applied biosciences

MDPI

Article

Biochar Extracts Can Modulate the Toxicity of Persistent Free Radicals in the Nematode *Caenorhabditis elegans*

Xuchao Zhang [1,2], Nadine Saul [3,4], Thora Lieke [3,5,6], Yi Chen [1], Min Wu [1,*], Bo Pan [1] and Christian E. W. Steinberg [1,3,*]

1 Yunnan Provincial Key Lab of Soil Carbon Sequestration and Pollution Control, Faculty of Environmental Science and Engineering, Kunming University of Science and Technology, Kunming 650500, China
2 Faculty of Life Sciences, Ecology Group, Humboldt Universität zu Berlin, Philippstraße 13, 10115 Berlin, Germany
3 Faculty of Life Sciences, Freshwater and Stress Ecology Group, Humboldt Universität zu Berlin, 12437 Berlin, Germany
4 Faculty of Life Sciences, Molecular Genetics Group, Humboldt Universität zu Berlin, 10115 Berlin, Germany
5 Institute of Aquaculture and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Na Sádkách 1780, 370 05 České Budějovice, Czech Republic
6 Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, 370 05 Ceske Budejovice, Czech Republic
* Correspondence: minwup@kust.edu.cn (M.W.); christian_ew_steinberg@web.de (C.E.W.S.); Tel.: +86-871-6510-2829 (M.W.); +49-30-614-2746 (C.E.W.S.)

Abstract: As an effective soil amendment, biochars require a comprehensive ecological evaluation before they can be widely used in agriculture because endogenous contaminants, such as environmentally persistent free radicals (EPFRs), certainly pose an ecological risk to soil invertebrates. In this study, *Caenorhabditis elegans* (*C. elegans*) was used as a model organism to investigate the neurotoxicity of two rice straw biochars pyrolyzed at 500 and 700 °C. After 24 h exposure to unwashed biochar, washed biochar, and leaching fluids (supernatants), the neurobehavioral parameters of *C. elegans* were determined in a liquid toxicity test. The results showed that the washed 700 °C biochar particles significantly impaired locomotion and prolonged the defecation interval at a biochar concentration of 4 g·well^{-1}, while the unwashed biochar and supernatants caused no apparent impairment. Supporting this, electron paramagnetic resonance (EPR) results showed that the intensity of EPFRs in unwashed 700 °C biochar was stronger than that of the corresponding washed particles. This indicates that, in the liquid test, the EPR signal alone is not indicative of particle toxicity. The accessibility and activity of the EPFRs should be considered. Dissolved organic matter (DOM) was observed in the leaching fluids. The neurotoxic activity of the washed biochar was alleviated after the re-addition of leaching fluids to the washed biochar, suggesting that the dissolved organic materials modulate the reactivity of the EPFRs in the liquid phase. This study suggests that the leaching process may increase the risk of biochar when used in the field environment.

Keywords: environmental persistent free radicals; *Caenorhabditis elegans*; neurotoxicity; biochar

Citation: Zhang, X.; Saul, N.; Lieke, T.; Chen, Y.; Wu, M.; Pan, B.; Steinberg, C.E.W. Biochar Extracts Can Modulate the Toxicity of Persistent Free Radicals in the Nematode *Caenorhabditis elegans*. *Appl. Biosci.* **2023**, *2*, 71–83. https://doi.org/10.3390/applbiosci2010007

Academic Editor: Robert Henry

Received: 30 November 2022
Revised: 10 February 2023
Accepted: 24 February 2023
Published: 28 February 2023

1. Introduction

Biochar is an environmentally friendly carbonaceous material to improve soil quality [1,2]. Biochar is expected to mitigate global warming through carbon sequestration [3]; enhance crop production by improving soil quality, including fertility and water balance [4,5]; and sequester contaminants due to its absorbability [6]. However, biochar can also be critical to organisms due to endogenous contaminants [7]. Although conventional endogenous contaminants in biomass-derived biochars, such as polycyclic aromatic hydrocarbons (PAHs) and metals, have not posed significant toxicity risks to soil organisms due to their relatively low concentrations [8–10]. An emerging contaminant, environmentally

persistent free radicals (EPFRs), has been detected in biochar and is of increasing concern due to its long-term existence and potential risks in environmental compartments such as soil, atmospheric particulate matter, and coal dust [11,12]. In general, EPFRs have typically long been overlooked as elicitors of adverse effects in individuals or populations, probably because traditional analytical techniques did not detect EPFRs and there was a lack of understanding of their fate in the environment [13]. In previous studies, EPFRs found in biochar were able to damage seed membranes and inhibit seedling growth and urease activity in soil [8,14], and were even neurotoxic to an invertebrate model organism, *Caenorhabditis elegans* (*C. elegans*) [15]. This implies that the potential adverse effects of exposure to biochar-containing EPFRs should be thoroughly evaluated before large-scale application.

Dissolved organic matter (DOM) is a ubiquitous component in terrestrial and aquatic environments. It plays a critical role in the fate, transport, and toxicity of environmental pollutants [2,16–18]. DOM has been recognized as a signaling compound in the environment due to its ability to interact with molecular signaling structures in invertebrates and fish [19–22]. Studies found that the nematode *C. elegans* prefers to live in DOM-rich environments, both in nature and under laboratory conditions, and that DOM extends their lifespan and increases their reproductive capacity [20,21]. Biochar, as a source of DOM, can release significant amounts of DOM into the soil under rainfall and irrigation conditions [23–26]. DOM has a positive effect on soil quality by providing essential nutrients and promoting the growth and diversity of microorganisms. Still, it could also have adverse effects, such as increased toxicity of environmental pollutants [15,27]. However, the effect of DOM and its impact on organisms has not yet been fully explored or understood. It is important to have a better understanding of the impact of DOM leaching from biochar on the environment and its biota, as this will provide a more comprehensive evaluation of using biochar in soil applications.

To further investigate the potential toxicity of EPFRs on organisms and the role of DOM, experiments were conducted using *C. elegans* and biochars with varying intensities of EPFR and DOM content. *C. elegans* has proven to be an effective and powerful tool in evaluating the risks of environmental pollutants, such as nanomaterials and xenobiotics [15,27]. First, neurobehavior, including autonomic behavior (movement length, body bend, defecation interval) and sensory behavior (touch response and chemotaxis), was determined to evaluate the neurotoxic potential of the biochar [15,27]. The results were compared to the effects of EPFRs to determine the cause of stress. Finally, DOM leached from the biochar was analyzed to verify its impact on the nematodes. We hypothesize that DOM reduces the direct neurotoxicity of biochar-derived EPFRs to *C. elegans*.

2. Material and Method

2.1. Strains and Maintenance

Wild-type *C. elegans* nematode N2 (var. Bristol) and its feed *Escherichia coli* strain OP50 were originally obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA). After culturing OP50 in 200 mL LB medium at 37 °C for 12 h, the optical density (OD) was detected at 600 nm spectrophotometrically (Techne Specgene, Model FSPECGE, Stone, UK). The OD_{600} value used in this study was 2.6 ± 0.3. Before exposure tests, all *C. elegans* were kept in nematode growth plates (NGM) at 20 °C and seeded with OP50 bacteria as a diet.

2.2. Preparation and Characterization of Biochars

Rice straw was collected from the Wujiaying Residential District, Chenggong, Kunming, China (24.8°N; 102.8°E) as raw biomass for biochar production. The biochar was produced as described in our previous study [15].

