The evaluation of the potential ecotoxicity of pyroligneous acid obtained from fast pyrolysis

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Abstract
Pyroligneous acid (PA) is a by-product of bio-oil, which is obtained by pyrolysis of the wood. This product has been tested for use in several areas, such as agriculture, as a promising green herbicide; however, there are few scientific data regarding its environmental impacts. For this study, an ecotoxicity testing battery, composed of *Daphnia magna* acute toxicity test, *Allium cepa* test and *in vitro* Comet assay with the rainbow trout gonad-2 cell fish line (RTG-2) were used to evaluate the acute toxicity and genotoxicity of PA obtained from fast pyrolysis of eucalyptus wood fines. The PA presented acute toxicity to *D. magna* (microcrustacea) with EC$_{50}$ of 26.12 mg/L, and inhibited the seed germination (EC$_{50}$ 5.556 g/L) and root development (EC$_{50}$ 3.436 g/L) of *A. cepa* (higher plant). No signs of genotoxicity (chromosomal aberrations and micronuclei in *A. cepa* and primary DNA lesions in RTG-2 cells) were detected to this product. The acute toxicity and absence of genotoxicity may relate to the molecules found in the PA, being the phenolic fraction the key chemical candidate responsible for the toxicity observed. In addition, daphnids seem to be more sensitivity to the toxicity of PA than higher plants based on their EC$_{50}$ values. This first ecotoxicological evaluation of PA from fast pyrolysis pointed out the need of determining environmental exposure limits to promote the safer agriculture use of this product, avoiding impacts to living organisms.

Keywords: Pyroligneous Acid, acute toxicity, DNA damages, *Allium cepa*, *Daphnia magna*, RTG-2 fish cell line.

1. Introduction

Bio-oil is a product that can be derived from pyrolysis of wood (Meier and Faix, 1999; Mohan et al., 2006; Roberts, 1970), which consists in decomposition of the biomass by heat under air controlled environment (Mohan et al., 2007). The resultant process is formed by vapor condensation. Bio-oil has many unique characteristics that make this product valuable in a number of applications, such as crop protection agent (Shihadeh and Hochgreb, 2000).
For the production of bio-oil, two main methods are employed (slow and fast pyrolysis) and they differ in the percentage of gas, char and liquid products obtained (Grewal et al., 2018). Slow pyrolysis consists of slow heating rates and yields equal quantities of gas, char and liquid while is heated at temperatures of 300 °C. Contrary, fast pyrolysis, which consists of high heating rates, usually yields larger quantity of liquid phase (60-75% of liquid bio-oil) heating at temperatures of 500 °C. The derived product via pyrolysis can be separated via distillation of the condensed liquid (Souza et al., 2012) and, although research have been focusing on the energy combustion of this product (pyroligneous tar) (Bridgwater, 2003; Honnery et al., 2008), the aqueous part - pyroligneous acid (PA) - is used in agriculture. The promising agriculture usage of PA relates to their antimicrobial, antioxidant and pesticidal activities; however, this product has not yet been properly investigated towards its safety to environmental organisms (e.g., non-target plants and aquatic life) (Kadota et al., 2002; Ma et al., 2013; Mathew and Zakaria, 2015; Mmojieje, 2016; MURAYAMA et al., 1995; Wei et al., 2010).

The two main routes to obtain PA, have its own attractiveness depending on the application intended (Yang et al., 2016). Fast pyrolysis seems to gather some attention as potential energy resources (Dabros et al., 2018), its biomass, but the aqueous part fraction of the bio-oil has yet to find a potential use rather than meat browning agents (Czernik and Bridgwater, 2004). For these reasons, researchers have been led to deduce that PA from fast pyrolysis can also be used in the agriculture field (Hossain et al., 2015).

PA can contain in its composition more than 200 compounds depending on the base material it was extracted from (Kadota et al., 2002); however, this aqueous phase is formed mainly by aldehydes and phenols (Loo et al., 2008; Marumoto et al., 2012; Underwood, 1992).

Although PA usage has positive aspects mainly to agriculture, there is a risk of environmental contamination and thus, the need to understand its adverse effects to non-target organisms (Zulkarami et al., 2011). Impacts caused by chemicals on the
environment and health of living organisms can often not be estimated visually; consequently, tests to evaluate their potential harmful effects on biological systems should be performed, contributing to a safer application (Tiilikka et al., 2010).

