Microbial Ecotoxicity of Biochars in Agricultural Soil and Interactions with Linear Alkylbenzene Sulfonates

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Abstract: Large-scale application of biochar on agricultural land offers the prospect of soil improvement and carbon sequestration for climate-change mitigation. However, negative side-effects on the soil microbial ecosystem are poorly understood, notably in relation to the functions of native microorganisms under realistic routes of biochar exposure. Due to divergent properties, different biochars might interact with soil in complex ways. This might result in decreased or increased ecotoxicity from biochar contaminants, such as heavy metals and polycyclic aromatic hydrocarbons (PAHs). Using five biochars produced from straw and wood under contrasting pyrolysis conditions, we traced their ecotoxicological dose-effect using a bioassay for potential ammonia oxidation (PAO), through microorganisms that are sensitive stress indicators. Assays were made after soil/biochar interaction for up to 3 weeks, where straw biochar with the lowest PAH content (<0.5 mg kg\(^{-1}\)) showed the most pronounced dose-effects to PAO, corresponding to a 10% effect concentration (EC\(_{10}\)) of 4.6% (dry weight biochar/dry weight soil). In comparison, straw biochar with the highest PAH content was least ecotoxic (EC\(_{10}\), 15.2% after 3 weeks) and wood biochars pyrolysed at high temperature (700–725 °C) showed no ecotoxicity to PAO. Interactions between biochars and anionic surfactants, i.e., linear alkylbenzene sulfonates, which are common soil pollutants, resulted in varying effects on PAO, but the effects were small and of limited ecological importance for soil-amended biochars. In conclusion, the results showed that short-term microbial side-effects of biochar in the soil ecosystem were minor at relevant field application rates (such as <30 Mg ha\(^{-1}\) mixed into a plough layer of 20 cm), and that inherent PAHs in biochar were not a likely source of short-term ecotoxicity. However, there were notable differences in the effects eventually observed at very high biochar rates, stressing that individual biochars need specific ecotoxicological assessment before their safe application at large scale in agricultural soils.

Keywords: biochar; soil; ecotoxicity; LAS; ammonia oxidation; microorganisms; PTE

1. Introduction

Biochars are solid carbonaceous residues from pyrolysed biomass, holding the potential to enhance ecosystem services in agricultural soils, such as water holding capacity, aggregate stability, and nutrient availability [1,2]. However, it is difficult to generalize the strength of these effects, because biochar properties depend on feedstocks and pyrolysis conditions [3,4]. Furthermore, the effect of individual biochars is modulated by edaphic factors, climate, and agricultural management [5]. A common finding is that biochar is more likely to stimulate ecosystem services in deprived soils than in fertile soils and well-managed cropping systems [6,7]. However, even in fertile soils, biochar application can be pursued to improve greenhouse gas balances, as biochar contributes to long-term carbon (C) storage [8,9] and might reduce soil emissions of nitrous oxide (N\(_2\)O), which often compromise the environmental sustainability of intensive cropping systems [9].

Any large-scale application of biochar to agricultural soils must consider the possible negative side-effects on biological processes, due to the potentially toxic elements
(PTEs) in biochar. These PTEs include heavy metals resulting from the feedstock, and polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds originating from re-condensation of pyrolysis liquids and gases [10,11]. Several studies of biochar ecotoxicity targeted soil mesofauna and earthworms, where varying effects on avoidance, reproduction, and mortality are reported [12–15]. These endpoints represent important behavioral and integral responses of soil animals, but should be supplemented by responses of soil micro-organisms, which are sensitive key organisms in soil ecosystem functioning and nutrient cycling [16–18]. Classical microbial ecotox tests have been performed with isolated bacteria exposed to water extracts of biochars [15], for example, Microtox® assays with Vibrio fisheri that show 12–99% inhibition of metabolic activity [19]. Yet, such data are challenging to interpret in an ecological context, since microbial responses in complex soil ecosystems depend on native microbiomes and realistic routes of toxicant exposure [18,20]. Therefore, ecotoxicity tests with biochar-amended soils is a necessary step to improve our understanding of the ecological and environmental impacts of biochar application.

In contrast to their potential ecotoxicity, many biochars are reported to mitigate harmful effects of PTEs already present in the soil [21]. This is explained by the high cation exchange capacity and surface properties of biochar, leading to immobilization and reduced bioavailability of cationic PTEs like heavy metals [22]. Little is known, however, about the interactions between biochar and anionic PTEs, although complex sorption mechanisms, involving, e.g., internal porosity [23,24], suggest that biochar might reduce anionic PTE availability in the soil. Surfactants from high-tonnage household detergents, in particular linear alkylbenzene sulfonates (LAS), are common anionic PTEs in sewage sludge [25] that is applied to agricultural soils, where LAS can be harmful to soil organisms and microbial processes [26]. However, there are no studies showing if biochars can reduce the bioavailability and harmful effects of LAS in soil or, in contrast, if LAS might increase the ecotoxicity of biochars due to surfactant-mediated desorption of PTEs.

