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Effect of pyrolysis temperature on potential toxicity of biochar if applied to the environment☆

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ABSTRACT

Biochars have increasingly been used as adsorbents for organic and inorganic contaminants in soils. However, during the carbonization process of pyrolysis, contaminants, including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated dioxins and furans (PCDD/DF) can be generated. In this study, biochars made from sawdust, were prepared at various temperatures ranging from 250 to 700 °C. The Microtox® and rat hepatoma cell line H4IIE-*luc* assays were used to characterize the general toxic and effects, mediated through the aryl hydrocarbon receptor (AhR), or dioxin-like potencies of organic extracts of biochars. The greatest total concentrations of PAHs (8.6×10^2 μg kg^{-1}) and PCDD/DF (6.1×10^2 pg g^{-1}) were found in biochar generated at 400 °C and 300 °C, respectively. Results of the H4IIE-*luc* assay, which gives total concentrations of 2,3,7,8-TCDD equivalents ($TEQ_{H4IIE\text{-}luc}$), indicated that total potencies of aryl hydrocarbon receptor (AhR) agonists were in decreasing order: 300 °C > 250 °C > 400 °C > 500 °C > 700 °C. The 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents (TEQ_{chem}) calculated as the sum of products of 16 PAHs and 17 PCDD/DF congers multiplied by their respective relative potencies (RePs) was less than that of $TEQ_{H4IIE\text{-}luc}$ determined by use of the bioanalytical method, with the H4IIE-*luc* assay, which measures the total dioxin-like potency of a mixtures. The ratio of $TEQ_{chem}/TEQ_{H4IIE\text{-}luc}$ was in the range of 0.7%–3.8%. Thus, a rather small proportion of the AhR-mediated potencies extracted from biochars were identified by instrumental analyses. Results of the Microtox test showed similar tendencies as those of the H4IIE-*luc* test, and a linear correlation between EC50 of Microtox test and EC20 of H4IIE-*luc* test was found. The results demonstrated that biochars produced at higher pyrolysis temperatures (>400 °C) were less toxic and had lower potencies of AhR-mediated effects, which may be more suitable for soil application.

1. Introduction

Biochar is now increasingly receiving attention as an "environmentally-friendly" approach for remediation of soil contaminated with either metals or organic pollutants (Tang et al., 2013). As a method to disposal wastes, biochar is generally produced by pyrolysis of biological waste materials under conditions of limited oxygen at temperatures lower than 700 °C (Mesa and Spokas, 2011). Biochar produced at higher temperature is characterized by stronger adsorption of metals, which can be 10-fold greater than that of commercial, activated carbon (Chen et al., 2014). When applied to soils, BC can enhance adsorption of dissolved organic compounds and reduce concentrations of trace metals in leachates, because of the porous structure and unique properties resulting from carbonization (Beesley et al., 2010). In addition, the porous nature of biochars and their ability to absorb soluble organic carbon provides spaces within which microorganisms can live on absorbed

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organic substrates and be protected from grazers, which can enhance rates of biotransformation of organic compounds by microbes (Beesley et al., 2011).

During the past few decades, effects of pyrolysis temperature on properties of biochar have been characterized. Temperature of pyrolysis affected surface area of biochar and as a consequence, had great influence on sorption capacities for pollutants, such as heavy metals and pesticides (Chun et al., 2004; Chen et al., 2008). A threshold for temperature of pyrolysis of 360 °C was suggested, above which the thermal and biological resistance to degradation increased dramatically (Mimmo et al., 2014). Biochars obtained at temperatures between 400 and 500 °C were composed of a highly ordered aromatic carbon structure (Kim et al., 2012). Also, loss of cellulose and crystalline mineral components were greater when biochar was produced at higher temperatures of pyrolysis (Al-Wabel et al., 2013). For this reason, biochars produced at higher temperatures of pyrolysis are recommended because of their greater capacities to adsorb heavy metals (Chen et al., 2014; Melo et al., 2013). However, unwanted, hazardous materials like PAHs and dioxin-like compounds that are ligands for the aryl-hydrocarbon receptor (AhR) can be produced during pyrolysis process. Total concentrations of PAHs (sum of EPA 16 PAHs) in biochar were as great as 33.7 mg kg^{-1}, dry mass (dm) which can result in contamination of large amounts of PAH in nearby soils (Kloss et al., 2012; Oleszczuk et al., 2014; Kusmierz and Oleszczuk, 2014). Temperatures of pyrolysis between 400 and 500 °C, which are commonly used to produce biochar in industry could result in maximum concentrations of PAHs in biochar (Keiluweit et al., 2012). Dioxins including polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDD/DF) were also found in various biochars produced at temperatures of pyrolysis from 250 to 900 °C. The greatest concentration of 17 congeners of PCDD/DF in biochar produced at 300 °C was 12.2 pg g^{-1} (Hale et al., 2012). Nitrogen-containing organic compounds (NCCs) and aliphatic components were also components of contaminants in biochar produced from poultry litter (Rombolà et al., 2015).