Ecotoxicity tests provide relevant information about the adverse effects of chemicals on living organisms at different levels of biological hierarchy (Zhou et al., 2018). Acute assays protocols to *Daphnia magna* (microcrustacean – planktonic invertebrate organism) have been recommended by several regulatory agencies because of its geographical distribution, central role in freshwater food webs and sensibility to a wide range of chemicals (Grintzalis K, Dai W., Panagiotidis, K, Belvgeni A., 2017).

Most of the ecotoxicological studies with *D. magna* are based on acute toxicity data of effective concentration (EC$_{50}$ – Half maximal effective concentration), for immobilisation of neonates, to estimate the acute mortality following short-term exposure (24-48 h) to a chemical (Bownik, 2017; De Coen and Janssen, 1997; Janssen and Persoone, 1993).

Study on fish toxicity is another common ecotoxicity test used to determine safe levels of chemicals to aquatic environments (Bols et al., 2005). These tests were primary conducted using juveniles or adults life stages of species (*in vivo*); however, there are considerable efforts to promote the use of fish cell lines in ecotoxicology (Bermejo-Nogales et al., 2017; Castaño et al., 2003; Franco et al., 2018; Lillicrap et al., 2016). The interaction of chemicals at cellular level is an important study to determine the cytotoxicity of a compound; thus, fish cell lines have been used to evaluate the effects of chemicals on processes, such as xenobiotic metabolism and DNA damages (genotoxicity) (Lillicrap et al., 2016). With respect to genotoxicity, the RTG-2 permanent fish cell line, derived from rainbow trout (*Oncorhynchus mykiss*) gonadal tissue, has been successfully used to detect aquatic genotoxicants, estimating their genotoxic effects on the reproductive system of fish (Castaño and Becerril, 2004; Felzenszwalb et al., 2018; Klingelfus et al., 2019; Llorente et al., 2012; Marabini et al., 2011; Munari et al., 2014; Oliveira et al., 2018; Sánchez-Fortún et al., 2005).

Plant toxicity tests are also essential test methods of ecotoxicological assessment
(Boutin et al., 2014; Egan et al., 2014; Felisbino et al., 2018). Different biomarkers of toxicity can be analysed on plant test systems in order to estimate their toxic effects. The higher plant Allium cepa presents good chromosomal condition (i.e., reduced number and large size of chromosomes), favouring its use to estimate the genetic damages induced by chemicals to plants (Leme and Marin-Morales, 2009; Silveira GL, Lima MGF, Reis GB, Palmieri MJ, 2017), aside from its use to evaluate other parameters of toxicity, such as seed germination and root growth (Rank, 2003; Silveira GL, Lima MGF, Reis GB, Palmieri MJ, 2017; Tkalec M, Malaric K, Pavlica, M, Pevalek-kozlina B, 2009).

The fact that PA is used in agriculture (Mmojieje, 2016) without proper evaluation of its toxicity to non-target organisms raises concerns. Therefore, the present work aimed to evaluate the acute toxicity and genotoxicity of PA extracted from fast pyrolysis of eucalyptus wood fines. To accomplish this, seed germination, root elongation, as well as chromosomal aberration (CA) and micronucleus (MN) tests were conducted on A. cepa to examine its potential on seed development and toxicity. In addition, D. magna acute toxicity assay and the in vitro Comet assay with RTG-2 fish cell line were used to determine its ecotoxicity potential.

2. Materials and Methods

The procedure for bio-oil production was derived from (Lourençon et al., 2016) which was obtained in a pilot-scale fast pyrolysis reactor (BIOWARE, Brazil), operating in a fluidized bed with nominal supply of 20 kg h\(^{-1}\), poor oxygen atmosphere, reaction temperature of 500°C and 100 mm H\(_2\)O of static pressure. The reaction temperature was achieved by partial combustion of the biomass products through preheated air injection. Then, the reactor was fed with hot non-condensable gases to maintain the reaction temperature constantly at 500°C. Eucalypt wood fines rejected from a Kraft pulp line (Suzano Papel e Celulose, São Paulo, Brazil) were used for obtaining the soluble bio-oil fraction.

2.1 Preparation of pyroligneous acid (PA)
To obtain the PA, 45 mL of chloroform (CHCl$_3$) and 45 mL of ultrapure water were added to 5 mL of the bio-oil (i.e., a mixture of aqueous and non-aqueous fractions). After 24 h, the aqueous fraction was collected and mixed again with chloroform. After complete separation of the phases, the aqueous fraction (PA) was withdrawn and stored at room temperature until the analyses were performed.

