Letter

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Cytotoxicity of biochar - a workplace safety concern?

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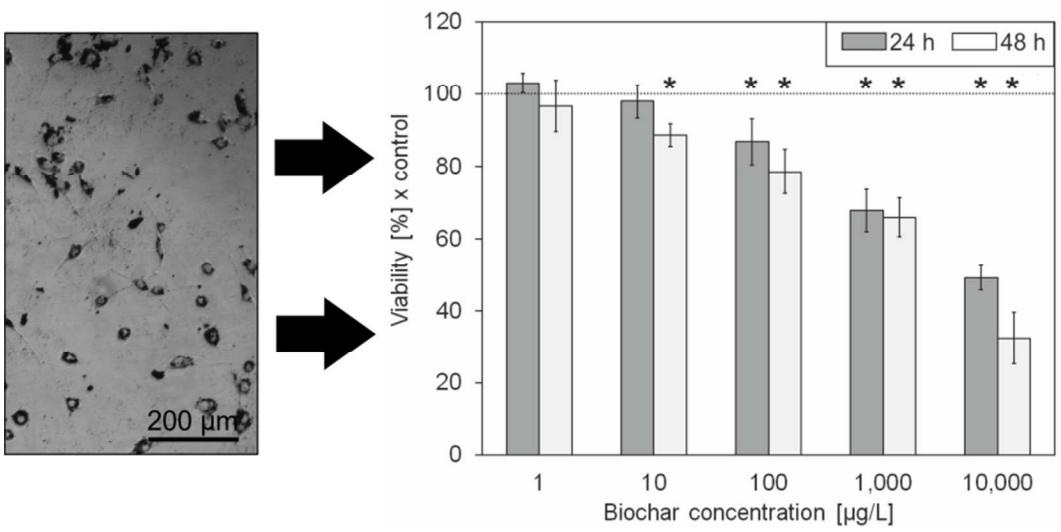

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Abstract

Biochar has a number of environmental applications including soil amendment for agriculture, remediation of contaminated soils and sediments, and climate change mitigation. Dust formed during its production and field application may pose a health risk but the cytotoxicity of biochar has, to our knowledge, not previously been investigated. Therefore, we measured the concentration-dependent cytotoxicity of biochar on an NIH 3T3 mouse fibroblast cell line. We used a contaminant trap experiment to measure the total and non-desorbable PAH fractions of the biochar. PAH release was found to be negligible due to the biochar's strong PAH sorption potential. The biochar was nevertheless observed to have a cytotoxic effect on the fibroblast cells; the EC10 values were 49.6 and 18.8 μg/ml after 24 hours and 48 hours of incubation, respectively. This cytotoxic effect is likely to relate to the particulate nature and size distribution of the biochar; the biochar had similar particle sizes to atmospheric particulate matter (PM2.5) that bound to the fibroblast cell surface. To minimize the risk of exposure, practitioners should wear respiratory protective equipment during biochar production. During field application, biochar should be applied in slurries and should always be mixed with a soil matrix to avoid secondary dust formation.

1. Introduction

Biochar is a carbon-rich product of biomass pyrolysis and is a valuable soil additive that can enhance a soil's water-holding capacity and counteract soil acidification caused by intensive agriculture. In addition, biochar is considered to be an environmentally friendly carbon sink due to its high stability[1]. The use of biochar for remediation of contaminated soils and sediments has also recently been proposed[2]. Due to the growing need to address environmental issues including soil acidification, climate change, and environmental pollution, biochar is expected to be increasingly used in a broad range of situations.

Fine biochar particles that can be dispersed as dust are commonly released during biochar production and application, especially in dry environments where biochar can have substantial agronomic benefits; dust formation may pose a risk to human health. Research on similar materials (carbon nanomaterials and soot) has indicated that agglomeration with cells may play a key role in the toxicity of carbonaceous materials and that biochar dust can be expected to have a cytotoxic effect[3–7]. However, to the best of our knowledge the cytotoxicity of biochar has not been investigated to date.

