Chemical and ecotoxicological properties of three bio-oils from pyrolysis of biomasses

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ABSTRACT

In view of the potential use of pyrolysis based technologies, it is crucial to understand the environmental hazards of pyrolysis derived products, in particular bio oils. Here, three bio oils were produced from fast pyrolysis of pine wood and intermediate pyrolysis of corn stalk and poultry litter. They were fully characterized by chemical analysis and tested for their biodegradability and their ecotoxicity on the crustacean *Daphnia magna* and the green alga *Raphidocelis subcapitata*. These tests were chosen as required by the European REACH regulation. These three bio oils were biodegradable, with 40 60% of biodegradation after 28 days, and had EC50 values above 100 mg L^{-1} for the crustacean and above 10 mg L^{-1} for the alga, showing low toxicity to the aquatic life. The toxic unit approach was applied to verify whether the observed toxicity could be predicted from the data available for the substances detected in the bio oils. The predicted values largely underestimated the experimental values.

1. Introduction

The thermochemical valorization of biomass and wastes can provide a relatively constant and storable amount of bio energy that can play a key role in the future renewable energy scenario, thus contributing to reduce the greenhouse gas emissions. Nevertheless, the low spatial density of biomass productivity, especially for agricultural wastes (< 10 t y^{-1} ha^{-1}, in comparison to more than 1000 t y^{-1} ha^{-1} obtainable for coal opencast mining), still hampers the large scale exploitation of this resource for energy purpose. Among the various thermochemical treatments, pyrolysis (conducted under inert conditions and at temperatures above 350 °C) converts dry solid biomass into three fractions whose yield and composition strictly depend on the heating rate (fast, intermediate or slow pyrolysis; Bridgwater, 2012): *i*) a solid bio char, potentially useful for soil amendment; *ii*) a pyrolysis gas, exploitable for auto sustaining the pyrolysis process or to generate heat, and *iii)* a liquid, named bio oil or pyro oil, that can be used as an energy carrier, which is storable, transportable and processable (e.g. upgraded to fuel or burnt for energy generation; Woolf et al., 2014). Pyrolysis performed in small scale and de centralized processing plants involves the risk of potential emissions from small scale facilities (in vapor, aerosol or liquid form), as well as spillages during transportation. Moreover, bio-oils are composed of a wide set of potentially noxious compounds (Diebold, 2000; Oasmaa and Peacocke, 2001; Garcia Perez et al., 2007; Nakai et al., 2007) and, even if some of them are recognized as individually harmful to human beings and ecosystems (Diebold, 1997), very little is known about the ecotoxicity of bio oils as a whole product (Pimenta et al., 2000; Hagner et al., 2010; Oasmaa et al., 2012; Chatterjee et al., 2014; Hossain et al., 2015).

Environmental management and control of toxic chemicals have gained significant attention from policy makers, researchers, and enterprises in Europe and abroad (Sharma et al., 2014); specifically, the implementation of the REACH (Registration, Evaluation and Authorization of Chemicals) regulation will result in a number of changes in the ERA (European Research Area) process in the entire European Union (European Commission, 2003a; Lahl et al., 2006; Christensen et al., 2011). The most significant of these changes is that a large number of previously untested chemical substances will undergo testing and risk assessment (European Commission, 2003b). In particular, the REACH regulation establishes the submission of eco toxicological information for all new substances to be notified and registered by the European Chemicals Agency (ECHA); the required data depend on the annual tonnage per registrant.

With respect to bio oils, in a recent study within the BIOTOX project (Oasmaa et al., 2012), a complete and detailed

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characterization of a spruce fast pyrolysis oil, considered as a "model" for wood derived fast pyrolysis oils, was performed. The authors provided information related to the acute toxicity, mutagenicity and ecotoxicological impact of the examined bio oil and found out that it had a negligible toxicity and considerable biodegradability. These findings confirm what Blin et al. (2007) reported for the biodegradability of various bio oils produced through fast and slow pyrolysis of wooden biomass and Hagner et al. (2010) for the ecotoxicity of birch tar oil.

The present study aims at increasing the knowledge about the environmental hazard of different bio oils, produced from various pyrolysis conditions and substrates. Specifically, we tested the ecotoxicity of three bio oils on two aquatic organisms (*Daphnia magna* and *Raphidocelis subcapitata*) and their ready biodegradability. Two of these bio oils (intermediate pyrolysis poultry litter and corn stalk bio oils) have never been tested before.

2. Methods

2.1. Chemical reagents

All solvents and chemicals used in this study were obtained from Sigma Aldrich (purities ≥ 98%) and used without further purification.

2.2. Bio oils

Three pyrolysis oils were tested:

1) A commercial bio oil (BTG®, kindly provided by BTG Biomass Technology Group BV, The Netherlands) obtained by flash pyrolysis of pine wood in a rotating cone reactor (temperature: 500 °C; residence time: 2 s): a homogenous liquid that flows freely, also when refrigerated, with no suspended solids and a dark brown homogenous color.
2) Corn stalk intermediate pyrolysis oil (500 °C for 10 min), produced by the authors of the present study, consisting of a non homogenous slurry of an aqueous syrup and a denser water insoluble fraction (9 wt%). This bio oil was shaken before all analyses and tests to avoid the separation of the two fractions so they could be tested simultaneously to reproduce a "spill like" scenario.
3) Poultry litter intermediate pyrolysis oil (500 °C for 10 min), produced by the authors of the present study, consisting in an emulsion of a reddish aqueous phase and a less dense grease like material. This bio oil was shaken before all analyses and tests to avoid the separation of the two fractions so they could be tested simultaneously.

