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Potential risk of biochar-amended soil to aquatic systems: an evaluation based on aquatic bioassays

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Abstract It is vital to address potential risks to aquatic ecosystems exposed to runoff and leachates from biochar-amended soils, before large scale applications can be considered. So far, there are no established approaches for such an assessment. This study used a battery of bioassays and representative aquatic organisms for assessing the acute toxicity of water-extractable fractions of biochar-amended soil, at reported application rates (80 t ha^{-1}). Biochar-amended aqueous soil extracts contained cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), zinc (Zn), nickel (Ni), lead (Pb), arsenic (As) and mercury (Hg) (Σmetals 96.3 μg l^{-1}) as well as the 16 priority PAHs defined by the U.S. Environmental Protection Agency (Σ_{16}PAHs 106 ng l^{-1}) at contents in the range of current EU regulations for surface waters. Nevertheless, acute exposure to soil-biochar (SB) extracts resulted in species-specific effects and dose–response patterns. While the bioluminescent marine bacterium *Vibrio fischeri* was the most sensitive organism to aqueous SB extracts, there were no effects on the growth of the microalgae *Pseudokirchneriella subcapitata*. In contrast, up to 20 and 25 % mobility impairment was obtained for the invertebrate *Daphnia magna* upon exposure to 50 and 100 % SB extract concentrations (respectively). Results suggest that a battery of rapid and cost-effective aquatic bioassays that account for ecological representation can complement analytical characterization of biochar-amended soils and risk assessment approaches for surface and groundwater protection.

Keywords Biochar · Soil · Aqueous extracts · Contaminants · Aquatic ecosystems · Bioassays

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Introduction

Biochar is the porous and carbon-rich product of pyrolysis of biomass under limited oxygen conditions that is used as a soil amendment (Lehmann and Joseph 2009). The literature provides compelling evidence that soil amendment with biochar can improve soil properties and processes linked to agronomic and environmental functions (e.g. water and nutrient retention, microbial abundance and activity, immobilization of soil contaminants, plant resistance to biotic stress), while contributing to carbon sequestration (Glaser et al. 2009; Beesley and Dickinson 2011; Chen and Yuan 2011; Jeffery et al. 2011; Elad et al. 2012; Awad et al. 2013; Khan et al. 2013; Domene et al. 2014; Prendergast-Miller et al. 2014). Biochar suitability for improving specific soil functions is dependent on its physical–chemical properties (Lehmann and Joseph 2009; Verheijen et al. 2010; Jeffery et al. 2013), which in turn, are a function of the feedstock and processing conditions (Antal and Grönli 2003; Demirbas 2004). This is the case for potentially toxic elements in biochar, including metals and polycyclic aromatic hydrocarbons (PAHs). Reported metal and PAH concentrations in biochars from commonly

used feedstocks often have been in the range of background soils or guideline concentrations for other traditional soil amendments (Freddo et al. 2012; Hale et al. 2012; Fabbri et al. 2013; Marks et al. 2014a, b; Luo et al. 2014). Further, low desorption rates from the char carrier are expected (Jonker et al. 2005), overall suggesting a reduced environmental risk associated with biochar application to soils. Recent evidence of charcoal mobilization from soils and dissolved charcoal fractions being subject to riverine transport into the ocean (Jaffé et al. 2013) demonstrate the likelihood of aquatic ecosystems being exposed to aqueous fractions of biochar enriched soils, over time. Nonetheless, experimental evidence remains scarce for the possible detrimental impacts on aquatic organisms exposed to aqueous amended-soil fractions, in the form of run-off or leachates.

