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

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19 **Abstract**

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

38

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

41 ## 1. Introduction

42 Bio-oil is a product that can be derived from pyrolysis of wood (Meier and Faix, 1999;
43 Mohan et al., 2006; Roberts, 1970), which consists in decomposition of the biomass
44 by heat under air controlled environment (Mohan et al., 2007). The resultant process
45 is formed by vapor condensation. Bio-oil has many unique characteristics that make
46 this product valuable in a number of applications, such as crop protection agent
47 (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

77 environment and health of living organisms can often not be estimated visually;
78 consequently, tests to evaluate their potential harmful effects on biological systems
79 should be performed, contributing to a safer application (Tiilikkala et al., 2010).

80 Ecotoxicity tests provide relevant information about the adverse effects of chemicals
81 on living organisms at different levels of biological hierarchy (Zhou et al., 2018).
82 Acute assays protocols to *Daphnia magna* (microcrustacean – planktonic invertebrate
83 organism) have been recommended by several regulatory agencies because of its
84 geographical distribution, central role in freshwater food webs and sensibility to a
85 wide range of chemicals (Grintzalis K, Dai W., Panagiotidis, K, Belvgeni A., 2017).
86 Most of the ecotoxicological studies with *D. magna* are based on acute toxicity data
87 of effective concentration (EC_{50} – Half maximal effective concentration), for
88 immobilisation of neonates, to estimate the acute mortality following short-term
89 exposure (24-48 h) to a chemical (Bownik, 2017; De Coen and Janssen, 1997; Janssen
90 and Persoone, 1993).

91 Study on fish toxicity is another common ecotoxicity test used to determine safe
92 levels of chemicals to aquatic environments (Bols et al., 2005). These tests were
93 primary conducted using juveniles or adults life stages of species (*in vivo*); however,
94 there are considerable efforts to promote the use of fish cell lines in ecotoxicology
95 (Bermejo-Nogales et al., 2017; Castaño et al., 2003; Franco et al., 2018; Lillicrap et
96 al., 2016). The interaction of chemicals at cellular level is an important study to
97 determine the cytotoxicity of a compound; thus, fish cell lines have been used to
98 evaluate the effects of chemicals on processes, such as xenobiotic metabolism and
99 DNA damages (genotoxicity) (Lillicrap et al., 2016). With respect to genotoxicity, the
100 RTG-2 permanent fish cell line, derived from rainbow trout (*Oncorhynchus mykiss*)
101 gonadal tissue, has been successfully used to detect aquatic genotoxicants, estimating
102 their genotoxic effects on the reproductive system of fish (Castaño and Becerril, 2004;
103 Felzenszwalb et al., 2018; Klingelfus et al., 2019; Llorente et al., 2012; Marabini et al.,
104 2011; Munari et al., 2014; Oliveira et al., 2018; Sánchez-Fortún et al., 2005).

105 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_2O of static pressure. The reaction temperature was achieved by partial combustion of the biomass products through preheated air injection. Then, the reactor was feed 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)

134 To obtain the PA, 45 mL of chloroform ($CHCl_3$) and 45 mL of ultrapure water were
135 added to 5 mL of the bio-oil (*i.e.*, a mixture of aqueous and non- aqueous fractions).
136 After 24 h, the aqueous fraction was collected and mixed again with chloroform. After
137 complete separation of the phases, the aqueous fraction (PA) was withdrawn and
138 stored at room temperature until the analyses were performed.

139 **2.2 Gas chromatography–mass spectrometry (GC–MS)**

140 For Bio-oil (100 μL) phase separation, was added water (2 mL) and chloroform (2
141 mL). The mixture was stirred and centrifuged (5 min, 3000 rpm). The water phase
142 was transferred to an Eppendorf tube and dried under vacuum for 18 h (Speed
143 Vacuum - Eppendorf). The residues were dissolved in acetone (100 μL) and analysed
144 by gas chromatography-mass spectrometry (GC-MS). The extracts were injected (1
145 μL, Thermo Triplus AS) into a Focus GC gas chromatography tandem to a Polaris Q
146 ion trap mass spectrometer (Thermo), equipped with a DB5ms capillary column (30m
147 x 0.25mm, 25μm film thickness). The GC oven temperature was programmed from
148 40 °C (held for 8 min) to 280 °C at 7°C min $^{-1}$, then held for 15 min. Helium, at a
149 constant flow of 1.0 mL min $^{-1}$, was the carrier gas. The inlet in split mode 1:100 was
150 set at 230 °C. The GC-MS interface and ion source temperatures were 250 °C and
151 200 °C, respectively.