A European Biochar Certificate (EBC) requires that the total polycyclic aromatic hydrocarbon (PAH) content of biochars should not exceed 12 mg/kg of the US EPA's 16 PAH Priority Pollutants[8]. Hilber et al.[9] have shown that PAH release from biochars that meet EBC quality criteria can generally be expected to be negligible due to the strong PAH sorption potential of biochar. No toxic effect from PAH release would therefore be expected, in contrast to other materials such as soot. Any potential cytotoxicity of biochar may therefore relate instead to its particulate nature and properties.

Fibroblast cell lines such as NIH 3T3 mouse fibroblasts are useful for evaluating the cytotoxic effects of biochar because of their known sensitivity to particulate toxins, such as TiO_2 particles[10] and soot[11]. To investigate possible risks to human health during biochar production and application we therefore measured the concentration-dependent cytotoxicity of a biochar that met EBC criteria on an NIH 3T3 mouse fibroblast cell line.

2. Experimental

2.1 Materials

The UK Biochar Research Center's (UKBRC) standard biochar with the highest nominal PAH content was selected for experimentation (SWP550, Edinburgh, United Kingdom, 4.39 ± 3.45 mg/kg 16 EPA PAHs). The SWP550 biochar is produced from mixed softwood pellets in a rotary kiln pyrolysis unit with a nominal peak temperature of 550°C. Two additional UKBRC biochars produced from *Miscanthus* straw pellet at 550°C and 700°C (MSP550, MSP700) were also used for experimentation. The biochars were crushed using a mortar and pestle and the < 250 µm sieved fraction used for all experiments. A (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT) reduction test kit was purchased from Sigma Aldrich (USA), the NIH 3T3 mouse fibroblast cell line was supplied by ATCC (USA), and Dulbecco's Modified Eagle Medium (DMEM) without phenol red was supplied by Biochrom GmbH (Berlin, Germany).

2.2 PAH composition and bulk properties

A contaminant trap was used in an approach developed by Mayer et al.[12] to determine the biochar's content of the US EPA's 16 PAH Priority Pollutants and its bio-accessible PAH

fraction. In short, the fraction of bio-accessible PAHs in the biochar was quantified as the difference between the total extractable PAHs and the contaminant trap desorption-resistant PAHs in a cyclodextrin suspension following incubation for 30 days. A detailed description of the method used can be found in Sigmund et al.[13] The limit of quantification (LoQ) was < 0.01 mg/kg for each of the all individual US EPA's 16 PAH Priority Pollutants.

Elemental compositions were determined for unmodified biochar and for biochar incubated in DMEM cell culture media (as used in the cell viability assay - see Section 2.3), following previously presented protocols[14]. Specific surface area and pore volume for unmodified biochar and for biochar incubated in DMEM cell culture media (filtered to > 0.45 μm and dried at 105°C) were derived from N_2 and CO_2 physisorption isotherms following degassing overnight at 105°C (Quantachrome Nova 2000 analyzer[15]). The colloidal size distributions for 1, 10, and 100 μg/mL biochar suspensions in DMEM media were determined using an EyeTech particle size and shape analyzer (Ambivalue, Netherlands), based on the time-of-transition principle (laser shading). All suspensions were measured in duplicate and for three measurement cycles (each cycle: > 60 sec and > 95% volume-based confidence). A magnetic stirrer was used to prevent settling of the larger biochar particles during measurement.

2.3 Cell viability assay

Biochar that had previously been autoclaved (at 121°C and 2 bar) was diluted in DMEM media buffered at pH 7.4 to the following concentrations: 0; 1; 10; 100; 1000; and 10000 μg/mL. The biochar stocks in DMEM media were sonicated in an ultrasonic bath for 1 hour to obtain homogeneous particle suspensions and avoid agglomeration. NIH 3T3 cells were seeded with a concentration of 1.0×10^4 cells per well in a 96-well plate. Media with the different biochar concentrations (ranging from 0 to 10,000 μg /mL) were added to the cells and incubated for 24

and 48 hours. Following incubation, the MTT assays were performed, which required 10 µL of MTT reagent to be added to each well and 3 hours of incubation. Formazan crystals were solubilized and the absorbance was measured spectrophotometrically with a Tecan microplate reader (Tecan, Austria) at a wavelength of 570 nm. Negative control values were measured for all biochar concentrations in DMEM media without the cell line and subtracted from values obtained for the corresponding media with the cell line, to account for the influence of biochar particles on the spectrophotometric measurement. The viability of the cells was calculated as a percentage of the viability in the control test, which was taken to be 100%. The morphology of the cells was investigated prior.

