applied sciences

MDPI

Article

High Rates of Biochar Soil Amendment Cause Increased Incidences of Neurotoxic and Oxidative Stress in *Eisenia fetida* (Oligochaeta) Exposed to Glyphosate

Nomasonto Portia Dlamini [1,2,*] and Patricks Voua Otomo [1,2]

1 Department of Zoology and Entomology, University of the Free State, Private Bag x13, Phuthaditjhaba 9866, South Africa; OtomoPV@ufs.ac.za
2 Afromontane Research Unit, University of the Free State, Private Bag x13, Phuthaditjhaba 9866, South Africa
* Correspondence: 2013096462@ufs4life.ac.za

Abstract: Despite several known beneficial attributes, biochar is suspected to cause harm to soil organisms when present in relatively high quantities in the soil. To determine the potential detrimental effects of biochar, for 96 h, we exposed the earthworm *Eisenia fetida* to 0, 2, 4 and 8 mg glyphosate (GLY) per kg in non-amended and biochar-amended soil at rates of 5, 10 and 15%. The results indicated that in non-amended soil, survival was significantly decreased in the highest GLY concentration. Although no median lethal concentration (LC_{50}) could be computed due to the lack of sufficient mortality, in the absence of biochar, a lethal concentration 10% (LC_{10}) of 5.540 mg/kg and a lethal concentration 20% (LC_{20}) of 7.067 mg/kg were calculated. In the biochar-amended soil, no mortality occurred in the control and GLY treatments for all three biochar amendment rates. Biomass results showed significant biomass loss in the highest GLY treatment in the absence of biochar, with an effective concentration of 10% (EC_{10}) of 5.23 mg/kg and an effective concentration of 20% (EC_{20}) of 6.848 mg/kg. In the amended soil, overall, slight non-significant increases in biomass were recorded and no effective concentrations could be calculated due to the lack of significant biomass loss. The assessment of neurotoxicity via the activity of acetylcholine esterase (AChE) showed no change in AchE due to GLY in all the non-amended treatments. However, in the biochar-amended treatments, statistically high levels of AchE occurred ($p < 0.05$) even in the control (in the absence of GLY). The assessment of oxidative stress through catalase (CAT) activity, showed similar results with no significant effects of GLY alone on CAT activity, but rather dramatic increases in activity in the control and GLY treatments in the biochar-amended soil, with one significant increase in the 10% amended in 8 mg GLY/Kg ($p < 0.05$). Such significant increases in both AChE and CAT were only observed in soil amended with 10 and 15% biochar. Our findings show that although seemingly beneficial for whole body endpoints, biomarker responses indicate that a biochar amendment higher than 5% adds considerable additional stress to earthworms and should be avoided.

Keywords: biochar effects; ecotoxicological effects; mechanistic biomarkers; herbicide toxicity; earthworm ecotoxicology

check for updates

Citation: Dlamini, N.P.; Voua Otomo, P. High Rates of Biochar Soil Amendment Cause Increased Incidences of Neurotoxic and Oxidative Stress in *Eisenia fetida* (Oligochaeta) Exposed to Glyphosate. *Appl. Sci.* **2022**, *12*, 2381. https://doi.org/10.3390/app12052381

Academic Editor: Manfred Lübken

Received: 18 October 2021
Accepted: 23 November 2021
Published: 24 February 2022

1. Introduction

In South Africa, as in many regions of the world, the leading products used to control weeds and invading alien plant species are glyphosate-based herbicides [1]. Eventually, both terrestrial and aquatic invertebrates are unintentionally exposed to glyphosate (GLY) residues after spraying events. Maharaj [2] and Dalvie et al. [3] both reported that high GLY concentrations were found in the Hex River Valley, an intensive grape-farming area in the Western Cape Province of South Africa.

Among other species, the harmful effects of GLY have been documented in earthworms [4–7], amphibians [8–11], and freshwater shrimps [1,12]. Glyphosate is also known to affect belowground interactions between earthworms and symbiotic mycorrhizal fungi [13].

In temperate ecosystems, earthworms and arbuscular mycorrhizal fungi are very important for nutrient recycling and overall ecosystem functioning.

The deleterious effects of GLY contrast with its known usefulness in agriculture. Yet, because of its widespread use and its documented harmful effects in several non-target species [1,4–12], it is imperative to find means to diminish its environmental impact. Biochar amendment of agricultural soil can be such a means.

Biochar results from biomass pyrolysis under the limited presence or complete absence of oxygen at temperatures greater than 250 °C. Because of its stability and absorbent properties, some view biochar amendment as a means to mitigate the effects of chemicals in soils [14,15]. Such toxicity mitigating abilities have been shown after soil amendment in the cases of the spring onion *Allium cepa* exposed to carbofuran and chlorpyrifos [16], the potworm *Enchytraeus albidus* exposed to imidacloprid and silver nanoparticles [17] and several earthworms species exposed to fomesafen, chlorantraniliprole, mesotrione, and imidacloprid [15,18–20]. Despite these beneficial attributes, some have found that biochar amendment reduces the efficacy of soil pest management efforts due to its absorbent capacities [21,22] or worse causes direct harm to soil organisms such as earthworms [23–26].

The aim of the study was to investigate the effects of different rates of biochar soil amendment on the survival, biomass, neurotoxic and oxidative stress biomarkers in the earthworm *Eisenia fetida* exposed to glyphosate.

2. Materials and Methods

2.1. Biochar and Glyphosate

Biochar from pine tree chips (in pellet form) was purchased from C FERT™ in Johannesburg, South Africa. According to the manufacturer, it was prepared at pyrolysis temperatures of 400–450 °C, at a rate of 100 kg per 12 h. The elemental composition of this biochar was as follows: 0.31% N, 0.04% P, 0.28 K, 22.22% Ca, 0.63 Mg, 0.26%, and 28.90% C. Glyphosate, commercially available as Roundup® Power 360 SL (containing 360 g GLY/L) was purchased from Game Store South Africa (www.game.co.za (accessed on 18 October 2021)).