2.3. Pyrolysis apparatus and conditions

Intermediate pyrolysis bio oils from corn stalk and poultry litter (5 g for each experiment) was performed in a fixed bed reactor at 500 °C for 10 min. The apparatus is composed by a quartz tubular reactor (30 mm inner diameter, 1000 mm length) where samples are introduced by a quartz sample holder. The reactor is positioned inside a furnace pre heated at the set temperature and connected downstream to two consecutive traps kept at a temperature of 25 °C and 0 °C respectively. During the experimental runs, the reactor was fluxed by a purge gas flow of 1000 mL min $^{-1}$. Each sample was maintained inside the heated zone at 500 °C for 10 min. Pyrolysis was performed on 5 g samples, and the entire procedure was repeated 5 times in order to collect the amount of oil needed for the toxicity tests and chemical characterization. Both corn stalk and poultry litter feedstock (dried pelletized samples with a length of 10 20 mm) were used as received.

2.4. Bio oil characterization

The elemental composition of the bio oils was determined by using an elemental analyzer (Thermo Scientific, Flash 2000, Organic Elemental Analyzer) by means of the flash combustion technique. The water content was determined by Karl Fischer titration (Scholz, 1984). The analytical characterization was based on the solvent fractionation procedure developed by Oasmaa et al.(2003) and Oasmaa and Kuoppala (2008), with minor modifications. The bio oil (0.5 g) was added drop wise to a 10 fold excess of water (5 mL) and then extracted with hexane (1:10 pyrolysis oil to solvent ratio). Hexane extractable material was indicated as "extractives" (HE). The heterogeneous mixture resulting from the addition of water to the bio oil was sonicated for 20 min. The fraction insoluble in water (WI) was recovered by centrifugation, filtered and weighted after drying. Pyrolytic lignin (PL) was calculated from the difference WI HE.

The aqueous supernatant (5 mL), derived from the procedure described above, was washed with ethyl acetate and pentane. The resulting water solution was purged with nitrogen for 30 min in order to eliminate the residual organic solvent, and analyzed by means of a Brix Refractometer (Sper Scientific, 8281 E. Evans Rd., Suite #103, Scottsdale, AZ 85260, USA) so the water soluble/ethyl acetate insoluble (EI WS) fraction could be obtained. This fraction represents an estimation of the total amount of the water soluble solutes. It was assumed that this fraction was mainly composed by sugar derivatives (Oasmaa et al., 2003). The amount of high molecular weight sugar derivatives (anhydro/oligosaccharides), not detectable by using GC MS, was calculated by subtracting the GC MS detectable silylated derivatives of the polar compounds (e.g. levoglucosan) from the total solutes (Busetto et al., 2011).

GC MS analyses of the semi volatile compounds were performed by using a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer (Agilent Technologies, 5301 Stevens Creek Blvd., Santa Clara, CA 95051, USA).

In order to achieve the highest mass balance closure, different GC columns and temperature programs were used for the various semi volatile compound classes.

For the polar volatile substances (e.g. volatile fatty acids) the injection port temperature was 250 °C. Analytes were separated by a DB FFAP capillary column (nitroterephthalic acid modified polyethylene glycol, 30 m, 0.25 mm i.d., 0.25 μm film thickness), with helium as carrier gas (at constant pressure, 33 cm s $^{-1}$ linear velocity at 200 °C). Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s $^{-1}$ within the 33 600 *m/z* range. The temperature of the column started from 50 °C and was held for 5 min, then increased up to 250 °C at 10 °C min $^{-1}$ and held for 12 min.

For the mildly apolar semi volatile substances (phenols and hydrocarbons), the injection port temperature was 280 °C. Analytes were separated by means of a 5HP MS (Agilent) fused silica capillary column (stationary phase: poly[5% diphenyl/95% dimethyl]siloxane, 30 m, 0.25 mm i.d., 0.25 μm film thickness) using helium as carrier gas (constant pressure, linear velocity of 33 cm s $^{-1}$ at 200 °C). The temperature program consisted of a ramp from 50 °C (5 min) to 325 °C (3 min) at 5 °C min $^{-1}$, and held at 325 °C for 10 min.

The heavy polar GC MS detectable substances (e.g. levoglucosan and other anhydrosugars) were determined by GC MS analysis after trimethylsilylation as described in Torri et al. (2009). An aliquot (0.1 mL) of the bio oil solution (1% by weight in acetonitrile) was added with internal standard (1 methyl α arabinopyranoside), bis trimethylsilyltrifluoroacetamide (0.1 mL) containing 1% of trimethylchlorosilane and pyridine (0.02 mL). The solutions were placed in an incubator at 80 °C for 30 min and then analyzed

by GC MS. Thereafter, the same thermal program used for mildly apolar semi volatile substances was used.

Reactive aldehydes, including hydroxyacetaldehyde, were obtained through a dimethyl acetalization with methanol (Busetto et al., 2011). The bio oil sample (40 mg) reacted at 60 °C for 2 h with excess methanol (4 mL) in the presence of Amberlyst 15 H (200 mg), it was then injected under the same GC column and conditions were used for the polar volatile substances. Extemal calibration was performed applying the same procedure with standard solutions of hydroxyacetaldehyde dimer.

2.5. 72 h algal growth inhibition assay with Raphidocelis subcapitata

Three test trials were conducted in accordance with the OECD (2006) 201 test protocol for algal growth inhibition. The test or ganism was the freshwater unicellular green alga *Raphidocelis subcapitata* (Chlorophyta, Chlorophyceae), purchased from SAG Goettingen University and then cultivated in the laboratory. For each test trial, stock solutions (10 g L^{-1}) of the three bio oils were prepared in the OECD growth medium (pH 8.1) and tested simultaneously using algae from the same batch culture. The controls, together with five concentrations of each oil, were prepared in 100 mL borosilicate glass flasks. The 70 mL samples were inoculated with an aliquot of exponentially growing algae to ensure an algal concentration of 10^4 cells mL^{-1} and then incubated for 72 h. During the incubation the flasks were placed on an orbital shaker operating at 100 rpm (see Table 1 for the experimental conditions). The number of algal cells in the flasks was determined by counting under the microscope (Nikon Eclipse E600, 400 ×) with a Burker haemocytometer, at the beginning and at the end of the test. The average specific growth rate μ (d^{-1}) for each treat ment was calculated as:

$$\mu = \frac{\ln N_{72} - \ln N_0}{t}$$

where: N_0 is the initial algal density (cell mL^{-1}), N_{72} is the algal density at the end of the 72 h (t=3 d) exposure. The EC50, i.e. the concentration giving a 50% reduction in the algal growth rate after 72 h compared to the controls, was calculated separately for each trial.

