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# Plant and algal toxicity of persistent free radicals and reactive oxygen species generated by heating anthracene-contaminated soils from 100 to 600 °C

Jinbo Liu<sup>1</sup> · Ning Gao<sup>1</sup> · Xin Wen<sup>1</sup> · Hanzhong Jia<sup>1</sup>  · Eric Lichtfouse<sup>2</sup> 

## Abstract

Persistent free radicals (PFRs) are emerging contaminants of increasing concern, yet their formation, fate, toxicity and health risk are poorly known. Thermal treatment, a common remediation technique to clean industrial soils, induces the formation of PFRs, which could paradoxically increase soil toxicity, contrary to the original objective of remediation. Actually, there is little knowledge on the formation and toxicity of PFRs in soils contaminated by polycyclic aromatic hydrocarbons (PAHs). Here we studied the generation of PFRs of soils spiked with anthracene and heated 1 h from 100 to 600 °C, using electron paramagnetic resonance. We also investigated the formation of reactive oxygen species (ROS), e.g. superoxide radical ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $\cdot OH$ ), in the aqueous phase of thermal-treated soil, and the impact of heating on soil oxidative potential, wheat growth and green algae activity. Results showed that PFRs, ROS, soil oxidative potential, plant toxicity and algal toxicity show a similar trend with an increase from 100 to 300 °C, followed by a decrease to 600 °C. Scavenger trapping tests reveal that algal toxicity is mainly due to the generation of  $O_2^{\cdot-}$ ,  $\cdot OH$  and  $H_2O_2$  induced by anthracene-PFRs and that anthracene and anthracene-PFRs have negligible direct algal toxicity. Overall, our findings reveal the unintended formation of toxic compounds peaking at 300 °C during the thermal remediation of PAH-contaminated soils. These results should help to assess the environmental risk of thermally treated PAH-contaminated soils.

**Keywords** Thermal treatment · Polycyclic aromatic hydrocarbons · Persistent free radicals · Reactive oxygen species · Oxidative potential · Wheat growth · Green algae

## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of priority organic pollutants displaying teratogenicity, carcinogenicity and mutagenicity (Haritash and Kaushik 2009). About 90% of environmental PAHs end up into soils and

sediments, owing partly to their excellent hydrophobic property (Eriksson et al. 2000). PAHs may then be transferred to plants, animals, milk and blood (Grova et al. 2000; Laurent et al. 2001, 2002; Crépineau et al. 2003). The concentration of PAHs in contaminated soils ranges from 1  $\mu g\ kg^{-1}$  to tens of thousands  $mg\ kg^{-1}$  (Bryselbout et al. 2000; Eriksson et al. 2000; Kanaly and Harayama 2000). The chemical stability of PAHs and soil conditions such as physical sequestration and anoxia inhibit the degradation of PAHs (Lichtfouse et al. 1997; Zhang et al. 2015). For instance, ancient gasworks soils contaminated by PAHs still display plant toxicity by 1–2 aromatic hydrocarbons several decades after gaswork closure (Henner et al. 1999). Therefore, rapid and effective methods are needed to remediate PAHs contaminated soil (Henner et al. 1997).

Remediation techniques of PAH-contaminated soils include chemical oxidation (Jia et al. 2011, 2018), soil washing (Pourfadakari et al. 2019), bioremediation (Haritash and Kaushik 2009) and thermal treatment (Peng et al.

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2018). Thermal treatment is widely applied due to its high removal efficiency, low cost and short time (Merino and Bucala 2007; Falciglia et al. 2011). The removal efficiency of the 16 priority PAHs increases with temperature, attaining 98.0% at 500 °C (Chen et al. 2020). Peng et al. (2018) found that phenanthrene was almost totally converted to H<sub>2</sub>, CO<sub>2</sub>, and CH<sub>4</sub> within the first 15 s at 800 °C. During thermal treatment, various mechanisms may explain the decrease in extractable PAHs, such as desorption (Peng et al. 2018), volatilization (Falciglia et al. 2011), degradation (Zhang et al. 2019) and formation of bound residues (Lichtfouse et al. 1998; Kronimus et al. 2006), the respective contribution of which being poorly known. Transformation, e.g. oxidation, has also been evidenced recently during the removal of benzo[a]pyrene-spiked soils at 100–200 °C, despite desorption being considered as the main removal mechanism (Jia et al. 2020). We also found that the transformation of benzo[a]pyrene in heated soils induces the formation of persistent free radicals (PFRs) (Liu et al. 2020). Since thermal treatment is commonly applied to clean PAH-contaminated soils (Kuppusamy et al. 2016), this remediation technique may pose potential health risks due to the formation of PFRs. Yet knowledge on the formation and toxicity of PFRs in soils is limited.