3. Results and discussion

3.1 PAH composition and bulk properties

The nominal content in the biochar of the US EPA's 16 PAH Priority Pollutants was 4.39 ± 3.45 mg/kg, according to the producer. The measured total content of 4.05 ± 0.07 mg/kg is in good agreement with the nominal content, and the majority of the PAHs had three or less fused aromatic rings (96 ± 2 %). The non-desorbable fraction of PAHs measured following incubation in a cyclodextrin solution (to increase desorption kinetics) in the contaminant trap was 3.83 ± 1.11 mg/kg and thus did not differ significantly from the total PAH content. Furthermore, Garza et al.[7] found that the toxicity of soot and surrogate black carbon to lung epithelial carcinoma cells was not related to the PAH content. PAHs can therefore be excluded from the toxicity assessment of this biochar. Due to its low ash content (1.25 ± 0.42 %) heavy metals and mineral components were also excluded from the toxicity assessment of this biochar.

The elemental composition, total micropore volume, and micro-specific surface area of the biochar did not change significantly following incubation in DMEM media (see Table 1). In contrast, the N_2-based specific surface area and total pore volume decreased slightly, possibly due to the adsorption of components from the DMEM media. This observation is consistent with the adsorption of bovine serum albumin previously described by Wang et al.[16] Nevertheless, since only a relatively small proportion of the total DMEM media in the experiment was adsorbed, the sorption of media components is unable to explain the observed cytotoxic effects and can only have had a minor effect (if any) on PAH desorption due to pore blockage.

Table 1: Bulk biochar properties before and after incubation in DMEM cell culture media, including elemental composition, specific surface area (SSA), total pore volume (TPV), micro specific surface area (μSSA), and total micropore volume (μPV).

	Fresh	DMEM media
H/C	0.335 ± 0.004	0.373 ± 0.004
N/C	0.004 ± 0.001	0.004 ± 0.001
SSA	30.8 ± 1.2	26.4 ± 1.2
TPV	0.029 ± 0.001	0.023 ± 0.001
μSSA	374.6 ± 11.4	363.6 ± 9.8
μPV	0.118 ± 0.005	0.115 ± 0.004

The particle/colloid size did not increase with concentration ($p > 0.01$) indicating that homo-aggregation only made a minor contribution to the particle size distribution. The number-based particle size distribution is shown in Figure 1. Although particles were sieved to < 250 μm, more than 95% of the particles measured were < 10 μm in all measurements. The median number-based particle size was 2.04 ± 0.20 μm and more than half of the biochar particles were therefore in the size range of regulated atmospheric particulate matter (PM2.5, < 2.5 μm). These trends in particle size distribution are consistent with measurements on *Miscanthus* based biochars (> 90%

of the particles measured were < 10 µm for MSP550 and MSP700). However, particle size distribution for biochars may vary.