2.6. 48 h immobilization assay with Daphnia magna

Four test trials were conducted in accordance with the OECD 202 guideline (2004). The test organism was the freshwater zooplankter *Daphnia magna* (Crustacea, Cladocera). Briefly, five neonates (< 24 h old) were transferred to each glass beaker containing 20 mL of control solution (4 replicates) or test solution (2 replicates). Five test concentrations were prepared for each bio oil. Test vessels were incubated for 48 h (Table 1) and the number of immobilized *D. magna* was recorded at 24 and 48 h. The animals that were not able to swim within 15 s, after gentle agitation of the test vessel, were considered as immobilized (even if they could still move their antennae). The EC50, i.e. the concentration giving a 50% reduction in the number of mobile *D. magna* after 48 h com pared to the controls, was calculated separately for each test trial.

The separation of the two phases of the com stalk and poultry litter bio oils occurred after several days even in the concentrated oils. For this reason, we assumed that the two phases were well mixed, either fully dissolved or finely suspended, in the dilution water for the entire duration of the test. Separation of phases was never observed in the test beakers.

Table 1
Operational conditions and bio-oil concentrations in the three tests.

Test condition	Algal assay	*Daphnia* assay	Biodegradability
Concentration range (mg L^{-1}):			
Pine wood	18–60[a]	50–191[b]	193
Corn stalk	33–109[a]	85–327[b]	193
Poultry litter	16–54[a]	172-658[b]	193
Duration	72 h	48 h	28 d
Temperature (°C)	23 ± 1	20 ± 1	20 ± 2
Illumination	continuous light, 4000 K, 6000–8000 lx	16 h light and 8 h dark cycle, 4000 K, 600–800 lx	Dark
Other incubation conditions	Continually shaken at 100 rpm (orbital shaker)	not fed	Continually shaken at 100 rpm (orbital shaker)

[a] Separation factor: 1.35.
[b] Separation factor: 1.25.

2.7. Ready biodegradability

The biodegradation of the three pyrolysis oils was determined by a ready biodegradability test in an aerobic aqueous medium according to the OECD (1992) guideline 301F, "Manometric respirometry". The test medium was prepared by adding specific concentrations of mineral components from stock solutions (potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulfate and iron (III) chloride) to distilled water. The bacterial inoculum, derived from an activated sludge taken from a treatment plant receiving domestic sewage located in Ravenna, Italy, was aerated in mineral medium for 5 days at the test temperature. The biodegradability tests were carried out in bottles for 28 days at 20 ± 2 °C; during the incuba tion the bottles were placed on an orbital shaker operating at 100 rpm (Table 1). The three bio oils and glucose (reference compound) were tested in duplicate, run in parallel with a blank (containing only inoculum) and a toxicity control (containing pine wood bio oil, glucose and inoculum) in duplicate.

The test concentration of the bio oils was 193 mg L^{-1}, corresponding to a Theoretical Oxygen Demand (ThOD) of 102, 220 and 123 mg O_2 L^{-1} for corn stalk, pine wood and poultry litter bio oil, respectively. The test concentration of glucose was 194 mg L^{-1}, corresponding to a ThOD of 207 mg O_2 L^{-1}. The ThOD values for the bio oils and glucose were calculated on the basis of the carbon content determined by elemental analysis and under the assumption that nitrogen was eliminated as ammonia (no nitrification).

The consumption of oxygen was determined by measuring the change in pressure in the apparatus. Evolved carbon dioxide was absorbed in a solution of potassium hydroxide. The amount of oxygen taken up by the microbial population during biodegradation of the test substance (corrected for uptake by blank inoculum) was expressed as a percentage of ThOD.

2.8. Data analysis

The 50% effect concentration (EC50) of each bio oil for the *R. subcapitata* growth inhibition tests and for the *D. magna* immobilization tests was estimated by fitting the experimental concentration response curves obtained in each test trial to a lo gistic model:

$$y = \frac{\max}{1 + \left(\frac{x}{\log_{10} EC50}\right)^{slope}}$$

where: y is the endpoint value (algal growth rate or number of mobile *D. magna*), x is the $\log_{10}$ of the oil concentration, *max* is the

expected endpoint value when the concentration of the bio oil is zero, *slope* is a parameter related to the steepness of the curve. The parameters of the equation, including the EC50 and their standard errors and confidence limits, were estimated using the non linear regression procedures implemented in Statistica (Statsoft, Tulsa, OK, USA). The difference among the EC50s of the three bio oils was tested by one way analysis of variance (ANOVA) and by the Student Newman Keuls (SNK) *post hoc* test, using the EC50s of the single test trials as replicates of the treatment. The analysis was performed on log transformed data, after checking the assumption of homogeneity of variance using the Cochran's C test.

The toxicity of the bio oil solutions and of the single substances making up the mixture was expressed as toxic units (TU) computed as:

$$TU = \frac{c_i}{EC50_i}$$

where c_i is the concentration (mg L ^{1}) of a bio oil or of a component substance and $EC50_i$ is the respective EC50 (mg L ^{1}). Assuming that the effects of the mixture are as described by the concentration addition model (Loewe and Muischnek, 1926), in absence of synergism or antagonism among component substances the TU value of a mixture is the sum of the TU values of all component substances.

3. Results and discussion

3.1. Chemical characterization

The three oils analyzed in this study represent an array of pyrolysis oils that could be produced in a delocalized scheme of biomass collection. Pine wood fast pyrolysis oil was characterized by relatively low water content (24%) and a high amount of water insoluble compounds (mainly pyrolytic lignin, 24%) and semi volatile compounds (e.g. phenols and aldehydes; Table 2 and S1). The elemental composition agrees with the chemical characteristics of different bio oils from various biomasses (Ingram et al., 2008).