2.2 Gas chromatography–mass spectrometry (GC–MS)

For Bio-oil (100 µL) phase separation, was added water (2 mL) and chloroform (2 mL). The mixture was stirred and centrifuged (5 min, 3000 rpm). The water phase was transferred to an Eppendorf tube and dried under vacuum for 18 h (Speed Vacuum - Eppendorf). The residues were dissolved in acetone (100 µL) and analysed by gas chromatography-mass spectrometry (GC-MS). The extracts were injected (1 µL, Thermo Triplus AS) into a Focus GC gas chromatography tandem to a Polaris Q ion trap mass spectrometer (Thermo), equipped with a DB5ms capillary column (30m x 0.25mm, 25µm film thickness). The GC oven temperature was programmed from 40 °C (held for 8 min) to 280 °C at 7°C min$^{-1}$, then held for 15 min. Helium, at a constant flow of 1.0 mL min$^{-1}$, was the carrier gas. The inlet in split mode 1:100 was set at 230 °C. The GC-MS interface and ion source temperatures were 250 °C and 200 °C, respectively.

The ion trap mass spectrometer was operated in the positive impact electronic mode at 70 eV scanning the range m/z 40–650 in a total scan time of 0.59 and emission current 250 mA. Mass spectral deconvolution and automated calculation of RI was performed by the automated mass spectral deconvolution and identification system (AMDIS, National Institute of Standards and Technology, Gaithersburg, MD, USA). Standard solutions of linear alkanes (C$_7$ – C$_{30}$, Sigma-Aldrich 49451-U) was used for Kováts RI calibration in the GC-MS. Data deconvolution was performed with the following specifications: component width = 12; adjacent peak subtraction = 2; resolution = low; sensitivity = very low; shape requirements = medium. Compounds were identified from the deconvoluted mass spectra by comparison with mass spectra published in the specialised literature.
2.3 *Daphnia magna* acute toxicity assay

*D. magna* acute toxicity assay was carried out according to the OECD guideline 202 (OECD Guideline, 1984). *D. magna* juveniles (<24 h), from a healthy stock (Laboratory of Ecotoxicology, Federal University of Technology – Paraná, Brazil), were maintained in reconstituted water and fed with *Desmodesmus subspicatus* until the exposure to PA. The daphnids (10 neonates/treatment) were initially exposure (48 h) to PA at 10.5 g/L, and due to the high toxicity they were exposure to the tested compound at 11.55, 17.33, 23.10, 28.88 and 34.65 mg/L (final concentration range). The experiments were carried out in triplicate per treatment, and, during the test, the organisms were maintained incubated at 20 ± 2 ºC without light and feeding. At the end of exposure period (48 h), organism immobility was assessed and toxicity calculated through Probit method and expressed in EC$_{50}$.

2.4 Bioassays with *A. cepa* seeds

2.4.1. Test system and exposure condition

*A. cepa* seeds, same batch and variety ("Baia Periform" onion) purchased from “Isla Sementes” company (Porto Alegre-RS/Brazil) were used. For the seed germination and root elongation toxicity test, seeds of *A. cepa* were placed into petri dishes covered with filter paper (100 seeds/plate) and submitted to germination (5 days) in different concentrations of PA - ultrapure water (negative control – NC) and 6 mg/L of zinc sulfate heptahydrate (CAS No. 7446-20-0, Sigma-Aldrich) (positive control – PC) (Santos-Filho et al., 2018). The range of concentrations of PA in this test was 0.81, 1.62, 3.24, 6.5, 13.07 g/L. For the chromosomal aberration and micronucleus test (genotoxicity), *A. cepa* seeds were also submitted to germination (5 days) at different test solutions of PA (0.85, 1.75 and 3.5 g/L – non-toxic concentrations) in Petri dishes covered with filter paper (100 seeds/plate). Ultrapure water and 10 mg/L of Methyl Methanesulfonate (MMS, CAS No. 66-27-3, Sigma-Aldrich) were used as NC and PC, respectively (Leme and...
Both experiments were kept under controlled temperature (25°C) and in the absence of light.

2.4.2. Seed germination and root elongation toxicity test

After five days of exposure, the number of germinated seeds was counted and the root length was measured. The relative seed germination percentage was calculated by dividing the number of seeds germinated in the exposed groups by the number of seeds germinated in the NC. The criterion for test validation was that at least 65% of the seeds from the NC should germinate, and 5 mm of radicular protrusion was regarded as germinated. The toxicity was expressed as effective percentage of 50% (EC_{50}) in seed germination or root growth inhibition. The data are presented as triplicate plates per treatment.