Soil particles containing PFRs generate reactive oxygen species (ROS) such as singlet oxygen <sup>1</sup>O<sub>2</sub>, superoxide radical O<sub>2</sub><sup>•-</sup>, hydrogen peroxide H<sub>2</sub>O<sub>2</sub> and hydroxyl radical ·OH, which are known to induce toxicity to living organisms by oxidative stress (Dellinger et al. 2001; Khachatryan et al. 2011, 2014; Jia et al. 2016; Zhu et al. 2019). Oxidative stress is usually caused by an imbalance of ROS in exposed cells or tissues (Paul et al. 2012). Oxidative stress can be measured by the oxidative potential, an indicator of ROS-generating potential of particles that correlates well with adverse effects in acellular assays (Paul et al. 2012; Chen et al. 2018). Since toxicity mechanisms of soil PFRs are poorly known, we hypothesized that measuring the oxidative potential of thermally treated PAH-contaminated soils would give some insight on the possible role of ROS. Therefore, here we heated an anthracene-spiked soil at 100–600 °C during 1 h to study the formation of PFRs and ROS, and to measure the toxicity for algae and wheat, because algal tests are highly sensitive and wheat grows in a wide range of soil conditions (Banks and Schultz 2005; Inckot et al. 2011; Wang et al. 2016). We used compound-specific radical scavengers to distinguish the toxicity contribution of PFRs and ROS. The obtained results would shed light on the potential environmental risk of thermally treated PAH-contaminated soil and underneath mechanisms.

## Materials and methods

### Chemicals

High purity of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, >97%), dithiothreitol (DTT, 99%), 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 98%), tert-butanol (TBA), anthracene (ANT, 98%), methanol (chromatographic grade), dimethyl sulfoxide (DMSO, 99%) and ascorbic acid (AA, >99%) were obtained from J&K Scientific Ltd. (Beijing, China). Superoxide dismutase (SOD, >2500 U/mg) and catalase (CAT, >2000 U/mg) were purchased from Beijing Solarbio Science & Technology Co., Ltd. Potassium phosphate monobasic-sodium hydroxide (KH<sub>2</sub>PO<sub>4</sub>) and disodium hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>) were guaranteed reagents, purchased from Kemiou Chemical Reagent Co., Tianjin. Dichloromethane and acetone were of analytical grade from Sinopharm Chemical Reagent Co., Shanghai.

### Preparation of soils contaminated by anthracene

Coastal solonchaks were sampled from surface-layer soil (0–20 cm) in Tianjin province of China (38° 45' N, 117° 06' E). Soil samples were air-dried for 24 h at about 23 °C and then sieved through 100-mesh before analysis. Soil physicochemical properties including organic carbon (OC), clay and metal contents, pH and cation exchange capacity (CEC) were analyzed. Detailed analysis methods are provided in supplementary material Text S1. Soil pH (8.29), OC (12.77 g kg<sup>-1</sup>), CEC (24.67), clay (6.37%), silt (78.14%), sand (15.49%), Fe (21,084 mg kg<sup>-1</sup>), Mn (397 mg kg<sup>-1</sup>), Cu (24 mg kg<sup>-1</sup>) and Zn (17 mg kg<sup>-1</sup>). Anthracene was below detection limits in original soils. The preparation of anthracene-contaminated soil was previously described (Liu et al. 2020). Briefly, 1 g of soil sample was mixed with 1 mL of acetone solution containing 1000 mg L<sup>-1</sup> of anthracene. Anthracene-containing soil samples were placed in the dark at about 23 °C until the acetone was completely evaporated. The anthracene-contaminated soils were then stored at -4 °C for subsequent experiments. The final concentration of anthracene in soil samples ranged from 983 to 1012 mg kg<sup>-1</sup>.