152 The ion trap mass spectrometer was operated in the positive impact electronic mode at
153 70 eV scanning the range m/z 40–650 in a total scan time of 0.59 and emission
154 current 250 mA. Mass spectral deconvolution and automated calculation of RI was
155 performed by the automated mass spectral deconvolution and identification system
156 (AMDIS, National Institute of Standards and Technology, Gaithersburg, MD, USA).
157 Standard solutions of linear alkanes (C 7 –C 30 , Sigma-Aldrich 49451-U) was used
158 for Kováts RI calibration in the GC-MS. Data deconvolution was performed with the
159 following specifications: component width = 12; adjacent peak subtraction = 2;
160 resolution = low; sensitivity = very low; shape requirements = medium. Compounds
161 were identified from the deconvoluted mass spectra by comparison with mass spectra
162 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

Marin-Morales, 2008).

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 geminated 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 (5×10^4 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 (H_2O_2, 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

247 mA. The slides were neutralized with Tris-HCl buffer (pH 7.5) for 20 min and fixed
248 in 100% ethanol. For the hOGG1-modified alkaline version, the comet slides were
249 washed (3 × 5 min) with enzyme buffer (hOGG1: 40 mM HEPES, 0.1 M KCl, 0.5
250 mM EDTA, 0.2 mg/mL BSA, pH 8) after lysis and then incubated with hOGG1 (0.08
251 U/slide, New England Biolabs) for 30 min at 37 °C, in a moistened chamber. After
252 enzyme incubation, the slides were rinsed with distilled water and placed into the
253 electrophoresis chamber for DNA unwinding and electrophoresis, as described above
254 (Felzenszwalb et al., 2018, Oliveira et al., 2018, Klingelfus et al., 2018).
255 The slides were stained with ethidium bromide solution (20 µg/mL, Sigma-Aldrich)
256 and analyzed under a fluorescence microscope (Axio Imager Z2, Carl Zeiss, Jena,
257 DE), equipped with Metafer 4/V Slide automated capture software (Metasystems,
258 Altlussheim, DE) and Camera Cool Cube 1 – Metasystems. DNA lesions were
259 quantified as DNA tail intensity (percentage of DNA in tail) (Azqueta et al. 2011)
260 using the computer-based image analysis Metafer CometScan v.2.8.0® (Metasystems,
261 Germany) on 100 selected nucleoids. The Comet data were evaluated using ANOVA
262 and Dunnett's post hoc tests. All experiments were carried out independently in
263 triplicate, using a single well per treatment.

264 3. Results and Discussion

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

274 The chemical composition of PA vary depending on their source of extraction and

275 process, and the chemical nature of a compound strictly relates to its potential toxicity.
276 From the chromatography analysis (GC-MS) of the PA, 45 elements were identified
277 out of 57 (Table 1). The most yielded compound was identified within the
278 anhydrosugar group, represented by levoglucosan with more than 60% of total GC
279 area; catechol and hydroquinone from hydroxybenzenes group represented 6.45% and
280 2.05% respectively. Although, hydroxyacetone was identified in large quantity due to
281 the procedure used in this technique, little represents to the PA composition.