Pollutants in biochar can result in toxicity to organisms during environmental application of biochar. When toxic potencies of biochar were assessed by several methods, a significant correlation between concentrations of PAHs and toxicity was observed (Oleszczuk et al., 2013). Genotoxicity of biochar was also observed with the *Tradescantia micronucleus* and Ames tests (Busch et al., 2013; Anjum et al., 2014). Re-condensation of volatile organic compounds (VOCs) during pyrolysis can result in biochar containing compounds that are bioavailable and phytotoxic (Oleszczuk et al., 2014). However, it remained unclear which specific compounds were responsible for this toxicity and how the temperature of pyrolysis affects toxic potency.

Most of the adverse effects of dioxin-like chemicals (DLCs) are mediated through the aryl hydrocarbon receptor (AhR) (Giesy and Kannan, 1998; Giesy et al., 2002), and a 2,3,7,8-TCDD equivalency factor (TEF) approach has been developed (Van den Berg et al., 1998). There are two ways of estimating 2,3,7,8-TCDD equivalents (TEQ). In the instrumental approach, the sum of the products of concentrations of individual DLCs and corresponding TEF or relative potencies (RePs) is defined as the TEQ_{chem}. This system allows simplification of complex mixtures to a single estimator of toxic effects mediated by the AhR (Hilscherova et al., 2000). One limitation of this approach is that concentrations of each DLCs must be known (Lee et al., 2013). The other approach is to use a bioassay, such as the rat hepatoma cell line H4IIE-*luc* bioassay as an integrative measure of potency of DLCs expressed as TEQ in a mixture (TEQ_{bio}) (Eichbaum et al., 2014). When comparing concentrations of TEQ_{bio} to concentrations expressed as TEQ_{chem} calculated from instrumental analyses, it is important to use bioassay-specific RePs (Giesy et al., 2002; Koh et al., 2004). However, comparison studies of the RePs of DLCs in H4IIE-*luc* are scarce.

Biochar can be produced from abundant feedstock materials, and it is considered an effective approach to sequester carbon (C) and reduce bioavailabilities of contaminants. However, toxicity of biochar is a significant obstacle that limits its application. In the case of toxicity in biochar, it is still not clear how temperature of pyrolysis affects toxicity of biochar products. The results until now suggested that pyrolysis temperature played a much more important role in determining the toxicity of biochar than the sources of biological wastes (Hale et al., 2012). Thus, the objective of this study is to use a combination of instrumental and bioanalytical techniques to assess hazardous components in pine tree wood biochars produced at various temperatures of pyrolysis and make clear the relationship between different parameters.

2. Materials and methods

2.1. Biochars produced under different temperatures

Sawdust from pine tree wood, which is viewed as forest biomass waste, was selected as a feedstock for biochar. Biochars were produced by pyrolyzing sawdust under oxygen-limited conditions following a revision of previously described method (Fang et al., 2014). In brief, sawdust was air dried for 7 days after cleaning with distilled water, oven-dried overnight at 80 °C, milled into powders of <1 mm, and then tightly packed into a ceramic crucible. After being covered with a fitting lid, the crucible was put into a muffle furnace under oxygen-limited conditions to prevent calcination of the sawdust, heated at a rate of 5 °C min^{-1}, and then held for 3 h when pyrolytic temperatures reached 250, 300, 400, 500 and 700 °C, respectively. Biochars were then cooled to room temperature before the mass was determined. Then biochars were ground in a mortar grinder, and passed through a 60 mesh sieve.

2.2. Quantification of PAHs

Soxhlet extraction: 2 g of biochar was Soxhlet-extracted for 16 h with 100 mL dichloromethane at 54 °C. The extract was evaporated to 1 mL using a rotary evaporator (R1001-VN, Zhengzhou Great Wall Scientific Industrial and Trade CO., LTD, Henan, China) at 50 rpm and 42 °C.