### Thermal treatment of anthracene-contaminated soils

Thermal treatment was performed in a quartz boat heated by a pipe furnace under a continuous flow of N<sub>2</sub> with a flow rate of 1 L/min. The thermocouple of the pipe furnace recorded the temperature and guaranteed that the required heating temperature was reached. Before the heating

started, the air inside the pipe furnace was exhausted by flushing  $N_2$  for 10 min. Then, 5 g of anthracene-contaminated soil was placed in a quartz boat, transferred into the pipe furnace, and then held there at a fixed temperature for 1 h. After cooling down to about 23 °C, the soil was stored in sealed polyethylene bags prior to analysis. A blank experiment with the original soil (without anthracene) was heated under the same conditions; this experiment did not show EPR signals (Figure S1). In addition, unheated anthracene-spiked soils were used as control (CT). Residual concentration of anthracene in soils after thermal treatments was quantified by high-performance liquid chromatography (HPLC–UV, Thermo Fisher Scientific U3000, USA). The specific extraction and determination methods are presented in Text S2.

The variations of soil properties, including soil pH, water-holding capacity, organic carbon and dissolved organic carbon, before and after thermal treatment are presented in Text S3 and Table S1.

### Persistent free radicals and reactive oxygen species

Thermally treated soils and control were extracted under various conditions to analyze persistent free radicals (PFRs) and reactive oxygen species (ROS) as described by Zhao et al. (2020) and Liu et al. (2020). PFRs and ROS were analyzed by an electron paramagnetic resonance (EPR) spectrometer (Bruker EMXmicro-6/1/P/L, Karlsruhe, Germany) at room temperature, about 23 °C. Specifically, 50 mg of thermally treated soil was placed into a 1.5 mL centrifuge tube, and mixed with 300  $\mu$ L of acetone and dichloromethane 1/1 v/v to extract the PFRs effectively (Zhao et al. 2020). The mixture was vortexed by Vortex Genie for 1 min and filtrated through a 0.45  $\mu$ m nylon membrane. The filtrate was sucked with a capillary tube then loaded in the EPR tube for PFRs analysis.

For ROS, analysis of hydroxyl radical  $\cdot$ OH involved extraction of 50 mg of soil by 300  $\mu$ L of 0.1 M 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) aqueous solution. Superoxide radical  $O_2^{\cdot-}$  involved extraction of 50 mg of soil by 300  $\mu$ L of 0.1 M DMPO/DMSO. Other steps were carried out under the same conditions as mentioned above. EPR parameters are provided in Text S4.

Determination of hydrogen peroxide  $H_2O_2$  concentration involved preparing a 10 g/L suspension of soils, filtering 5 mL of suspension and analysis using the hydrogen peroxide assay kit from Solarbio Life Sciences Co., Beijing. Control experiments were paralleled by measuring the signals in the 250  $\mu$ L of 1.0 mM hydrogen peroxide standard and 250  $\mu$ L of ultrapure water. The concentration of  $H_2O_2$  produced by thermal treated soil samples

was calculated by comparing the absorbance with control experiments.

### Soil oxidative potential

The oxidative potential of the soil samples was determined by an acellular in vitro assay, the dithiothreitol assay (Tong et al. 2018). The oxidative potential was calculated as mass-normalized dithiothreitol and ascorbate decay rates. Specifically, 5.0 mg of soil was dispersed in 9.8 mL of 0.05 M potassium phosphate buffer made with 0.01 M  $KH_2PO_4$  and 0.04 M  $Na_2HPO_4$  (pH 7.4). After 5 min of incubation at 37 °C, 200  $\mu$ L of 1.0 mM dithiothreitol solution was added, and the mixture was incubated 10 min in a thermostatic bath set at 37 °C with constant shaking. Then, 400  $\mu$ L of the mixture was mixed with 20  $\mu$ L of 1.0 mM DTNB solution and filtered. The absorbance of the filtrate was measured to determine the residual dithiothreitol concentration at 412 nm using an Evolution 201 UV–Visible spectrophotometer (Thermo Scientific, USA). Following the same procedures, blank and control experiments (without soils) were run in parallel. The depletion rate of dithiothreitol was calculated with a zero-order kinetic equation, and the control value subtracted.

### Soil toxicity to plant germination and growth

To investigate the possible reuse of the thermally treated PAHs soil, wheat vegetation germination and growth experiments were conducted. Wheat seed germination was determined by placing 30 seeds in layers of moistened soil within a glass petri dish. The germination dishes were placed in a growth chamber at 25 °C under dark conditions until the seed germination percentage was constant. Then, the wheat germination rates in different soils were determined.

For wheat growth tests, six seedlings of uniform size were transferred to a brown polypropylene bottle, which was filled with 250 mL of sterilized ultrapure waters containing 12.5 g soil. The polypropylene bottles were placed in the growth chamber, and the climate chamber was adjusted to 16 h of daylight, day/night temperature of 25/20 °C. On day 21, the wheat plants were harvested, and the root length, shoot length, wet weight and dry weight of each plant were measured.