Here, we report the ecotoxicity of five biochars, after their addition to soil, using a bioassay for microbial ammonia (NH₃) oxidation. The assay quantifies potential ammonia oxidation (PAO), mediated by chemoautotrophic bacteria and archaea that provide an early and sensitive response to chemical soil stresses and play an essential role in the biogeochemical cycling of soil nutrients [27–29]. Further, we assessed the effect of biochar on the ecotoxicity of anionic LAS surfactants, which are known to inhibit microbial ammonia oxidizers, in particular [30].

### 2. Materials and Methods

#### 2.1. Soil and Biochars

Soil was sampled at the Foulumgaard Experimental Station (Foulum, Denmark, 56°30’ N, 9°34’ E) from a field in arable rotation with annual crops, including winter wheat (Triticum aestivum L.) and spring barley (Hordeum vulgare L.). The Foulum soil was characterized as a Mollic Luvisol according to the WRB soil taxonomy [31], and represents a sandy loam with 7.6–8.8% clay, 10–13% silt, 74–79% sand, 1.7–1.9% organic C, and pH(CaCl₂) of 5.4 [32,33]. Topsoil (0–10 cm) was sampled at two occasions (November 2010 and September 2013) from more than 10 sampling points across the field, after the crop harvest. The composite samples (>10 kg) were sieved (<4 mm) and homogenized using a cone-and-quarter technique [34], after removal of the visible roots and plant parts. The soil was stored field-moist at 2°C and was used for ecotoxicalogical assays within five weeks after sampling [35].

Biochars were obtained by thermal conversion of feedstocks from wheat straw (SB1, SB2), pine wood (WB1, WB2), and oak wood (WB3). Thermal conversion conditions and physico-chemical properties of the biochars are shown in Table 1. Particle sizes of the bio-chars were homogenized to pass a 4-mm sieve after crushing, if needed.
Table 1. Pyrolysis conditions and chemical composition for the five biochars from wheat straw (SB1, SB2), pine wood (WB1, WB2), and oak wood (WB3). Chemical analyses were performed by certified laboratories (WESSLING, Hungary; FORCE Technology, Denmark; AGROLAB, Germany); na—not available.

| Property          | Unit   | SB1 (Dry wt.) | SB2 (Dry wt.) | WB1 (Dry wt.) | WB2 (Dry wt.) | WB3 (Dry wt.) |
|-------------------|--------|---------------|---------------|---------------|---------------|---------------|
| Provenance        | -      | DK            | DK            | DE            | FR            |
| Pyrolysis type    | -      | slow          | flash         | slow          | slow          |
| Residence time    | -      | ~1 h          | few s         | ~1 h          | na            |
| Pyrolysis temperature | -     | 725 °C        | 750 °C        | 725 °C        | 700 °C        |
| Ash %             | %      | 16.3          | na            | 1.8           | na            |
| Carbon (C) %      | %      | 79.8          | 91.2          | 81.0          | 81.1          |
| Nitrogen (N) %    | %      | 0.53          | 0.65          | 0.42          | <0.08         |
| Phosphorous (P) g kg⁻¹ | 1.2   | 3.4           | 0.2           | 1.6           | 0.4           |
| Potassium (K) g kg⁻¹ | 35   | 34            | 2.3           | 7.0           | 3.0           |
| pH                | -      | 10.2          | 8.5           | 9.6           | 9.4           | 9.5           |
| ΣPAH ¹ mg kg⁻¹    |        | 385           | 0.9           | 68            | 0.7           | 10.3          |
| Cadmium (Cd) mg kg⁻¹ | 0.03  | <0.01         | <0.01         | <1            | <0.03         |
| Chromium (Cr) mg kg⁻¹ | 1.0   | 14.7          | 0.53          | 26            | 9             |
| Mercury (Hg) mg kg⁻¹ | <0.02 | <0.02         | <0.02         | <1            | 0.04          |
| Nickel (Ni) mg kg⁻¹ | 0.6   | 29.7          | 0.44          | 17            | 13            |
| Lead (Pb) mg kg⁻¹ |        | 0.3           | <3.0          | 1.4           | 7             |

¹ Sum of 16 US EPA polycyclic aromatic hydrocarbons (PAH). The high PAH concentrations in SB1 and WB1 were mainly due to phenanthrene with 161 and 37 mg kg⁻¹, respectively.