Purification and quantification of PAHs: The extract was purified by use of previously described methods (Liu et al., 2015). In brief, concentrated extracts were eluted from an alumina-silica gel column (dimensions: 20 mm × 400 mm, anhydrous Na_2SO_4 granules: neutral aluminum: silica gel = 2 g:6 g:12 g, soaked with hexane) using 20 mL of hexane, 70 mL of hexane and dichloromethane (volume ratio = 1:1), 50 mL of methanol to separate saturated hydrocarbons, PAHs, and polar components. After purification and separation, concentrations of PAHs were determined by use of a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP mass spectrometer (Agilent, CA, USA). Aliquots of 1 μL each were injected at 280 °C in pulsed, splitless mode (1 min, then split ratio 1:50 to the end of analysis). The GC oven temperature was held at 60 °C for 3 min, increased by 15 °C min^{-1} from 60 °C to 180 °C, followed by 6 °C min^{-1} from 180 °C to 300 °C, and then held at 300 °C for 10 min. The mass spectrometer was operated with the ion source at 220 °C with energy of ionization of 70 eV. Five concentrations of mixtures containing 16 US EPA target PAHs mixture (Naphthalene 1.0 mg mL^{-1}, Acenaphthene 1.0 mg mL^{-1}, Acenaphthylene 2.0 mg mL^{-1}, Anthracene 0.1 mg mL^{-1}, Fluorene 0.2 mg mL^{-1}, Fluoranthene 0.2 mg mL^{-1}, Phenanthrene 0.1 mg mL^{-1}, Pyrene 0.1 mg mL^{-1}, Benzo(a)anthracene 0.1 mg mL^{-1}, Chrysene 0.1 mg mL^{-1}, Benzo(b)fluoranthene 0.2 mg mL^{-1},

Benzo(k)fluoranthene 0.1 mg mL^{-1}, Benzo(a)pyrene 0.1 mg mL^{-1}, Indeno(1,2,3-cd)pyrene 0.1 mg mL^{-1}, Dibenzo(a,h)anthracene 0.2 mg mL^{-1}, Benzo(g,hi)perylene 0.2 mg mL^{-1}) were used as external standards (J&K Scientific Ltd., Beijing, China). PAHs were quantified in single ion monitoring (SIM) mode, with the molecular ion of each PAH corresponding to the elution retention time of the external standard.

2.3. Quantification of dioxins and furans

Soxhlet extraction: Dioxins were extracted and purified following a revised method by Sundqvist et al. (Sundqvist et al., 2009). Briefly, 2 g of biochar and ^{13}C labelled recovery standard EPA 1613LCS (obtained from J&K Scientific Ltd., Beijing, China) were mixed, then they were extracted by Soxhlet with toluene for at least 18 h. Tetradecane (40–50 μL) was added to the extracts as a keeper and samples were then evaporated as described in the extraction of PAHs.

Purification and quantification of Dioxin: The extract was purified through a multilayer silica column (dimensions: 16 mm × 300 mm, anhydrous Na_2SO_4 granules: 40% (*w*/*w*) H_2SO_4-silica: neutral silica = 3 g:6 g:3 g), which was rinsed with 100 mL n-hexane and then eluted with 60 mL n-hexane. A carbon/Celite column was used to fractionate the extract by elution with 40 mL dichloromethane and n-hexane (volume ratio = 1:1). PCDD/DF was recovered by eluting with 40 mL toluene in the reverse direction.

A congener-specific analysis was conducted by use of a high resolution gas chromatography-high resolution mass spectrometry (HRGC-HRMS) system consisting of an Agilent 6890 N/Autospec Ultima NT GC (Santa Clara, CA, USA) with a 60 m × 0.25 mm × 0.25 μm DB560m capillary column (J & W Scientific, Agilent Technologies, USA), which was connected to a double focusing magnet sector mass spectrometer (Autospec Ultima HRMS Waters, Milford, MA, USA) operating in EI^+ selected ion monitoring mode. Samples were injected splitless (3 μL, split opened after 2 min) at an injector temperature of 250 °C. The temperature of the oven was held at 200 °C for 2 min, increased by 3 °C min^{-1} to 290 °C, held for 5 min, then raised by 10 °C min^{-1} to 320 °C. Testing conditions were as follows: helium carrier gas at a constant flow of 1.2 mL min^{-1}, transfer line temperature of 275 °C, ion source temperature of 250 °C, with resolution of 10,000 and electron energy of 36 eV.

2.4. AhR agonistic activity test using H4IIE-luc cell line

Extract preparation: 3 g of biochar sample was extracted with 100 mL of methanol using Soxhlet extraction for 16 h in a water bath temperature of 70 °C, followed by condensation using rotary evaporation with water bath temperature of 35 °C and speed of 50 rpm. After evaporation, 4 mL of chromatographic purity hexane was used to transfer the dried extract to a 5 mL brown sample bottle and the rotary evaporation bottle was washed three times with hexane. The sample bottle was blown to dryness with Nitrogen and then re-dissolved in 500 μL DSMO.