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

RT	Identity	m/z	m/z	m/z (1)	%	Class
8,68	hydroxyacetone	43	74	73	3.42	small molecules
11,77	2-methoxytetrahydrofuran	41	72	101	0.10	furans
12,48	butanedial	43	57	58	0.35	small molecules
14,25	2-methyl-2-pentenal	54	69	98	0.13	small molecules
14,36	furfural	67	95	96	0.45	furans
15,96	tetrahydro-2,5-dimethoxy-furan	69	101	131	0.54	furans
16,09	monoacetate-1,2-ethanediol	43	61	74	0.52	small molecules
16,42	tetrahydro-2,5-dimethoxy-furan (isomer)	69	101	131	0.53	furans
16,76	2(3H)-Furanone	41	55	84	0.45	furans
16,96	unidentified	55	71	115	0.10	unknown
17,58	dihydro-3-methylene-2,5-Furan dione	40	53	68	0.11	furans
18,25	unidentified	69	75	101	0.16	unknown
18,28	3-ethyl-2-pentanone	55	86	114	0.08	small molecules
19,23	4-hydroxy-2,3-dihydropyran-6-one	58	85	114	0.16	pyrans
20,00	hydroxy - methyl - cyclopentenone	55	84	112	0.26	cyclopentenones
20,31	1,2-Cyclohexanediol	57	85	116	0.58	small molecules
20,38	unidentified	43	73	128	0.49	unknown
20,67	2-furancarboxylic acid	84	95	112	0.35	furans
20,83	isobutyric anhydride	41	43	71	0.34	small molecules
21,15	unidentified	53	81	97	0.25	unknown
21,64	unidentified	43	57	69	2.82	unknown
21,80	isobutyric anhydride (isomer)	41	43	71	0.37	small molecules
22,10	dimethyl ester tetrahydro-2,5-furandicarboxylic acid	59	69	101	0.17	furans

22,47	dimethyl ester tetrahydro-2,5-furandicarboxylic acid (isomer)	59	69	101	0.15	furans
22,82	unidentified	43	55	74	0.48	unknown
22,85	unidentified	75	85	115	0.15	unknown
23,28	unidentified	59	69	101	0.37	unknown
23,42	unidentified	59	69	101	0.38	unknown
23,48	2,3-Dihydroxybenzaldehyde	120	137	138	0.32	hydroxybenzenes
23,71	5-(Hydroxymethyl)dihydro-2(3H)-furanone	57	70	85	0.80	furans
23,80	unidentified	57	85	83	0.07	unknown
23,90	catechol	81	92	83	6.45	hydroxybenzenes
24,32	1,4:3,6-Dianhydro-α-d-glucopyranose	69	98	144	0.34	anhydrosugars
24,54	5-(Hydroxymethyl)furfural	69	97	126	0.34	furans
24,60	anhydrosugar	43	71	97	0.72	anhydrosugars
24,76	anhydrosugar	43	71	97	0.53	anhydrosugars
25,13	unidentified	43	57	73	0.62	unknown
25,19	anhydrosugar	60	81	97	0.36	anhydrosugars
25,31	methyl -catechol	78	106	124	0.68	hydroxybenzenes
25,38	Hydroquinone	55	81	110	2.05	hydroxybenzenes
25,53	methoxy-catechol	97	125	140	1.58	hydroxybenzenes
25,88	methyl -catechol (isomer)	78	106	124	1.26	hydroxybenzenes
26,19	4-hydroxy-benzaldehyde	65	93	121	0.15	hydroxybenzenes
26,64	unidentified	57	73	81	0.15	unknown
26,91	methyl -catechol (isomer)	78	106	124	0.60	hydroxybenzenes
27,73	anhydrosugar	60	73	97	0.99	anhydrosugars
28,29	vanillin	109	123	151	1.55	hydroxybenzenes
28,64	unidentified	101	116	129	0.25	unknown
28,81	unidentified	101	116	129	0.35	unknown
28,92	anhydrosugar	60	73	97	0.65	anhydrosugars
30,05	Levoglucosan	60	73	97	60.24	anhydrosugars
30,57	6-hydroxy-hydrocoumarin	122	136	164	0.19	hydroxybenzenes
30,80	anhydrosugar	69	73	115	1.46	anhydrosugars
31,62	anhydrosugar	73	97	115	0.78	anhydrosugars
32,31	anhydrosugar	69	73	115	0.16	anhydrosugars
32,86	Syringaldehyde	139	167	182	1.40	hydroxybenzenes
33,56	Homosyringaldehyde	123	167	196	0.69	hydroxybenzenes
					100.00	

283 A summary of the amounts obtained by GC grouped by class of molecules (Table 2)
284 evidences that the highest amount yielded for the investigated pyroligneous acid
285 consisted of anhydrosugar and hydroxybenzenes following by small molecules, furans

286 cyclopentenones and pyrans (Tamburini et al., 2017).