The intermediate pyrolysis of corn stalk and poultry litter produced in both cases phase separated oils with high water content (about half of the weight) and a lower amount of water insoluble compounds, which consisted of pyrolytic lignin for corn stalk and of oily like matter for poultry litter. It is interesting to notice the considerable N content in the bio oil from poultry litter, which indicates a high amount of protein derived compounds such as N based heterocycles and nitriles, 0.1 and 0.001 wt%, respectively (Table 2 and S1).

3.2. Ecotoxicity tests

The results of the ecotoxicity tests are summarized in Table 3. To the best of our knowledge, this is the first time that point estimates of EC50 for bio oils have been identified with an algal growth inhibition assay. The EC50s for the three bio oils for *R. subcapitata* were significantly different, with values below 40 mg L ^{1} for pine wood and poultry litter bio oils, while com stalk bio oil showed the lowest toxicity (highest EC50, 67 mg L ^{1}). The immobilization test on *D. magna* showed that the EC50 for poultry litter bio oil was significantly higher than that for corn stalk and pine wood bio oils. The results of com stalk and pine wood bio oils were not significantly different form each other.

The toxicity values found in this study for *D. magna* are consistent with previous experimental studies, which reported EC50s above 100 mg L ^{1} for wood derived bio oils (Pimenta et al., 2000; Hagner et al., 2010; Oasmaa et al., 2012).

On the other hand, the results of this study on the inhibition of algal growth differ largely from previous findings. Oasmaa et al. (2012) did not observe any significant inhibition of algal growth up to 100 mg L ^{1} (the highest tested concentration) and Hagner et al. (2010) observed only a slight inhibition of the cell growth at the highest tested concentration (381 mg L ^{1}). Consequently neither authors estimated an EC50. These differences could be possibly ascribed to the different algal species previously used for the test, *Scenedesmus gracilis* (Hagner et al., 2010) or not specified (Oasmaa et al., 2012). A possible explanation could be that some species belonging to the Scenedesmaceae family, because of their very thick cell wall made of algaenans that give them a certain physical resistance, are protected from the action of toxic compounds (Torri et al., 2012).

Moreover, contrary to the trend previously reported by Hagner et al. (2010), in the present study the alga was more sensitive than the crustacean; however, the higher sensitivity of algae agrees with the findings of studies on other materials. In particular, the OSPAR commission (2005) reported a very large variation in sen sitivity to effluents among different taxa: in many cases algae were the most sensitive organisms, followed by crustaceans (in the

Table 2
Chemical characterization of corn stalk, poultry litter and pine wood bio-oils in terms of elemental analysis (wt%; O amount calculated as difference), fractionation (wt%) and GC-MS detectable compounds (wt%). Values are reported as mean ± standard deviation. EI-WS: substances soluble in water and insoluble in ethyl acetate; PL: pyrolytic lignin; HE: hexane soluble fraction; ES-WS: substances soluble in water and ethyl acetate.

	Bio-oil		
Pyrolysis	Pine wood Fast	Corn stalk Intermediate	Poultry litter Intermediate
C	42 ± 2	20 ± 2	24 ± 2
H	5 ± 1	7 ± 1	8 ± 1
N S O	–	0.3 ± 0.1	4.8 ± 0.5
	–	–	–
	52 ± 3	73 ± 7	64 ± 6
Water	24 ± 1	56 ± 1	49 ± 1
EI-WS	36 ± 2	26 ± 2	36 ± 2
PL	24 ± 3	8 ± 1	2 ± 1
HE	2.8 ± 0.5	1.0 ± 0.1	7 ± 2
ES-WS	14 ± 2	9 ± 1	5 ± 1
Aldehydes	14 ± 2	1.7 ± 0.3	0.2 ± 0.3
Amides	0.05 ± 0.01	0.04 ± 0.0	0.2 ± 0.1
Anhydrosugars	3.0 ± 0.1	0.4 ± 0.2	0.10 ± 0.01
Carboxylic acids	2.6 ± 0.4	4.3 ± 0.0	6.6 ± 0.2
Furans Hydrocarbons	0.6 ± 0.1	0.3 ± 0.1	0.1 ± 0.0
Ketones and alcohols	–	–	0.2 ± 0.0
Nitriles	1.1 ± 0.3	1.2 ± 0.1	0.4 ± 0.0
Phenols	0.002 ± 0.00	0.0004 ± 0.0	0.01 ± 0.0
Nitrogen heterocycles	6.6 ± 2.0	1.5 ± 0.2	0.7 ± 0.1
Sum GC-MS detected	–	0.04 ± 0.0	0.1 ± 0.00
	27.6 ± 0.8	9. 6 ± 0.7	8.6 ± 0.5

Table 3
EC50 of the tested bio-oils for *Raphidocelis subcapitata* and *Daphnia magna*. The mean values from three and four test trials (*n*) and their 95% confidence limits are reported.

Test species and source of bio-oil	*n*	EC50 (mg L ^{1})	95% confidence limit	
			lower	upper
R. subcapitata growth inhibition				
Pine wood	3	38	31	45
Corn stalk	3	67	48	93
Poultry litter	3	27	26	28
D. magna immobilization				
Pine wood	4	118	76	183
Corn stalk	4	143	117	175
Poultry litter	4	383	207	706

chronic tests), bacteria and fishes (in the acute tests).

Considering the threshold of 100 mg L^{-1} for aquatic in vertebrates and 10 mg L^{-1} for algae (Weyers and Vollmer, 2000; Directive 91/414/EEC, 1991), all tested bio oils showed low toxicity to the aquatic life. According to the transportation guideline (ADR, 2011), the tested bio oils should be classified as "non environmentally hazardous substances (aquatic environment)", since they do not satisfy the criteria for the category Acute 1 (EC50 < 1 mg L^{-1}). It is, however, important to note that neither this nor previous studies have assessed the long term toxicity of bio oils. Acute tests are valuable because they allow for a rapid acquisition of screening information on scarcely studied materials such as bio oils. However, a comprehensive characterization of the hazard to the aquatic environment will have to consider also chronic tests.