2.2. Dose-Effects of Biochar on Potential Ammonia Oxidation

For SB1 and WB1, the dose-effects on PAO were tested after biochar addition to field-moist soil (110 g) at rates of 0, 0.4, 0.7, 2.2, 5.9, 15, and 37% (wt./wt.; calculated as dry weight biochar/dry weight soil), where the highest biochar concentrations, far beyond realistic field application rates, were included to obtain data points at the lower response level (i.e., low levels of PAO). The soil and biochar was mixed in 1-L glass jars (n = 3) using a spatula, and PAO was measured after 1 day, 1 week, and 3 weeks of soil/biochar interaction (closed jars, 20 °C, darkness). Soil water content during incubation was monitored by weighing (relative water loss, <5%) and soil pH(CaCl₂) was measured by electrode at the final sampling [36]. Assays of PAO were performed using the chlorate (ClO₃⁻) inhibition technique of Belser and Mays [37], following the protocol of Elsgaard et al. [38]. In brief, subsamples of 5 g soil were suspended in 100 mL of PAO buffer (0.5 mM (NH₄)₂SO₄ in 1 mM K₂HPO₄; pH 7.2), where 1 mL of 1 M NaClO₃ was added to inhibit nitrite (NO₂⁻) oxidation. After 15 min (T₁) and again after 4 h (T₂), 3 mL of soil suspension was withdrawn, mixed with 3 mL of 4 M KCl (to stop microbial activity), centrifuged (3000 × g, 5 min), and filtered (0.45 μm). The concentration of NO₂⁻ in the filtrate was measured colorimetrically [38] and rates of PAO (nmol NO₂⁻ g⁻¹ dry wt. soil h⁻¹) were calculated from the amount of NO₂⁻ produced from T₁ to T₂, after (negligible) correction for blanks without soil.

For SB2, WB2, and WB3, the dose-effects on PAO were tested after biochar addition to field-moist soil (200 g) at rates of 0, 0.6, 1.1, 2.8, 5.6, and 11% (wt./wt.), which were selected on the basis of results of the assays with SB1 and WB1. The soil/biochar treatments were incubated in closed 1-L glass jars (n = 2), as described above, and the assays of PAO were performed with subsamples taken after 2 days, 1 week, and 3 weeks of soil/biochar interaction.

2.3. Effect of Biochar on LAS Ecotoxicity

Linear alkylbenzene sulfonate (Marlon A350; Hüls AG, Marl, Germany) was obtained as a stock solution with 50% active substance (C₁₀-C₁₃ LAS) with an average alkyl chain length of 11.7 C atoms and average molar mass of 344 g mol⁻¹ (Figure 1). The stock solution
was gently heated (35 °C) and swirled, before subsamples were diluted in demineralized water to nominal concentrations of 0.87, 1.75, 3.5, 7.0, 14, and 28 mg LAS mL$^{-1}$.

Figure 1. Structure of the anionic surfactant linear alkylbenzene sulfonate (LAS) with hydrophobic (alkyl chain) and hydrophilic (sulfonate) moieties. The C$_{10}$–C$_{13}$ LAS homologues are characterized by $x + y = 7–10$.

The effect of biochar on LAS ecotoxicity was tested with field-moist soil (200 g) with or without 1.1% biochar (SB1, SB2, WB1, WB2) that was added as previously described. The concentration of 1.1% biochar was chosen to represent a high realistic field application rate, corresponding to ca. 30 Mg ha$^{-1}$ for biochar incorporation to a tillage depth of 20 cm in arable soil, with a bulk density of 1.35 g cm$^{-3}$. Biochar and soil was mixed in 1-L glass jars and 2 mL of LAS (or H$_2$O) was added dropwise to the resulting nominal concentrations of 0, 10, 20, 40, 80, 160, and 320 mg LAS kg$^{-1}$ dry wt. soil ($n = 3$). The soil treatments were mixed (using a spatula), incubated for 1 week (20 °C), and then subsampled for assays of PAO.

In a separate assay, the acute ecotoxicity of LAS to PAO was tested by (i) adding LAS directly to soil suspended in 100 mL PAO buffer, and by (ii) adding the same amount of LAS to SB1 biochar, which was then added to 100 mL PAO buffer with soil. In practice, counterparts of 15 g field-moist soil in 100 mL of PAO buffer ($n = 3$) were amended with either (i) 150 µL of LAS solutions (0, 0.87, 1.75, 3.5, 7.0, 14, and 28 mg LAS mL$^{-1}$) or with (ii) samples of SB1 biochar (0.15 g) that were first equilibrated (15 min) with 150 µL of the seven LAS solutions ($n = 3$ for all treatments). The resulting nominal concentrations in the PAO assays corresponded to 0, 10, 20, 40, 80, 160, and 320 mg LAS kg$^{-1}$ dry wt. soil, with or without 1.1% biochar.