The samples were dried, ground, and passed through a 60-mesh sieve. They were then pyrolyzed at 500 and 700 °C for 2 h under N_2 atmosphere in a muffle furnace. The resulting biochars were ground and sieved through a 300-mesh sieve. According to the pyrolysis temperature, the unwashed biochars were labeled B500 and B700. The washed

particles were prepared by adding 40 mg of unwashed biochar to 30,000 µL of sterilized double-distilled water (sddH_2O), then dried and labeled as P500 and P700. To obtain the supernatant, 40 mg of unwashed biochar was added to 15,000 µL of sddH_2O and shaken for 24 h at 20 °C without light; the leachate was passed through a 0.45 µm filter and labeled as S500 and S700. All supernatants were stored at 4 °C.

Hydroxyl radicals (•OH) were scavenged with 5,5-dimethyl-1-pyrroline N-oxide (DMPO, Aladdin). Briefly, for unwashed and washed biochar, 4 mg of solid powder was mixed with 300 µL of sddH_2O, vortexed for 1 min, and the solid powder was allowed to settle. Then, 100 µL of 0.3 M fresh DMPO was mixed with 100 µL supernatant of the solid samples; for liquid samples, 100 µL of the sample was mixed with 100 µL of fresh DMPO. Finally, 50 µL of each sample was used to determine the free radical content by absorption in a micropipette (1.5 mm in outer diameter, 0.5 mm in wall thickness, and 125 mm in length). Electron paramagnetic resonance (EPR) signals were recorded using an EPR spectrometer (X-Band A300-6/1, Bruker, Billerica, MA, USA) [8]. All EPR detection experiments were performed at room temperature.

The DOM concentration in the supernatants was determined with a fluorescence excitation emission matrix (EEM). Spectra were recorded with an excitation wavelength range of 200–400 nm and an emission wavelength range of 250–550 nm using an F-7000 FL spectrophotometer (HITACHI, Hitachi, Ibaraki, Japan) at a scan speed of 12,000 nm·min^{-1} and a voltage of 550 V. All supernatants were freshly prepared before testing, as described above.

2.3. Exposure Condition

C. elegans individuals were exposed to three treatments: unwashed biochar, washed biochar, and supernatant, respectively. The unwashed and washed biochars were mixed directly with the exposure medium (K medium: M9 buffer and OP50 in a 3:3:1 ratio, see Text S1 for more details). In each well of a sterile 24-well tissue culture plate (Costar, Corning, New York, NY, USA), 300 µL of exposure medium was added, including 0, 0.5, 1, 2, or 4 mg of unwashed and washed biochar according to our previous experiment [15] and the germination assay of biochar at the ratio of 0, 0.5, 1, 2, 4 g per Petri dish [8]. For unwashed biochar of 500 °C (B500), it equals about 0, 550, 1100, 2200, 4400 mg C·L^{-1}; for unwashed biochar of 700 °C (B700), it equals about 0, 400, 800, 1600, 3200 mg C·L^{-1}. All the carbon data are based on the elemental analyses (Table S2).

For the supernatant tests, 150 µL was added to 150 µL of exposure medium (to obtain the same solid–liquid ratio of 40 mg: 30,000 µL was used to obtain the washed biochar). *C. elegans* worms were synchronized and cultured to L4 stage at 20 °C on NGM plates before being transferred to the 24-well exposure plates. All test plates were cultured at 20 °C for 24 h, and *C. elegans* were transferred to new NGM plates before neurotoxic assays.

2.4. Neurotoxic Assays

Neurobehavioral experiments, including autonomic and sensory behaviors, were performed as previously described [15,27]. For the autonomic behavioral tests, a single worm was transferred to a new NGM plate covered with OP50, and the crawling path was observed for 20 s under a microscope (Nikon SMZ 1500, Tokyo, Japan). Body bends were counted as changes in the direction of movement in the sinusoidal motion of the worm, and the relative length of movement was measured using the NIS-Elements D software (Nikon Eclipse E100, Tokyo, Japan) and normalized to the average body size of each group. The defecation intervals were calculated as the time between two contractions of the posterior body wall under the microscope. For the mechanical sensory behavior, the anterior tip of the worm was touched 10 times with a fine hair, and each stimulus response was recorded as a reversal movement. The chemotaxis assay is based on the worm's attraction to sodium chloride (NaCl), as its memory is associated with feeding. Ninety-six mm chemotaxis assay plates were prepared without NaCl and stored at 4 °C (Text S2). Twenty-four hours before an assay, an agar plug (approximately 5 mm × 5 mm × 3 mm) containing 100 mM NaCl was placed on the NaCl spot, with the NaCl spot and the control spot equidistant

from the starting spot (3 cm), and their distance was 4 cm. The agar plug was removed just before the assay, then 1 µL 0.5 M sodium azide was added to the NaCl spot and the control spot. Thirty worms were transferred to the starting spot and incubated at 20 °C for 1 h. Then, the number of nematodes in the circular area (r = 1.5 cm) of the NaCl spot and the control spot was counted as N_n and N_c, respectively. The chemotaxis index was calculated as the difference between the number of N_n and N_c divided by the total number. The test order for each day was body bending, relative movement length, touch response, and defecation; the chemotaxis test was constantly tested on a different day and was not performed with the four endpoints mentioned above. For the body bending, defecation, and mechanical sensory assays, at least 10 worms were randomly selected from each exposure concentration. All experiments were repeated at least three times.

2.5. The Autonomic Behavior Assay of Selected Supernatant

Supernatants S500 and S700 were selected to test their potential effects after a short exposure time (12 h, test 1 in Figure 1). Autonomous behavior was performed as described in Section 2.4. Next, S500 or S700 was added to the M9 buffer containing OP50 (see Section 2.3), and body curvature and relative movement length were determined after 12 h of exposure. In test 2 (Figure 1), *C. elegans* were first treated with S500 or S700 for 12 h, then P500 and P700 were added to the appropriate portion of the supernatant (S+P500 and S+P700). Additional controls were exposed to P500 and P700 only (C+P500 and C+P700). The concentration of P500 and P700 was 4 mg·well^{-1}, and the corresponding supernatants were used.

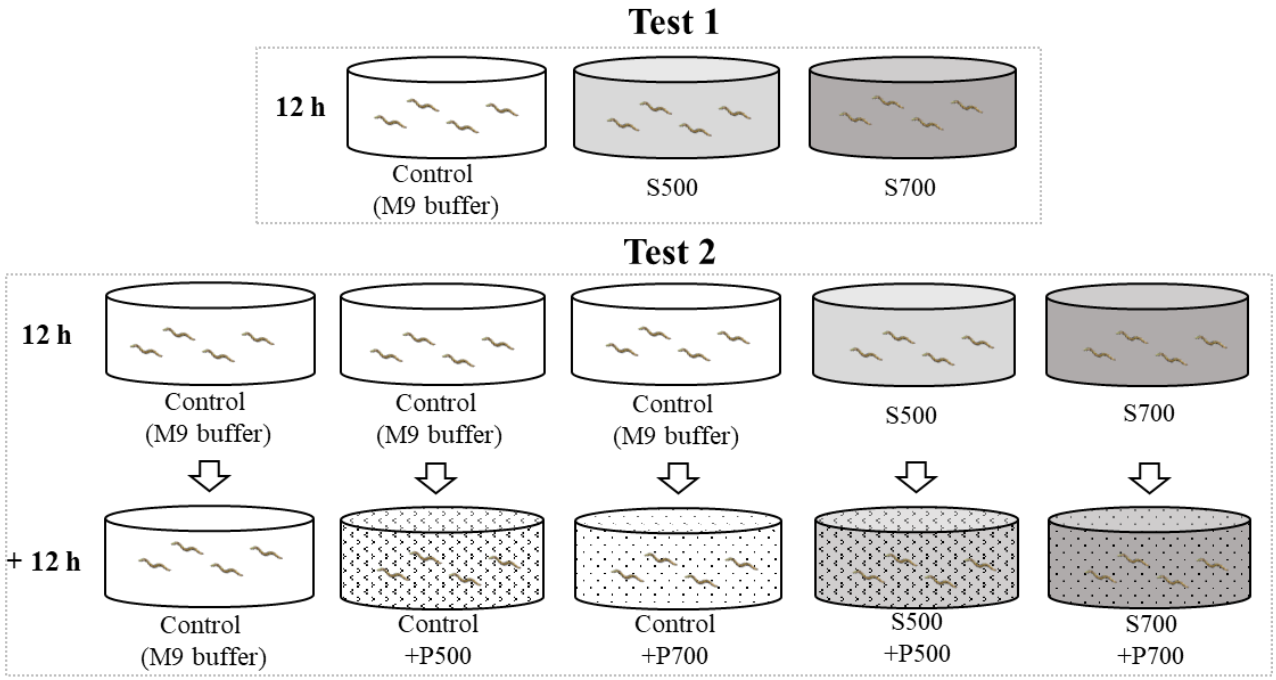

Figure 1. Diagram of the autonomic assay with supernatant (S500 and S700) and washed biochar particles (P500 and P700). Test 1: Exposure to the supernatant for 12 h. Test 2: Exposure to the supernatant for 12 h with the subsequent addition of washed particles.