The H4IIE-*luc* assay was conducted based on previously developed methods with minor modification (Hong et al., 2012). Briefly, H4IIE-*luc* cells in logarithmic growth phase were harvested and seeded into a 96-well plate, with each well containing 250 μL of a cell suspension containing approximately 8.0 × 10^4 cells. After overnight incubation, 2.5 μL of dilute of sample extract of biochar was added into each well. All extracts were previously diluted to a concentration of 3 g biochar L^{-1} equivalents with phosphate buffered saline (PBS) solution and serial diluted to 2, 1, 0.5 and 0.25 g biochar L^{-1}, respectively. The final tested concentrations of each extract in wells were 3, 2, 1, 0.5 and 0 g equivalents of biochar L^{-1}. Luciferase assays were conducted after 72 h of exposure using Steadylite plus™ Reporter Gene Assay System (Perkin Elmer, MA, USA). Luminescence was measured using Polar Star OPTIMA microplate reading luminometer (BMG Labtech, Ortenberg, Germany).

2.5. Microtox® test

The Microtox bioassay was conducted using a M500 Toxicity Analyzer (Modern Water Inc, DE, USA) with previously described methods (Romero et al., 2008). Extract solution was diluted to 30 mg biochar equivalents L^{-1} by PBS solution and then diluted serially with 2-fold dilutions to give 10 series concentrations. Acute toxicity was assessed by determining inhibition of luminescence generated by the marine, gram-negative bacterium *Vibrio fischeri*. The bacterium was purchased in freeze-dried form from Modern Water Inc. and activated by rehydratation with reconstitution solution to provide a ready-to-use suspension. Concentrations of samples (g L^{-1}) that produce a 50% decrease in light intensity after exposure for 5 min and 15 min were designated as the Effective Concentrations (EC50). Results are presented as EC50-5min and EC50-15min (g L^{-1}), respectively. EC50 values were calculated by use of the gamma distribution.

2.6. Potency balance calculation

TCDD equivalents (TEQ) for dioxin-like PAHs (TEQ_{PAHs}) including BaA, Chr, BbF, BkF, BaP, InP and DBA were calculated using the toxic equivalency factor (TEF) by use of previously described methods (Larsson et al., 2012), TEQ for the sum of 17 congers of PCDD/DF ($TEQ_{PCDD/DF}$) was calculated based on TEF described previously (Lee et al., 2013; Eichbaum et al., 2014).

2.7. Statistical analysis

The H4IIE-*luc* and Microtox were performed in quadruplicates. All other tests were conducted in triplicates. Data were presented as mean ± standard deviation. T-test was performed to analyze the significant difference. All mathematical and statistical computations were conducted using SPSS 16.0 (IBM, New York, USA). Data were tested for statistical significance with One-Way analysis of variance (ANOVA). Normality was confirmed by the Kolmogorov-Smirnov test and homogeneity of variance was confirmed by use of Levine's test. A value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Concentrations of PAHs and PCDD/DF in biochars

Concentrations of 16 PAHs in biochar produced at various temperatures of pyrolysis are presented (Table 1). Total concentrations of PAHs in biochar directly proportional to temperature of pyrolysis and reached to a maximum concentration of 8.6 × 10^2 μg kg^{-1} at 400 °C, and then moderately decreased at higher temperatures of pyrolysis and reached a maximum of 5.9 × 10^2 μg kg^{-1} at 700 °C. PAHs were classified into 3 groups based on the method of Devi and Saroha (Fig. 1) (Larsson et al., 2012). The group of 2&3-ring PAHs represented the majority of PAH in all biochars, and followed trends similar to those of total concentrations of PAHs. The group of 4-ring PAHs also demonstrated a similar maximum concentration in biochar produced at 400 °C but with a lower concentration (8.6 × 10^2 μg kg^{-1} for total PAHs vs. 1.5 × 10^2 μg kg^{-1} for 4-ring PAHs). The concentration of 4-ring PAHs decreased from 1.5 × 10^2 μg kg^{-1} to 51 μg kg^{-1} with an increase of pyrolysis temperature from 400 to 700 °C. The group of

5&6-ring PAHs exhibited a different relationship with temperature of pyrolysis, reaching a maximum concentration between 300 and 400 °C with a maximum production which was approximately 20% of that of 4-ring PAHs produced at 400 °C. Concentrations of 5&6-ring PAHs decreased dramatically to 6.3 and 2.7 μg kg^{-1} at pyrolysis temperatures of 500 and 700 °C, respectively. These results demonstrated that maximum concentrations of PAHs containing fewer rings were produced at 400 °C, with lower concentrations of PAHs formed at higher pyrolysis temperatures. However, maximum production of PAHs with high rings was observed at lower temperatures of pyrolysis, and much less production at higher temperatures. Concentrations of PAHs in biochar observed in this study were consistent with the range of concentrations of 0.07–4 μg g^{-1} for most biochar products reported in other studies (Oleszczuk et al., 2014; Devi and Saroha, 2014; Wiedner et al., 2013). Greater concentrations of PAHs were also found with maximum concentrations of 17 and 27 μg g^{-1} in biochars formed from paper mill sludge and wood, respectively (Devi and Saroha, 2014; Keiluweit et al., 2012).