A number of studies suggests the absence of a direct link between total concentrations of potentially toxic elements in biochar and its observed effects on plants (Li et al. 2011; van Zwieten et al. 2010; Ahmad et al. 2012; Busch et al. 2012; Marks et al. 2014b), earthworms (Li et al. 2011; Tammeorg et al. 2014), aquatic bacteria, algae (*Selenastrum capricornutum*, also known as *P. subcapitata*) and the protozoan *Tetrahymena thermophile* (Oleszczuk et al. 2013). Bridging the gap between total and bioavailable contaminant fractions in soils, the Equilibrium Partitioning Theory posits that both edaphic and aquatic organisms are affected only by the bioavailable fraction of soil contaminants (DECHEMA 1995; Sijm et al. 2000, 2007). Water-extractable soil components are regarded as potentially bioavailable, due to the link between measured contaminant concentrations in aqueous soil extracts and detrimental effects on organisms exposed via the aqueous phase (e.g. Maxam et al. 2000; Frische 2002; Hund-Rinke and Kördel 2003; van Gestel and Koolhaas 2004). When compared to solvent extractions, water-based extractions are considered to better simulate biological extraction processes and provide an estimate of potentially bioavailable contaminants in pore-water or that may be mobilized to groundwater systems (Kelsey et al. 1997; Maxam et al. 2000; Frische 2002; Hund-Rinke et al. 2002; Sheehan et al. 2003). As a complement to traditional chemical analysis of aqueous soil fractions, aquatic bioassays have proven to be crucial for assessing the water-extractable toxicity of contaminated or wildfire-impacted soils and sediments, the ecotoxicological impact of runoff and leachates, while helping to unravel the effects of multiple stressors on aquatic organisms (Doherty 2001; Loibner et al. 2004; Loureiro et al. 2005; Lampi et al. 2006; Eom et al. 2007; Berglind et al. 2010; Schäfer et al. 2010; Rocha et al. 2011; Campos et al. 2012; Gonçalves et al. 2013).

Two recent studies showed ecotoxicological effects on two photosynthetic algae (Smith et al. 2013) and on the bacteria *V. fischeri* (Oleszczuk et al. 2013) exposed to water and solvent-based extracts respectively, from various biochar materials. In both cases, the extent of effects was dependent on biochar characteristics and on extract concentration (Oleszczuk et al. 2013; Smith et al. 2013). Despite representing advances in characterization of potentially bioavailable biochar elements, the reported work excluded the soil component.

This study used a battery of established and standardized aquatic bioassays to evaluate the potential risk to aquatic organisms posed by aqueous fractions of soil amended with a representative biochar, at reported application rates (80 t ha^{-1}). Responses of aquatic organisms to water-extractable biochar-amended soil fractions are likely to be representative of that for run-off or leachates from amended soils. A number of aquatic bioassays using marine and/or freshwater species have been traditionally used under the International Standard Organization (ISO/DIS 17402:2008; 15799:2003) and the Organization for Economic Cooperation and Development (OECD 201: 2006; 202: 2004) strategies for the rapid screening of numerous soil contaminants and aqueous soil extracts. Examples include *V. fischeri* bioluminescence inhibition test, growth inhibition of the microalgae *P. subcapitata* and immobilization of the invertebrate *Daphnia magna* (Straus). The use of multiple assays with different test organisms can provide complementary information and account for specific physiological and ecological traits (Bierkens et al. 1998; van Gestel 2008). The rapid, short-term and cost-effective character of such an approach, if effective, could complement physical–chemical characterization of potentially bioavailable components in biochar and/or amended soils and contribute to routine or site-specific biochar risk assessment, in a range of biochar application scenarios and soil properties.