2.6. Statistical Analysis

The statistical significance of the changes was determined using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) and the one-way ANOVA test (Holm–Sidak's multiple comparison test). Probability levels of 0.05 (*) and 0.01 (**) were considered statistically significant. All neurobehavioral data are expressed as mean ± SEM (standard error of the mean). Neurobehavioral figures were generated using Graphpad Prism 7. For

the EPR signal and fluorescence of DOM, the figures were generated using Origin 2018 (OriginLab Corporation, Northampton, MA, USA).

3. Results and Discussion

3.1. Only Washed Particles from 700 °C Pyrolysis Caused Neurobehavioral Changes

To investigate the neurotoxic potential of different biochars on *C. elegans*, two different pyrolysis temperatures (500 and 700 °C) were chosen for biochar preparation. *C. elegans* were exposed to unwashed biochars, washed biochar particles, and supernatants. After 24 h exposure to unwashed biochars and supernatants, none of the neurological parameters tested was significantly altered by any biochar concentration (Figure S1 in Supplementary). For washed biochar particles, body curvatures decreased by 15.1% ($p < 0.01$) and 21.0% ($p < 0.01$) after treatment with 1 mg·well^{-1} and 4 mg·well^{-1} of P700, respectively (Figure 2B). After exposure to 2 and 4 mg·well^{-1} of P700, the defecation interval increased by 13.3% ($p < 0.01$) and 20.9% ($p < 0.01$), respectively (Figure 2C). Relative movement length, touch response, and chemotaxis did not change for any particle exposures tested (Figure 2A,D,E).

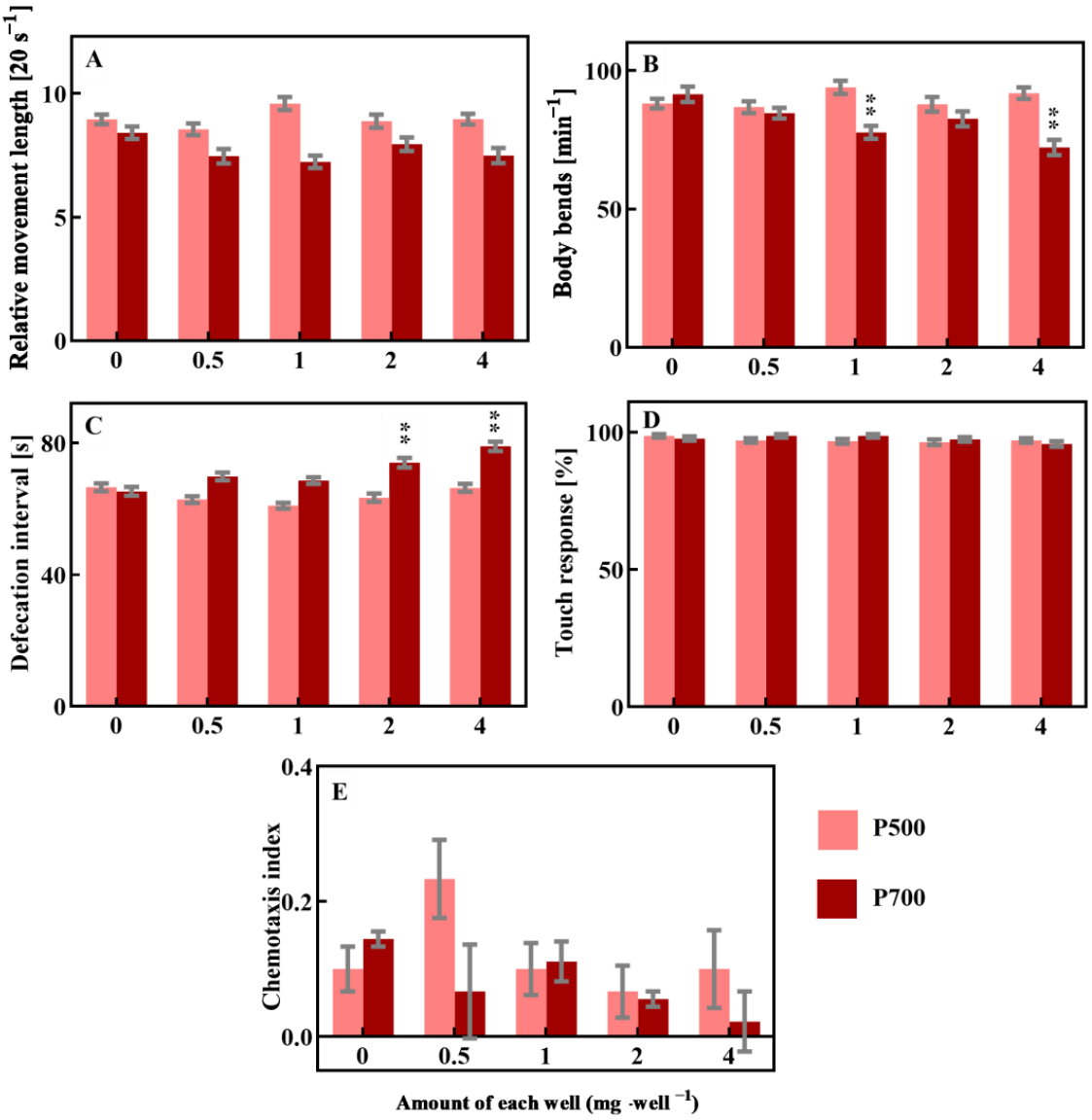

Figure 2. (**A**–**E**) Neurobehavior of *Caenorhabditis elegans* after 24 h exposure to washed biochar particles of 500 °C and 700 °C (P500 and P700). ** means $p < 0.01$, which indicates a significant difference.

In the literature, the avoidance behavior of earthworms to biochar could be alleviated in the liquid phase [28], and the phytotoxicity of corncob biochar could also be reduced by washing out the water-soluble toxic compounds [29]. However, the question arises: how does biochar change during the washing process? Thus, to find the underlying cause of the phenotypical observations, we compare the different characteristics before and after washing the biochar (details are shown in Figure S2, Tables S1 and S2).

The consequences of the reduced rate of body bends could be an impaired ability to reach food and avoid stressful or risky places. The prolonged defecation interval was considered a possibility of neuronal damage [30]. In this study, unwashed biochar had no adverse effects on nematodes, while the washed particle fraction at 700 °C caused significant inhibition of the autonomic behavior. These results contradict our initial assumption that EPFRs could trigger the production of free oxygen radicals (•OH) in solution and, therefore, exhibit higher toxicity in solution than on the NGM plate. Compared to our previous study using solid agar surface plates for exposure, in which 500 °C rice biochar with high-intensity EPFRs decreased locomotion behavior and prolonged the defecation interval of *C. elegans* [15], the present study found that the two unwashed biochars did not significantly affect the behavioral parameters tested. According to the literature, earthworm avoidance behavior towards biochar was mitigated in the liquid phase [28], and the phytotoxicity of biochar was also reduced by washing [29]. However, the question remains on how biochar changes during the washing process. To find the reason for the phenotypic observations, we compare the different traits before and after washing the biochar (details are shown in Figure S2, Tables S1 and S2).

