase	4
				Disturbed	22
				Fragment	5
				Polyploid	1
				Sticky	1
				Vagrant	1
			Anaphase	Bridge	1
				Laggard	1
				Vagrant	1
			Telophase	Bridge	4
				Laggard	2
				Vagrant	1

		2	Metaphase	C-metaphase	16
				Disturbed	11
			Anaphase	Bridge	3
				Fragment	1
				Laggard	1
				Vagrant	2
			Telophase	Bridge	2
				Fragment	1
				Laggard	2
				Vagrant	1
		3	Metaphase	C-metaphase	3
				Disturbed	25
				Fragment	3
				Sticky	1
			Anaphase	Bridge	2
				Vagrant	2
			Telophase	Bridge	5
				Laggard	4
				Vagrant	4
Pos. ctr.		1	Metaphase	C-metaphase	11
				Disturbed	37
				Fragment	2
				Polyploid	1
				Sticky	2
			Anaphase	Bridge	12
				Fragment	2
				Laggard	14
				Vagrant	18
			Telophase	Bridge	5
				Fragment	1
				Laggard	9
				Vagrant	13
		2	Metaphase	C-metaphase	20
				Disturbed	36
				Fragment	1
				Sticky	2
			Anaphase	Bridge	8
				Fragment	2
				Laggard	9
				Star	2
				Vagrant	9
			Telophase	Bridge	8
				Laggard	8
				Vagrant	11
		3	Metaphase	C-metaphase	12
				Disturbed	45
				Sticky	1
			Anaphase	Bridge	4

				Laggard	5
				Star	2
				Vagrant	5
			Telophase	Bridge	1
				Laggard	7
				Vagrant	11

Appendix D: Statistics

Table D: Results from a two-sample Student t-test (p<0.05), including critical t-values and degrees of freedom (df), testing if the mean (root length, mitotic index, damaged cells (%), chromosomal aberrations (%)) of three individuals of onions is equal between two different treatment-groups (1 and 2). Different symbols denote the means of the different treatment-groups that are not significantly different (ns) or significantly different (weakly (-/+), clearly (--/++) or strongly (---/+++)).

Root inhibition test					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Bl. - 0.00004	Bl. - 0.00001	0.729	4.000	0.506	ns
Bl. - 0.0001	Bl. - 0.00001	0.859	4.000	0.439	ns
Bl. - 0.0004	Bl. - 0.00001	1.313	4.000	0.259	ns
Bl. - 0.001	Bl. - 0.00001	1.477	4.000	0.214	ns
Bl. - 0.004	Bl. - 0.00001	0.138	4.000	0.897	ns
Bl. - 0.01	Bl. - 0.00001	1.501	4.000	0.208	ns
Bl. - 0.04	Bl. - 0.00001	2.079	4.000	0.106	ns
Bl. - 0.0001	Bl. - 0.00004	0.002	4.000	0.999	ns
Bl. - 0.0004	Bl. - 0.00004	-0.080	4.000	0.940	ns
Bl. - 0.001	Bl. - 0.00004	0.335	4.000	0.754	ns
Bl. - 0.004	Bl. - 0.00004	-0.715	4.000	0.514	ns
Bl. - 0.01	Bl. - 0.00004	0.133	4.000	0.901	ns
Bl. - 0.04	Bl. - 0.00004	0.628	4.000	0.564	ns
Bl. - 0.0004	Bl. - 0.0001	-0.100	4.000	0.925	ns
Bl. - 0.001	Bl. - 0.0001	0.385	4.000	0.720	ns
Bl. - 0.004	Bl. - 0.0001	-0.874	4.000	0.431	ns
Bl. - 0.01	Bl. - 0.0001	0.158	4.000	0.882	ns
Bl. - 0.04	Bl. - 0.0001	0.737	4.000	0.502	ns
Bl. - 0.001	Bl. - 0.0004	0.716	4.000	0.513	ns
Bl. - 0.004	Bl. - 0.0004	-1.922	4.000	0.127	ns
Bl. - 0.01	Bl. - 0.0004	0.531	4.000	0.624	ns
Bl. - 0.04	Bl. - 0.0004	1.428	4.000	0.226	ns
Bl. - 0.004	Bl. - 0.001	-1.642	4.000	0.176	ns
Bl. - 0.01	Bl. - 0.001	-0.347	4.000	0.746	ns
Bl. - 0.04	Bl. - 0.001	0.377	4.000	0.725	ns
Bl. - 0.01	Bl. - 0.004	1.918	4.000	0.128	ns
Bl. - 0.04	Bl. - 0.004	2.485	4.000	0.068	ns
Bl. - 0.04	Bl. - 0.01	0.893	4.000	0.422	ns
P. - 0.001	P. - 0.0004	4.318	4.000	0.012	-
S. - 0.004	S. - 0.001	4.468	4.000	0.011	-
Mitotic index					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	1.820	4.000	0.143	ns
B. - 0.00004	Neg. ctr.	4.321	4.000	0.012	-
B. - 0.0004	Neg. ctr.	4.293	4.000	0.013	-
B. - 0.001	Neg. ctr.	4.176	4.000	0.014	-
Pos. ctr.	Neg. ctr.	3.984	4.000	0.016	-
P. - 0.00004	Neg. ctr.	3.605	4.000	0.023	-