2.4.3 Chromosomal aberration and micronucleus test (genotoxicity)

Roots of ~ 2 cm in length (5 days of exposure) were collected, fixed in alcohol-acetic acid (3:1-v/v) and stored at 4°C until analysis. Cytological slides were prepared according to Leme and Marin-Morales (2008); cells carrying changes in the genetic material were quantified by light microscope, analysing 5000 cells per treatment (500 cells/slide, 10 slides/treatment). Different types of abnormalities were considered for chromosome aberration (CA) (losses, fragments, bridges, delays, chromosomal adhesions, among others) in different phases of cell division (prophase, metaphase, anaphase, telophase). However, for the evaluation of CA as a single endpoint (genotoxicity), all different abnormalities found were put together into one group. The analysis of micronucleus in these cells is considered as another parameter of evaluation (mutagenicity), as well as the Mitotic Index (MI), which is related to the number of dividing cells and constitutes as a third parameter of evaluation (cytotoxicity). The data were statistically analysed using the Mann-Whitney non-parametric test and significant differences related to control were considered at p <0.05.
2.5 In vitro Comet assay with RTG-2 fish cell line (genotoxicity)

2.5.1 Cell culture and exposure

The gonadal lineage of rainbow trout (*Onchorhyncus mykiss*) named as RTG-2 (Rainbow trout gonad-2 cell line, European Collection of Authenticated Cell Cultures [ECACC] 90102529) was used and maintained in Leibovitz-15 (L-15) medium supplemented with 10% fetal bovine serum, 2 mM of L-glutamine, and addition of 1% penicillin-streptomycin (all from Gibco®), at 22°C. Cells were subcultured when reached ~80% of confluence.

RTG-2 cells were seeded into 24-well plates (5×10⁴ cells/well) and incubated at 22 °C for 24 h before exposure. These cells were exposed to different non-cytotoxic concentrations of pyroligneous acid (0.2, 0.4, 0.85, 1.75 and 3.5 g/L) for 3 h at 22 °C. Sterile deionized water at 10%-v/v was used as NC and MMS at 0.5 mM and hydrogen peroxide (H₂O₂, Sigma-Aldrich) at 80 μM (15 min via culture medium) was used as PC of the alkaline (standard) and hOGG1-modified alkaline (oxidative) versions of the Comet assay, respectively.

After exposure, RTG-2 cells were harvested, and the single cell suspensions was obtained to each treatment. 10 µL of these single cell suspensions were used in Trypan Blue Dye Exclusion Test to verify cell viability. The Trypan Blue results indicated cell viability higher than 90% to all treatments. The remaining cell suspensions were processed for the in vitro Comet assay.

2.5.2 In vitro Comet assay procedure

50 µL of the single cell suspension was resuspended in low melting agarose (120 µL – 0.5%-w/v in pyroligneous acid). Cell suspension was spread on two 1.5% agarose-coated slides, which, after solidification at 4 °C, were immersed in a cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% lauryl sarcosinate, 1% Triton X-100 and 10% DMSO, pH 10) for 2 h. The slides were then transferred to an electrophoresis chamber and then filled with electrophoresis buffer (200 mM EDTA, 10 M NaOH, pH > 13) for 25 min at 4°C for DNA unwinding. Electrophoresis was carried out using the same buffer for 25 min at 1 V/cm and 300
m. The slides were neutralized with Tris-HCl buffer (pH 7.5) for 20 min and fixed in 100% ethanol. For the hOGG1-modified alkaline version, the comet slides were washed (3 × 5 min) with enzyme buffer (hOGG1: 40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8) after lysis and then incubated with hOGG1 (0.08 U/slide, New England Biolabs) for 30 min at 37 °C, in a moistened chamber. After enzyme incubation, the slides were rinsed with distilled water and placed into the electrophoresis chamber for DNA unwinding and electrophoresis, as described above (Felzenszwalb et al., 2018, Oliveira et al., 2018, Klingelfus et al., 2018).