2.2. Test Substrate, Biochar Amendment, and Glyphosate Contamination

All experiments were performed in the artificial soil recommended by the Organization of Economic and Co-operation and Development (OECD) [27]. OECD soil was prepared by thoroughly mixing 10% sphagnum peat, 20% kaolin clay and 70% air-dried quartz sand (pH 5.5–6.5) [27]. To amend the OECD soil, the biochar pellets were ground to a fine powder and passed through a 2 mm sieve. Different amounts of the dry artificial soil were then replaced by 5, 10, and 15% of the sieved biochar in separate soil batches. Thereafter, the biochar-amended soils and a non-amended batch were spiked with GLY, to make a concentration range of 0 (control), 2, 4, and 8 mg of GLY/kg soil. Aqueous solutions of GLY were thoroughly mixed with the prepared substrates before the introduction of the worms. This concentration range was chosen to be largely sublethal based on Stanley and Joy [28] who reported a 96-h glyphosate LC_{50} of 10.4 mg GLY/kg soil using the earthworm *Nsukkadrilus mbae*.

2.3. Test Species and Exposure to the Contaminated, Amended, and Non-Amended Soils

Eisenia fetida, bred in the Ecotoxicology Research Laboratory at the University of the Free State, Qwaqwa Campus (Phuthaditjhaba, South Africa), were used as experimental organisms. Adult worms with visible clitellum were used in this study. Prior to exposure, the earthworms were allowed to empty their guts overnight, and were weighed. Ten adult *E. fetida* were exposed to 500 g of each treatment of glyphosate in biochar-amended and non-amended soils, which were then incubated at 20 ± 1 °C (in a Labcon low temperature incubator) for 96 h. This exposure duration was chosen not only to fill a gap in the literature, because similar studies on biochar typically last longer [17,20,23,24] but also to increase our likelihood of observing biomarker responses, which can peak in the early days of such an exposure and vanish after relatively longer exposure durations [18]. All exposures were made in triplicates. No feeding was performed during the exposure period. After 96 h, the earthworms were weighed once again. The surviving adult worms were counted and stored at −80 °C until further processing for biomarker analysis.

2.4. Biomarker Analysis

2.4.1. Determination of Acetylcholinesterase Activity

Acetylcholinesterase (AChE) was measured to find out whether the tested treatments had neurotoxic effects on the earthworms. To prepare tissue homogenates for acetylcholinesterase activity, two worms from each exposure treatment were thawed. Three segments of the earthworms' tail ends were sectioned. Tris-buffer (pH 7.4) was used to homogenize the tissues, which were centrifuged at 9500× *g* for 10 min at 4 °C. Supernatants were then used for AChE and protein analyses. Ellman's method [29] was used for the assessment of AChE activity. The assay mixture contained the sample homogenate (5 µL), in 210 µL of 0.09 M phosphate buffer (pH 7.4), 10 µL of 30 mM Acetylthiocholine iodide, and 10 µL of 10 mM of 5,5′-dithio-bis-(2-ni-trobenzoicacid) (DTNB). The reaction proceeded for 5 min at 37 °C, before spectrophotometric reading at 412 nm in 1-min intervals over a 6-min period. Acetylcholinesterase activity was expressed as absorbance/min/mg protein. The protein determination of the homogenates was performed using the Bradford method [30].

2.4.2. Determination of Catalase (CAT) Activity

The catalase activity was determined following to the method of Cohen et al. [31]. The reaction mixture contained the sample homogenate (10 µL) in 10 µL 0.09 M phosphate buffer (pH 7.0), 93 µL of 6 mM (30%) hydrogen peroxide, 19 µL of 6 N sulfuric acid, and 130 µL of 0.01 N potassium permanganate. The degradation of hydrogen peroxide by the catalase present in the samples was measured within 60 s at 490 nm and expressed in µmol H_2O_2/min/mg protein. The protein determination of the homogenates was performed using the Bradford method [30].

2.5. Statistical Analysis

Statistical analyses of the survival, biomass, acetylcholinesterase activity, and catalase activity data were performed in GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA, www.graphpad.com (accessed on 18 October 2021)). Parametric data were analysed using a One-way ANOVA, with Bonferroni post-test. Non-parametric data were analysed using the Kruskal–Wallis ANOVA followed by Dunns' test. The level of significance was $p < 0.05$. ToxRat® version 2.10.05 (Toxicity Response Analysis and Testing; ToxRat solutions GmbH, Alsdorf, Germany) was used to calculate median lethal concentrations (LC_{50}) and half maximal effective concentrations (EC_{50}) whenever possible.

3. Results

3.1. Survival

After 96 h of exposure to non-biochar amended OECD soil spiked with GLY, earthworm mortality was significant in the highest concentration (8 mg/kg) compared with the control and other concentrations ($p < 0.05$; Figure 1). No mortality was observed in the control and the lowest concentration (2 mg/kg). However, there was about 3% mortality in the 4 mg/kg treatment.

In the biochar-amended treatments, 100% survival was observed in all the biochar amendment rates. When homologous treatments were compared with each other, there were no significant differences between treatments apart from the highest non-biochar amended concentration (8 mg/kg) where mortality was significantly higher ($p < 0.05$) than in the similar treatments amended with biochar.