Dioxin-like compounds can also be produced during pyrolysis of biochar. Table 1 shows the concentrations of PCDD/DF varied among biochars produced at various temperatures of pyrolysis. Maximum concentrations of PCDD/DF of 6.1 × 10^2 pg g^{-1} were observed in biochar produced at 300 °C. Significantly lower concentrations of PCDD/DF were observed in biochar produced at pyrolysis temperatures higher than 400 °C. However, the effect of temperature of pyrolysis was less for concentrations of TEQ_{WHO} than the effect for total concentrations of PCDD/DF which had a maximum concentration of 9.6 pg g^{-1} at 300 °C. PCDD/Fs were formed more easily at lower temperatures of pyrolysis compared to PAHs. Although concentrations of TEQ of most chars were less than the detection limit (<6 ng kg^{-1}), but greater concentrations of TEQ (14 ng kg^{-1}) were observed in biochar made from sewage sludge (Wiedner et al., 2013). Concentrations of TEQ in biochar produced at 250, 400, 500 and 700 °C were less than that reported by International Biochar Initiative (IBI) (9 ng kg^{-1}) or European Biochar Certificate (EBC) (20 ng kg^{-1}). The results indicate that biochar produced in the study, results of which are presented here were within the safe level for environmental risk. Biochar produced at 300 °C contained greater concentrations of TEQ which exceeded the suggested IBI value.

3.2. AhR activity from H4IIE-luc test

A standard curve was generated by exposing H4IIE-*luc* cells to TCDD (0–150 pM) (Fig. 2). Measurement of cytotoxicity suggested that cells could be exposed to a maximum extract concentration of 3 g equivalents L^{-1} of biochar produced at 400 °C, 500 °C, and 700 °C, and 2 g equivalents L^{-1} for biochar produced at 250 °C and 300 °C. These latter two products resulted in higher concentrations of $TEQ_{H4IIE\text{-}luc}$. AhR potencies of extracts of biochar reported as $TEQ_{H4IIE\text{-}luc}$ differed among biochars produced at varying temperatures of pyrolysis (Fig. 3). Biochar produced at 300 °C exhibited the most potent AhR agonistic ability. Exposed to 2, 1 and 0.5 g equivalents L^{-1} produced significant AhR-mediated expression of the reporter gene of 5.1 ± 0.67, 3.6 ± 0.94, and 2.3 ± 0.26 fold greater values than that of the control (*t*-test, $p < 0.05$), respectively. Exposed to extract of biochar produced at 700 °C did not cause any significant induction of AhR-mediated expression of reporter gene. Generally, potencies of AhR agonists in extracts of biochar were in the following decreasing order: 300 °C > 250 °C > 400 °C > 500 °C > 700 °C.

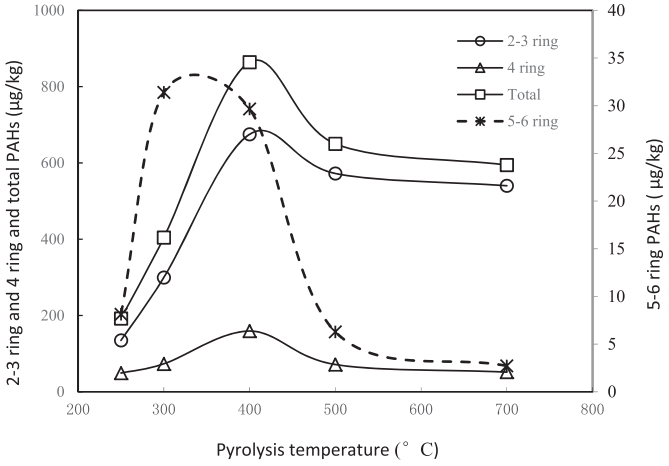

Fig. 1. Concentrations of 2&3-ring, 4-ring, 5&6-ring and total PAHs in extracts of biochar produced at various temperatures of pyrolysis.

Table 1
Concentrations of 16 indicator PAHs and dioxins (PCDD/DF) in extracts of biochar produced at various temperatures of pyrolysis.