The slides were stained with ethidium bromide solution (20 μg/mL, Sigma-Aldrich) and analyzed under a fluorescence microscope (Axio Imager Z2, Carl Zeiss, Jena, DE), equipped with Metafer 4/V Slide automated capture software (Metasystems, Altlussheim, DE) and Camera Cool Cube 1 – Metasystems. DNA lesions were quantified as DNA tail intensity (percentage of DNA in tail) (Azqueta et al. 2011) using the computer-based image analysis Metafer CometScan v.2.8.0® (Metasystems, Germany) on 100 selected nucleoids. The Comet data were evaluated using ANOVA and Dunnett’s post hoc tests. All experiments were carried out independently in triplicate, using a single well per treatment.

3. Results and Discussion

To date, few studies have evaluated the toxicological responses of bio-oils in biological systems (Chatterjee et al., 2013; Pekol et al., 2012). Some previous studies performed with different bio-oils have shown their ability to induce adverse effects, both at the cellular and genetic levels. Cell responses include the reduction of cell viability and the increase of cell death by apoptosis in human and rodent cells along with increasing concentrations of bio-oil (Chatterjee et al., 2013). Contrarily, the extracted components of bio-oil, such as PA, is used as pesticide and growth stimulating activity in , but its uses have not yet been investigated towards its safety to non-target organisms (Mathew and Zakaria, 2015; Mmogieje, 2016).

The chemical composition of PA vary depending on their source of extraction and
process, and the chemical nature of a compound strictly relates to its potential toxicity.

From the chromatography analysis (GC-MS) of the PA, 45 elements were identified out of 57 (Table 1). The most yielded compound was identified within the anhydrosugar group, represented by levoglucosan with more than 60% of total GC area; catechol and hydroquinone from hydroxybenzenes group represented 6.45% and 2.05% respectively. Although, hydroxyacetone was identified in large quantity due to the procedure used in this technique, little represents to the PA composition.

**Table 1.** Pyroligneous acid (PA) compounds obtained by GC-MS.

<table>
<thead>
<tr>
<th>RT</th>
<th>Identity</th>
<th>m/z</th>
<th>m/z</th>
<th>m/z (%)</th>
<th>%</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.68</td>
<td>hydroxyacetone</td>
<td>43</td>
<td>74</td>
<td>73</td>
<td>3.42</td>
<td>small molecules</td>
</tr>
<tr>
<td>11.77</td>
<td>2-methoxytetrahydrofuran</td>
<td>41</td>
<td>72</td>
<td>101</td>
<td>0.10</td>
<td>furans</td>
</tr>
<tr>
<td>12.48</td>
<td>butanediol</td>
<td>43</td>
<td>57</td>
<td>58</td>
<td>0.35</td>
<td>small molecules</td>
</tr>
<tr>
<td>14.25</td>
<td>2-methyl-2-pentenal</td>
<td>54</td>
<td>69</td>
<td>98</td>
<td>0.13</td>
<td>small molecules</td>
</tr>
<tr>
<td>14.36</td>
<td>furfural</td>
<td>67</td>
<td>95</td>
<td>96</td>
<td>0.45</td>
<td>furans</td>
</tr>
<tr>
<td>15.96</td>
<td>tetrahydro-2,5-dimethoxy-furan</td>
<td>69</td>
<td>101</td>
<td>131</td>
<td>0.54</td>
<td>furans</td>
</tr>
<tr>
<td>16.09</td>
<td>monoacetate-1,2-ethanediol</td>
<td>43</td>
<td>61</td>
<td>74</td>
<td>0.52</td>
<td>small molecules</td>
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<tr>
<td>16.42</td>
<td>tetrahydro-2,5-dimethoxy-furan (isomer)</td>
<td>69</td>
<td>101</td>
<td>131</td>
<td>0.53</td>
<td>furans</td>
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<tr>
<td>16.76</td>
<td>2(3H)-Furanone</td>
<td>41</td>
<td>55</td>
<td>84</td>
<td>0.45</td>
<td>furans</td>
</tr>
<tr>
<td>16.96</td>
<td>unidentified</td>
<td>55</td>
<td>71</td>
<td>115</td>
<td>0.10</td>
<td>unknown</td>
</tr>
<tr>
<td>17.58</td>
<td>dihydro-3-methylene-2,5-Furanone dione</td>
<td>40</td>
<td>53</td>
<td>68</td>
<td>0.11</td>
<td>furans</td>
</tr>
<tr>
<td>18.25</td>
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<td>69</td>
<td>75</td>
<td>101</td>
<td>0.16</td>
<td>unknown</td>
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<td>18.28</td>
<td>3-ethyl-2-pentanone</td>
<td>55</td>
<td>86</td>
<td>114</td>
<td>0.08</td>
<td>small molecules</td>
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<td>19.23</td>
<td>4-hydroxy-2,3-dihydropyran-6-one</td>
<td>58</td>
<td>85</td>
<td>114</td>
<td>0.16</td>
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<tr>
<td>20.00</td>
<td>hydroxy - methyl - cyclopentenone</td>
<td>55</td>
<td>84</td>
<td>112</td>
<td>0.26</td>
<td>cyclopentenones</td>
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<tr>
<td>20.31</td>
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<td>57</td>
<td>85</td>
<td>116</td>
<td>0.58</td>
<td>small molecules</td>
</tr>
<tr>
<td>20.38</td>
<td>unidentified</td>
<td>43</td>
<td>73</td>
<td>128</td>
<td>0.49</td>
<td>unknown</td>
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<tr>
<td>20.67</td>
<td>2-furancarboxylic acid</td>
<td>84</td>
<td>95</td>
<td>112</td>
<td>0.35</td>
<td>furans</td>
</tr>
<tr>
<td>20.83</td>
<td>isobutyric anhydride</td>
<td>41</td>
<td>43</td>
<td>71</td>
<td>0.34</td>
<td>small molecules</td>
</tr>
<tr>
<td>21.15</td>
<td>unidentified</td>
<td>53</td>
<td>81</td>
<td>97</td>
<td>0.25</td>
<td>unknown</td>
</tr>
<tr>
<td>21.64</td>
<td>unidentified</td>
<td>43</td>
<td>57</td>
<td>69</td>
<td>2.82</td>
<td>unknown</td>
</tr>
<tr>
<td>21.80</td>
<td>isobutyric anhydride (isomer)</td>
<td>41</td>
<td>43</td>
<td>71</td>
<td>0.37</td>
<td>small molecules</td>
</tr>
<tr>
<td>22.10</td>
<td>dimethyl ester tetrahydro-2,5-furancarboxylic acid</td>
<td>59</td>
<td>69</td>
<td>101</td>
<td>0.17</td>
<td>furans</td>
</tr>
</tbody>
</table>
A summary of the amounts obtained by GC grouped by class of molecules (Table 2) evidences that the highest amount yielded for the investigated pyroligneous acid consisted of anhydrosugar and hydroxybenzenes following by small molecules, furans.
cyclopentenones and pyrans (Tamburini et al., 2017).