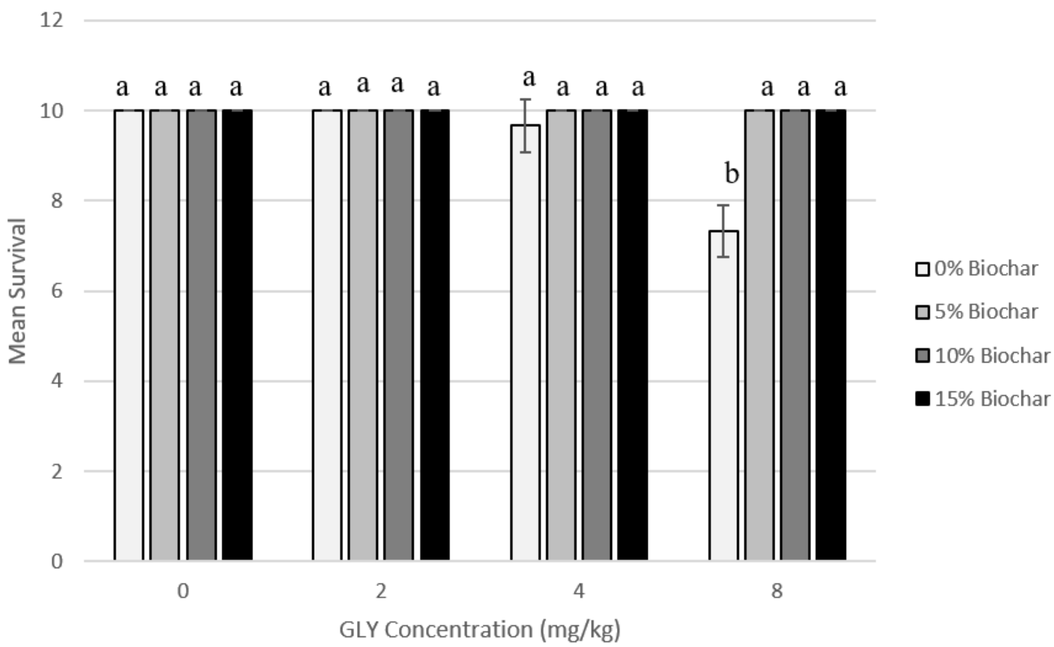

Figure 1. Number of surviving *Eisenia fetida* adults after 96-h exposure to glyphosate in biochar-amended (5, 10 and 15%) and 0% biochar-amended OECD artificial soil. Data represented as mean of 3 replicates (*n* = 30 per treatment). Error bars represent standard deviations and different letters represent significant differences.

The lethal concentrations (LC_{10}, LC_{20} and LC_{50}) for GLY in non-biochar amended treatments after 96 h were found to be 5.54, 7.067, and >8 mg/kg, respectively (Table 1). In all biochar-amended treatments, they were found to be >8 mg/kg due to the lack of mortality.

Table 1. Lethal concentrations (LC_S) calculated after 96-h exposure of *Eisenia fetida* to glyphosate in biochar-amended (5, 10 and 15%) and non-amended OECD soil.

LC_S (mg/kg)	0% Biochar	5% Biochar	10% Biochar	15% Biochar
LC_{10}	5.54 (2.889–7.003)	>8	>8	>8
LC_{20}	7.067 (5.292–10.517)	>8	>8	>8
LC_{50}	>8	>8	>8	>8

3.2. Biomass

The assessment of the effect of GLY on the biomass of adult *Eisenia fetida*, in the absence of biochar, revealed that consistent biomass loss occurred in the treatments, with significant loss ($p < 0.05$) in the 8 mg/kg treatment (Figure 2).

In the biochar-amended treatments, only in the 2 mg/kg treatment amended with 5% biochar was a slight non-significant biomass loss observed ($p > 0.05$). In all the other treatments and for all biochar amendment rates, the worms experience non-significant biomass gains ($p > 0.05$). Despite clear variations in earthworm biomass, within the GLY concentration range studied, no significant gains or losses in biomass were observed in the presence of biochar (Figure 2).

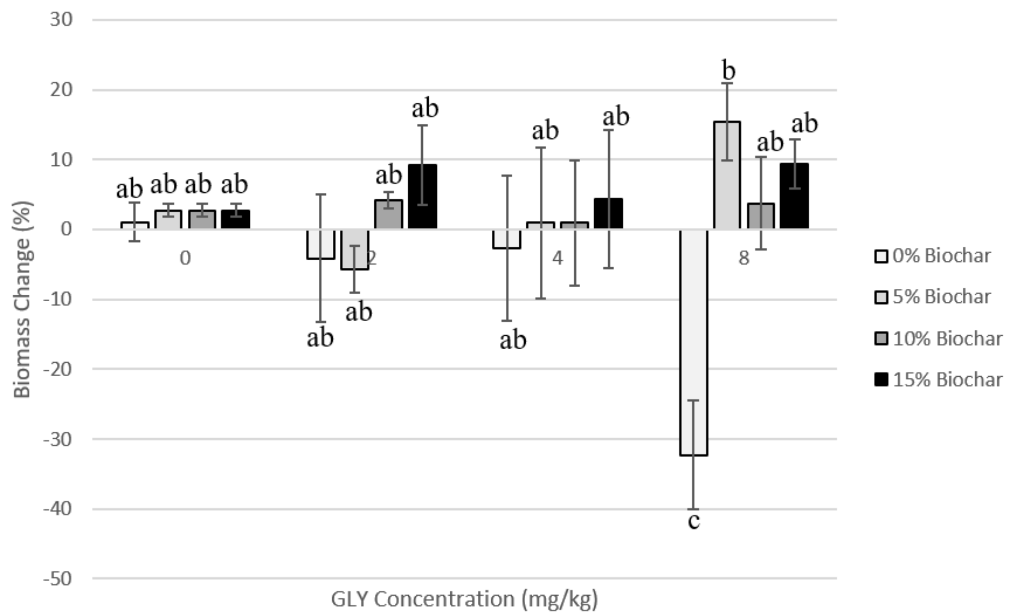

Figure 2. Change in biomass (%) of adults *Eisenia fetida* after 96-h exposure to glyphosate in biochar-amended (5, 10 and 15%) and non-biochar amended OECD artificial soil. Data represented as mean of 3 replicates (n = 30 per treatment). Error bars represent standard deviations and different letters represent significant differences.

Based on biomass variation data in *E. fetida*, the effective concentrations (EC_{10}, EC_{20} and EC_{50}) of GLY in the absence of biochar were 5.874, 6.973, and >8 mg/kg, respectively (Table 2). In worms exposed to 5, 10, and 15% biochar-amended, the reduction in biomass was not high enough to allow the computation of any effective concentrations within the GLY concentration range investigated.

Table 2. Effective concentrations (ECs) of glyphosate on *Eisenia fetida* biomass calculated after 96-h exposure in biochar-amended (5, 10 and 15%) and non-amended OECD soil.

EC_S (mg/kg)	0% Biochar	5% Biochar	10% Biochar	15% Biochar
EC10	5.238 (2.168–12.657)	>8	>8	>8
EC20	6.848 (2.643–18.317)	>8	>8	>8
EC50	>8	>8	>8	>8

3.3. Biomarker Results

3.3.1. Acetylcholinesterase Activity

In the negative control, in the absence of GLY, the activity of AChE was similar in the 0, 5, and 10% amended controls but significantly higher in the 15% biochar-amended control (Figure 3). The addition of GLY at a rate of 2 mg/kg did not change the AChE levels observed in the controls; a significantly higher AChE activity was present in the 15% biochar-amended treatment, whereas AChE activity was similar in the 0, 5 and 10% biochar-amended treatments.