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

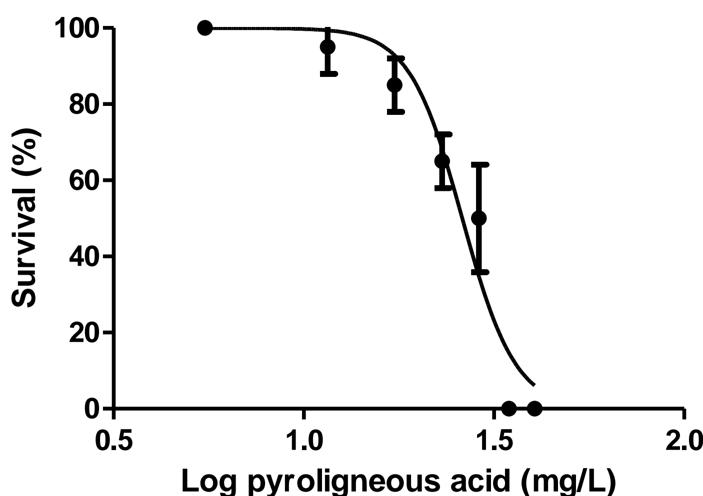
Class	%
small molecules	5,79
Cyclopentenones	0,26
Pyrans	0,16
Furans	4,75
Anhydrosugars	66,23
Hydroxybenzenes	16,92
Unknown	5,88

289 From the detected compounds obtained by GC-MS, it can be seen that the lowest
290 concentrations are cyclopentenones and pyrans. More specifically cyclopentenone,
291 according to United States Environmental Protection Agency (US EPA) are already
292 used in the market as pesticidal agent and present no signs of ecotoxicity (EPA
293 004049), as well as pyrans which the subclass have various FDA approved inhibitor
294 against mycobacterium tuberculosis (Bhat et al., 2017). For furan classes, the majority
295 of the compounds presents low ecotoxicity, such as furanone, furancarboxylic acid
296 and furandione derivatives (Atkins et al., 1981; Paulus, 2005; Pilgård et al., 2010;
297 Reynolds, 1989; Ventura et al., 2016) at the same concentration detected in the tested
298 PA; however 2,5-Furandione, furfural and 5-Hydroxymethylfurfural are considered
299 slightly ecotoxicants (Ventura et al., 2016). Nonetheless, reports on
300 5-Hydroxymethylfurfural and furfural for *Daphnia magna* suggests a moderate
301 toxicity (Hessov, 1975). Catechol and hydroquinone induces chromosome aberrations
302 in *Allium cepa* (Devillers et al., 1990; Petriccione et al., 2013). Coumarin and vanillin
303 also shows moderate acute toxicity (Palmer and Maloney, 1958; Podbielkowska et al.,
304 1995); the other available components of the hydroxybenzene groups are considered
305 harmless (Staver et al., 2014).

306 Therefore, to better understand the potential ecotoxicity (acute toxicity and
307 genotoxicity) of PA from fast pyrolysis, tests using the higher plant *A. cepa*
308 (monocotyledon), the microcrustacean *D. magna* and the *in vitro* fish model RTG-2
309 (cell line-derived from gonad tissue) were performed.