### Toxicity to algae and contribution of persistent free radicals versus reactive oxygen species

Green algae *Chlorella* sp. was obtained from the Institute of Oceanology, Chinese Academy of Sciences, Qingdao. Preparation of the algae cultivation medium is detailed in Text

S5. In an Erlenmeyer, 20 mL of algae cultivation medium is mixed with 1 g of soil. After 1 h incubation at 37 °C, the growth rate was monitored by optical density (OD), as follows 200 µL reaction mixture containing green algae was transferred into a costar 3590 ELISA plate and then analyzed with the microplate reader SpectraMax M5 at 680 nm.

To distinguish the effects of PFRs and ROS on the inhibition of algae, we added chemical scavengers in the medium before incubation: either 10 mM ascorbic acid (AA) to trap  $O_2^{\cdot-}$ ,  $H_2O_2$  and  $\cdot OH$ , or 0.5 mg/L superoxide dismutase (SOD) to scavenge  $O_2^{\cdot-}$ , or 10 mM catalase (CAT) to scavenge  $H_2O_2$ , or 0.5 mM tert-butanol (TBA) to scavenge  $\cdot OH$  (Zhu et al. 2020) (Figure S2).

### Statistical analysis

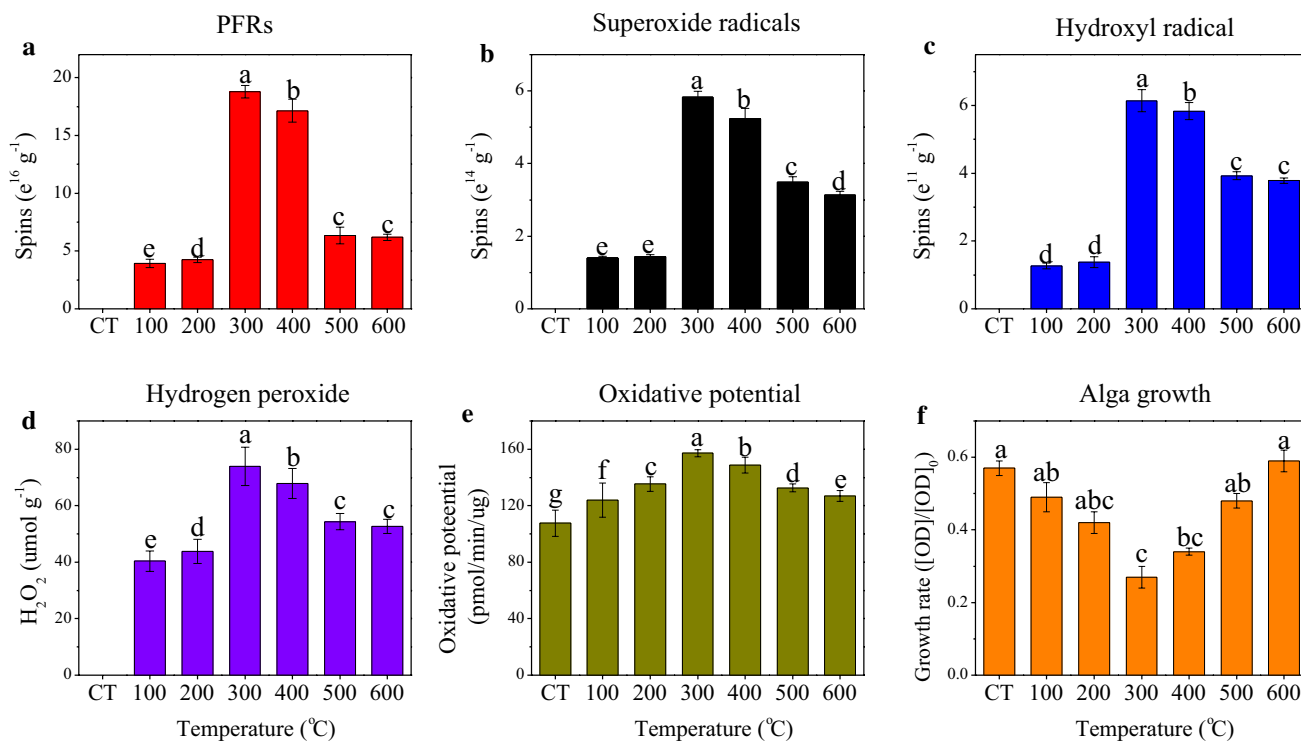
All experiments were repeated for three times, and the results were analyzed with ANOVA. The correlation analyses were conducted by SPSS 19.0 (SPSS Software, USA), with significance levels of  $P < 0.05$  and  $P < 0.01$ , respectively.