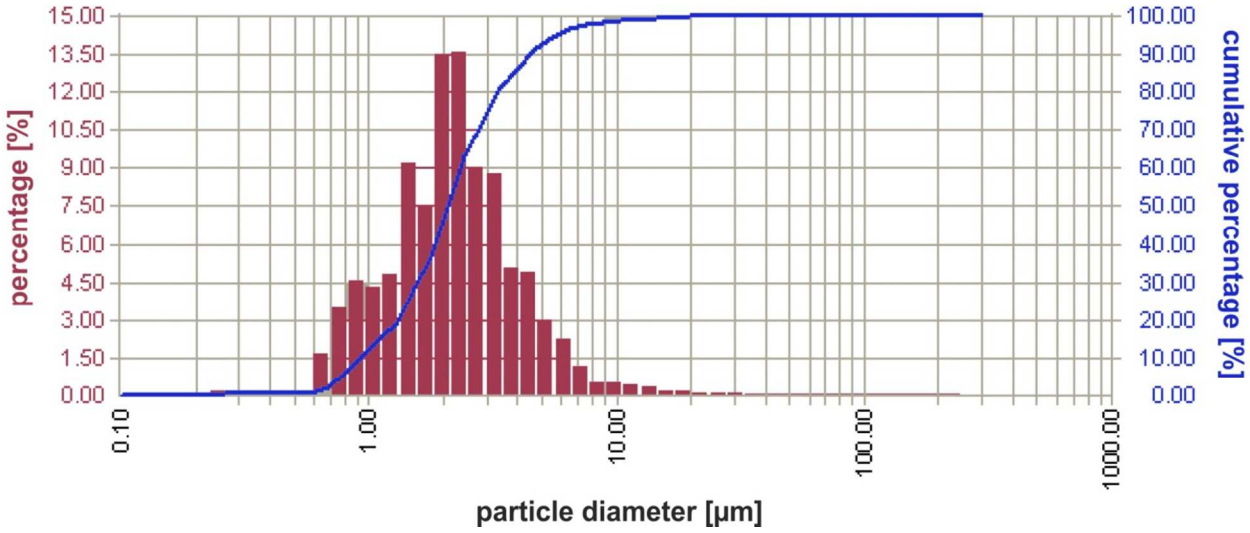

Figure 1: Number-based size distribution of biochar particles sieved to < 250 µm and suspended in DMEM cell culture media buffered at pH 7.4 under the same conditions used in the cytotoxicity test.

3.2 Cytotoxicity of biochar

The proliferation of NIH 3T3 mouse fibroblast cells in response to various concentrations of biochar was investigated by MTT assay. The NIH 3T3 cells were incubated for 24 and 48 hours and a negative control with no biochar was performed and taken to represent 100% viability. The cell viability was expressed as a percentage of the viability in the control test (Figure 2). A biochar concentration of 1 µg/mL did not exhibit any cytotoxic effect after 24 hours, or even after 48 hours. However, a biochar concentration of > 100 µg/mL produced a marked reduction in cell viability after 24 hours of incubation ($p < 0.05$). The cytotoxicity increased further after 48 hours, with cytotoxic effects at biochar concentrations as low as 10 µg/mL ($p < 0.05$, see Table 2 for calculated EC_X values).