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

319

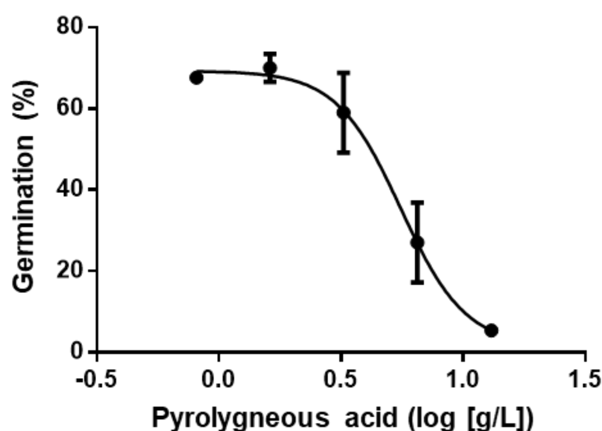


320

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

324 The PA also caused toxic effects on *A. cepa*. The results of the first toxicity test with

325 the highest concentrations of PA (13.07, 26.25, 52.5, 78.75 and 105 g/L) exhibited a
326 complete inhibition of seed germination of *A. cepa*, except for the concentration of
327 13.07 g/L for which a low germination rate was observed (5%). From these results, a
328 new concentration range was determined (0.81, 1.62, 3.24, 6.5, 13.07 g/L), and the
329 seed germination of *A. cepa* was inhibited by PA with EC_{50} of 5.556 g/L (Fig. 2A).
330 This product also inhibited the root growth, showing an EC_{50} of 3.436 g/L (Fig. 2B).
331 However, PA presented lower toxicity on the higher plant *A. cepa* compared to *D.*
332 *magna*. Terrestrial and aquatic organisms may display different sensitivities to the
333 toxic effects of chemicals, and in most cases there are greater impact of aquatic
334 environment than the terrestrial environment (Oliveira et al., 2018). These findings
335 reinforce the need of using an ecotoxicity test battery, comprising test organism's
336 representative of different trophic levels and ecosystems in order to accurately predict
337 the chemical hazard.



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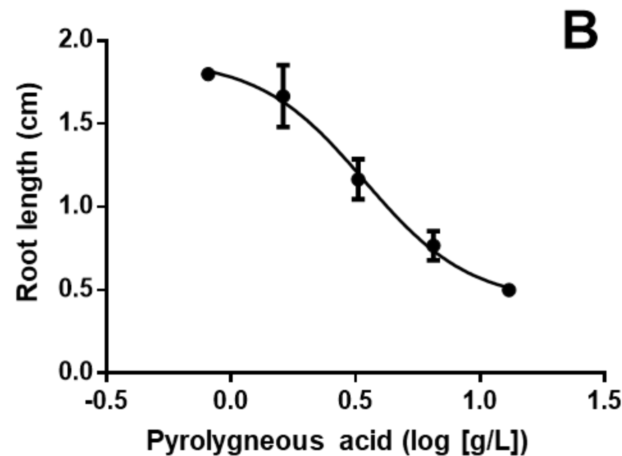
339 

340 **Figure 2.** Concentration-response curves of the effects of pyroligneous acid (PA) on
341 seed germination (A) and root elongation (B) of *Allium cepa*. Parameter log (g/L) PA
342 vs percentage of seed germination (A) or root length (cm) (B) were used to determine
343 these concentration-response curves.

344 Although the acute toxicity evaluated by endpoints, such as mortality and growth
345 rates, are the mostly used parameter for estimating the toxic effects of a chemical to
346 environmental organisms (Hanson et al., 2017), endpoints of genetic changes
347 represent an important category of the adverse effects of pollutants (Oliveira et al.,
348 2018). The consequences of genotoxic damages in ecotoxicology may also have later
349 manifestation in life, and can be associated with reduced fitness in individuals and
350 genetic diversity in populations and communities (Depledge, 1994; Jha, 2004).