2.4. Calculations and Statistics

Calculations of 10 and 50% effects concentrations (EC$_{10}$ and EC$_{50}$, respectively) were performed using the extension package drc for the statistical environment R [39]. Dose-responses were fitted with a log-logistic model (Equation (1)):

$$f(x) = c + \frac{d - c}{1 + \exp(b \times (\log x - \log E))}$$  \hspace{1cm} (1)

where $x$ is the dose, $d$ is the maximum response, $b$ is the slope around the inflection point, and $E$ is the response halfway between the upper limit $d$ and lower limit $c$ [40]. When hormesis was indicated (i.e., stimulatory effect at sub-inhibitory doses), a modified model was used (Equation (2)):

$$f(x) = c + \frac{d - c + f \times \exp(-1/x^a)}{1 + \exp(b \times (\log x - \log E))}$$  \hspace{1cm} (2)
where $\alpha$ describes the rate at which hormesis was manifested, and $f$ measures the rate of stimulation at doses close to zero [41]. The lower limit, $c$, was set to 0 in both models and the upper limit, $d$, was set to 100 in Equation (1), resulting in response boundaries from 0 to 100% in this model.

Effect concentrations were compared between treatments, using the confidence interval (CI) ratio test [42]:

$$CI\ ratio = EC_D \pm (F \times SE_D)$$

where $EC_D$ is the ratio between the two compared EC values, $F$ is the degrees-of-freedom-corrected 95% quantile, calculated with the Welch-Satterthwaite formula [43], and $SE_D$ is the standard error (SE) of the difference between the two compared EC values calculated using the delta method [44]. If the CI ratio interval included the value 1, the EC values were considered not to be different with approximately 95% certainty [42].

In Equations (1) and (2), it was assumed that measurement errors were independent, normally distributed, and homoscedastic. This was found to be adequately satisfied by inspection of residuals and quantile plots for each model fit [45].

3. Results

3.1. Dose-Effects of Biochar on Potential Ammonia Oxidation and pH

The five biochars had different dose-effects on PAO (Figure 2) in terms of both initial effects (1–2 days after biochar amendment) and effects after soil/biochar interaction for 1 and 3 weeks, as indicative of apparent recovery or persistence of stress.

Initial biochar effects were negligible at the lowest concentration tested (Figure 2), but at higher concentrations, SB1, SB2, and WB3 caused significant decreases in PAO. Yet, for SB1, a slight hormetic effect occurred, whereas inhibitory effects were absent for WB1 and WB2, even at the highest biochar concentrations.

The patterns of dose-effects after soil/biochar interaction for 1 and 3 weeks were mostly similar to the initial effects for each biochar (Figure 2). However, for SB2 the initial decrease in PAO at intermediate biochar levels (2.8–5.6%) showed an apparent recovery after 1 and 3 weeks (Figure 2d–f).

Modeling of the initial dose-effects for SB1, SB2, and WB3 indicated $EC_{10}$ values of 23.8, 1.3, and 2.6%, respectively, with $EC_{50}:EC_{10}$ ratios of 2.4, 6.8, and 12.3 (Table 2). The high EC values for SB1 were caused by the hormetic effect at intermediate biochar concentrations of 2.2–5.9% (Figure 2), but this effect gradually decreased with time after biochar amendment. Dose-effects of WB3 never exceeded 50% PAO inhibition and the high $EC_{50}$ was therefore based on extrapolation. For WB1 and WB2, it was unfeasible to model dose-effects, as the biochars caused no inhibition of PAO. Accordingly, $EC_{10}$ and $EC_{50}$ were considered to exceed the highest doses tested, which were 37% for WB1 and 11% for WB2 (Table 2).