3.2. EPFRs Reactivity Might Play a Crucial Role in Neurotoxicity

Many contaminants, such as PAHs and metals, remain or are formed during the pyrolysis of biochar [11]. Our previous work with plate cultures showed that PAHs and metals were not the main cause of the observed neurotoxicity due to their low content in biochar [15]. EPFRs have been found in biochars and have been shown to have the potential to inhibit grain seedling growth and modulate *C. elegans* behavior [8,14,15]. In this word, EPFRs were also determined in all samples before and after washing. The *g*-factor and EPR signal intensity parameters can be used to identify the type of free radicals and the concentration of EPR-active species [8,31]. As shown in Figure 3, both the unwashed biochar and the washed biochar particles exhibited significantly higher intensities than the blank control. The supernatants did not show any EPFR signals. With increasing pyrolysis temperature, the EPR intensity of the unwashed biochar decreased from 4.8×10^5 spins·mg^{-1} (500 °C) to 5.9×10^4 spins·mg^{-1} (700 °C), and the same phenomenon was observed for the washed particles (Figure 3A,C). The toxicity experiments showed that only P700 decreased body bends and prolonged defecation intervals (Figure 2B,C). At the same time, P500 and B500, with the strongest EPR intensities, did not affect the neurobehavior of *C. elegans*. Moreover, Yang, et al. [32] showed that the degradation of 700 °C biochar with low EPR intensity was even better than that of 500 °C biochar with relatively high intensity of EPR in the first 100 h, indicating that the 700 °C biochar is likely to be more reactive. Thus, a stronger EPR signal does not necessarily indicate higher toxicity; rather, the reactivity of EPFRs should be considered.

The EPFRs could stimulate the formation of •OH in an aqueous solution, causing a deleterious effect [8,33,34]. In this work, •OH was trapped using DMPO, and the typical spectrum of the DMPO-OH adduct is a quartet shape with a relative intensity ratio of 1:2:2:1 [8]. As shown in Figure 3B,D, both unwashed (B500 and B700) and washed biochar (P500 and P700) exhibit strong DMPO-OH signals. This indicates that both 500 °C and 700 °C biochar can induce the production of •OH. Researchers shows that •OH is the main cause of organic chemical degradation and biochar toxicity [8,35], indicating that •OH might be the reason for the toxicity. However, only P700 showed a significant inhibitory effect (Figure 2), suggesting that •OH may not be responsible for this observation.

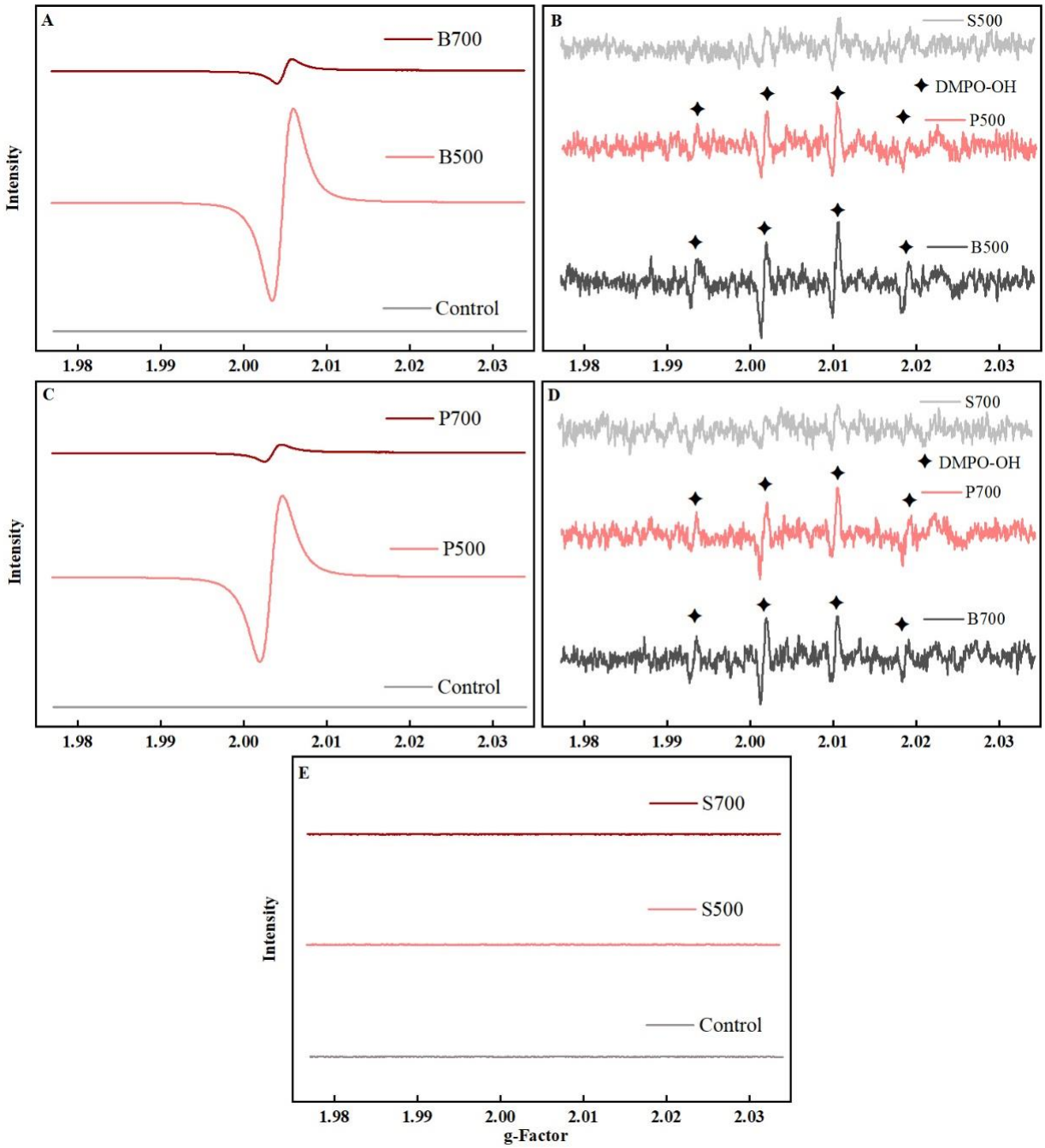

Figure 3. The EPR spectra of biochar at 500 and 700 °C. The resulting intensity of unwashed biochar (**A**), washed particle (**C**), and supernatant (**E**) was divided by their mass (mg^{-1}). EPR signals of DMPO-OH (**B**,**D**) are marked as four-point stars (✦).

3.3. DOM Can Modulate the Toxicity of EPFRs

Dissolved organic material plays an essential role in the formation and stability of EPFRs and can affect EPFR activity [13,36,37]. In this work, DOM was observed in all supernatants leached from biochar using the EEM spectrometer, and the peaks at the excitation/emission wavelength pair (Ex/Em) of S500 and S700 were 270 nm/395 nm and 250 nm/380 nm, respectively (Figure 4). Peaks at these two Ex/Em pairs are commonly associated with the presence of humic acid-like substances [38,39]. The relative fluorescence intensity of S500 was stronger than that of S700, showing that the amount of DOM leached from B500 was higher than that from B700.