Table 2. General classes of molecules and percentual area obtained from pyroligneous acid (PA).

<table>
<thead>
<tr>
<th>Class</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>small molecules</td>
<td>5,79</td>
</tr>
<tr>
<td>Cyclopentenones</td>
<td>0,26</td>
</tr>
<tr>
<td>Pyrans</td>
<td>0,16</td>
</tr>
<tr>
<td>Furans</td>
<td>4,75</td>
</tr>
<tr>
<td>Anhydrosugars</td>
<td>66,23</td>
</tr>
<tr>
<td>Hydroxybenzenes</td>
<td>16,92</td>
</tr>
<tr>
<td>Unknown</td>
<td>5,88</td>
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</table>

From the detected compounds obtained by GC-MS, it can be seen that the lowest concentrations are cyclopentenones and pyrans. More specifically cyclopentenone, according to United States Environmental Protection Agency (US EPA) are already used in the market as pesticidal agent and present no signs of ecotoxicity (EPA 004049), as well as pyrans which the subclass have various FDA approved inhibitor against mycobacterium tuberculosis (Bhat et al., 2017). For furan classes, the majority of the compounds presents low ecotoxicity, such as furanone, furancarboxylic acid and furandione derivatives (Atkins et al., 1981; Paulus, 2005; Pilgåråd et al., 2010; Reynolds, 1989; Ventura et al., 2016) at the same concentration detected in the tested PA; however 2,5-Furandione, furfural and 5-Hydroxymethylfurfural are considered slightly ecotoxicants (Ventura et al., 2016). Nonetheless, reports on 5-Hydroxymethylfurfural and furfural for *Daphnia magna* suggests a moderate toxicity (Hessov, 1975). Catechol and hydroquinone induces chromosome aberrations in *Allium cepa* (Devillers et al., 1990; Petriccione et al., 2013). Coumarin and vanillin also shows moderate acute toxicity (Palmer and Maloney, 1958; Podbielkowska et al., 1995); the other available components of the hydroxybenzene groups are considered harmless (Staver et al., 2014).
Therefore, to better understand the potential ecotoxicity (acute toxicity and genotoxicity) of PA from fast pyrolysis, tests using the higher plant *A. cepa* (monocotyledon), the microcrustacean *D. magna* and the *in vitro* fish model RTG-2 (cell line-derived from gonad tissue) were performed.