Table 2. Effect concentrations ($EC_{10}$ and $EC_{50}$) for ecotoxicity of biochars to potential ammonia oxidation (PAO) in agricultural soil. PAO was measured after soil/biochar interaction for 1–2 days, 1 week, and 3 weeks. Small letters indicate significant differences ($p < 0.05$) between biochars and capital letters indicate significant differences between interaction times.

| Biochar        | $EC_{10}$ (%) Biochar, wt./wt. | $EC_{50}$ (%) Biochar, wt./wt. |
|----------------|-------------------------------|---------------------------------|
|                | 1–2 d | 1 Week | 3 Weeks | 1–2 d | 1 Week | 3 Weeks |
| Straw biochar, SB1 | 23.8 aA | 16.6 abA | 15.2 ab | 57.1 aA | 43.1 aA | 29.2 abA |
| Straw biochar, SB2 | 1.3 cB   | 4.2 bA   | 4.6 bA | 10.1 bA | 9.5 bA | 8.8 bA   |
| Wood biochar, WB3 | 2.6 bA   | 2.6 bA   | 3.9 bA | 32.0 aA | 56.5 aA | 36.9 aA   |

1 Effect concentrations are shown as weight (wt.) of biochar in percent of soil dry wt.
Figure 2. Dose-effect of biochars on potential ammonium oxidation (PAO) in agricultural soil. PAO assays were made after soil/biochar interaction for 1–2 days ($t_1$; left panels), 1 week ($t_2$; middle panels), and 3 weeks ($t_3$; right panels). Data are shown as percent of the means of uninhibited references (100%). SB1, SB2—straw biochars; WB1, WB2—pine wood biochars; WB3—oak wood biochar.

The fitted model parameters in Equations (1) and (2) were always significant ($p < 0.05$), except for the $E$ parameter ($EC_{50}$ estimation) for WB3 after 1 and 3 weeks of soil/biochar interaction ($p > 0.11$). Too few data points at the lower response level caused this uncertainty.

Soil $pH(CaCl_2)$ systematically increased with increasing rates of biochar, notably for the two straw biochars, SB1 and SB2 (Figure 3). At biochar rates of less than 1.1%, which are realistic for field application to the plough layer (0–20 cm), the $pH$ increase was generally moderate (0.1–0.3 units), yet with an increase of 0.9 $pH$ units for SB2. At the extraordinarily high rates of biochar (>10%), both SB1 and SB2 increased the soil $pH$ to 7.5–8.5. The pine wood biochars WB1 and WB2 caused an intermediate $pH$ response, whereas the low-temperature oak wood biochar WB3 caused minor increases (<0.4 $pH$ units), even at >10% biochar (Figure 3).
Effect of biochar concentration on soil pH after ≥1 week of soil/biochar interaction. Data are means ± standard error (n = 2–3). SB1, SB2—straw biochars; WB1, WB2—pine wood biochars; WB3—oak wood biochar. Soil pH of 8.6 and 6.8 was measured at biochar concentrations of 37% for SB1 and WB1, respectively (data not shown).

3.2. Effect of Biochar on LAS Ecotoxicity

Ecotoxicity of LAS to PAO showed a clear dose-effect (Figure 4a) with EC\textsubscript{10} and EC\textsubscript{50} of 43 and 141 mg LAS kg\textsuperscript{-1}, respectively (Table 3). When LAS was added to the soils amended with 1.1% biochar, the clear dose-effects on PAO persisted (Figure 4b–e), but the EC parameters slightly changed with EC\textsubscript{10} from 36–62 mg LAS kg\textsuperscript{-1} and EC\textsubscript{50} from 119–178 mg LAS kg\textsuperscript{-1} (Table 3). However, only SB2 treatments showed consistently higher EC\textsubscript{10} and EC\textsubscript{50} than the reference soil (p < 0.05), thus, signifying decreased ecotoxicity of LAS (Table 3). For SB1, the EC\textsubscript{10} was higher than that in reference soil, but the differences were not significant for EC\textsubscript{50} (Table 3). The wood biochars WB1 and WB2 caused no decrease in LAS ecotoxicity to PAO (Table 3). Rather, EC\textsubscript{50} for LAS was lower in soil with WB1 than in reference soil, indicating increased LAS toxicity in the presence of WB1 biochar. Yet, this effect was not significant for EC\textsubscript{10}.

| Biochar Addition | EC\textsubscript{10} (mg LAS kg\textsuperscript{-1} Soil) | EC\textsubscript{50} (mg LAS kg\textsuperscript{-1} Soil) | Soil pH (CaCl\textsubscript{2})