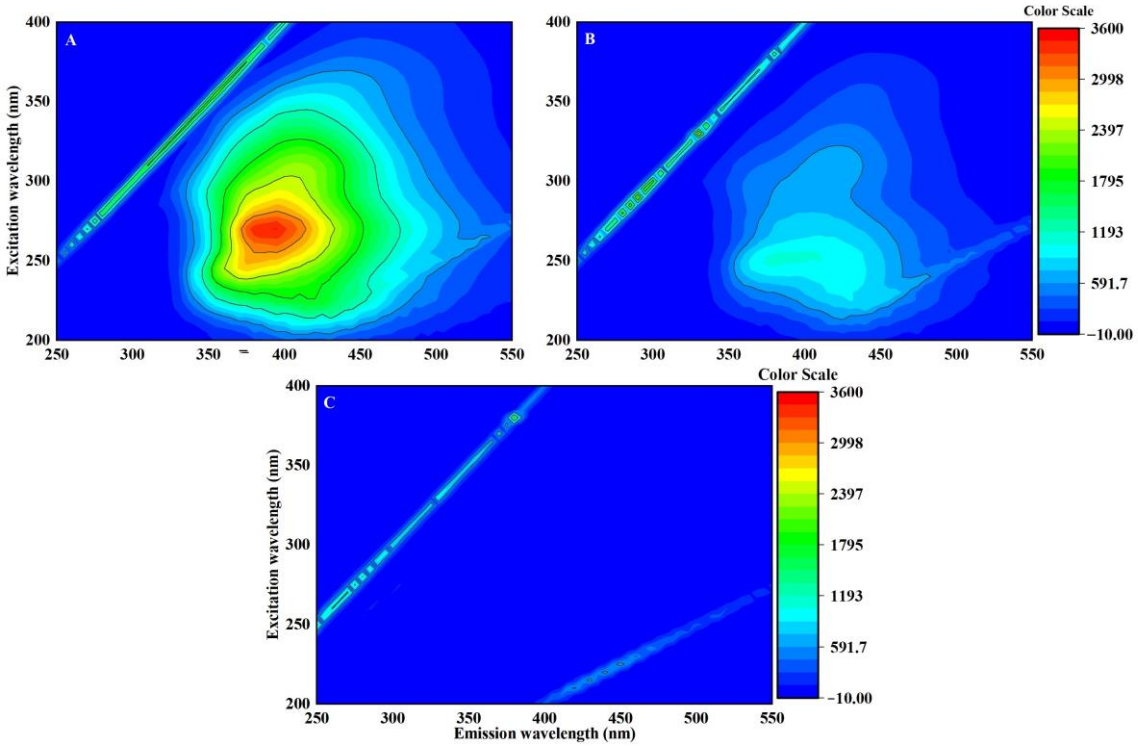

Figure 4. Fluorescence excitation-emission matrices of S500 (**A**), S700 (**B**), and H_2O (**C**).

Applying solid-state ^{13}C NMR, Liu, et al. [40] showed that biochar DOM contains abundant small aromatics with fused rings, aliphatic C, and carboxyl-C, and that water-extractable DOM can reach up to 0.5–40 mg C g^{-1} biochar. This coarse characterization is similar to that of humic substances. Pyrolyzing manure, protein-like and humic acid-like substances were the major components of DOM. The proteinaceous matter first decreased and then increased with an increase in the pyrolysis temperature, while an opposite trend was observed for the humic acid-like matter [41]. By sequential extraction, Liu, et al. [42] confirmed the humic-like character of DOM extractable from biochars. They showed that water-extractable DOM components are mostly composed of low aromatic and highly hydrophilic fulvic acid-like substances, which are the most labile and diversified components. This statement was confirmed by recent studies [43–45], which also detected fulvic acid-like compounds. In contrast, Tomczyk, et al. [46] found that extractable DOM from biochars produced from potato and raspberry stems was predominantly constituted of substances of large molecular weight and high aromaticity. This illustrates that there is (still) no unifying model that describes under which conditions, and of what quality, leachable organic carbon is produced by the pyrolysis of biowaste. However, some effects of humic and humic-like substances on soil organisms can be predicted, which are relevant for the present study since mainly fulvic acids interact with organisms [45,47].

Humic substances primarily reduce the bioavailability of organic chemicals, metals, and nanomaterials to *C. elegans*, and the extent of the reduction depends on the quantity and quality of the humic substances [21,46,48,49]. In addition, Lieke, et al. [50] found that fulvic acid protects fish gills from oxidative stress, probably by directly scavenging reactive oxygen species (ROS). Based on the above studies, DOM in unwashed biochar and supernatant can be expected to modulate the fate and reactivity of EPFRs. However, humic substances in higher internalized concentrations can block exporter proteins [51] and disproportionately increase bioconcentrations of persistent organic pollutants [52]. This could be detrimental if these persistent organic pollutants can also be toxic via the release of EPFRs.

To test whether DOM can modulate the toxicity of EPFRs, supernatants and particles of 500 °C and 700 °C biochar were selected to determine the effect of DOM. In test 1,

the nematodes were directly exposed to the supernatant for 12 h. The aim is to check whether the nematode adapts to the DOM environment after 24 h exposure since no impairments were observed (unshown data). The relative movement length and body bends of nematodes exposed to S700 increased significantly compared to the control (Figure 5A,C). In addition, there was a significant difference between S500 and S700, and exposure to S700 improved locomotion. In test 2, nematodes were first exposed to supernatants for 12 h, then corresponding particles were added for another 12 h. After exposure to C+P500 and C+P700, the rate of body bends decreased compared to the control, but there was no significant difference between P500 and P700 (Figure 5B). The results demonstrate that the washed particles are harmful to the nematodes. After 12 h exposure to the supernatant and 12 h exposure to the particles, the body bends of the nematodes exposed to S+P500 were significantly different from C+P500 but not significantly different from the control. The supernatant S500 significantly reduced the damage caused by EPFRs in the particles. A comparison of DOM levels and EPFR intensities in biochar, particles, and supernatants indicates that DOM can modulate the toxicity of EPFRs.