P. - 0.0004	Neg. ctr.	3.878	4.000	0.018	-
P. - 0.001	Neg. ctr.	4.369	2.013	0.048	-
S. - 0.00004	Neg. ctr.	3.822	4.000	0.019	-
S.- 0.0004	Neg. ctr.	4.214	4.000	0.014	-
S. - 0.001	Neg. ctr.	4.895	4.000	0.008	--
Damaged cells (%)					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Pos. ctr.	B. - 0.0004	-2.682	4.000	0.055	ns
Pos. ctr.	B. - 0.001	-1.836	4.000	0.140	ns
Blind	Neg. ctr.	-1.486	4.000	0.212	ns
B. - 0.00004	Neg. ctr.	-4.760	4.000	0.009	++
B. - 0.0004	Neg. ctr.	-9.039	4.000	0.001	++
B. - 0.001	Neg. ctr.	-6.332	4.000	0.003	++
Pos. ctr.	Neg. ctr.	-9.769	4.000	0.001	++
P. - 0.00004	Neg. ctr.	-7.649	4.000	0.002	++
P. - 0.0004	Neg. ctr.	-6.606	4.000	0.003	++
P. - 0.001	Neg. ctr.	-12.964	4.000	0.000	+++
S. - 0.00004	Neg. ctr.	-4.330	4.000	0.012	+
S.- 0.0004	Neg. ctr.	-3.935	4.000	0.017	+
S. - 0.001	Neg. ctr.	-7.016	4.000	0.002	++
Pos. ctr.	P. - 0.0004	-2.182	4.000	0.095	ns
Pos. ctr.	P. - 0.001	-0.389	4.000	0.717	ns
Pos. ctr.	S. - 0.00004	-5.494	4.000	0.005	++
Pos. ctr.	S. - 0.0004	-7.860	4.000	0.001	++
Pos. ctr.	S. - 0.001	-6.085	4.000	0.004	++
Damaged cells in metaphase (%)					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	-0.940	4.000	0.400	ns
B. - 0.00004	Neg. ctr.	-2.341	4.000	0.079	ns
B. - 0.0004	Neg. ctr.	-6.596	4.000	0.003	++
B. - 0.001	Neg. ctr.	-6.068	4.000	0.004	++
Pos. ctr.	Neg. ctr.	-9.253	4.000	0.001	++
P. - 0.00004	Neg. ctr.	-4.535	4.000	0.011	+
P. - 0.0004	Neg. ctr.	-4.259	4.000	0.013	+
P. - 0.001	Neg. ctr.	-6.007	4.000	0.004	++
S. - 0.00004	Neg. ctr.	-4.183	4.000	0.014	+
S.- 0.0004	Neg. ctr.	-1.464	4.000	0.217	ns
S. - 0.001	Neg. ctr.	-4.518	4.000	0.011	+
Damaged cells in anaphase (%)					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	-3.308	4.000	0.030	+
B. - 0.00004	Neg. ctr.	-7.784	4.000	0.001	++
B. - 0.0004	Neg. ctr.	-3.383	4.000	0.028	+
B. - 0.001	Neg. ctr.	-6.743	4.000	0.003	++
Pos. ctr.	Neg. ctr.	-8.169	4.000	0.001	++
P. - 0.00004	Neg. ctr.	-5.400	4.000	0.006	++
P. - 0.0004	Neg. ctr.	-2.720	2.125	0.106	ns
P. - 0.001	Neg. ctr.	-10.065	4.000	0.001	++