Comparing the results of the present study to the results of other studies on petroleum derived oils and green fuels (Table 4), it can be observed that pine wood and corn stalk bio oils are less toxic than conventional fuels. Conversely, poultry litter bio oil seems to be as toxic as creosote oil and diesel to *R. subcapitata*, whereas its toxicity on *D. magna* was similar to that of biodiesel, considerably less toxic to aquatic organisms than conventional diesel (Khan et al., 2007).

Bio oils are complex mixtures of several substances, which can be individually responsible for adverse biological effects. In order to evaluate the role of the single components, several online databases were searched for the EC50s of the identified substances on *Daphnia* spp. immobilization or mortality and on algal growth. The selected values have been reported in Table 5.

Point estimates of *Daphnia* EC50 were available only for 18 compounds of the nearly 90 substances identified by the chemical characterization (Table 2 and S1) and only 11 values were found for algal growth inhibition. Moreover, no values were found for compounds belonging to three out of ten identified classes (amides, nitriles and N based heterocyclic compounds). It is also important to note that the quantified substances (Table 2 and S1) do not sum up to 100% of the mass of the bio oils and that a significant fraction of unidentified components is present.

Table 5 also shows the toxic units (TU) of each substance, when the three bio oils are at their respective EC50, i.e. when 1 TU of bio-oil is present. In all cases, acetic acid was present with the highest value of TU among all substances for which the EC50 was available. In the case of the pine wood oil, guaiacol had the same TU as acetic acid for *Daphnia*. However, the sum of all TUs of the single substances ranges from 0.06 to 0.50, depending on the bio oil and the organism. Thus, the data available for the single com ponents only account for a fraction of the experimentally mea sured value of 1 TU of the mixtures and their observed toxicity remains largely unexplained; the most obvious explanation for this seems to be the incompleteness of the available information. Specifically, with regard to the algal growth inhibition, acetic acid is the compound that mainly contributes to the calculated TU.

Cordella et al. (2012) calculated that the ecotoxicity EC50s of three slow pyrolysis bio oils (cornstalks, poplar and switchgrass) were between 6.5 and 25 mg L^{-1}. These values were calculated, with a procedure similar to the one adopted here, using the measured concentrations of the component substances and their EC50s for crustaceans and fish. In this case, a direct comparison with the experimental EC50s is not possible since the authors did not carry out any toxicity test. However, the predicted values are notably lower than all the experimental values for *Daphnia* reported here and in previous studies (Pimenta et al., 2000; Hagner et al., 2010; Oasmaa et al., 2012). It seems thus possible that the values calculated by Cordella et al. (2012) overestimated toxicity, contrary to the outcome of the present study.

The main difference between the two studies is the higher

Table 4
Short-term toxicity values (EC50) and biodegradability of several petroleum-derived oils and green fuels.

Fuel type	CAS#	EC50 (mg L^{-1})		Biodegradation in water (%)	References
		Algae and cyanobacteria	Aquatic invertebrates		
Creosote oil	61789-28-4	25	2.7–69	n/a	ECHA
Gas oil, light catalytic-cracked (CCGO)	64741-59-9	0.7	0.45	n/a	ECHA; Swigert et al. (2014)
Gas oil, light hydro-cracked (HCGO)	64741-77-1	0.85	1.0	n/a	Swigert et al. (2014)
Fuel oil (US EPA Api oil)	68476-31-3	15–22	0.22–10	57.5	ECHA; El-Dib et al. (1997); Redman et al. (2012)
Diesel	68334-30-5	22	1.78	24–28	ECHA; Blin et al. (2007); Khan et al. (2007); Lu et al. (2009)
Neat biodiesel B100 (made from recycled cooking oils and fats)	n/a	n/a	1.65	n/a	Khan et al., (2007)
Biodiesel (Soy methyl ester)	68990-52-3	>100	332	87.4	ECHA 2015, Birchall et al. (1995); Peterson and Reece (1994); Zhang et al., (1998)

Table 5
Available EC50 values of substances detected in the three tested pyrolysis oils and toxic units (TU) of each substance, when the three oils are at their respective EC50, i.e. when 1 TU of bio-oil is present.