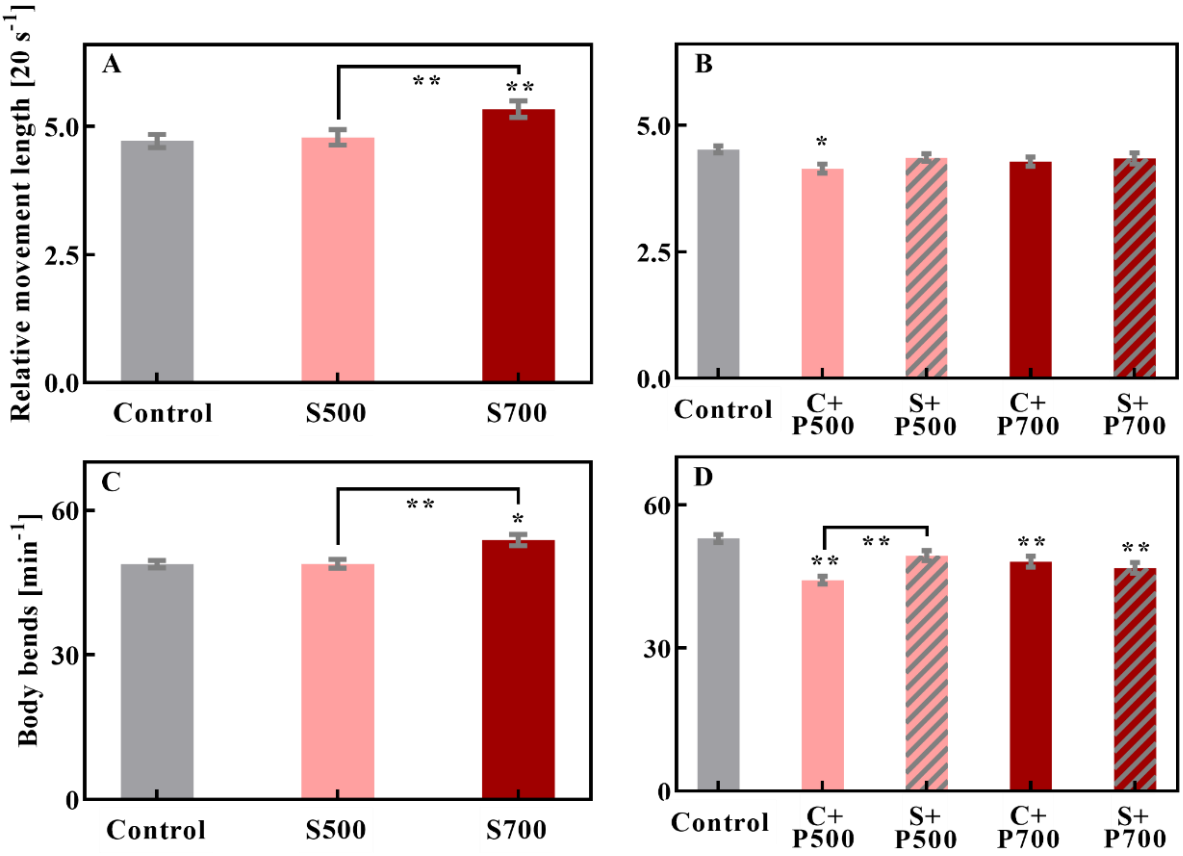

Figure 5. The autonomic behaviors of S500 and S700. Test 1: Exposure to the supernatant for 12 h (**A**,**C**). Test 2: Exposure to the supernatant for 12 h with the subsequent addition of washed particles (**B**,**D**). After exposure, the relative movement length (**A**,**B**) and body bend (**C**,**D**) were determined. * $p < 0.05$, ** $p < 0.01$.

It is evident that DOM has modulatory effects on environmental substances and their effects on organisms; however, the direction of modulation is not uniform. In a model analysis, Steinberg and Brüggemann [53] showed that humic substances can react in seemingly opposite ways, depending on their structure and chemical characterization. In fact, the effect of organic matter is a trade-off between promotion and inhibition due to its diverse nature, and this contrasting effect appeared to be intrinsic. Moreover, dissolved humic substances could facilitate fish life in rather acidic environments and prolong the

lifespan of *C. elegans* [17]. Therefore, it is not unlikely that DOM, which is leachable from biochar, can protect nematodes from damage by EPFRs. However, a promoting effect of S700 was observed in test 1, while no significant reduction in toxicity was observed in test 2. The reason may be the different nature and reactivity of EPFRs in the solid particles and the leached DOM between 500 °C and 700 °C, which should be clarified in future studies

4. Conclusions

The present study aimed to determine the reactivity of EPFRs in different biochars with or without the presence of different DOM. Increased neurotoxicity was only present in EPFRs with relatively high reactivity (P700). Although the potential risks of EPFRs in biochar have been a growing concern, our findings suggest that the presence of organic matter in the environment can significantly influence the toxicity of EPFRs, potentially overestimating the danger they pose. The DOM released from biochar can alleviate the harm from biochar particles, such as through (1) decreasing the probability of direct contact between biochar and *C. elegans*, and (2) partially quenching EPFRs by some active components of DOM. This may be the reason why EPFRs have been overlooked and understudied. To better understand how environmental factors affect the reactivity of EPFRs, future research should focus on using different types of biochar and developing methods to quantify reactivity. In addition, this study was only considering short-term exposure; long-term exposure may lead to chronic toxicity or affect the next generations, which also should be of more concern in further studies. This could contribute to developing effective strategies for reducing the risks associated with EPFRs and promoting the safe use of biochar in various applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applbiosci2010007/s1, Figure S1. Neurobehavior of *C. elegans* after 24 h exposure to unwashed biochars (A, D, G, J, and M), washed biochar particles (B, E, H, K, and N, these data were copied from Figure 2A–E for a better comparison), and supernatants (C, F, I, L, and O) at 20 °C. Relative move length (A, B, and C), body bend (D, E, and F), defecation interval (G, H, and I), touch response (J, K, and L), and chemotaxis index (M, N, and O) were determined. * $p < 0.05$, ** $p < 0.01$; Figure S2. Fourier transform infrared (FTIR) spectra of unwashed biochar and washed biochar. Samples were prepared with potassium bromide (mass ratio = 1:1500) and detected by ThermoFisher spectrometer (NICOLET iS50 FT-IR, USA). The dark line means unwashed biochar, the grey line means washed biochar particles.; Table S1. Total of carbon concentration in the supernatant; Table S2. The elemental composition in unwashed biochar and washed biochar.

Author Contributions: Conceptualization, X.Z. and M.W.; Data curation, B.P.; Formal analysis, X.Z., N.S., T.L., Y.C., M.W., B.P. and C.E.W.S.; Funding acquisition, M.W. and B.P.; Investigation, X.Z. and Y.C.; Methodology, X.Z., N.S., T.L., B.P. and C.E.W.S.; Project administration, M.W. and B.P.; Resources, X.Z., N.S., T.L. and C.E.W.S.; Supervision, M.W. and B.P.; Validation, X.Z.; Visualization, M.W. and B.P.; Writing—original draft, X.Z.; Writing—review and editing, X.Z., N.S., T.L., Y.C., M.W., B.P. and C.E.W.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Natural Scientific Foundation of China (41977334, and 42067055), the Yunnan Province Basic Research Project (202001AS070015), and the joint fund between NSFC-NCN (41961134002).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no competing interests.