\textsuperscript{1} |
|-----------------|---------------------------------|---------------------------------|-----------------|
| Reference (no biochar) | 42.8 \textsuperscript{bc} (±5.0) | 140.5 \textsuperscript{b} (±6.9) | 5.4 \textsuperscript{c} |
| Straw biochar, SB1 | 59.9 \textsuperscript{a} (±6.2) | 146.9 \textsuperscript{b} (±6.2) | 5.7 \textsuperscript{b} |
| Straw biochar, SB2 | 61.9 \textsuperscript{a} (±7.3) | 178.1 \textsuperscript{a} (±8.2) | 6.1 \textsuperscript{a} |
| Wood biochar, WB1 | 35.5 \textsuperscript{c} (±4.8) | 118.6 \textsuperscript{c} (±5.8) | 5.3 \textsuperscript{c} |
| Wood biochar, WB2 | 49.8 \textsuperscript{b} (±5.7) | 151.3 \textsuperscript{b} (±7.2) | 5.7 \textsuperscript{b} |

\textsuperscript{1} Data are means (n = 6–8) across treatments in the absence and presence of the highest dose of LAS (320 mg kg\textsuperscript{-1}). Differences in pH were tested by one-way analysis of variance (ANOVA), followed by post-hoc Holm-Sidak multiple pairwise comparisons with α = 0.05 [45].
Figure 4. Dose-effects of linear alkylbenzene sulfonate (LAS) on potential ammonia oxidation (PAO) in agricultural soil without (a) or with (b–e) added biochar (1.1%, wt./wt.). SB1, SB2—straw biochars; WB1, WB2—pine wood biochars. Assays of PAO were performed after 1 week of soil interaction with biochar and LAS.

Soil pH(CaCl$_2$) in treatments with LAS (0–320 mg kg$^{-1}$) were similar within a range of ±0.15 pH units, showing that LAS had no consistent effects on soil pH (data not shown). Additionally, the soil-amended biochars at concentrations of 1.1% (with or without LAS) only caused minor increases in soil pH (<0.3 pH units), except for SB2, which caused an increase of 0.7 pH units (Table 3).

3.3. Effect of SB1 Biochar on Acute LAS Ecotoxicity

LAS added directly to soil suspensions in PAO buffer (Figure 5a) caused similar dose-effects than after 1 week of preceding LAS/soil interaction (Figure 4a), with EC$_{10}$ of 36 mg LAS kg$^{-1}$ and EC$_{50}$ of 212 mg LAS kg$^{-1}$ (Table 4). When LAS was added to biochar (SB1) prior to the PAO assay (Figure 5b), EC$_{10}$ and EC$_{50}$ were lowered by 44% and 27%, respectively (Table 4), indicating that the LAS/biochar interaction resulted in a higher acute ecotoxicity towards microbial ammonia oxidation.
Figure 5. Acute dose-effects of linear alkylbenzene sulfonate (LAS) and LAS/biochar mixture to potential ammonia oxidation (PAO). LAS was added (a) directly to PAO buffer with soil (LAS/direct) or (b) to SB1 straw biochar, which was then added to PAO buffer with soil (LAS/biochar).

Table 4. Effect concentrations (EC_{10} and EC_{50}) for acute ecotoxicity of linear alkylbenzene sulfonate (LAS) to potential ammonia oxidation (PAO). LAS was added either directly to PAO buffer with soil (LAS/direct) or to SB1 straw biochar, which was then added to PAO buffer with soil (LAS/biochar). Data are model estimates ± standard error. Differences between treatments were significant at p < 0.01.

| LAS Addition   | EC_{10} (mg LAS kg^{-1} Soil) | EC_{50} (mg LAS kg^{-1} Soil) |
|----------------|-------------------------------|-------------------------------|
| LAS/direct     | 36.2 ± 6.3                    | 212 ± 15                      |
| LAS/biochar    | 20.4 ± 3.6                    | 155 ± 11                      |

4. Discussion

Biochar currently attracts attention as a negative carbon emission technology in countries that are committed to ambitious climate goals, such as Denmark, with a goal of 70% reduction in CO_{2} emissions by 2030. Therefore, large-scale application of biochar to agricultural land could become a megatrend. This is further supported by updated EU rules on fertilizing products [46], which will apply from 16 July 2022, making biochar available on the market for soil amendment across the EU, according to compliance with simple quality criteria [47]. Yet, these quality criteria do not include biological endpoints of ecosystem functioning, which is also the case for voluntary biochar standards, such as the European Biochar Certificate [48] and the British Biochar Quality Mandate [49]. It is, therefore, timely to strengthen the research emphasis on ecotoxicological tests and assays with biochar-amended soils both for the short- and long-term [15].

The bioassay applied in the present study (PAO) measured the rate-limiting step of nitrification in soil under conditions of non-limiting availability of electron donors (NH_{3})
and electron acceptors (O₂) for chemoautotrophic energy metabolism of ammonia oxidizers. In unamended reference soils, PAO provides an estimate of nitrifier biomass [50], whereas PAO in toxicant amended soils depends on the nitrifier biomass as well as the direct effects of the added toxicant on the ammonia oxidizing microorganisms.