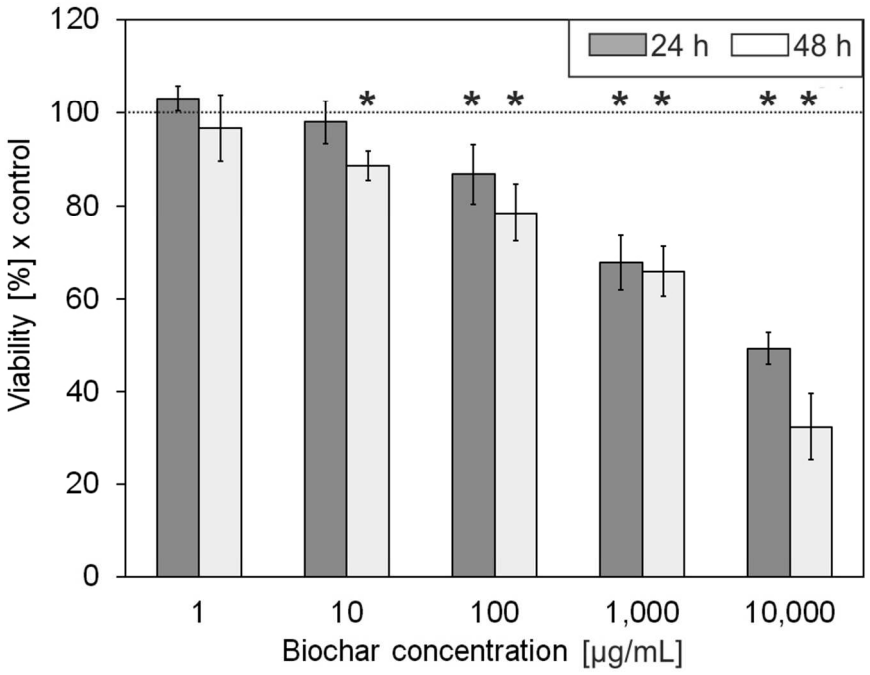

Figure 2: NIH 3T3 mouse fibroblast cell viability as a percentage of the viability in the control test. Biochar concentration varied from 1 to 10,000 µg/mL. The viability (%), as determined by MTT assay, is shown after 24 hours (grey bars) and 48 hours (white bars) of incubation. The analyses were carried out in triplicate and the results are shown with the corresponding standard deviations. Viabilities that differ significantly from that in the control test are marked with an asterisk.

Table 2: Cell viability based effective concentrations (ECx, µg/mL) and their 95%-confidence limits, according to Fieller`s theorem.

		EC_{10}	EC_{20}	EC_{50}
24 hours	value	49.6	286.0	8163.5
	lower 95%	28.5	200.8	6179.8
	upper 95%	77.7	385.5	11295.2
48 hours	value	18.8	103.1	2669.3
	lower 95%	8.8	61.9	1977.7
	upper 95%	33.8	154.9	3742.2

Microscopy images of the NIH 3T3 cell line showed changes in cell morphology starting from a biochar concentration of 100 µg/mL (see Figure 3), confirming the measured reduction in cell viability. At concentrations of 1,000 and 10,000 µg/mL (Figure 3, E and F), binding of biochar to the cell surface could be observed, as has previously been reported for iron oxide nanoparticles in a study with a HeLa cell line[17]. The sizes of the observed biochar particles increased considerably compared to those measured in pure media (see Figures 1 and 3), indicating that attachment to the cell surfaces occurred. Carbon nanotubes have also been reported to induce cell death at concentrations of less than 0.2 µg/mL (i) if they are in contact with a lung tumor cell line, or (ii) after their internalization[18]. Sgro et al.[11] observed cytotoxic cell internalization of soot particles in the 1-3 nm size range, using the NIH 3T3 cell line. However, no internalization of biochar in the NIH 3T3 cell line was seen in our study, which can be explained by the larger size of the biochar particles. Particle-induced oxidative stress has also previously been reported as a key mechanism for nanocarbon blacks[4] and soot[7] cytotoxicity, increasing with decreasing particle size[4].

Our data show a strong overall reduction in cell viability at all concentrations ≥ 100 µg/mL, after both 24 hours and 48 hours. The cytotoxic effect can be related to the particulate nature of the biochars, and to the biochar particles binding to the surfaces of the mouse fibroblast cells. Cytotoxicity of biochar may thus be explained by (i) induction of oxidative stress, (ii) physical changes of the cell morphology, and (iii) inhibition of cell membrane transport by reduction of the accessible outer cell surface.