## Results and discussion

### Effect of soil heating on persistent free radicals

We analyzed persistent free radicals (PFRs) in soils spiked with anthracene and heated 1 h from at 100–600 °C, using electron paramagnetic resonance (EPR). Results show strong PFRs signals in heated soils, maximizing at 300 °C, and almost no signal for the unheated anthracene-spiked soils (Figure S3) and heated raw soils without anthracene (Figure S1). This finding confirms that PFRs signals are induced by both heating and the presence of PAHs, as shown for benzo[a]pyrene-spiked soils heated at 100–200 °C (Liu et al. 2020). Rising the temperature was also necessary to form PFRs during adsorption of dichlorobenzene on cupric oxide-containing particles at 100–400 °C (Lomnicki et al. 2008).

Our data show that the PFRs intensity increases from 100 to 300 °C, reaching  $1.88 \times 10^{17}$  spins  $g^{-1}$  at 300 °C, and then decreases to 600 °C (Fig. 1a). The increase in PFRs from 100 to 300 °C is explained partly by the decreasing concentration of anthracene (Figure S4), which suggests the transformation of anthracene into anthracene-PFRs (Liu et al. 2020). A decline of PFRs above 300 °C may be due to radical decomposition, recombination and transformation



**Fig. 1** Effect of soil heating on **a** persistent free radicals (PFRs) and reactive oxygen species (ROS), **b** superoxide radical  $O_2^{\cdot-}$ , **c** hydroxyl radical  $\cdot OH$ , **d** hydrogen peroxide  $H_2O_2$ , **e** oxidative potential, and **f** growth of the green algae *Chlorella*. Error bars and letters denote

standard deviations and significant differences at  $P$  below 0.05. CT: control unheated soil with anthracene.  $[OD]_0$  and  $[OD]$  are the initial optical density of algae and the optical density after 1 h growth, respectively



into stable compounds, similarly to what is observed in the biomass charring process (Gao et al. 2018; Odinga et al. 2019). Noteworthy, similar upward-downward trends with temperature have been observed for phenolic-contaminated soils (Zhang et al. 2019) and for coal (Chang et al. 2017). Furthermore, our results show that the formed PFRs are rather stable with less than 3% concentration decline after 44 d (Figure S5), which confirms the persistency of PAH-PFRs (Liu et al. 2020) and suggests possible long-term toxicity.

Overall, our results show that thermal treatment induces the formation of PFRs; PFRs concentration is peaking at 300 °C; and PFRs display persistence over weeks, which implies potential toxicity. The next section focuses on reactive oxygen species.

### Effect of soil heating on reactive oxygen species

The concentration of reactive oxygen species (ROS) is also showing a successive upward-downward trends peaking at 300 °C (Fig. 1b–d). For instance, the concentrations of  $O_2^{\cdot-}$  and  $\cdot OH$  increased with temperature, reaching  $5.83 \times 10^{14}$  spins  $g^{-1}$  for  $O_2^{\cdot-}$  and  $6.13 \times 10^{11}$  spins  $g^{-1}$  for  $\cdot OH$  at 300 °C, then decreased. A same trend was observed for  $H_2O_2$  extracted from thermally-treated soil suspensions. Here we hypothesize that ROS formation is linked to the formation of anthracene-PFRs. This hypothesis is strengthened by the occurrence of typical 6-line spectra of DMPO – OOH adducts and 4-line spectra of DMPO – OH adducts in heated soils, and their absence in unheated anthracene-spiked soils (Figure S6), which proves that the generation of  $O_2^{\cdot-}$  and  $\cdot OH$  is induced on the formation of PFRs. We therefore suggest the following mechanism: 1. Electrons are transferred from anthracene-PFRs to  $O_2$ , forming the superoxide radical  $O_2^{\cdot-}$ ; 2.  $O_2^{\cdot-}$  dismutates with  $H^+$  to form  $H_2O_2$ ; 3.  $H_2O_2$  is reduced by soil transition metals and transformed to  $\cdot OH$  (Khachatryan et al. 2011; Liao et al. 2014), which is supported by the high levels of metals in our soil: 21,084 mg  $kg^{-1}$  Fe and 24 mg  $kg^{-1}$  Cu.