References

1. El-Naggar, A.; Lee, S.S.; Rinklebe, J.; Farooq, M.; Song, H.; Sarmah, A.K.; Zimmerman, A.R.; Ahmad, M.; Shaheen, S.M.; Ok, Y.S. Biochar application to low fertility soils: A review of current status, and future prospects. *Geoderma* **2019**, *337*, 536–554. [CrossRef]
2. Yu, S.; Liu, J.; Yin, Y.; Shen, M. Interactions between engineered nanoparticles and dissolved organic matter: A review on mechanisms and environmental effects. *J. Environ. Sci. (China)* **2018**, *63*, 198–217. [CrossRef]
3. Spokas, K.A.; Cantrell, K.B.; Novak, J.M.; Archer, D.W.; Ippolito, J.A.; Collins, H.P.; Boateng, A.A.; Lima, I.M.; Lamb, M.C.; McAloon, A.J.; et al. Biochar: A synthesis of its agronomic impact beyond carbon sequestration. *J. Environ. Qual.* **2012**, *41*, 973–989. [CrossRef] [PubMed]
4. Beesley, L.; Moreno-Jimenez, E.; Gomez-Eyles, J.L.; Harris, E.; Robinson, B.; Sizmur, T. A review of biochars' potential role in the remediation, revegetation and restoration of contaminated soils. *Environ. Pollut.* **2011**, *159*, 3269–3282. [CrossRef] [PubMed]
5. Mohan, D.; Sarswat, A.; Ok, Y.S.; Pittman, C.U., Jr. Organic and inorganic contaminants removal from water with biochar, a renewable, low cost and sustainable adsorbent–a critical review. *Bioresour. Technol.* **2014**, *160*, 191–202. [CrossRef]
6. Hussain, M.; Farooq, M.; Nawaz, A.; Al-Sadi, A.M.; Solaiman, Z.M.; Alghamdi, S.S.; Ammara, U.; Ok, Y.S.; Siddique, K.H.M. Biochar for crop production: Potential benefits and risks. *J. Soils Sediments* **2017**, *17*, 685–716. [CrossRef]
7. Hale, S.E.; Lehmann, J.; Rutherford, D.; Zimmerman, A.R.; Bachmann, R.T.; Shitumbanuma, V.; O'Toole, A.; Sundqvist, K.L.; Arp, H.P.; Cornelissen, G. Quantifying the total and bioavailable polycyclic aromatic hydrocarbons and dioxins in biochars. *Environ. Sci. Technol.* **2012**, *46*, 2830–2838. [CrossRef]
8. Liao, S.; Pan, B.; Li, H.; Zhang, D.; Xing, B. Detecting free radicals in biochars and determining their ability to inhibit the germination and growth of corn, wheat and rice seedlings. *Environ. Sci. Technol.* **2014**, *48*, 8581–8587. [CrossRef]
9. Malev, O.; Contin, M.; Licen, S.; Barbieri, P.; De Nobili, M. Bioaccumulation of polycyclic aromatic hydrocarbons and survival of earthworms (*Eisenia andrei*) exposed to biochar amended soils. *Environ. Sci. Pollut. Res.* **2016**, *23*, 3491–3502. [CrossRef]
10. Wang, P.; Pan, B.; Li, H.; Huang, Y.; Dong, X.; Ai, F.; Liu, L.; Wu, M.; Xing, B. The overlooked occurrence of environmentally persistent free radicals in an area with low-rank coal burning, Xuanwei, China. *Environ. Sci. Technol.* **2018**, *52*, 1054–1061. [CrossRef]
11. Lian, F.; Xing, B. Black carbon (biochar) in water/soil environments: Molecular structure, sorption, stability, and potential risk. *Environ. Sci. Technol.* **2017**, *51*, 13517–13532. [CrossRef] [PubMed]
12. Zhang, C.; Zeng, G.; Huang, D.; Lai, C.; Chen, M.; Cheng, M.; Tang, W.; Tang, L.; Dong, H.; Huang, B.; et al. Biochar for environmental management: Mitigating greenhouse gas emissions, contaminant treatment, and potential negative impacts. *Chem. Eng. J.* **2019**, *373*, 902–922. [CrossRef]
13. Pan, B.; Li, H.; Lang, D.; Xing, B. Environmentally persistent free radicals: Occurrence, formation mechanisms and implications. *Environ. Pollut.* **2019**, *248*, 320–331. [CrossRef] [PubMed]
14. Liu, Y.; Dai, Q.; Jin, X.; Dong, X.; Peng, J.; Wu, M.; Liang, N.; Pan, B.; Xing, B. Negative impacts of biochars on urease activity: High pH, heavy metals, polycyclic aromatic hydrocarbons, or free radicals? *Environ. Sci. Technol.* **2018**, *52*, 12740–12747. [CrossRef] [PubMed]
15. Lieke, T.; Zhang, X.; Steinberg, C.E.W.; Pan, B. Overlooked risks of biochars: Persistent free radicals trigger neurotoxicity in *Caenorhabditis elegans*. *Environ. Sci. Technol.* **2018**, *52*, 7981–7987. [CrossRef]
16. Steinberg, C.E.W.; Sturm, A.; Kelbel, J.; Lee, S.K.; Hertkorn, N.; Freitag, D.; Kettrup, A.A. Changes of Acute Toxicity of Organic Chemicals to Daphnia magnain the Presence of Dissolved Humic Material (DHM). *Acta Hydrochim. Hydrobiol.* **1992**, *20*, 326–332. [CrossRef]
17. Steinberg, C.E.; Saul, N.; Pietsch, K.; Meinelt, T.; Rienau, S.; Menzel, R. Dissolved humic substances facilitate fish life in extreme aquatic environments and have the potential to extend the lifespan of Caenorhabditis elegans. *Ann. Environ. Sci.* **2007**, *1*, 81–90.
18. Yang, X.; Jiang, C.; Hsu-Kim, H.; Badireddy, A.R.; Dykstra, M.; Wiesner, M.; Hinton, D.E.; Meyer, J.N. Silver nanoparticle behavior, uptake, and toxicity in Caenorhabditis elegans: Effects of natural organic matter. *Environ. Sci. Technol.* **2014**, *48*, 3486–3495. [CrossRef]
19. Lutz, I.; Jie, Z.; Opitz, R.; Kloas, W.; Ying, X.; Menzel, R.; Steinberg, C.E. Environmental signals: Synthetic humic substances act as xeno-estrogen and affect the thyroid system of *Xenopus laevis*. *Chemosphere* **2005**, *61*, 1183–1188. [CrossRef]
20. Menzel, R.; Rodel, M.; Kulas, J.; Steinberg, C.E. CYP35: Xenobiotically induced gene expression in the nematode Caenorhabditis elegans. *Arch. Biochem. Biophys.* **2005**, *438*, 93–102. [CrossRef]
21. Menzel, R.; Sturzenbaum, S.; Barenwaldt, A.; Kulas, J.; Steinberg, C.E. Humic material induces behavioral and global transcriptional responses in the nematode Caenorhabditis elegans. *Environ. Sci. Technol.* **2005**, *39*, 8324–8332. [CrossRef] [PubMed]
22. Menzel, R.; Menzel, S.; Tiedt, S.; Kubsch, G.; Stosser, R.; Bahrs, H.; Putschew, A.; Saul, N.; Steinberg, C.E. Enrichment of humic material with hydroxybenzene moieties intensifies its physiological effects on the nematode Caenorhabditis elegans. *Environ. Sci. Technol.* **2011**, *45*, 8707–8715. [CrossRef] [PubMed]
23. Duan, W.; Oleszczuk, P.; Pan, B.; Xing, B. Environmental behavior of engineered biochars and their aging processes in soil. *Biochar* **2019**, *1*, 339–351. [CrossRef]
24. Rajapaksha, A.U.; Ok, Y.S.; El-Naggar, A.; Kim, H.; Song, F.; Kang, S.; Tsang, Y.F. Dissolved organic matter characterization of biochars produced from different feedstock materials. *J. Environ. Manag.* **2019**, *233*, 393–399. [CrossRef] [PubMed]