In the two highest concentrations of GLY (4 and 8 mg/kg), the pattern of AChE activity observed in the controls and lowest GLY concentration was different; the activity of AChE was now similar in all treatments and biochar amendments, including in the 15% amendment. Overall, as the concentration of GLY increased, the initially high AChE activity in the 15% amendment decreased to the point that no difference in activity could be found at 4 and 8 mg/kg.

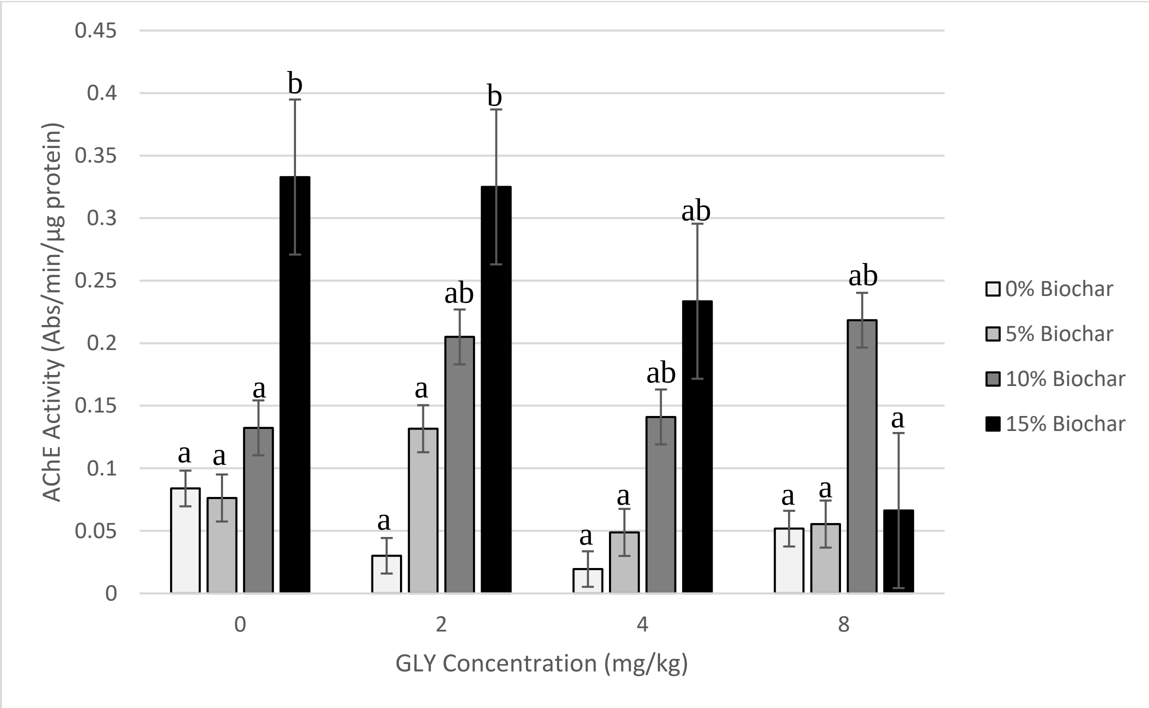

Figure 3. Acetylcholine esterase activity in *Eisenia fetida* after 96-h exposure to glyphosate in biochar-amended (5, 10, and 15%) and non-biochar amended OECD artificial soil. Data represented as mean of 3 replicates. Error bars represent standard deviations and different letters represent significant differences.

3.3.2. Catalase Activity

Catalase activity was similar in most treatments, except in the 10% biochar at 8 mg/kg, where the activity was significantly higher than in the controls with 0% and 5% biochar. CAT activity in the 10% biochar at 8 mg/kg was also significantly higher than in the worms exposed to 2 and 4 mg/kg treatments with 0% and 5% biochar (Figure 4).

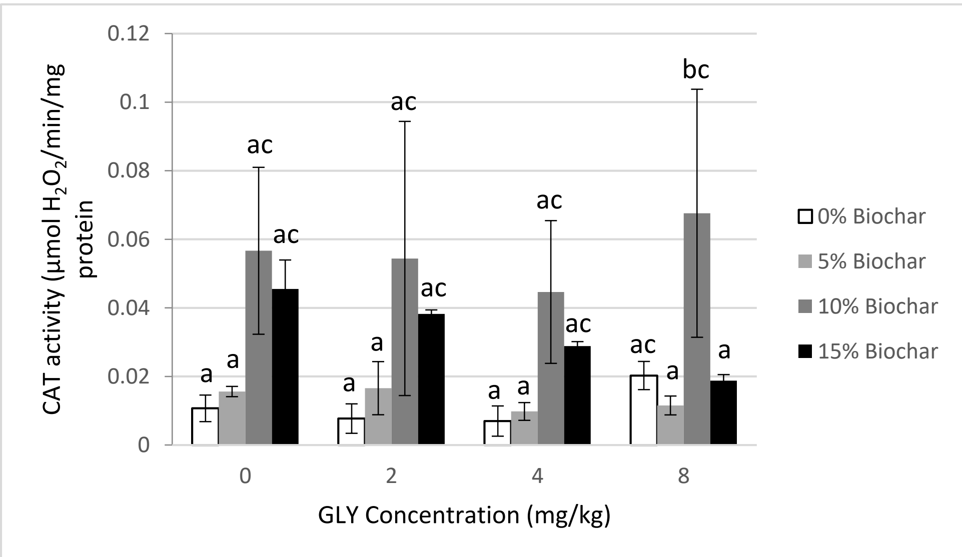

Figure 4. Catalase activity of *Eisenia fetida* after 96-h exposure to glyphosate in biochar-amended (5, 10, and 15%) and 0% biochar-amended OECD artificial soil. Data represented as mean of 3 replicates. Error bars represent standard deviations and different letters represent significant differences.

4. Discussion

Mortality data showed a significant effect in the highest GLY concentration (8 mg/kg) in the non-amended soil (Figure 1). The corresponding LC_{10}, LC_{20}, and LC_{50} were 5.54 mg/kg, 7.067 mg/kg, and greater than 8 mg/kg (Table 1). This means that, as the concentrations of GLY were increased, deleterious effects on the survival of the earthworms also increased. These results are in conformity with those of Stanley and Joy [28] who observed significant mortality after a 96-h exposure of the earthworm *Nsukkadrilus mbae* to GLY and reported an LC_{50} of 10.40 mg GLY/kg soil (i.e., LC_{50} > 8 mg/kg). Others have reported variable findings depending on the duration of exposure and the glyphosate-based product assessed. García-Torres et al. [32] reported 71% mortality in *E. fetida* exposed to 50,000 mg GLY/kg for 7 days. Stellin et al. [33] reported an LC_{10} and an LC_{50} of 0.496 (0.389–0.527) and 26.804 (23.437–27.821) g/m^2, respectively, after 21 days exposure of the earthworms *Lumbricus terrestris* to GLY. After 72-h filter paper contact exposures of *Eisenia andrei* to Roundup FG and Mon 8750, Piola et al. [34] reported glyphosate LC_{50} values of 66.0 (54.6–82.1) µg GLY/cm and 293.9 (254.0–339.8) µg GLY/cm, respectively.