Class	Substance	CAS number	EC50 (mg L^{-1})		TU of the single substance at 1 TU of bio-oil					
					D. magna			R. subcapitata		
			D. magna	R. subcapitata	pine wood	corn stalk	poultry litter	pine wood	corn stalk	poultry litter
Carboxylic acids	4-hydroxybenzoic acid	99-96-7	541[a]	138[a]	$1.8 \cdot 10^{-4}$	$8.6 \cdot 10^{-6}$	$9.0 \cdot 10^{-6}$	$2.2 \cdot 10^{-4}$	$1.6 \cdot 10^{-5}$	$2.5 \cdot 10^{-6}$
	3-methoxybenzoic acid	586-38-9	500[b]	84[b]	$8.0 \cdot 10^{-5}$	$2.3 \cdot 10^{-6}$	$5.1 \cdot 10^{-7}$	$1.5 \cdot 10^{-4}$	$6.3 \cdot 10^{-6}$	$2.2 \cdot 10^{-7}$
	oleic acid	112-80-1	97[a]	n/a	$1.6 \cdot 10^{-4}$	$1.2 \cdot 10^{-6}$	$5.1 \cdot 10^{-3}$	—	–	
	acetic acid	64-19-7	32[b]	8.1[b]	$4.4 \cdot 10^{-2}$	$1.2 \cdot 10^{-1}$	$3.4 \cdot 10^{-1}$	$5.4 \cdot 10^{-2}$	$2.2 \cdot 10^{-1}$	$9.4 \cdot 10^{-2}$
	propionic acid	79-09-4	23[c]	49[c]	$8.7 \cdot 10^{-3}$	$2.3 \cdot 10^{-2}$	$7.6 \cdot 10^{-2}$	$1.3 \cdot 10^{-3}$	$4.9 \cdot 10^{-3}$	$2.5 \cdot 10^{-3}$
	iso-butyric acid butyric	79-31-2	270[a]	n/a	$2.7 \cdot 10^{-4}$	$3.3 10^{-4}$	$2.0 \cdot 10^{-3}$	—	–	
	acid	107-92-6	1950[a]	180[a]	$2.9 \cdot 10^{-5}$	$1.1 \cdot 10^{-4}$	$3.0 \cdot 10^{-3}$	$1.0 \cdot 10^{-4}$	$5.3 \cdot 10^{-4}$	$2.3 \cdot 10^{-3}$
	valeric acid dodecanoic	109-52-4	240[c]	3500[c]	$5.8 \cdot 10^{-4}$	$2.7 10^{-4}$	$2.5 \cdot 10^{-3}$	$1.3 \cdot 10^{-5}$	$8.7 10^{-6}$	$1.2 \cdot 10^{-5}$
	acid furfural	143-07-7	13[a]	n/a	$1.6 \cdot 10^{-2}$	$7.5 \cdot 10^{-5}$	$2.1 \cdot 10^{-4}$	—	–	
Furans	5-hydroxymethyl-2-	98-01-1	20[a]	232[a]	$1.8 \cdot 10^{-2}$	$1.8 \cdot 10^{-2}$	$8.1 \cdot 10^{-4}$	$5.0 \cdot 10^{-4}$	$7.3 \cdot 10^{-4}$	$5.1 \cdot 10^{-6}$
	furfural	67-47-0	34[a]	n/a	$1.3 \cdot 10^{-2}$	$1.4 \cdot 10^{-3}$	$9.9 \cdot 10^{-4}$	—	–	
	alkanes									
Hydrocarbons	glycerol	97862-82-3	32[b]	90[b]	0	0	$1.8 \cdot 10^{-2}$	0	0	$4.6 \cdot 10^{-4}$
Ketones and alcohols		56-81-5	10,000[b]	n/a	$3.2 \cdot 10^{-6}$	$2.8 \cdot 10^{-5}$	$3.2 \cdot 10^{-5}$	—	–	
	phenol									
Phenols	4-methylphenol	108-95-2	21[a]	197[a]	$2.1 \cdot 10^{-3}$	$1.4 \cdot 10^{-2}$	$2.5 \cdot 10^{-2}$	$7.0 \cdot 10^{-5}$	$6.8 \cdot 10^{-4}$	$1.9 \cdot 10^{-4}$
	guaiacol	106-44-5	7.7[c]	21[c]	$2.7 \cdot 10^{-2}$	$1.0 \cdot 10^{-1}$	$1.7 \cdot 10^{-2}$	$3.2 \cdot 10^{-3}$	$1.8 \cdot 10^{-2}$	$4.3 \cdot 10^{-4}$
	4-methylguaiacol	90-05-1	26[a]	n/a	$2.6 \cdot 10^{-2}$	$4.7 \cdot 10^{-3}$	$8.2 \cdot 10^{-3}$	—	–	
	vanillin	93-51-6	150[a]	n/a	$9.4 \cdot 10^{-3}$	$1.5 \cdot 10^{-4}$	$2.6 \cdot 10^{-4}$	—	–	
		121-33-5	180[c]	n/a	$4.4 \cdot 10^{-4}$	$4.1 \cdot 10^{-4}$	$1.3 \cdot 10^{-3}$	—	–	
Total					0.17	0.29	0.50	0.06	0.24	0.10

Source:
[a] *USEPA ECOTOX database (http://cfpub.epa.gov/ecotox/).*
[b] *University of Hertfordshire Pesticide Properties Data Base (http://sitem.herts.ac.uk/aeru/ppdb/).*
[c] *OECD Existing Chemicals Database (http://webnet.oecd.org/HPV/UI/Search.aspx).*

number of component substances induded in the calculations by Cordella et al. (2012), due to the use of different databases and the use of QSAR models.

3.3. Biodegrodobility

At the end of the 28 day test, the biodegradability of corn sta/I<, poultry Jitter and pine wood bio oils were 60 ± 2, 51 ± 2 and 37 ± 3%, respective/y (mean of two rep/icates ± standard devia tion, Fig. 1). The amount of readily degradable carbon was well correlated to the amount of water so/uble organic substances in each bio oil (El WS calculated on dry basis, Table 2), quick/y metabolizable by a mixed of bacterial consortia, such as small oxygenated molecu/es (e.g. hydroxyacetaldehyde, volatile fatty adds, li.Jrans) and small o/igomers (e.g. sugar o/igomers or protein fragments). The degradation began immediately, without any lag phase and thus suggesting that a fast biologica/ adaptation and

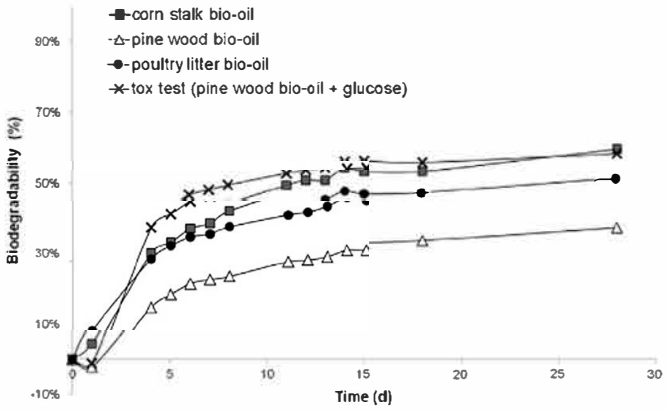

Fig. 1. Biodegradation curves of fast pyrolysis oil from pine wood, intermediate pyrolysis of corn stalk and poultry litter and toxicity test performed with both pine wood bio-oil and glucose.

response occurred The biodegradation rurves, very similar among the three bio oils, showed quite rapid degradation kinetics during the first 8 days, alter which a plateau was reached during the following 20 days. This highlights that, even if a substantial fraction is readily biodegradable, more persistent components are present in the bio oils, which reinforces the need to assess the chronic toxidty of these materials in the li.Jture.