	250 °C	300 °C	400 °C	500 °C	700 °C
Naphthalene	35	1.5 × 10^2	2.4 × 10^2	3.6 × 10^2	3.0 × 10^2
Acenaphthylene	5.9	9.1	13	11	12
Acenaphthene	6.9	8.9	17	17	23
Fluorene	16	25	99	35	40
Phenanthrene	63	98	2.6 × 10^2	1.4 × 10^2	1.4 × 10^2
Anthracene	6.9	10	43	10	17
Fluoranthene	26	29	59	31	22
Pyrene	18	29	68	32	24
Benzo(a)anthracene	1.8	5.5	12.7	3.4	2.7
Chrysene	3.8	9.6	19.2	4.6	2.7
Benzo(b)fluoranthene	3.4	16.4	14.7	2.7	1.3
Benzo(k)fluoranthene	1.2	3	1.2	1.2	0.48
Benzo(a)pyrene	1.6	5.3	8.6	1.5	0.44
Indeno(1,2,3-cd)pyrene	0.94	3.2	2.6	0.42	0.24
Dibenzo(a,h)anthracene	0.27	0.86	0.15	0.06	0.05
Benzo(g,hi)perylene	0.74	2.8	2.5	0.40	0.24
Total PAHs (μg kg^{-1})	1.9 × 10^2	4 × 10^2	8.6 × 10^2	6.5 × 10^2	5.9 × 10^2
Total PCDD/Fs (pg g^{-1})	2.7 × 10^2	6.1 × 10^2	3.6 × 10^2	67	50
TEQ_{WHO} (pg g^{-1})	7.0	9.6	4.9	2.1	1.7

Biochar produced at all temperatures of pyrolysis, except for 700 °C exhibited significant AhR-mediated potency. This is the first time that a transactivation, reporter gene assay such as the H4IIE-*luc* test was used to measure the total dioxin-like potencies of biochars produced at various temperatures.

3.3. Potency balance analysis

Concentration of TCDD-EQ and TEQ_{chem} varied among extracts of biochar produced at various pyrolysis temperatures (Table 2). TCDD-EQ_{20} values generated from responses to 20%-$TCDD_{max}$ were greater than concentrations of TEQ_{chem}, which were the sum of TCDD equivalents calculated as the sum of the products of concentrations of PAHs and PCDD/DF multiplied by their respective relative potency (ReP) values. The $TEQ_{chem}/TEQ_{H4IIE\text{-}luc}$ ratio was in the range of 0.7%–3.8% and was directly proportional to temperature of pyrolysis from 300 to 700 °C. These observations are consistent with the fact that using nonspecific RePs in calculations of the mass-balance could lead to significant error and inaccurate conclusions (Villeneuve, Khim, Kannan, Giesy). It is more likely that there are additional chemicals that were not measured by the instrumental analysis (Maier et al., 2014). Concentrations of $TEQ_{H4IIE\text{-}luc}$ in sediments and soils from the Shiawassee River were 100 to 200- fold greater than concentrations of TEQ_{chem} (Kannan et al., 2008). TEQ_{chem} contributed by PAHs accounted for between 0 and 34% of $TEQ_{H4IIE\text{-}luc}$, which indicated the presence of additional unidentified AhR ligands in sediments (Hong et al., 2012). The ratio of the concentration of TEQ_{chem} to that of $TEQ_{H4IIE\text{-}luc}$ ($TEQ_{chem}/TEQ_{H4IIE\text{-}luc}$) ranged from $<1.88 \times 10^{-16}$ to 7.77×10^{-5} when using only individual analogues of PBDEs to calculate TEQ_{chem} (Su et al., 2012). However, results of some studies have suggested that the difference between TEQ_{chem} and $TEQ_{H4IIE\text{-}luc}$ values is not large if PCDD/DF were the primary source of contaminants in the sediment extracts (Koh et al., 2004; Hilscherova et al., 2003). The reason for differences between TEQ_{chem} and $TEQ_{H4IIE\text{-}luc}$ observed in this study could be due to two factors. First, there might be other pollutants that contributed to the activity of $TEQ_{H4IIE\text{-}luc}$ assay. For example, 2,3-diB-7,8-DiCDD and 3-B-2,7,8-TriCDF were found to have RePs values of 1.15 and 1.09, respectively, in the H4IIE-*luc* transactivation assay (Behnisch et al., 2003). The other factor is the fact that RePs can vary among different studies of the same chemical compounds. For example, the calculated value of TEQ for PAHs (TEQ_{PAHs}) in extracts of biochar formed at a pyrolysis temperature of 300 °C were as great as 59 pg TCDD g^{-1} when RePs given by Behnisch et al (Behnisch et al., 2003) were used instead of those given by Larsson et al. and Villeneuve et al. (Larsson et al., 2012; Villeneuve, Khim, Kannan, Giesy). In addition, the different extract solvents used for the analysis of PAHs and PCDD/DF and for the assay of the toxicity may also be the reason causing the difference values between TEQ_{chem} and $TEQ_{H4IIE\text{-}luc}$.