As for biochar amendment, no significant mortality was experienced in all biochar-amended treatments (Figure 1). Our survival data suggest that in the presence of biochar, regardless of the amendment rate, GLY toxicity was significantly reduced as evidenced by the lack of mortality and failure to compute LC_{10}, LC_{20}, and LC_{50} values (Table 1). This is an indication that biochar was able to alleviate the toxicity of this herbicide. Biochar has been shown to improve survival rates in the manner reported in our study. Jing et al. [35] observed no earthworm death in 5% biochar-amended soil after 28 days of exposure of *E. fetida* to fenoxaprop-ethyl. Nyoka et al. [20] found that *E. fetida* exposed to imidacloprid in 10% biochar-amended soils experienced significant improvement in survival rate. These authors already reported similar findings after exposing the potworm *Enchytraeus albidus* to silver nanoparticles in soils amended with 10% biochar for 21 days [17]. It is worth noting that depending on the toxicant and the concentration range, biochar has its limitations in improving survival rates. For instance, Nyoka et al. [17] reported finding no difference in the mortality of *E. albidus* between a 10% biochar-amended substrate and a non-amended control, after exposure to imidacloprid for 21 days. The literature shows that such limitation can depend on the origin of the biochar itself. Liesch et al. [23] assessed the effects of different rates of application of biochar alone on earthworms. These authors used a poultry litter biochar and a pine chip biochar and found 100% mortality in 68 and 90 Mg (metric ton)/ha using the poultry litter biochar. At these same amendment rates, the pine chip biochar showed no difference in mortality with the non-amended control.

Our biomass results, as in the case of survival, showed significant effects in the highest GLY concentration in the absence of biochar (Figure 2). It is only in the non-amended soil that an EC_{10} and an EC_{20} for biomass can be computed (Table 2). These results indicate that higher concentrations of GLY exerted greater negative effects on the biomass of the earthworms. Several studies have demonstrated that *E. fetida* and *E. andrei* exposed to GLY lose a variable amount of weight [32,34,36–38]. On the contrary, Pochron et al. [39] reported no significant differences in *E. fetida* biomass after 7, 14, and 21 days of exposure to 60.7 mg GLY/kg. Santadino et al. [7] also reported no significant difference in the weight of *E. fetida* exposed for up to 40 days to twice the recommended amount of GLY in Roundup (Monsanto, St. Louis, MO, USA, 48% formula).

In the biochar-amended treatments, there was no significant weight loss in the earthworms (Figure 2). In addition, EC values could not be determined in all the biochar-amended soils because of insufficient weight loss (Table 2). The results indicate that biochar amendment helps preserve earthworm biomass. Nyoka et al. [20] recently reported similar findings after exposing *E. fetida* to imidacloprid in 10% biochar-amended soils for 28 days.

Despite evidence suggesting that biochar helps to alleviate the effects of chemical toxicity on earthworm biomass, there are recurring literature findings indicating that biochar amendment on its own (in the absence of a chemical toxicant) is capable of causing weight loss in earthworms. This usually seems to happen in a high amendment rate. For instance, Li et al. [24] reported significant weigh loss in *E. fetida* after exposure to 10 and 20% apple wood sawdust biochar-amended soil for 28 days. Liesch et al. [23] reported significant weight loss in *Eisenia fetida* exposed to soil amended with 68 and 90 Mg/ha biochar from poultry litter. According to Li et al. [24], 90 t of biochar/ha at a tillage depth of 10 cm corresponds to 10% amendment. This means that 68 and 90 Mg biochar/ha by Liesch et al. [19] represents 7.55 and 10% amendment. Interestingly, together with the poultry litter biochar, Liesch et al. [23] also tested a biochar prepared from pine chips and found no adverse effects on earthworm biomass at the same amendment rates. These findings suggest that the adverse effects of biochar on earthworms depend not only on the rate of amendment but also on the feed used to make the biochar.

After a 96-h exposure of *E. fetida* to GLY in the absence of biochar, we found no differences in the AChE level, regardless of the GLY concentrations (Figure 3). This implies that, within the concentration range assessed, GLY alone did not cause any neurological stress to the earthworms. These results are in conformity with those of Zhou et al. [40] who found that AChE activity remained unchanged after a 48-h exposure of *E. fetida* to GLY concentration as high as 2000 mg/L. Owagboriaye et al. [41] also observed no significant differences in AChE activity in three earthworm species (*Alma millsoni, Eudrilus eugeniae,* and *Lybyodrilus violaceus*) contained in pots planted with weeds (*Tridax procumbense, Ludwigia pasturis,* and *Pannicum maximum*) and sprayed with 83.2 g of GLY/m^2. Contrary, at day 7 of a 28-day experiment, Hackenberger et al. [42] found a significant increase in relative AChE activity in the earthworm *Dendrobaena veneta* exposed to GLY at concentrations overlapping those used in our study (i.e., $\leq$2160 µg/kg). By day 28, however, this initial increase in activity vanished. Wang et al. [18] also observed significant increases at day 3, 7, and 14 in the AChE activity of *E. fetida* exposed to GLY concentrations ($\leq$450 mg/kg) but by day 21, no significant differences in AChE activity were observed.