The toxicity test, performed by testing simultaneous/y glucose and pine wood bio oi/ reached 40% degradation (based on ThOD) within 4 days, thus pine wood bio oi/ did not result in inhibition of bacteria at the tested concentration (100 mg L^{-1}) as stated by the OECD protoml

Glucose degraded to approximately 60% by day 6, validating the test and mnfirming the viability of the microbial inocu/ums. The reproducibility of the measurements was satisfactory since the standard deviation between the two rep/icates of each tested compound (including blank) was from 1 to 8%.

The only two previous detailed papers onthe biodegradability of pyro/ysis oi/s (Blin et al., 2007; Oasmaa et al., 2012) evaluated the biodegradation rate of bio oils from wooden biomass (mainly spruce, beech and pine) under various pyro/ysis mnditions (flash/ sfow pyro/ysis, temperature, reactor of pyro/ysis). Tothe best of our knowledge, this is the first paper that explored the biade gradability of bio oils from intermediate pyrolysis of other biomasses, such a s corn stalk and poultry Jitter.

The biodegradability of the bio oils analyzed in the present study agrees with previous results for both slow and fast pyro/ysis oils. The corn stalk bio oi/, obtained with a fixed bed intermediate pyro/ysis at ***500*** °c degraded at a similar rate to that ofthe single slow pyro/ysis bio oil studied by Blin et al. (2007), that achieved 62% biodegradation after 28 d. The intermediate pyro/ysis poultry Jitter bio oil had a s/ightly Jower biodegradability. On the other hand, the biodegradability of the fast pyro/ysis pine bio oi/ was at the Jowest limit of the range reported by Blin et al. (2007) and Oasmaa et al. (2012) for sevem/ lignocel/ulosic fast pyro/ysis bio -

oils (biodegradability after 28 d: 40 50%). As a whole, the results from the present study and from previous studies suggest that pyrolysis conditions play a major role on the biodegradability of bio oils, possibly more than that of the feedstock, and that fast pyrolysis bio oils are less degradable than those produced by slow and intermediate pyrolysis. However, a definite conclusion is not possible, because none of the studies adopted an experimental design that allowed an independent assessment of the influence of feedstock and pyrolysis conditions. Furthermore, most of the available biodegradation data concern fast pyrolysis bio oils from wood feedstock and only very few data are available for slow and intermediate pyrolysis.

4. Conclusions

The tested bio oils were biodegradable and had low toxicity. On the whole, their environmental hazard is comparable to that of biodiesel.

It was not possible to reliably predict the observed toxicity from the data on the single substances that made up the mixture, due to the scarce availability of toxicity values for the single compounds and to the high fraction of undetectable compounds.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2016.05. 027.

References

ADR, 2011. European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR). Annex A: General provisions and provisions concerning dangerous substances and articles. Part 2: Classification. United Nations, 2010 ISBN 978-92-1-139140-4.

Birchall, C., Newman, J., Greaves, M., 1995. Degradation and Phytotoxicity of Biodiesel oil. Institute of Arable Crops Research Center for Aquatic Plant Management, Bristol, UK.

Blin, J., Volle, G., Girard, P., Bridgwater, T., Meier, D., 2007. Biodegradability of biomass pyrolysis oils: Comparison to conventional petroleum fuels and alternatives fuels in current use. Fuel 86, 2679–2686.

Bridgwater, A.V., 2012. Review of fast pyrolysis of biomass and product upgrading. Biomass Bioenergy 38, 68–94.

Busetto, L., Fabbri, D., Mazzoni, R., Salmi, M., Torri, C., Zanotti, V., 2011. Application of the Shvo catalyst in homogeneous hydrogenation of bio-oil obtained from pyrolysis of white poplar: new mild upgrading conditions. Fuel 90, 1197–1207.

Chatterjee, N., Eom, H.J., Jung, S.H., Kim, J.S., Choi, J., 2014. Toxic potentiality of bio-oils, from biomass pyrolysis, in cultured cells and *Caenorhabditis elegans*. En-viron. Toxicol. 29, 1409–1419.

Christensen, F.M., Eisenreich, S.J., Rasmussen, K., Riego Sintes, J., Sokull-Kluettgen, B., Erik van de Plassche, J., 2011. European experience in chemicals management: integrating science into policy. Environ. Sci. Technol. 45, 80–89.

Cordella, M., Torri, C., Adamiano, A., Fabbri, D., Barontini, F., Cozzani, V., 2012. Bio-oils from biomass slow pyrolysis: a chemical and toxicological screening. J. Hazard. Mater. 231–232, 26–35.

Diebold, J.P., 1997. A Review of the Toxicity of Biomass Pyrolysis Liquids Formed at Low Temperature. NREL/TP-430-22739, Golden, Colorado.

Diebold, J.P., 2000. A Review of the Chemical and Physical Mechanisms of the Storage Stability of Fast Pyrolysis Bio-Oils. NREL/SR-570-27613, Golden, Colorado.

Directive 91/414/EEC, 1991. Annex VI "Uniform principles for the evaluation of plant protection products".

ECHA database of registered substances: http://echa.europa.eu/it/information-on-chemicals/registered-substances (accessed on 02.07.15).

El-Dib, M.A., Abou-Waly, H.F., el-Naby, A.M., 1997. Impact of fuel oil on the freshwater alga *Selenastrum capricornutum*. Bull. Environ. Contam. Toxicol. 59, 438–444.

European Commission, 2003a. Technical guidance document in support of commission directive 93/67/EEC on risk assessment for new notified substances and commission regulation (EC) No. 1488/94 on risk assessment for exiting substances. Luxembourg, ISBN: 92-827-8011-2.