Materials and methods

Soil and biochar characteristics

The natural standard Lufa 2.2 soil (Lufa Speyer, Germany) with a sandy loam texture was selected as the test soil as recommended under the environmental risk assessment framework, aiming for reproducibility and accurate risk extrapolation. Soil pre-treatments following collection (0–20 cm depth), included sieving (<2 mm) and air-drying (Lufa Speyer, Germany, specification sheet). The main physico-chemical characteristics of the test soil are summarised as follows: pH (0.01 M, $CaCl_2$): 5.5 ± 0.1; soil organic C (%): 1.93 ± 0.2; cation exchange capacity (CEC) (100 $cmol^+$ kg^{-1}): 10.0 ± 0.8; sand (%): 81.3 ± 2.3; silt (%): 12.1 ± 1.3; clay (%): 6.60 ± 1.3;

water holding capacity (g 100 g^{-1}): 45.2 ± 5.0; bulk density (g ml^{-1}): 1.13 ± 0.045. A representative biochar produced by slow pyrolysis (highest treatment temperature, 500–550 °C) of mixed pine-wood chips was used, with a particle size range of 50–100 µm and the following characteristics: pH (0.01 M $CaCl_2$) 10.3 ± 0.01 (alone) and 7.65 ± 0.03 (mixed with soil, 80 t ha^{-1}); CEC 194 mS m^{-1}; 81.4 % C, 0.20 % N, 0.31 % S, 0.8 % P (by elemental analysis and Dumas inductively coupled plasma-optical emission spectrometry, respectively); 95.9 % dry matter and 3.98 % moisture (by gravimetry); particle size distribution (expressed as % weight): <0.05 mm (10.6 %), 0.05–0.1 mm (30.1 %), 0.1–2.0 mm (59.6 %), 2.0–5.0 mm (1.10 %), >5.0 mm (0.03 %). Concentrations of the 16 priority PAHs, metals and metalloids in respect to Cd, Co, Cr, Cu, Mn, Zn, Ni, Pb and As, as well as Hg are depicted in Table 1 for Lufa soil and the selected biochar. The sum of metals (∑metals) and of the 16 PAHs (∑16PAHs) is thereafter referred to, respectively, as total metal and PAH concentrations.

Table 1 Individual and total contents (dry weight) of metals and metalloids, as well as of the 16 priority PAHs in Lufa 2.2 soil and in the selected pine-wood biochar, as a mean of duplicate measurements. When available, standard errors of means are given as ± SE. Analytical detection limit is given by d.l.

Parameters	Lufa soil[a]	Biochar
Metals (µg kg^{-1})		
Cr	8831	82770
Mn	139.0	<d.l.
Co	4.500	<d.l.
Ni	2452	82310
Cu	1531	20846
Zn	17220	76600
As	11.60	1800
Cd	140.1	430
Pb	15981	15504
Hg	67.70	918
∑metals	46378	281178
PAHs (µg kg^{-1})		
NAP	43.9 ± 3.59	94.3 ± 1.02
ACY	30.1 ± 0.33	10.2 ± 0.90
ACE	29.3 ± 0.10	38.2 ± 0.44
FLU	31.7 ± 0.43	1.90 ± 0.01
PHE	34.8 ± 0.23	23.3 ± 1.76
ANT	22.4 ± 0.42	63.5 ± 0.25
FLT	41.8 ± 0.94	67.1 ± 1.09
PYR	43.2 ± 0.04	88.7 ± 1.15
CHR	40.9 ± 0.11	42.0 ± 0.28
BaA	15.3 ± 0.10	<d.l.
BbF	7.70 ± 0.15	48.7 ± 0.71
BkF	6.80 ± 0.61	72.5 ± 1.12
BaP	7.10 ± 0.24	<d.l.
DBA	6.40 ± 0.33	55.4 ± 0.61
BgP	2.33 ± 0.41	72.1 ± 0.74
IND	2.65 ± 0.13	34.0 ± 0.70
$\sum_{16}$PAHs	366 ± 1.27	712 ± 8.64

[a] Metal and PAH contents in Lufa 2.2 soil as reported by Bastos et al. (2014)

NAP naphthalene, *ACY* acenaphthylene, *ACE* acenaphthene, *FLU* fluorene, *PHE* phenanthrene, *ANT* anthracene, *FLT* fluoranthene, *PYR* pyrene, *CHR* chrysene, *BaA* benz(a)anthracene, *BbF* benzo(b)fluoranthene, *BkF* benzo(k)fluoranthene, *BaP* benzo(a)pyrene, *DBA* dibenz(a,h)anthracene, *BgP* benzo(g,h,i)perylene, *IND* indeno(1,2,3-cd)pyrene