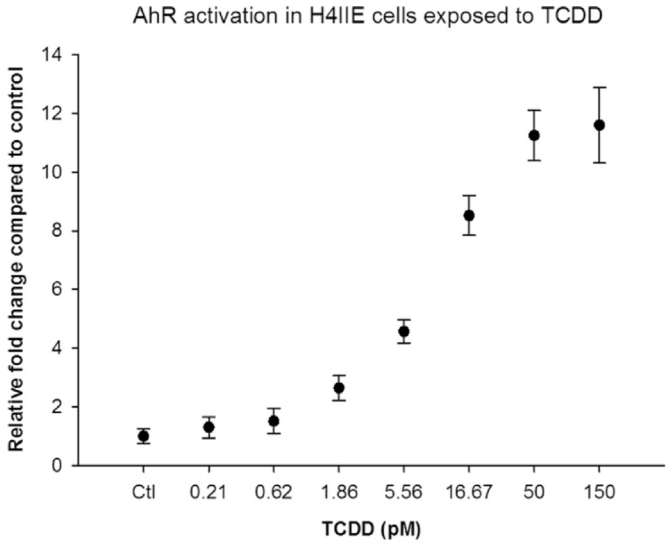

Fig. 2. 2,3,7,8-TCDD standard curve for response of luciferase reporter gene under the control of the AhR and dioxin response enhancer (DRE) in H4IIE-*luc* rat hepatoma cells.

3.4. Microtox analysis

EC50 values were used based on inhibition of luminescence in the Microtox test. Biochar produced at 300 °C (0.39 g L^{-1}) and 250 °C (0.65 g L^{-1}) (Fig. 4) had low EC50 values in the 5 min assay, suggesting greater toxicity to bacteria of extracts of biochar formed at lower temperatures of pyrolysis. For biochar produced at 700 °C, EC50 values were 6 and 3.3 g L^{-1} in the 5-min and 15-min Microtox tests, respectively, which are 15.5- and 16-fold greater (less potent) than that of biochar produced at 300 °C. This result is consistent with results of cytotoxicity to H4IIE-*luc* cells, where exposed to 3 g equivalents L^{-1} of biochar produced at 300 °C and 250 °C resulted in mortalities of 83.2± 5.5% and 16.2± 5.2%, respectively (Data not shown). However, results on toxicity to cells are not completely in accordance with results of instrumental analyses, which showed greater concentrations of PAHs in extracts of biochar formed at 400 °C. Greater contents of 5&6-ring PAHs were produced mostly at temperature of 300 °C and 250 °C (Fig. 1), which were more toxic and might contribute more to the toxicity of extracts of that biochar. It is also possible that some unidentified substances produced at lower temperatures contributed to greater toxicity to the bacteria and rat cells (Gao et al., 2015). For example, propyl phenols might be produced during breakdown of lignin. It has been reported that phenols can have greater toxic potency in the *Microtox* test than Fluoranthene (Hauser et al., 1997).

Results of the Microtox test were consistent with those based on viability of H4IIE-*luc* cells. A positive correlation between EC50 of

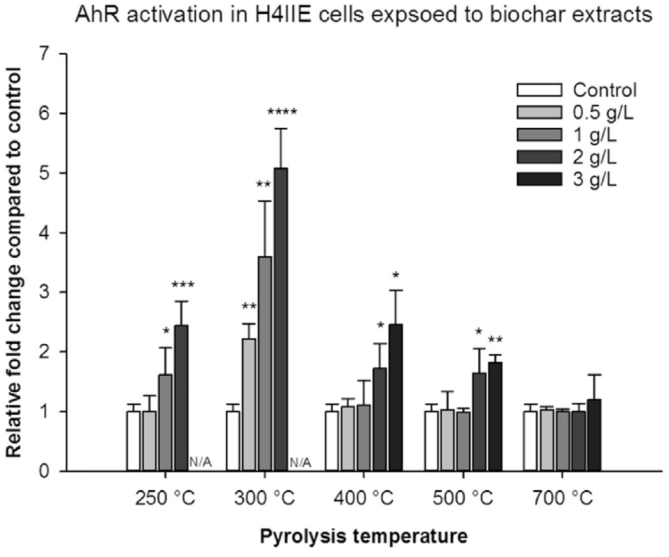

Fig. 3. Fold change in expression of luciferase reporter gene under control of the AhR in H4IIE-*luc* cells exposed to various concentrations of extracts of biochar produced at various temperatures of pyrolysis. N/A represents "not available" because dosages of 3 g L^{-1} at 250 °C and 300 °C were not performed due to high cytotoxicity. Asteroids represent significant difference compared to control within the same temperature group (*<0.05; **<0.01; ***<0.001; ****<0.0001).

Table 2
Comparison of concentrations (gram equivalents) of TCDD-EQ and TEQ_{chem}[a] in extracts of biochar produced at various temperatures of pyrolysis.