Overall, our results show that ROS concentrations show the same trends as PFRs in heated anthracene-spiked soils, and that ROS formation is most likely linked to PFRs formation. The next section focuses on soil oxidative potential.

### Effect of soil heating on soil oxidative potential

The oxidative potential of heated anthracene-spiked soils was measured by the dithiothreitol assay to determine the effect of heating on the oxidative stress (Fig. 1e). Results show that the oxidative potential increases from 100 to 300 °C and then decreases to 600 °C. This upward-downward trend is similar to trends observed for PFRs and ROS (Fig. 1a–d), thus indicating that the oxidative potential is closely linked to the generation of PFRs and ROS. This is in

agreement with previous studies that show that the oxidative potential is linked to ROS, free radicals and adverse effects in acellular assays for urban aerosols (Paul et al. 2012; Chen et al. 2018). The oxidative potential is indicative of the consumption of antioxidants, e.g. dithiothreitol, by particle-associated active components, which mainly include organic compounds, PFRs and ROS (Strak et al. 2012). Oxidative stress occurs when the production of PFRs or ROS exceeds the available antioxidant capacity (Paul et al. 2012).

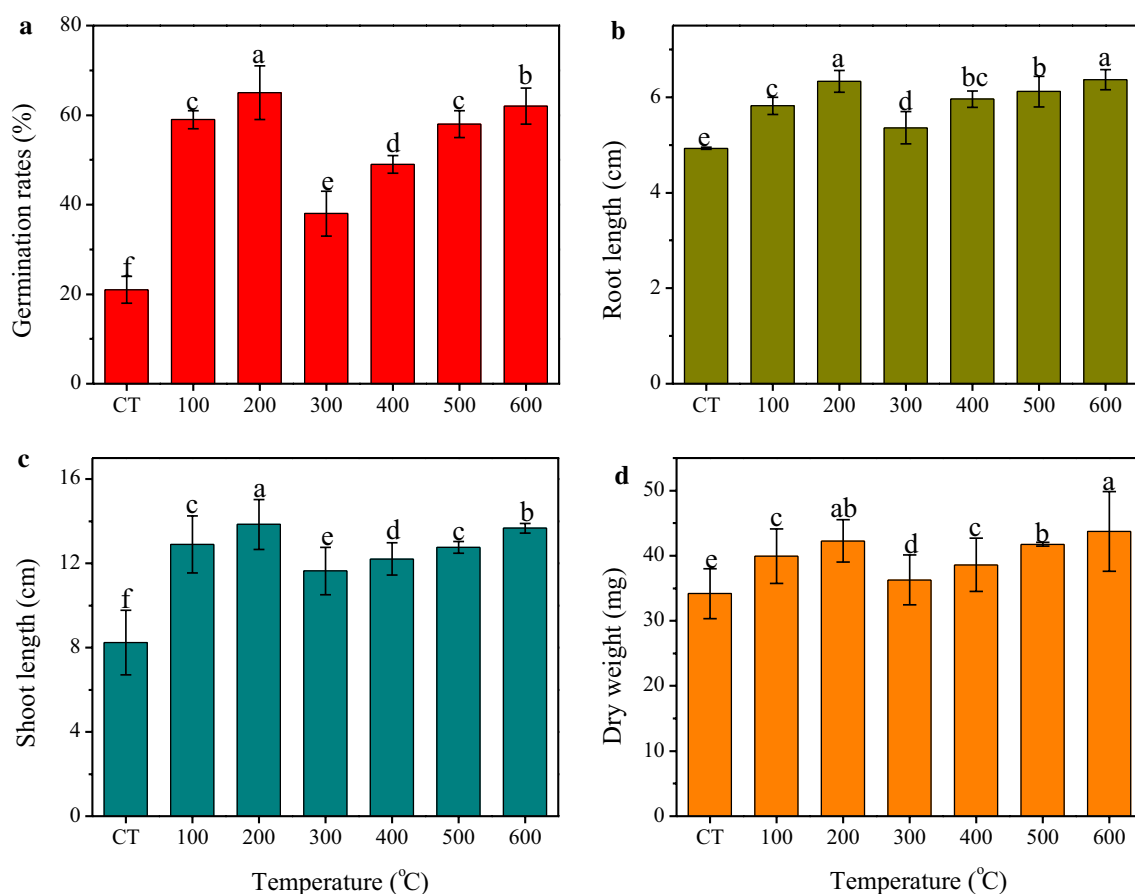
Moreover, the oxidative potential of heated anthracene-spiked soils, ranging from 123.8 to 157.2  $pmol\ min^{-1}\ \mu g^{-1}$ , are much higher than atmospheric particulate matter from biomass burning, of 4–37  $pmol\ min^{-1}\ \mu g^{-1}$ , and coal burning, of  $40 \pm 3\ pmol\ min^{-1}\ \mu g^{-1}$  (Jin et al. 2016; Fan et al. 2018). This suggests that heated anthracene-contaminated soils have much higher toxicity, e.g. upon inhalation of soil particulates. Results also show that the oxidative potential of the unheated control soils is  $107.6 \pm 9.4\ pmol\ min^{-1}\ \mu g^{-1}$ , which is in the same order of magnitude as an atmospheric organic aerosol (Tong et al. 2018; Chen et al. 2019).