The present biochars were alkaline and increased the soil pH, which is a general property of most biochars, due to the base cations and inorganic carbonates in the ash fraction [51]. Such liming effects might enhance the growth and activity of ammonia oxidizers, since NH₃, rather than NH₄⁺ (NH₃ + H⁺ ⇌ NH₄⁺, pKₐ = 9.3), is the substrate for ammonia mono-oxygenase, which is the rate-limiting enzyme in nitrification [52], converting NH₃ to hydroxylamine (NH₂OH). It follows that pH increase mediated by biochar to some extent could compensate for the negative effects of biochar PTEs in the soil environment; in our case during the period of soil/biochar interaction (1–2 days to 3 weeks) that preceded the PAO assay, which was buffered to pH 7.2. The role of pH stimulation was likely minor at least for PAO assays after 1–2 days of soil/biochar interaction and for moderate rates of biochar. Nevertheless, the response of PAO should be considered as an integral response to biochar in the soil ecosystem.

4.1. Dose-Effects of Biochar on Potential Ammonia Oxidation

A priori, contents of heavy metals and PAHs are considered as the main PTEs in biochar, which could lead to inhibition of microbial activity. However, heavy metal concentrations in the five tested biochars were generally low and did not exceed various suggested biochar quality standards [47,53] or comparable cut-off limits in Danish regulations, for application of bio-ash or sewage sludge to agricultural soils [54,55]. Moreover, the biochar (WB2) with the highest total content of heavy metals showed no inhibition of PAO, even at the highest tested biochar dose. On the other hand, some of the tested biochars had excessive concentrations of PAHs exceeding the current biochar quality standards by one (WB1) or two orders of magnitude (SB1), notably due to high levels of phenanthrene. These elevated concentrations are unusual but not unprecedented when compared to literature values [56], and might result from improper separation of biomass and vapors during pyrolysis [57]. Nevertheless, these biochars provide a framework of worst-case scenarios to understand microbial ecotoxicity due to PAHs. Yet, SB1 biochar with the highest PAH content showed the lowest ecotoxicity to PAO and even a hormetic effect was indicated. Whether this was caused by PAHs could not be concluded from the data, but hormetic effects of phenanthrene on PAO were previously reported for a number of soils [58], although the mechanism was not elucidated. Wood-based biochars (WB1 and WB2) pyrolysed at high temperature (700–725 °C) also showed no ecotoxicity to PAO, despite the high contents of PAH in WB1. Indeed, the biochar causing the most pronounced dose-effects on PAO was SB2, which had the lowest content of PAHs. In summary, the results of the PAO assays indicated that inherent PAHs in biochar were not a prime source of ecotoxicity. However, some other biochar properties or interactions that were not identified in the present study, might be inhibitory to PAO at very high biochar application rates.

Ecotoxicity of soil contaminants are generally poorly predicted from total concentrations, but depends on the bioavailable or bioaccessible fractions [15]. Previous studies showed strong adsorption of PAH to biochar, interpreted as a result of steric and chemical interactions between the planar PAH molecules and the biochar surface [59]. Thus, the bioavailable fraction might only represent few per mill of the total PAHs in biochar [56,60], which might help explain the weak effects of high-PAH biochars in the present study. Yet, there is limited data on how the bioavailability and bioaccessibility of PAHs and other PTEs might evolve over time in biochar-amended soils. Notably, the bioaccessible fraction, which might become bioavailable (and toxic) as a result of biotic or abiotic interactions, needs to be better documented in longer-term studies, for example in relation to interactions with surface-active compounds in the soil ecosystem [15].
4.2. Ecotoxicity of LAS and Interactions with Biochar

The ecotoxicity of LAS to PAO in Foulum soil (EC$_{50}$, 141 mg LAS kg$^{-1}$) was less severe than previously reported for a Danish coarse sandy soil, where EC$_{50}$ was 40 mg LAS kg$^{-1}$ [38]. Brandt et al. [61] found a similar effect of soil type on LAS toxicity to PAO, with EC$_{50}$ of 149 and 63 mg LAS kg$^{-1}$ for PAO in sandy loam and more sandy soil, respectively, which they attributed to interactions between LAS and clay soil particles or organic matter. Particular sensitivity of ammonia oxidizing bacteria to LAS was substantiated for cultures of *Nitrosomonas* and *Nitrosospira*, which were severely inhibited with EC$_{50}$ in the range of 6–38 mg LAS L$^{-1}$, whereas heterotrophic soil bacteria tolerated up to 300 mg LAS L$^{-1}$ [30]. The high sensitivity of ammonia oxidizing bacteria to LAS was most likely due to the energetic constraints of these autotrophs [62]. As the mechanism of LAS toxicity is known to involve disturbance of the cell membrane function [63,64], causing increased cell permeability and impeded energy metabolism, this might be critical for the energy balance of ammonia oxidizers. However, if the interaction between LAS and cell membranes is reduced by sorption of LAS to biochar (or other soil constituents), the reduced bioavailability would expectedly reduce the toxicity of LAS to ammonia oxidizers, although some studies suggest that the adsorbed LAS could still be inhibitory to microorganisms [61].