S. - 0.00004	Neg. ctr.	-11.196	4.000	0.000	+++
S.- 0.0004	Neg. ctr.	-6.450	4.000	0.003	++
S. - 0.001	Neg. ctr.	-5.103	4.000	0.007	++
Damaged cells in telophase (%)					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	0.758	4.000	0.491	ns
B. - 0.00004	Neg. ctr.	-0.647	4.000	0.553	ns
B. - 0.0004	Neg. ctr.	-6.430	4.000	0.003	++
B. - 0.001	Neg. ctr.	-4.544	4.000	0.010	+
Pos. ctr.	Neg. ctr.	-3.696	4.000	0.021	+
P. - 0.00004	Neg. ctr.	-4.280	4.000	0.013	+
P. - 0.0004	Neg. ctr.	-7.806	4.000	0.001	++
P. - 0.001	Neg. ctr.	-3.699	4.000	0.021	+
S. - 0.00004	Neg. ctr.	2.132	4.000	0.100	ns
S.- 0.0004	Neg. ctr.	-2.576	4.000	0.062	ns
S. - 0.001	Neg. ctr.	-1.160	4.000	0.311	ns
Specific chromosomal aberration as percentage of total dividing cells					
Bridge					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	-2.064	4.000	0.108	ns
B. - 0.00004	Neg. ctr.	-1.678	4.000	0.169	ns
B. - 0.0004	Neg. ctr.	0.192	4.000	0.857	ns
B. - 0.001	Neg. ctr.	-0.413	4.000	0.701	ns
P. - 0.00004	Neg. ctr.	-12.821	4.000	0.000	+++
P. - 0.0004	Neg. ctr.	2.895	4.000	0.044	-
P. - 0.001	Neg. ctr.	-0.961	4.000	0.391	ns
C-metaphase					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	-0.298	4.000	0.781	ns
B. - 0.00004	Neg. ctr.	-0.725	4.000	0.509	ns
B. - 0.0004	Neg. ctr.	-2.146	4.000	0.098	ns
B. - 0.001	Neg. ctr.	-0.940	4.000	0.401	ns
P. - 0.00004	Neg. ctr.	0.489	4.000	0.650	ns
P. - 0.0004	Neg. ctr.	0.776	4.000	0.481	ns
P. - 0.001	Neg. ctr.	0.324	4.000	0.762	ns
Disturbed metaphase					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	0.746	4.000	0.497	ns
B. - 0.00004	Neg. ctr.	-1.409	4.000	0.232	ns
B. - 0.0004	Neg. ctr.	-2.044	4.000	0.110	ns
B. - 0.001	Neg. ctr.	-1.711	4.000	0.162	ns
Fragment					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	1.392	4.000	0.236	ns
B. - 0.00004	Neg. ctr.	0.878	4.000	0.430	ns
B. - 0.0004	Neg. ctr.	-0.228	4.000	0.831	ns
B. - 0.001	Neg. ctr.	-0.822	4.000	0.457	ns

P. - 0.00004	Neg. ctr.	0.398	4.000	0.711	ns
P. - 0.0004	Neg. ctr.	0.104	4.000	0.922	ns
P. - 0.001	Neg. ctr.	-0.118	4.000	0.911	ns
Laggard					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	-1.869	4.000	0.135	ns
B. - 0.00004	Neg. ctr.	-5.798	4.000	0.004	++
B. - 0.0004	Neg. ctr.	-2.173	2.011	0.161	ns
B. - 0.001	Neg. ctr.	-4.054	2.018	0.055	ns
S. - 0.00004	Neg. ctr.	-1.682	4.000	0.168	ns
S. - 0.0004	Neg. ctr.	-5.196	4.000	0.007	++
S. - 0.001	Neg. ctr.	-3.772	4.000	0.020	+
Polyploidy					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	-0.582	4.000	0.592	ns
Star anaphase					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	-1.000	2.000	0.423	ns
Stickiness					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	-1.099	4.000	0.334	ns
B. - 0.00004	Neg. ctr.	0.651	4.000	0.551	ns
B. - 0.0004	Neg. ctr.	-4.046	4.000	0.016	+
B. - 0.001	Neg. ctr.	-2.369	4.000	0.077	ns
P. - 0.00004	Neg. ctr.	-7.444	4.000	0.002	++
P. - 0.0004	Neg. ctr.	-5.300	4.000	0.006	++
P. - 0.001	Neg. ctr.	-4.357	4.000	0.012	+
S. - 0.00004	Neg. ctr.	-0.588	4.000	0.588	ns
S. - 0.0004	Neg. ctr.	-2.870	4.000	0.045	+
S. - 0.001	Neg. ctr.	-1.315	4.000	0.259	ns
Vagrant					
Treatment (1)	**Treatment (2)**	**t**	**df**	**Sig. (2-tailed)**	**Symbol**
Blind	Neg. ctr.	-3.388	4.000	0.028	+
B. - 0.00004	Neg. ctr.	-3.858	4.000	0.018	+
B. - 0.0004	Neg. ctr.	-8.098	4.000	0.001	++
B. - 0.001	Neg. ctr.	-3.679	4.000	0.021	+
S. - 0.00004	Neg. ctr.	-2.377	4.000	0.076	ns
S. - 0.0004	Neg. ctr.	-9.450	4.000	0.001	++
S. - 0.001	Neg. ctr.	-13.811	4.000	0.000	+++