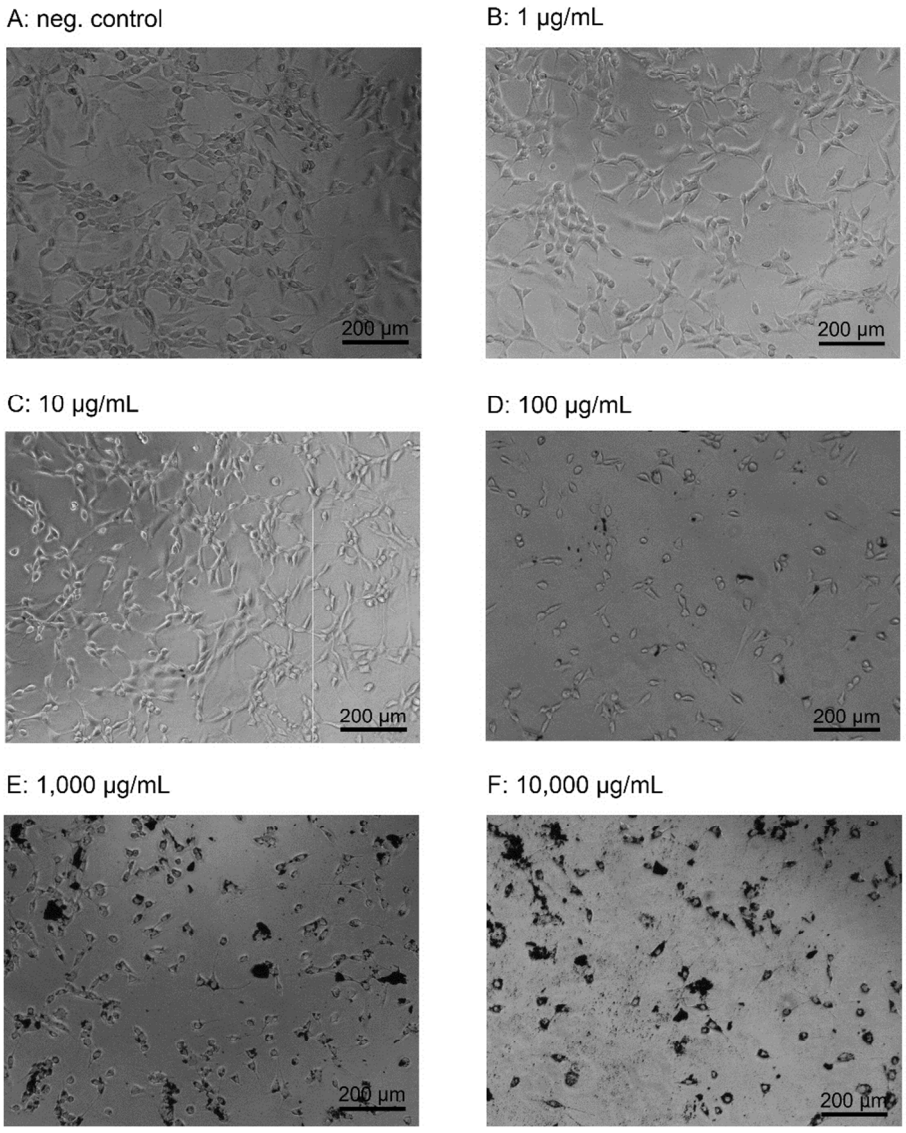

Figure 3: Morphology of NIH 3T3 cell line treated with different biochar concentrations: (A) negative control, (B) 1 µg/mL, (C) 10 µg/mL, (D) 100 µg/mL, (E) 1,000 µg/mL, and (F) 10,000 µg/mL.

3.3 Implications for biochar application

To the best of our knowledge this is the first direct observation of the cytotoxicity of biochar particles. Lyu et al.[19] have previously investigated the cytotoxic effect of Soxhlet extractions

from biochar on a rat hepatoma cell line. However, the study did not consider the direct contact with biochar particles and the toxicity of extracted PAHs is most likely not representative of biochar toxicity in the field, as PAHs in biochar fulfilling EBC quality guidelines are commonly not bio-accessible[9]. The biochar in this study appeared to be less toxic than the reported cytotoxicities of other particulate carbon-based materials such as soot, vehicle exhaust particles[11], carbon nanotubes[18] and nanocarbon blacks[4]. This may be partially explained by (i) the lower potential for PAH release from the biochar used in this study (as confirmed by the contaminant trap experiment), compared to soot and vehicle exhaust particles, and (ii) the larger size (and different shape) of biochar particles compared to carbon nanotubes and nanocarbon blacks, which reduces the potential for cell internalization and oxidative stress induced by biochar particles. Safety precautions are nevertheless recommended during both production and application of biochar due to toxicity associated with the biochar particle size (more than 95% < 10 μm, more than 50% < 2.5 μm). To minimize the risk of exposure, practitioners should wear respiratory protective equipment during biochar production. During field application, biochar should be applied in slurries and should always be mixed with a soil matrix to avoid secondary dust formation, as is recommended by the EBC.

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