With regard to the effects of biochar on AChE, our results reveal that dramatic increases in AChE levels were only observed in the biochar-amended treatments. In the 15% biochar amendment, a significant increase occurred in the control (in the absence of GLY) and the lowest GLY concentrations (Figure 3). However, no significant differences in AChE were observed in the 5 and 10% biochar amendment. These results show that high biochar amendment (especially 15%) caused a spike in AChE activity even in the negative control. As in our study, Nyoka et al. [20] documented a pattern of low inhibition of AChE (i.e., high AChE activity) in biochar-amended soil contaminated with imidacloprid. As in our case, this lack of inhibition (this high activity), although observed predominantly in the amended treatments, was statistically significant only in the lowest concentration of the toxicant. Nyoka et al. [20] argued that by promoting greater AChE activity, biochar was demonstrating its ability to help reduce the neurotoxic effects of imidacloprid, especially because AChE is the main cholinesterase in earthworms [20]. Similar to imidacloprid, glyphosate is a neurotoxicant capable of inducing nerve cell death via the activation of pathways leading to autophagy and apoptosis [43]. This might justify the similarities found between the two studies.

As in the case of AChE, there were no significant differences in the CAT activity in *E. fetida* after a 96-h exposure to GLY in the absence of biochar (Figure 4). This means that the concentration range of GLY investigated in the present study was not high enough to cause oxidative stress in the test organisms. Our results are similar to those of Hackenberger et al. [42] who found no significant changes in the CAT activity in *Dendrobaena veneta* exposed to GLY ($\leq$2160 µg/kg) for up to 28 days. Contrary, Owagboriaye et al. [41] found that GLY exposure significantly increased the CAT activity in the earthworms *Alma millsoni, Eudrilus eugeniae,* and *Lybyodrilus violaceus* exposed in pots with weed plants sprayed with 83.2 g GLY/m^2.

Our CAT results further revealed a single significant increase in CAT activity in the 8 mg/kg treatment amended with 10% biochar (Figure 4). As in the case of AChE, Figure 4 revealed that in the presence of biochar, CAT activity increased especially in the 10 and 15% amendment. Nyoka et al. [20] also found that in the presence of biochar, CAT activity in *E. fetida* exposed to imidacloprid were much higher than in the non-amended treatments. Similarly, Huang et al. [44] reported greater CAT activity in *E. fetida* exposed to metal contamination in biochar-amended soil than in non-amended, and that a month after the start of the exposure CAT levels were back to pre-exposure levels. Nyoka et al. [20] argued that such significant increases in the biochar-amended treatment, when there seemed to be a CAT inhibition in the non-amended treatments, showed the beneficial effects of biochar amendment.

It is worth noting that as in the case of AChE, Figure 4 showed dramatic increases in CAT activity in the biochar-amended control, in the absence of GLY. Han et al. [45] reported no significant differences in the CAT activity of *E. fetida* exposed to 0–5% biochar from rice straws but found skin change (from 2.5% amendment) and digestive deterioration (at 5% amendment). Contrary, Shi et al. [26] who looked at the effects of biochar from cow dung on *E. fetida* and found a significant increase in CAT activity in amendment percentages $\leq$5.5%. Such low biochar amendment rates did not cause any significant variations in CAT in our study, pointing to the fact that different biochar feeds may influence how such endpoints are impacted. Shi et al. [26] also found that a temperature pyrolysis of 550 °C caused much higher CAT activity than 350 and 750 °C. A similar study by Kim et al. [25] investigated the effects of biochar prepared from perilla, sesame, and pumpkin seed biomass on the earthworm *E. fetida.* These authors also found that at 5% amendment, the biochar prepared at a pyrolysis temperature of 550 °C caused higher CAT activity than the one pyrolyzed at 300 °C. In comparison, the biochar used in the present study was produced at 400–450 °C falling within the temperature bracket inducing comparatively lower CAT activity (although, our pine tree biochar did cause noticeable CAT increases on its own, Figure 4). It is believed that the pyrolysis temperature of biochar influences its physical and chemical attributes, including surface area and pore size distribution which have an impact on the sorption rate of chemicals [46,47].

Beside oxidative stress and neurotoxic effect, biochar has also been found to cause genotoxic effects in earthworms. Zhang et al. [19] studied the combined effects of wheat straw-derived biochar amendment and the herbicide mesotrione on *E. fetida* and reported that 10% biochar amendment caused both a significant decrease in earthworm growth and DNA damage even in the absence of mesotrione. As it was the case for the present study, these authors recorded some beneficial effects of biochar amendment at lower rates (1–3%, especially on earthworm biomass and the alleviation mesotrione toxicity). The growing body of data, therefore, suggests that a biochar amendment of 5% and higher add additional stress to earthworms [23–26,45]. Han et al. [45] went as far as recommending an amendment rate of no more than 1%.

5. Conclusions

Our biomarker results reveal that biochar on its own causes increases in AChE and CAT activity. Although pervious authors have argued that such increases might be beneficial to the organisms, it is worth noting that typically, toxicants are the ones expected to alter these biomarkers in such a manner. Our results indicate that beyond a certain amendment threshold, biochar can cause the sort of neurotoxic and oxidative stress typically induced by “classic toxicants”. Although findings on life cycle parameters indicated no obvious differences between the 5, 10, and 15% biochar amendment rates, the biomarkers shows that amendment rates higher than 5% can be detrimental to these soil organisms. Research evidence so far indicates that both the quality of the feed and the rate of amendment play a role on the ultimate effects of biochar on earthworms. Our experience with Pinetree biochar (pyrolyzed at 400–450 °C) prompts us to recommend amendment rates lower than or equal to 5%.