Soil-amended biochars showed modest and oppositely directed effects on LAS toxicity to PAO. The straw biochar SB2 consistently decreased LAS toxicity (as seen from both EC$_{10}$ and EC$_{50}$), but interpretation in relation to decreased bioavailability of LAS was inconclusive, since this biochar, in particular, increased the soil pH (from 5.4 to 6.1), which might stimulate microbial ammonia oxidation [52]. Thus far, little is known about sorption of LAS to soil-amended biochar, but adsorption mechanisms of the acid form of C$_{12}$-LAS (dodecylbenzene sulfonic acid) to biochar were described, including partition, anion exchange, and hydrogen bonds [65]. For the present biochars, no general decrease in LAS toxicity was observed, which could otherwise have indicated a sorption of LAS to soil-amended biochar.

The only significant increase in LAS toxicity was caused by WB1, where EC$_{50}$ decreased from 141 to 119 mg LAS kg$^{-1}$ soil, but the EC$_{10}$ was not significantly affected. Thus, even though SB1 and WB1 had aberrantly high concentrations of PAHs [56], this generally did not increase exotoxicity in the presence of LAS, suggesting that ecotoxicity of biochars in the soil ecosystem due to surfactant-mediated desorption of PTEs might be of limited importance. Nevertheless, in the acute toxicity assay, where LAS and biochar was allowed to interact directly, a desorption of PAHs from SB1 biochar could not be excluded as a possible explanation for the increased ecotoxicity to PAO, although this represented a worst-case scenario. In short, we conclude that the assay procedures, which artificially enhanced the conditions for desorption of biochar PTE’s might result in increased ecotoxicity to sensitive microbial bioindicators, but this was not prevalent for soil-amended biochars.

4.3. Ecological Relevance and Conclusions

The present ecotox assays were performed with soil-amended biochars and targeted the native soil ammonia oxidizers, in order to provide an ecologically relevant assessment of biochar effects [61]. Still, the results represented short-term laboratory experiments, which may be uncertain to extrapolate to field conditions. However, a high realistic field application rate of 30 Mg biochar ha$^{-1}$ would correspond to ca. 1.1% (wt./wt.) in the present study, which was below the lowest EC$_{10}$ value (1.3% for initial EC$_{10}$ for SB2). Further, the lowest EC$_{10}$ values after 1–3 weeks of soil/biochar interaction corresponded to 70–105 Mg ha$^{-1}$, which was much higher than the realistic field application rates. Therefore, we conclude that the biochars showed negligible short-term ecotoxicity to ammonia oxidizing microorganisms, at realistic field application rates. In addition, the two high-temperature wood biochars never caused negative effects on PAO, even at extremely high rates corresponding to >200 Mg ha$^{-1}$. These results were in line with field
studies where wood biochar at experimental rates of 10–50 Mg ha\(^{-1}\) showed negligible effects on soil microbial activity, including PAO, after more than 6 months of soil/biochar interaction, and following repeated biochar amendments [66], which could potentially lead to accumulated ecotoxicity that was not addressed in the present study.

In conclusion, using PAO as a microbial bioindicator, we found that negative short-term effects of biochar in the soil ecosystem were minor at relevant field application rates, and that inherent PAHs in biochar was not a likely source of short-term ecotoxicity. This fits with a general absence of data showing that biochar toxicity is determined by PAHs [15].

Interactions between biochar and surface-active anionic LAS contaminant showed varying effects on PAO, but the effects were moderate and of limited ecological consequence. Yet, worst-case assay procedures, which likely facilitated desorption of biochar PTEs, indicated an increased ecotoxicity to PAO, but this was not prevalent for the soil-amended biochars. In their entirety, the data support that biochars at normal rates were of minor risk to the soil microbial ecosystem [15]. However, it was also observed that among the tested biochars there were notable differences in the effects eventually observed at very high rates, stressing that individual biochars need distinct ecotoxicological assessment, also in longer-term studies, before their safe application at a large scale in agricultural soils.

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