The results of the *D. magna* acute toxicity assay exhibited that at the highest test concentrations of 40.43 mg/L and 34.65 mg/L (preliminary dose-finding experiment) the PA presented high toxicity with 100% of immobilization. Toxic effects were still observed to *D. magna* exposed to PA after 48 hr, showing an EC$_{50}$ of 26.12 mg/L (Fig. 1). The toxicity of this product started to decrease at 28.88 mg/L and there was no toxicity below the concentration of 11.55 mg/L. Compounds with 48 hr EC$_{50}$ (for crustacean) of 10 > but ≤ 100 mg/L are classified in the acute category 3 according to the Globally Harmonized System of classification and labelling of chemicals (GHS), and thus the PA from fast pyrolysis is harmful to aquatic life.

![Figure 1](image-url)  
*Figure 1.* Concentration-response curve of the effects of pyroligneous acid (PA) on *Daphnia magna*. Parameter log (mg/L) PA vs percentage of survival organisms was used to determine the concentration-response curve.

The PA also caused toxic effects on *A. cepa*. The results of the first toxicity test with
the highest concentrations of PA (13.07, 26.25, 52.5, 78.75 and 105 g/L) exhibited a complete inhibition of seed germination of *A. cepa*, except for the concentration of 13.07 g/L for which a low germination rate was observed (5%). From these results, a new concentration range was determined (0.81, 1.62, 3.24, 6.5, 13.07 g/L), and the seed germination of *A. cepa* was inhibited by PA with EC$_{50}$ of 5.556 g/L (Fig. 2A). This product also inhibited the root growth, showing an EC$_{50}$ of 3.436 g/L (Fig. 2B). However, PA presented lower toxicity on the higher plant *A. cepa* compared to *D. magna*. Terrestrial and aquatic organisms may display different sensitivities to the toxic effects of chemicals, and in most cases there are greater impact of aquatic environment than the terrestrial environment (Oliveira et al., 2018). These findings reinforce the need of using an ecotoxicity test battery, comprising test organism’s representative of different trophic levels and ecosystems in order to accurately predict the chemical hazard.
Figure 2. Concentration-response curves of the effects of pyroligneous acid (PA) on seed germination (A) and root elongation (B) of Allium cepa. Parameter log (g/L) PA vs percentage of seed germination (A) or root length (cm) (B) were used to determine these concentration-response curves.

Although the acute toxicity evaluated by endpoints, such as mortality and growth rates, are the mostly used parameter for estimating the toxic effects of a chemical to environmental organisms (Hanson et al., 2017), endpoints of genetic changes represent an important category of the adverse effects of pollutants (Oliveira et al., 2018). The consequences of genotoxic damages in ecotoxicology may also have later manifestation in life, and can be associated with reduced fitness in individuals and genetic diversity in populations and communities (Depledge, 1994; Jha, 2004).

The data referring to genotoxicological evaluation using A. cepa can be visualized in Table 3. Pyroligneous acid significant inhibited the MI at all tested concentrations, but this product did not increase the levels of CAs and MN under the condition tested.

Based on these findings, PA can be considered a cytotoxicant (slight effect) to A. cepa test system, but do not damage the genetic material of this higher plant.

Table 3. Frequencies of chromosomal aberration (CA) and micronuclei (MN) observed in meristematic cells of Allium cepa exposed to different concentration of pyroligneous acid (PA).
<table>
<thead>
<tr>
<th>Concentrations (g/L)</th>
<th>Mitotic Index</th>
<th>Genotoxic alterations</th>
<th>Mutagenic alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% M ± sd</td>
<td>% M ± sd</td>
<td>% M ± sd</td>
</tr>
<tr>
<td>NC</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.85</td>
<td>167 ± 40.58</td>
<td>0.47 ± 0.66</td>
<td>0.87 ± 1.52</td>
</tr>
<tr>
<td>1.7</td>
<td>112 ± 20.38*</td>
<td>0.95 ± 0.78</td>
<td>1.88 ± 1.91</td>
</tr>
<tr>
<td>3.5</td>
<td>108 ± 32.43*</td>
<td>0.67 ± 0.65</td>
<td>2.02 ± 1.86</td>
</tr>
<tr>
<td>PC</td>
<td>94 ± 27.50*</td>
<td>3.34 ± 1.64*</td>
<td>41.95 ± 22.99*</td>
</tr>
</tbody>
</table>

NC: negative control (ultrapure water); PC: positive control (MMS at 100 mg/L).