Appendix E: Pictures of chromosomal aberrations

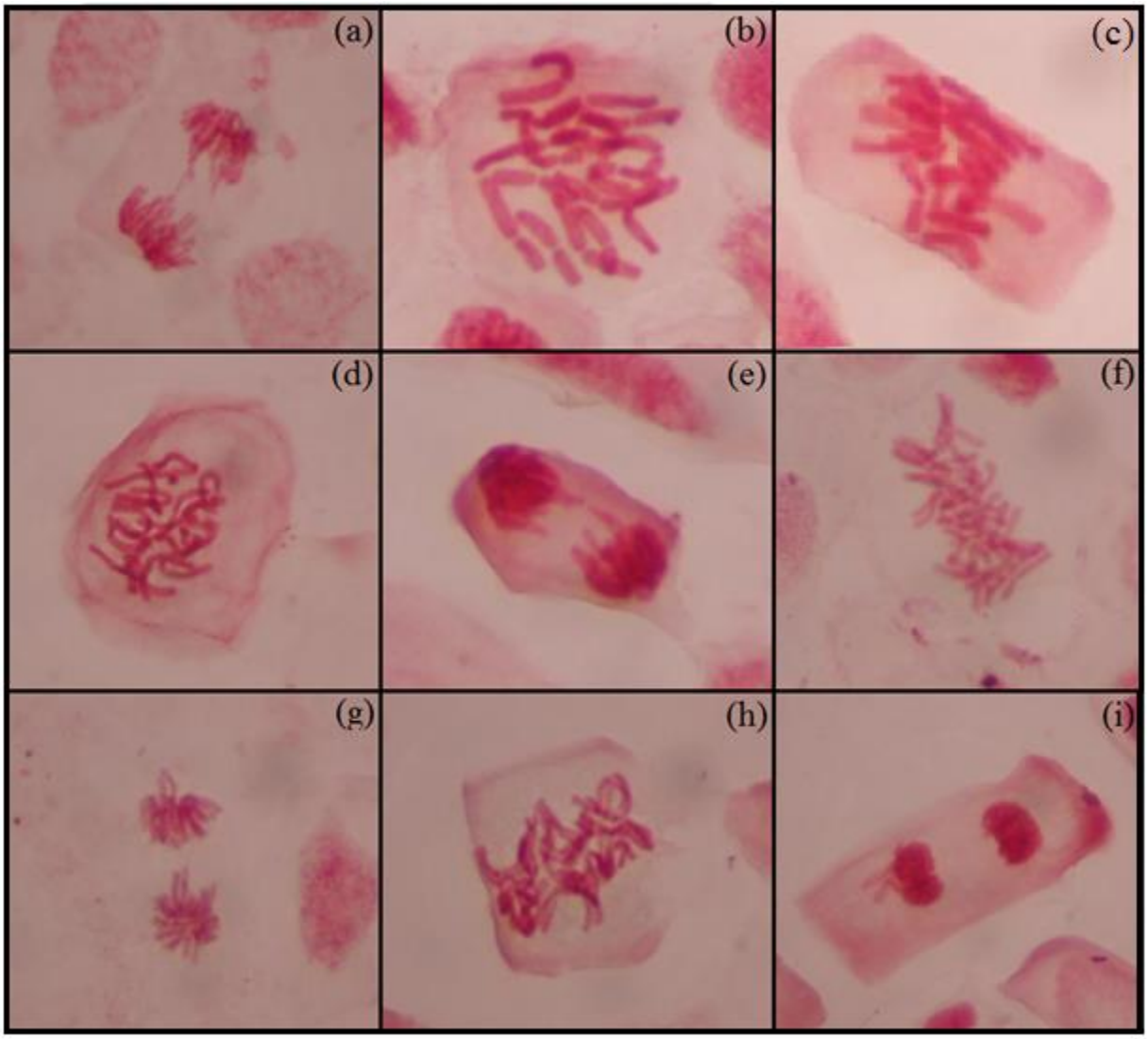

Figure E. Different chromosomal aberrations detected in meristematic root cells of *Allium cepa* onions after exposure to different treatment-groups. A: Bridge found in anaphase within an onion exposed to tap water. B: C-metaphase found in an onion exposed to beech-oil (0.0004 ml oil/ml solution). C: Disturbed metaphase found in an onion exposed to poplar-oil (0.0004 ml oil/ml solution). D: Fragment found in a disturbed metaphase within an onion exposed to poplar-oil (0.0004 ml oil/ml solution). E: Laggards found in telophase within an onion exposed to poplar-oil (0.00004 ml oil/ml solution). F: Polyploid cell found in an onion exposed to beech-oil (0.0004 ml oil/ml solution). G: Star-anaphase found in an onion exposed to spruce-oil (0.001 ml oil/ml solution). H: Sticky metaphase found in an onion exposed to poplar-oil (0.00004 ml oil/ml solution). I: Vagrants found in telophase within an onion exposed to poplar-oil (0.00004 ml oil/ml solution).