Author Contributions: Conceptualization, P.V.O.; methodology, P.V.O. and N.P.D.; software, P.V.O.; validation, P.V.O.; formal analysis, P.V.O. and N.P.D.; investigation N.P.D.; resources, P.V.O.; data curation, N.P.D.; writing—original draft preparation, N.P.D.; writing—review and editing, P.V.O.; visualization, P.V.O. and N.P.D.; supervision, P.V.O.; project administration, P.V.O.; funding acquisition, P.V.O. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported financially by the Afromontane Research Unit (University of the Free State, South Africa). Any opinion, conclusion and recommendations expressed in this work are those of the authors and are not to be attributed to the Afromontane Research Unit.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of The University of the Free State, Project Number: UFS-AED2016/0067, approved on 3 May 2016.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data and calculations tools are available on request from the corresponding author otomopv@ufs.ac.za.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mensah, P.K.; Palmer, C.G.; Muller, W.J. Derivation of South African water quality guidelines for Roundup using species sensitivity distribution. *Ecotoxicol. Environ. Saf.* **2013**, *96*, 26–31. [CrossRef]
2. Maharaj, S. *Modelling the Behaviour and Fate of Priority Pesticides in South Africa. Master of Science in Environmental and Water Science. Department of Earth Sciences*; University of the Western Cape: Cape, South Africa, 2005. Available online: http://etd.uwc.ac.za/xmlui/bitstream/handle/11394/1583/Maharaj_MSC_2005.pdf?sequence=1 (accessed on 2 October 2021).
3. Dalvie, M.A.; Naik, I.; Channa, K.; London, L. Urinary dialkyl phosphate levels before and after first season chlorpyrifos spraying amongst farm workers in the Western Cape, South Africa. *J. Environ. Sci. Health Part B* **2011**, *46*, 163–172. [CrossRef]
4. Martin, N.A. The effects of herbicides used on asparagus on the growth rate of the earthworm *Allobophora caliginosa*. In Proceedings of the 34th New Zealand Weed and Pest Control Conference, Blenheim, UK, 11–13 August 1981; pp. 328–331.
5. Springett, J.A.; Gray, R.A.J. Effect of repeated low doses of biocides on the earthworm *Aporrectodea caliginosa* in laboratory culture. *Soil Biol. Biochem.* **1992**, *24*, 1739–1744. [CrossRef]
6. Morowati, M. Histochemical and histopathological study of the intestine of the earthworm (*Pheretima elongata*) exposed to a field dose of the herbicide glyphosate. *Environmentalist* **2000**, *20*, 105–111. [CrossRef]
7. Santadino, M.; Coviella, C.; Momo, F. Glyphosate sublethal effects on population dynamics of the earthworm *Eisenia fetida*. *Water Air Soil Pollut.* **2014**, *225*, 2207. [CrossRef]
8. Relyea, R.A. The lethal impact of roundup on aquatic and terrestrial amphibians. *Ecol. Appl.* **2005**, *15*, 1118–1124. [CrossRef]
9. Berger, G.; Graef, F.; Pfeffer, H. Glyphosate applications on arable fields considerably coincide with migrating amphibians. *Sci. Rep.* **2013**, *3*, 2622. [CrossRef] [PubMed]
10. Brühl, C.A.; Schmidt, T.; Pieper, S.; Alscher, A. Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline. *Sci. Rep.* **2013**, *3*, 1135. [CrossRef] [PubMed]
11. Yadav, S.S.; Giri, S.; Singha, U.; Boro, F.; Giri, A. Toxic and genotoxic effects of Roundup on tadpoles of the Indian skittering frog (*Euflictis cyanophlyctis*) in the presence and absence of predator stress. *Aqua. Tox.* **2013**, *132–133*, 1–8. [CrossRef]
12. Mensah, P.K.; Muller, W.J.; Palmer, C.G. Using growth measures in the freshwater shrimp *Caridina nilotica* as biomarkers of Roundup® pollution of South African freshwater systems. *Phys. Chem. Earth Parts A/B/C* **2012**, *50–52*, 262–268. [CrossRef]
13. Zaller, J.G.; Heig, L.F.; Ruess, L.; Grabmaier, A. Glyphosate herbicide affects belowground interactions between earthworms and symbiotic mycorrhizal fungi in a model ecosystem. *Sci. Rep.* **2014**, *4*, 5634. [CrossRef] [PubMed]
14. Xu, G.; Lv, Y.; Sun, J.; Shao, H.; Wei, L. Recent advances in biochar applications in agricultural soils: Benefits and environmental implications. *Clean* **2012**, *40*, 1093–1098. [CrossRef]
15. Khorram, M.S.; Zhang, Q.; Lin, D.; Zheng, Y.; Fang, H.; Yu, Y. Biochar: A review of its impact on pesticide behavior in soil environments and its potential applications. *J. Environ. Sci.* **2016**, *44*, 269–279. [CrossRef] [PubMed]
16. Yu, X.-Y.; Ying, G.-G.; Kookana, R.R. Reduced plant uptake of pesticides with biochar additions to soil. *Chemosphere* **2009**, *76*, 665–671. [CrossRef]
17. Nyoka, N.W.-K.; Kanyile, S.N.; Bredenhand, E.; Prinsloo, G.J.; Voua Otomo, P. Biochar alleviates the toxicity of imidacloprid and silver nanoparticles (AgNPs) to *Enchytraeus albidus* (Oligochaeta). *Environ. Sci. Pollut. Res.* **2018**, *25*, 10937–10945. [CrossRef] [PubMed]
18. Wang, Y.; Cang, T.; Zhao, X.; Yu, R.; Chen, L.; Wu, C. Comparative acute toxicity of twenty-four insecticides to earthworm, *Eisenia fetida*. *Ecotoxicol. Environ. Saf.* **2012**, *79*, 122–128. [CrossRef] [PubMed]
19. Zhang, Q.; Saleem, M.; Wang, C. Effects of biochar on the earthworm (*Eisenia foetida*) in soil contaminated with and/or without pesticide mesotrione. *Sci. Total Environ.* **2019**, *671*, 52–58. [CrossRef]