Data expressed in percentage (%) related to control and mean (M) ± standard deviation (sd). 5000 cells analysed per treatment. *Significant difference related to NC (p< 0.05), according to Mann-Whitney non-parametric test.

The capacity of PA to induce DNA damage (genotoxicity) was also evaluated by the standard and oxidative Comet assay (primary DNA lesions), with the fish cell line RTG-2. No significant genotoxic effects were observed for the PA in both genotoxic mode of action (DNA strand breaks – standard Comet assay, oxidized bases – oxidative Comet assay) (Fig. 3).

**Figure 3.** Genotoxicity evaluation of the pyroligneous acid (PA) using the *in vitro* Comet assay with gonad fish cell line (RTG-2). The y-axis shows the mean ± standard deviation (sd) of DNA damages measured by the tail intensity parameter (% of DNA in tail). 100 cells per treatment were analyzed in each experiment (three independent experiment). NC: negative control (sterile deionized water at 10% v/v); MMS: methyl methanesulfonate (0.5 mM); H$_2$O$_2$: hydrogen peroxide (80 μM). *Indicates significant
differences related to NC at p<0.05.

Reports on literature seems contradictory about the potential use on germination improvement by the use of PA (Kadota et al., 2002; Mmojieje and Hornung, 2015). However, most reports describe an inhibitory effect when PA is used in the early stages of germination; the positive usages of this product as herbicide seems to be related to the topical application, on plant growth as post-germination (Mmojieje, 2016).

Nonetheless, few studies performed genotoxicity of liquid products from pyrolysis of wood; on slow pyrolysis of Eucalyptus grandis wood, values of D. magna assay toxicity are reported to be as EC50 of 170 mg/L attributing the acute toxicity mainly to the phenolic fraction (concentration about 8% w/w) (Pimenta et al., 2000). Comparing the EC50 values, our PA presented higher acute toxicity to D. magna (EC50 26.12 mg/L). These variations on toxicity is possibly related to the different process to obtain PA, fast and slow pyrolysis, which vary in composition and consequently may differ regarding their potential toxicity. We deduce that the phenolic compounds (hydroxybenzenes) presented in the PA could be the main factor that might have contributed to the positive values of ecotoxicity, as discussed before, which may explain the high toxicity observed to D. magna.

It is worth mention that the amount of pyroligneous acid used in the agriculture field is really low - the dilution is in the rate of 0.3% to 0.1% (Grewal et al., 2018; Souza et al., 2018). Even though the tested PA at these concentrations presented to be weak ecotoxic effects to higher plants, this compound is harmful for aquatic life based on GSH.

Although the PA is a product derived from carbonized wood with subsequent trap and condensation of the gases generated, no signs of polycyclic aromatic hydrocarbons (PAHs) – chemicals recognized by their capacity of damage the DNA (Leme et al., 2008; Leme and Marin-Morales, 2008) – was found among the list of compounds obtained from GC-MS analysis. This could probably be due to the distillation process.
that occurred in the separation of PA, which have the most PAHs products in its dense
part. The absence of PAHs may explain the absence of genotoxicity observed; however, other constituent molecules of the PA can also explain the no genotoxicity of
this product.

3. Conclusions

PA of *eucalyptus* obtained from fast pyrolysis presented, as the largest number of
components in its molecular structure, anhydrosugars and hydroxibenzenes classes
with levoglucosan having more than 60% of the total solution. This product present
low toxicity to terrestrial plants (test organism: higher plant *A. cepa*) but may pose
risks to aquatic life due to the acute toxicity observed to *D. magna* (crustacean). These
toxic effects may be attributed to the phenolic content of the PA; however additional
studies testing PA with reduced amounts of phenols should be carried out in order to
confirm this hypothesis. Although PA present acute toxicity to *D. magna*, this
compound caused no concerns regarding its potential of damaging the genetic
material (non-genotoxicant) of terrestrial and aquatic organisms. In conclusion, our
findings pointed out that there is a need of determining environmental exposure limits
to promote the safer agriculture use of PA from fast pyrolysis, avoiding impacts (acute
toxicity) to aquatic environments.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of
this manuscript.

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