25. Rombola, A.G.; Torri, C.; Vassura, I.; Venturini, E.; Reggiani, R.; Fabbri, D. Effect of biochar amendment on organic matter and dissolved organic matter composition of agricultural soils from a two-year field experiment. *Sci. Total Environ.* **2022**, *812*, 151422. [CrossRef] [PubMed]
26. Sun, Y.; Xiong, X.; He, M.; Xu, Z.; Hou, D.; Zhang, W.; Ok, Y.S.; Rinklebe, J.; Wang, L.; Tsang, D.C.W. Roles of biochar-derived dissolved organic matter in soil amendment and environmental remediation: A critical review. *Chem. Eng. J.* **2021**, *424*, 130387. [CrossRef]
27. Ju, J.; Saul, N.; Kochan, C.; Putschew, A.; Pu, Y.; Yin, L.; Steinberg, C.E. Cyanobacterial xenobiotics as evaluated by a *Caenorhabditis elegans* neurotoxicity screening test. *Int. J. Environ. Res. Public Health* **2014**, *11*, 4589–4606. [CrossRef]
28. Li, D.; Hockaday, W.C.; Masiello, C.A.; Alvarez, P.J.J. Earthworm avoidance of biochar can be mitigated by wetting. *Soil Biol. Biochem.* **2011**, *43*, 1732–1737. [CrossRef]
29. Intani, K.; Latif, S.; Islam, M.; Müller, J. Phytotoxicity of Corncob Biochar before and after Heat Treatment and Washing. *Sustainability* **2018**, *11*, 30. [CrossRef]
30. Lieke, T.; Steinberg, C.E.; Ju, J.; Saul, N. Natural Marine and Synthetic Xenobiotics Get on Nematode's Nerves: Neuro-Stimulating and Neurotoxic Findings in Caenorhabditis elegans. *Mar. Drugs* **2015**, *13*, 2785–2812. [CrossRef]
31. Dellinger, B.; Lomnicki, S.; Khachatryan, L.; Maskos, Z.; Hall, R.W.; Adounkpe, J.; McFerrin, C.; Truong, H. Formation and stabilization of persistent free radicals. *Proc. Combust. Inst.* **2007**, *31*, 521–528. [CrossRef]
32. Yang, J.; Pan, B.; Li, H.; Liao, S.; Zhang, D.; Wu, M.; Xing, B. Degradation of *p*-nitrophenol on biochars: Role of persistent free radicals. *Environ. Sci. Technol.* **2016**, *50*, 694–700. [CrossRef]
33. Khachatryan, L.; Dellinger, B. Environmentally persistent free radicals (EPFRs)-2. Are free hydroxyl radicals generated in aqueous solutions? *Environ. Sci. Technol.* **2011**, *45*, 9232–9239. [CrossRef] [PubMed]
34. Khachatryan, L.; Vejerano, E.; Lomnicki, S.; Dellinger, B. Environmentally persistent free radicals (EPFRs). 1. Generation of reactive oxygen species in aqueous solutions. *Environ. Sci. Technol.* **2011**, *45*, 8559–8566. [CrossRef] [PubMed]
35. Wen, G.; Wang, S.J.; Ma, J.; Huang, T.L.; Liu, Z.Q.; Zhao, L.; Xu, J.L. Oxidative degradation of organic pollutants in aqueous solution using zero valent copper under aerobic atmosphere condition. *J. Hazard. Mater.* **2014**, *275*, 193–199. [CrossRef] [PubMed]
36. Paul, A.; Hackbarth, S.; Vogt, R.D.; Roder, B.; Burnison, B.K.; Steinberg, C.E. Photogeneration of singlet oxygen by humic substances: Comparison of humic substances of aquatic and terrestrial origin. *Photochem. Photobiol. Sci.* **2004**, *3*, 273–280. [CrossRef] [PubMed]
37. Huang, D.; Luo, H.; Zhang, C.; Zeng, G.; Lai, C.; Cheng, M.; Wang, R.; Deng, R.; Xue, W.; Gong, X.; et al. Nonnegligible role of biomass types and its compositions on the formation of persistent free radicals in biochar: Insight into the influences on Fenton-like process. *Chem. Eng. J.* **2019**, *361*, 353–363. [CrossRef]
38. Chen, W.; Westerhoff, P.; Leenheer, J.A.; Booksh, K. Fluorescence excitation-emission matrix regional integration to quantify spectra for dissolved organic matter. *Environ. Sci. Technol.* **2003**, *37*, 5701–5710. [CrossRef]
39. Helms, J.R.; Stubbins, A.; Perdue, E.M.; Green, N.W.; Chen, H.; Mopper, K. Photochemical bleaching of oceanic dissolved organic matter and its effect on absorption spectral slope and fluorescence. *Mar. Chem.* **2013**, *155*, 81–91. [CrossRef]
40. Liu, C.-H.; Chu, W.; Li, H.; Boyd, S.A.; Teppen, B.J.; Mao, J.; Lehmann, J.; Zhang, W. Quantification and characterization of dissolved organic carbon from biochars. *Geoderma* **2019**, *335*, 161–169. [CrossRef]
41. Gui, X.; Liu, C.; Li, F.; Wang, J. Effect of pyrolysis temperature on the composition of DOM in manure-derived biochar. *Ecotoxicol. Environ. Saf.* **2020**, *197*, 110597. [CrossRef] [PubMed]
42. Liu, H.; Zhao, B.; Zhang, X.; Li, L.; Zhao, Y.; Li, Y.; Duan, K. Investigating Biochar-Derived Dissolved Organic Carbon (DOC) Components Extracted Using a Sequential Extraction Protocol. *Materials* **2022**, *15*. [CrossRef] [PubMed]
43. Yu, S.; Zhang, H.; Ni, J.; Xiang, Y.; Wei, R.; Qian, W.; Chen, W. Spectral characteristics coupled with self-organizing maps analysis on different molecular size-fractionated water-soluble organic carbon from biochar. *Sci. Total Environ.* **2023**, *857*, 159424. [CrossRef]
44. Zhang, H.; Qian, W.; Wu, L.; Yu, S.; Wei, R.; Chen, W.; Ni, J. Spectral characteristics of dissolved organic carbon (DOC) derived from biomass pyrolysis: Biochar-derived DOC versus smoke-derived DOC, and their differences from natural DOC. *Chemosphere* **2022**, *302*, 134869. [CrossRef] [PubMed]
45. Zhang, P.; Huang, P.; Xu, X.; Sun, H.; Jiang, B.; Liao, Y. Spectroscopic and molecular characterization of biochar-derived dissolved organic matter and the associations with soil microbial responses. *Sci. Total Environ.* **2020**, *708*, 134619. [CrossRef] [PubMed]
46. Tomczyk, A.; Kubaczynski, A.; Szewczuk-Karpisz, K. Assessment of agricultural waste biochars for remediation of degraded water-soil environment: Dissolved organic carbon release and immobilization of impurities in one- or two-adsorbate systems. *Waste Manag.* **2023**, *155*, 87–98. [CrossRef]
47. Reemtsma, T.; These, A.; Linscheid, M.; Leenheer, J.; Spitzy, A. Molecular and structural characterization of dissolved organic matter from the deep ocean by FTICR-MS, including hydrophilic nitrogenous organic molecules. *Environ. Sci. Technol.* **2008**, *42*, 1430–1437. [CrossRef]
48. Collin, B.; Tsyusko, O.V.; Starnes, D.L.; Unrine, J.M. Effect of natural organic matter on dissolution and toxicity of sulfidized silver nanoparticles to Caenorhabditis elegans. *Environ. Sci. Nano* **2016**, *3*, 728–736. [CrossRef]
49. Haitzer, M.; Abbt-Braun, G.; Traunspurger, W.; Steinberg, C.E.W. Effects of humic substances on the bioconcentration of polycyclic aromatic hydrocarbons: Correlations with spectroscopic and chemical properties of humic substances. *Environ. Toxicol. Chem.* **1999**, *18*, 2782–2788. [CrossRef]

50. Lieke, T.; Steinberg, C.E.W.; Pan, B.; Perminova, I.V.; Meinelt, T.; Knopf, K.; Kloas, W. Phenol-rich fulvic acid as a water additive enhances growth, reduces stress, and stimulates the immune system of fish in aquaculture. *Sci. Rep.* **2021**, *11*, 174. [CrossRef]
51. Timofeyev, M.A.; Shatilina, Z.M.; Bedulina, D.S.; Menzel, R.; Steinberg, C.E. Natural organic matter (NOM) has the potential to modify the multixenobiotic resistance (MXR) activity in freshwater amphipods *Eulimnogammarus cyaneus* and *E. verrucosus*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2007**, *146*, 496–503. [CrossRef] [PubMed]
52. Haitzer, M.; Hoss, S.; Traunspurger, W.; Steinberg, C. Effects of dissolved organic matter (DOM) on the bioconcentration of organic chemicals in aquatic organisms–a review. *Chemosphere* **1998**, *37*, 1335–1362. [CrossRef] [PubMed]
53. Steinberg, C.E.W.; Brüggemann, R. Ambiguous Ecological Control by Dissolved Humic Matter (DHM) and Natural Organic Matter (NOM): Trade-offs between Specific and Non-specific EffectsWe dedicate this paper to Prof. Dr. Fritz H. Frimmel on the occasion of his 60th birthday anniversary. *Acta Hydrochim. Et Hydrobiol.* **2001**, *29*, 399. [CrossRef]