Overall, our results showed that the oxidative potential of heated anthracene-spiked soils displays an upward-downward trend peaking at 300 °C, which is similar to the trends of PFRs and ROS. In addition, the oxidative potential values of heated anthracene-spiked soils are much higher than coal and charred biomass, which suggests a notable toxicity. The next section focuses on the toxicity for plant and algae.

### Effect of soil heating on soil toxicity for plant and algae

We studied the toxicity of heated anthracene-spiked soils for wheat germination and growth (Fig. 2) and for the growth of green algae (Fig. 1f). The low wheat germination rate of the unheated anthracene-spiked soil is probably due to the presence of phytotoxic compounds that may induce hypoxia, inner dehydration, impaired biochemical function, and embryo death (Tang et al. 2011). We speculate that these phytotoxic compounds are degraded upon heating at 100 °C and above. The presence of anthracene, a 3-ring PAH, is unlikely to cause toxicity at room temperature, as suggested by the absence of toxic effects of 3–5 ring PAHs on plant germination (Henner et al. 1999).

Upon heating of anthracene-spiked soils, all wheat factors such as germination, root and shoot length, and dry weight, show an overall cratering trend dropping first from 100 to 300 °C, then rising to 600 °C (Fig. 2). We observe a similar cratering trend for algae (Fig. 1f). These findings mean that soil toxicity for plants and algae increase with temperature to reach a maximum at 300 °C. Moreover, the growth of algae and the growth indicators of wheat are significant negatively correlated to PFRs and ROS concentration, and to the levels of oxidative potential, with correlation coefficients ranging



**Fig. 2** Effect of soil heating on wheat **a** germination rate, **b** root length, **c** shoot length and **d** dry weight. Error bars and letters denote standard deviations and significant differences at *P* below 0.05. CT: control unheated soil with anthracene

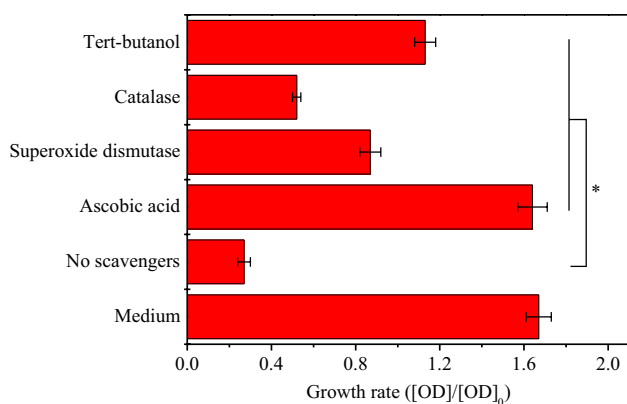
from  $-0.57$  to  $-0.93$  and  $-0.49$  to  $-0.93$  for algae and wheat, respectively (Table S3). In other words, the highest levels of PFRs, ROS and oxidative potential induce the highest toxicity for plants and algae. Plant toxicity was previously observed in PFRs-containing biochars, which inhibited wheat germination and growth (Odinga et al. 2019; Ruan et al. 2019). In the same vein, biochar PFRs damage plasma membranes when spins intensity reach about  $10^{16}$  spins  $g^{-1}$  (Liao et al. 2014). By comparison, our results display higher spin intensities, of  $3.93$ – $18.8 \times 10^{16}$  spins  $g^{-1}$ , which may partly explain wheat inhibition by membrane rupture. Moreover, PFRs-containing soils have been shown to exhibit acute toxicity to luminescent bacteria (Bastos et al. 2014; Zhang et al. 2019) and particles having oxidative potential usually induce a higher cellular response (Ahmed et al. 2019; Chowdhury et al. 2019).