20. Nyoka, N.W.-K.; Ogbeide, O.; Voua Otomo, P. Reproduction and biomarker responses of *Eisenia fetida* after exposure to imidacloprid in biochar-amended soil. *Biochar* **2021**, *3*, 615–624. [CrossRef]
21. Graber, E.R.; Tsechansky, L.; Gerstl, Z.; Lew, B. High surface area biochar negatively impacts herbicide efficacy. *Plant Soil* **2011**, *353*, 95–106. [CrossRef]
22. Graber, E.R.; Tsechansky, L.; Khanukov, J.; Oka, Y. Sorption, volatilization and efficacy of the fumigant 1,3-dichloropropene in a biochar-amended soil. *Soil Sci. Soc. Am. J.* **2011**, *75*, 1365–1373. [CrossRef]
23. Liesch, A.M.; Weyers, S.L.; Gaskin, J.W.; Das, K.C. Impact of two different biochars on earthworm growth and survival. *Ann. Environ. Sci.* **2010**, *4*, 1–9.
24. Li, D.; Hockaday, W.; Masiello, C.; Alvarez, P.J.J. Earthworm avoidance of biochar can be mitigated by wetting. *Soil Biol. Biochem.* **2011**, *43*, 1732–1737. [CrossRef]
25. Kim, W.I.; Kunhikrishnan, A.; Go, W.R.; Jeong, S.H.; Kim, G.J.; Lee, S.; Yoo, J.H.; Cho, N.; Lee, J.H. Influence of various biochars on the survival, growth, and oxidative DNA damage in the earthworm *Eisenia fetida*. *Korean J. Environ. Agric.* **2014**, *33*, 231–238. [CrossRef]
26. Shi, Z.; Yan, J.; Ren, X.; Wen, M.; Zhao, Y.; Wang, C. Effects of biochar and thermally treated biochar on *Eisenia fetida* survival, growth, lysosomal membrane stability and oxidative stress. *Sci. Total Environ.* **2021**, *770*, 144778. [CrossRef]
27. OECD. *Organization for Economic Cooperation and Development, Earthworm Reproduction Test*; Guideline for Testing Chemicals no. 222; OECD Publishing: Paris, France, 2004.
28. Stanley, O.N.; Joy, O.M. Histopathological Effects of Glyphosate and Its Toxicity to the Earthworm *Nsukkadrilus mbae*. *Brit. Biotech. J.* **2013**, *4*, 149–163. [CrossRef]
29. Ellman, G.L.; Courtney, K.D.; Andres, V., Jr.; Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **1961**, *7*, 88–95. [CrossRef]
30. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
31. Cohen, G.; Dembiec, D.; Marcus, J. Measurement of catalase activity in tissue extracts. *Anal. Biochem.* **1970**, *34*, 30–38. [CrossRef]
32. García-Torres, T.; Giuffré, L.; Romaiuk, R.; Ríos, R.P.; Pagano, E.A. Exposure Assessment to Glyphosate of Two Species of Annelids. *Bull. Environ. Contam. Toxicol.* **2014**, *93*, 209–214. [CrossRef]
33. Stellin, F.; Gavinellib, F.; Stevanatoa, P.; Concheria, G.; Squartinia, A.; Paoletti, M.G. Effects of different concentrations of glyphosate (Roundup 360®) on earthworms (*Octodrilus complanatus, Lumbricus terrestris and Aporrectodea caliginosa*) in vineyards in the North-East of Italy. *Appl. Soil. Ecol.* **2018**, *123*, 802–808. [CrossRef]
34. Piola, L.; Fuchs, J.; Oneto, M.L.; Basack, S.; Kesten, E.; Casabe, N. Comparative toxicity of two glyphosate-based formulations to *Eisenia andrei* under laboratory conditions. *Chemosphere* **2013**, *91*, 545–551. [CrossRef]
35. Jing, X.; Wang, T.; Yang, J.; Wang, Y.; Xu, H. Effects of biochar on the fate and toxicity of herbicide fenoxaprop-ethyl in soil. *R Soc. Open Sci.* **2018**, *5*, 171875. [CrossRef]
36. Yasmin, S.; D'Souza, D. Effect of pesticides on the reproductive output of *Eisenia fetida*. *Bull. Environ. Contam. Toxicol.* **2007**, *79*, 529–532. [CrossRef] [PubMed]
37. Correia, F.V.; Moreira, J.C. Effects of glyphosate and 2,4-D on earthworms (*Eisenia fetida*) in laboratory tests. *Bull. Environ. Contam.Toxicol.* **2010**, *85*, 264–268. [CrossRef]
38. Santos, M.J.G.; Ferreira, M.F.L.; Cachada, A.; Duarte, A.C.; Sousa, J.P. Pesticide application to agricultural fields: Effects on the reproduction and avoidance behaviour of *Folsomia candida* and *Eisenia Andrei*. *Ecotoxcicology* **2012**, *21*, 2113–2122. [CrossRef]
39. Pochron, S.T.; Mirza, A.; Mezic, M.; Chung, E.; Ezedum, Z.; Geraci, G.; Mari, J.; Meiselbach, C.; Shamberger, O.; Smith, R.; et al. Earthworms *Eisenia fetida* recover from Roundup exposure. *Appl. Soil Ecol.* **2021**, *158*, 103793. [CrossRef]
40. Zhou, C.F.; Wang, Y.J.; Yu, Y.C.; Sun, R.J.; Zhu, X.D.; Zhang, H.L.; Zhou, D.M. Does glyphosate impact on Cu uptake by, and toxicity to, the earthworm *Eisenia fetida*? *Ecotoxicology* **2012**, *21*, 2297–2305. [CrossRef]
41. Owagboriaye, F.; Dedeke, G.; Bamidele, J.; Aladesida, A.; Isibor, P.; Feyisola, R.; Adeleke, M. Biochemical response and vermiremediation assessment of three earthworm species (*Alma millsoni, Eudrilus eugeniae and Libyodrilus violaceus*) in soil contaminated with a glyphosate-based herbicide. *Ecol. Indic.* **2020**, *108*, 105678. [CrossRef]
42. Hackenberger, D.K.; Stjepanović, N.; Lončarić, Z.; Hackenberger, B.K. Acute and subchronic effects of three herbicides on biomarkers and reproduction in earthworm *Dendrobaena veneta*. *Chemosphere* **2018**, *208*, 722–730. [CrossRef] [PubMed]
43. Gui, Y.X.; Fan, X.N.; Wang, H.M.; Wang, G.; di Chen, S. Glyphosate induced cell death through apoptotic and autophagic mechanisms. *Neurotoxicol. Teratol.* **2012**, *34*, 344–349. [CrossRef] [PubMed]
44. Huang, K.; Chen, J.; Guan, M.; Xia, H.; Li, L. Effects of biochars on the fate of antibiotics and their resistance genes during vermicomposting of dewatered sludge. *J. Hazard. Mater.* **2020**, *397*, 12767.
45. Han, J.; Huang, Y.; Meng, J.; Fan, C.; Yang, F.; Tan, H.; Zhang, J. Exposure of earthworm (*Eisenia fetida*) to rice straw biochar: Ecotoxicity assessments for soil-amended programmes. *Sci. Total Environ.* **2021**, *794*, 148802. [CrossRef] [PubMed]
46. Yu, X.-Y.; Mu, C.-L.; Gu, C.; Liu, C.; Liu, X.-J. Impact of woodchip biochar amendment on the sorption and dissipation of pesticide acetamiprid in agricultural soils. *Chemosphere* **2011**, *85*, 1284–1289. [CrossRef] [PubMed]
47. Tang, J.; Zhu, W.; Kookana, R.; Katayama, A. Characteristics of biochar and its application in remediation of contaminated soil. *J. Biosci. Bioeng.* **2013**, *116*, 653–659. [CrossRef] [PubMed]