	Temperature of pyrolysis (°C)				
	250	300	400	500	700
TEQ_{PAHs} (pg g^{-1})[b]	0.14	0.44	0.42	0.13	0.07
$TEQ_{PCDD/DF}$(pg g^{-1})[c]	7.3	12	6.8	2.2	1.5
TCDD-EQ_{20}(pg g^{-1})[d]	5.9×10^2	1.7×10^2	3.1×10^2	7.5×10^2	4.2×10^2
$TEQ_{chem}/TEQ_{H4IIE\text{-}luc}$ (%)	1.3	0.70	2.3	3.2	3.8

[a] TEQchem = TEQ_{PAHs} + $TEQ_{PCDD/DF}$.
[b] Calculated based on Larsson et al., 2012.
[c] Calculated based on Lee et al., 2013 and Brown et al., 2002
[d] TCDD-EQ_{20} refer to the TCDD-EQs generated from responses to 20%- TCDDmax.

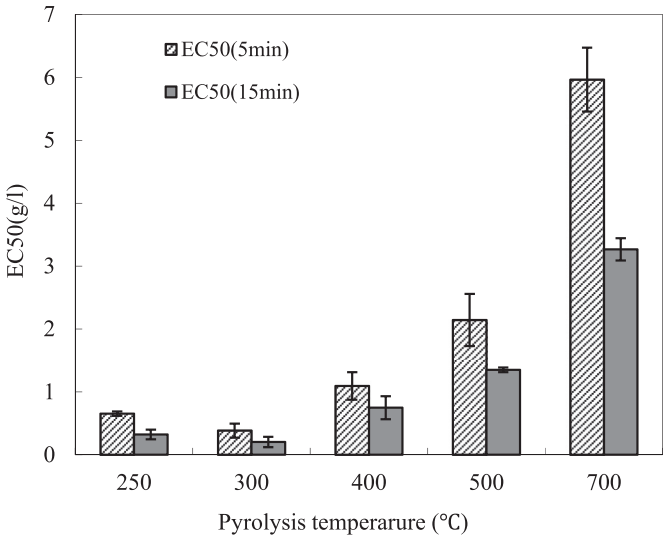

Fig. 4. EC50 values for inhibition of luminescence in the Microtox test, used as a general measure of toxicity.

Microtox and EC20 of H4IIE-*luc* analysis was observed (Fig. 5), which suggests that Microtox was more sensitive than H4IIE-*luc* for determining toxic potency of chemicals in extracts of biochar. This indicates that a simple Microtox test can be used to characterize toxicity and even predict AhR agonistic potencies of pollutants rapidly and cost-effectively as compared to H4IIE-*luc* assay and HRGC-HRMS.

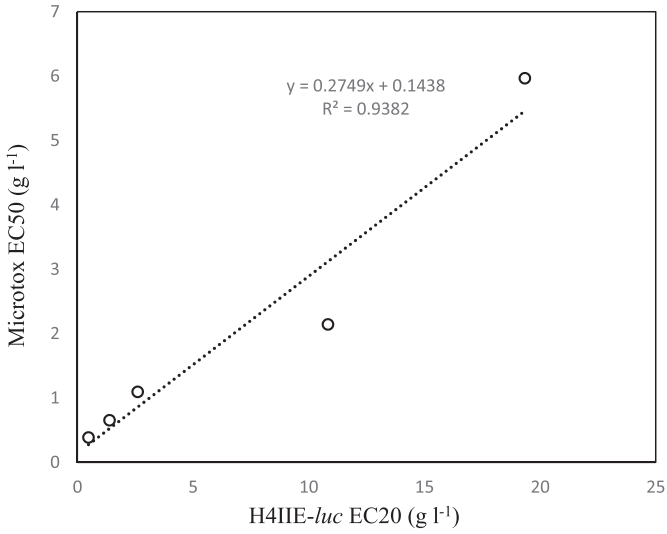

Fig. 5. Relationship between EC20 for induction of reporter gene luciferase under control of the AhR in H4IIE-*luc* cells and EC50 for toxicity as determined in the Microtox test.

4. Conclusions

In the present study, for the first time, toxicities of biochars produced at various temperatures of pyrolysis were characterized by use of H4IIE-*luc* and Microtox test. PCDD/DF and PAHs were mostly produced at temperatures of 300–400 °C. However, both H4IIE-*luc* and Microtox tests showed that the greatest potency was in extracts of biochar produced at 300 °C, followed by that produced at 250 °C. Thus, in general, biochar produced at lower temperatures of pyrolysis were more toxic and had greater potencies of AhR-mediated effects. Concentrations of $TEQ_{H4IIE\text{-}luc}$ determined by use of the H4IIE-*luc* test were greater than that of TEQ_{chem} calculated from instrumental analytical data and RePs values. This result indicates that there are other AhR-ligands produced during pyrolysis of organic matter in biochar that need to be identified in addition to PAHs and PCDD/DF. Positive correlation between EC50 of Microtox analysis and EC20 of H4IIE-*luc* test was observed, which suggested similar mechanism of effect for the toxicity to microorganisms and induction of AhR-mediated responses.

Notes

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