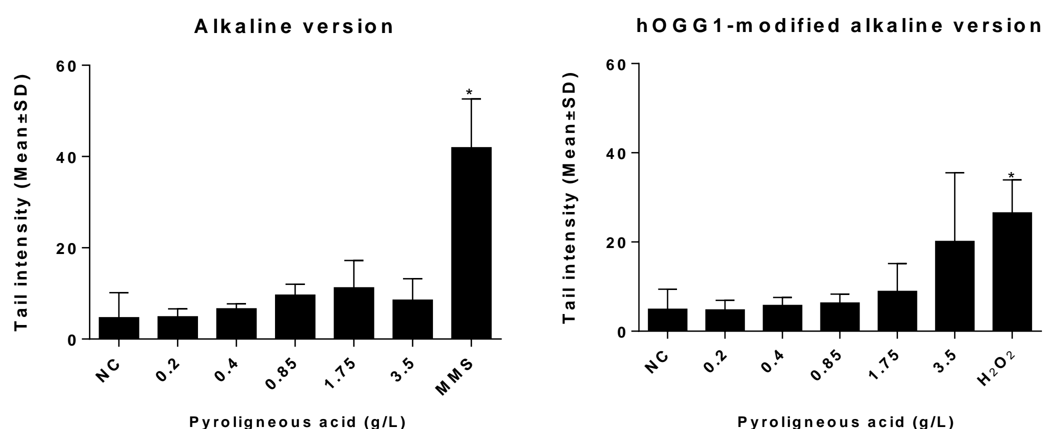
351 The data referring to genotoxicological evaluation using *A. cepa* can be visualized in
352 Table 3. Pyroligneous acid significant inhibited the MI at all tested concentrations, but
353 this product did not increase the levels of CAs and MN under the condition tested.
354 Based on these findings, PA can be considered a cytotoxicant (slight effect) to *A.*
355 *cepa* test system, but do not damage the genetic material of this higher plant.

356 **Table 3**. Frequencies of chromosomal aberration (CA) and micronuclei (MN)
357 observed in meristematic cells of *Allium cepa* exposed to different concentration of
358 pyroligneous acid (PA).

Concentrations (g/L)	Mitotic Index		Genotoxic alterations		Mutagenic alterations	
	%	M ± sd	%	M ± sd	%	M ± sd
NC	100	167 ± 40.58	100	0.47 ± 0.66	100	0.87 ± 1.52
0.85	61	102 ± 24.96*	78	0.37 ± 0.65	150	1.31 ± 1.50
1.7	67	112 ± 20.38*	203	0.95 ± 0.78	216	1.88 ± 1.91
3.5	65	108 ± 32.43*	142	0.67 ± 0.65	232	2.02 ± 1.86
PC	59	94 ± 27.50*	711	3.34 ± 1.64*	4822	41.95 ± 22.99*

359 NC: negative control (ultrapure water); PC: positive control (MMS at 100 mg/L).
360 Data expressed in percentage (%) related to control and mean (M) ± standard
361 deviation (sd). 5000 cells analysed per treatment. *Significant difference related to
362 NC ($p < 0.05$), according to Mann-Whitney non-parametric test.

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



368

369 **Figure 3.** Genotoxicity evaluation of the pyroligneous acid (PA) using the *in vitro*
370 Comet assay with gonad fish cell line (RTG-2). The y-axis shows the mean ± standard
371 deviation (sd) of DNA damages measured by the tail intensity parameter (% of DNA
372 in tail). 100 cells per treatment were analyzed in each experiment (three independent
373 experiment). NC: negative control (sterile deionized water at 10%-v/v); MMS: methyl
374 methanesulfonate (0.5 mM); H_2O_2: hydrogen peroxide (80 μM). *Indicates significant

375 differences related to NC at $p<0.05$.

376 Reports on literature seems contradictory about the potential use on germination
377 improvement by the use of PA (Kadota et al., 2002; Mmojieje and Hornung, 2015).
378 However, most reports describe an inhibitory effect when PA is used in the early
379 stages of germination; the positive usages of this product as herbicide seems to be
380 related to the topical application, on plant growth as post-germination (Mmojieje,
381 2016).

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

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

398 Although the PA is a product derived from carbonized wood with subsequent trap and
399 condensation of the gases generated, no signs of polycyclic aromatic hydrocarbons
400 (PAHs) – chemicals recognized by their capacity of damage the DNA (Leme et al.,
401 2008; Leme and Marin-Morales, 2008) – was found among the list of compounds
402 obtained from GC-MS analysis. This could probably be due to the distillation process

403 that occurred in the separation of PA, which have the most PAHs products in its dense
404 part. The absence of PAHs may explain the absence of genotoxicity observed;
405 however, other constituent molecules of the PA can also explain the no genotoxicity of
406 this product.

407 3. Conclusions

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

421 Conflicts of Interest

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

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