Overall, our findings demonstrate that plant and algal toxicity is peaking at  $300$  °C in anthracene-spiked soils, and that this trend is significantly correlated to the concentration of PFRs and ROS, and to the levels of oxidative potential.

## Toxicity mechanism

Our results above have revealed that plant and algal toxicity are correlated to persistent free radicals (PFRs) and reactive oxygen species (ROS). In order to decipher the respective contributions of PFRs and ROS we grew algae in the presence of the anthracene-spiked soil heated at  $300$  °C since the concentration of PFRs and ROS was the highest and the inhibition of the growth of algae was the most significant. We added the following scavengers: either  $10$  mM ascorbic acid to trap  $O_2^{\cdot-}$ ,  $H_2O_2$  and  $\cdot OH$ ;  $0.5$  mg/L superoxide dismutase to scavenge  $O_2^{\cdot-}$ ; or  $10$  mM catalase to scavenge  $H_2O_2$ , or  $0.5$  mM tert-butanol to scavenge  $\cdot OH$ . Results show that addition of soil without scavenger induces a sharp decrease in algal growth from  $1.67$  to  $0.27$  (Fig. 3). All scavengers induce an increase in algal growth, which demonstrate the involvement of ROS to various extent in the mechanisms of toxicity.

Ascorbic acid re-establishes the maximum algal growth of  $1.64$  because ascorbic acid scavenges all ROS. Superoxide dismutase induces an algal growth of  $0.87$ , which means that  $\cdot OH$  and  $H_2O_2$  are responsible of  $47.9\%$  of the toxicity



**Fig. 3** Effect of scavengers on the growth rate of green algae *Chlorella* in 300 °C thermal-treated soil. Medium denotes without adding thermal-treated anthracene-contaminated soil. The error bars and star symbol (\*) in the figure represent the standard deviations and the statistically significant difference between linked data points ( $P < 0.05$ ), respectively.  $[OD]_0$  and  $[OD]$  are the initial optical density of algae and the optical density after 1 h growth, respectively

versus algal growth without soil, of 1.67. The growth rate of algae decreases to 0.52 in the presence of catalase, representing 68.9% contribution of  $\cdot\text{OH}$  and  $\text{O}_2^{\cdot-}$  to the toxicity. Tert-butanol induces an algal growth of 1.13, indicating 32.3% contribution of  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  to the toxicity. Contrastively, ascorbic acid induces an algal growth of 1.64, which means that anthracene-PFRs are only responsible of 1.8% of the observed toxicity, or less. This is confirmed by a complementary experiment showing that unheated soil with anthracene do not modify algal toxicity versus unheated soil alone. In other words, both anthracene and heating are necessary to induce algal toxicity. Therefore, anthracene transformation products must be indirectly involved in algal toxicity by producing ROS, on two grounds: first, there is no PFRs signal when soils are heated without anthracene, which means that  $\cdot\text{OH}$  and  $\text{O}_2^{\cdot-}$  are not produced, and second, less than 25% of anthracene is recovered after heating at 300 °C (Figure S4), which means that 75% of anthracene is transformed by various possible mechanisms, e.g. trapping, degradation, polymerization, and reaction with other mineral or organic compounds.

Overall, our findings show that ROS induced algal toxicity with a contribution decreasing as follows:  $\cdot\text{OH} > \text{O}_2^{\cdot-} > \text{H}_2\text{O}_2$ . The highest contribution of  $\cdot\text{OH}$  to toxicity may be due to its strong oxidative potential  $E_0$  of 2.8 V, which can cause adverse effects to organisms (Gehling et al. 2014). The direct effect of anthracene and anthracene-PFRs on algal toxicity is negligible, yet anthracene transformation products are indirectly involved in algal toxicity because they induce ROS generation.

## Conclusion

This study investigated the potential risk of thermal treated anthracene-contaminated soil. Results clearly showed that PFRs with the relatively long lifetime were formed during thermal treatment of anthracene-contaminated soil, and they could induce the generation of ROS. The formed PFRs and ROS promoted the levels of oxidative potential, and posed negative effects to wheat germination and growth, algae activity. The scavengers suggested the toxicity of PFRs was mainly from the generation of ROS. Our findings indicate that thermal treatment of PAH-contaminated soil may not be so sustainable as previously assumed since PFRs may induce toxicity for living organisms. Further investigations are currently needed to quench these PFRs in thermal treatment of PAH-contaminated soil, thus ensuring the safe application of thermal treatment.

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