European Commission, 2003b. Proposal for a regulation of the European parliament and of the council concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). Brussels, 29.10.2003. COM (2003) 644 final.

Garcia-Perez, M., Chaala, A., Pakdel, H., Kretschmer, D., Roy, C., 2007. Characterization of bio-oils in chemical families. Biomass Bioenergy 31, 222–242.

Hagner, M., Penttinen, O.P., Pasanen, T., Tiilikkala, K., Setälä, H., 2010. Acute toxicity of birch tar oil on aquatic organisms. Agric. Food Sci. 19, 24-33.

Hossain, M.: M., Scott, I.M., McGarvey, B.D., Conn, K., Ferrante, L., Berruti, F., Briens, C., 2015. Insecticidal and anti-microbial activity of bio-oil derived from fast pyrolysis of lignin, cellulose, and hemicellulose. J. Pestic. Sci. 88, 171–179.

Ingram, L., Mohan, D., Bricka, M., Steele, P., Strobel, D., Crocker, D., Mitchell, B., Mohammad, J., Cantrell, K., Pittman, C.U., 2008. Pyrolysis of wood and bark in an auger reactor: physical properties and chemical analysis of the produced bio-oils. Energy Fuels 22, 614–625.

Khan, N., Warith, M., Luk, G., 2007. A comparison of acute toxicity of biodiesel, biodiesel blends, and diesel on aquatic organisms. JA&WMA 57, 286-296.

Lahl, U., Hawxwell, K.A., 2006. REACH-–the new european chemicals law. Environ. Sci. Technol. 40, 7115-7121.

Loewe, S., Muischnek, H., 1926. Über kombinationswirkungen. I. Mitteilung: Hilfsmittel der Fragestellung. Naunyn-Schmiedebergs. Arch. Exp. Pathol. Pharmakol. 114, 313–326.

Lu, Q., Li, W.Z., Zhu, X.F., 2009. Overview of fuel properties of biomass fast pyrolysis oils. Energy Convers. Manag. 50, 1376-1383.

Nakai, T., Nami Kartal, S., Hata, T., Imamura, Y., 2007. Chemical Characterization of pyrolysis liquids of wood-based composites and evaluation of their bio-efficiency. Build. Environ. 42, 1236–1241.

Oasmaa, A., Peacocke, C., 2001. A Guide to Physical Property Characterization of Biomass-derived Fast Pyrolysis Liquids. VTT Publications P450, Espoo (Finland).

Oasmaa, A., Kuoppala, E., Solantausta, Y., 2003. Fast pyrolysis of forestry residue. 2. Physicochemical composition of product liquid. Energy Fuels 17, 433–443.

Oasmaa, A., Kuoppala, E., 2008. Solvent fractionation method with brix for rapid characterization of wood fast pyrolysis liquids. Energy Fuels 22, 4245-4248. Oasmaa, A, Källi, A., Lindfors, C., Elliott, D.C., Springer, D., Peacocke, C., Chiaramonti, D., 2012. Guidelines for transportation, handling, and use of fast pyrolysis bio-oil. 1. Flammability and toxicity. Energy Fuels 26, 3864–3873.

OECD, 2006. Alga growth inhibition test. OECD Guideline for testing chemicals, no. 201. Paris, France.

OECD, 2004. *Daphnia* sp., acute immobilization test and reproduction test. OECD Guideline for testing chemicals, no.202. Paris, France.

OECD, 1992. Ready Biodegradability. OECD Guideline for testing chemicals, no. 301. Paris, France.

OSPAR Commission, 2005. Whole effluent assessment report.

Peterson, C.L., Reece, D., 1994. Toxicology, Biodegradability and Environmental Benefits of Biodiesel. University of Idaho, United States.

Pimenta, A.S., Bayona, J.M., Garcia, M.T., Solanas, A.M., 2000. Evaluation of acute toxicity and genotoxicity of liquid products from pyrolysis of Eucalyptus grandis wood. Arch. Environ. Contam. Toxicol. 38, 169-175.

Redman, A.D., Parkerton, T.F., McGrath, J.A., Di Toro, D.M., 2012. PETROTOX: an aquatic toxicity model for petroleum substances. Environ. Toxicol. Chem. 31, 2498–2506.

Scholz, E., 1984. Karl-Fischer-Titration. Springer-Verlag, Berlin.

Sharma, B.M., Bharat, G.K., Tayal, S., Nizzetto, L., Larssen, T., 2014. The legal framework to manage chemical pollution in India and the lesson from the Per-sistent Organic Pollutants (POPs). Sci. Total Environ. 490, 733–747.

Swigert, J.P., Lee, C., Wong, D.C., Podhasky, P., 2014. Aquatic hazard and biodegradability of light and middle atmospheric distillate petroleum streams. Chemosphere 108, 1–9.

Torri, C., Lesci, I.G., Fabbri, D., 2009. Analytical study on the pyrolytic behavior of cellulose in the presence of MCM-41 mesoporous materials. J. Anal. Appl. Pyrolysis 85, 192–196.

Torri, C., Garcia Alba, L., Samorì, C., Fabbri, D., Brilman, D.W.F., 2012. Hydrothermal treatment (HTT) of microalgae: detailed molecular characterization of HTT oil in view of HTT mechanism elucidation. Energy Fuels 26, 658-671.

Weyers, A., Vollmer, G., 2000. Algal growth inhibition: effect of the choice of growth rate or biomass as endpoint on the classification and labeling of new substances notified in the EU. Chemosphere 41, 1007–1010.

Woolf, D., Lehmann, J., Fisher, E.M., Angenent, L.T., 2014. Biofuels from pyrolysis in perspective: trade-offs between energy yields and soil-carbon additions. Environ. Sci. Technol. 48, 6492–6499.

Zhang, X., Peterson, C., Reece, D., Möller, G., Haws, R., 1998. Biodegradability of biodiesel in the aquatic environment. Trans. ASAE 41, 1423–1430.