Preparation and characterization of aqueous soil extracts

Biochar at the original gravimetric moisture content was mixed homogeneously with 100 g (oven-dry) of Lufa 2.2 soil, at 80 t ha^{-1} (4 % ww^{-1}), corresponding to the approximate mid-point of the reviewed range (1–150 t ha^{-1}) for which agricultural benefits (increased crop yield) have been observed (Jeffery et al. 2011). The short equilibration period (24 h) of the soil-biochar (SB) mixture, aimed at enhancing metal and PAH extraction. Controls contained un-amended Lufa 2.2 soil. De-ionised water was added to soil, biochar and the biochar-amended soil as a 1:2 ww^{-1} (soil:water) ratio (adapted from Eisentraeger et al. 2004), in triplicate, based on a water–adsorption curve previously developed to ensure soil saturation and sufficient volume of elutriates (data not shown). Following wetting and homogenisation, treatments were allowed to equilibrate (4 °C, 24 h), after which mild agitation (overnight, 150 rpm, 20 ± 2 °C) was performed using a bench top orbital shaker (adapted from Loureiro et al. 2005). Soil samples settled overnight (4 °C) in the dark before centrifugation (3000 rpm, 15 min) and collection of the supernatant through vaccuum-driven filtration (Millipore® cellulose filters 0.45 µm pore size; adapted from Eisentraeger et al. 2004). For removal of the finest suspended biochar particles, centrifugation of the supernatant (4000 rpm, 10 min) followed by vacuum filtration were performed using a Buckner devise equipped with glass microfiber-filters (Whatmann GFC Ø 47 mm, 1.2 µm pore size). Aqueous extracts were characterized for pH (H_2O; 1:5), dissolved organic carbon (DOC; TOC analyser 5050A, Shimadzu), oxygen content and electrical conductivity in a filtered suspension (inoLab, TetraCon, WTW). Potentially bioavailable concentrations of the 16 priority PAHs (by SPME-solid phase micro-extraction coupled to GC/MS-gas chromatography/mass spectrometry; Campos et al. 2012) were also measured, as well as metals and metalloids (by ICP/AES-inductively coupled

plasma-atomic emission spectroscopy; Campos et al. 2012) in respect to cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), zinc (Zn), nickel (Ni), lead (Pb) and arsenic (As). Mercury (Hg) was quantified by AAS-atomic absorption spectroscopy (Campos et al. 2012). Concentrations of metals, metalloids and PAHs in aqueous soil (S), biochar (B) and SB extracts are depicted in Table 2. Subsequent steps were performed always using fresh extract (<1 week storage, 4 °C).

Bioassays

Vibrio fischeri bioluminescence inhibition test

The bioluminescent marine bacteria *V. fischeri* was supplied in the form of a freeze-dried reagent, followed by reconstitution (Microbics Corporation 1992). Microtox® Basic Test was used to assess inhibition of bioluminescence, in which the bacteria were exposed to the test extracts up to an extract concentration of 81.9 % (Microbics Corporation 1992). Serial dilutions of S, B and SB extracts were performed by transferring sample aliquots and adjusting the salinity with MOAS (Microtox Osmotic Adjusting Solution, Azur Environmental, Carlsbad, CA, US). The bacteria were transferred into the extract vials and the response was measured after 5 or 15 min of exposure, using Microtox Data Collection and Reduction software (Microbics). Results were reported as % of extract concentration.

Pseudokirchneriella subcapitata growth inhibition assay

Prior to the test, unicellular algae cultures of *P. subcapitata* were prepared in 250 ml Erlenmeyer flasks containing Hoods Hole-MBL medium (Stein 1973) and stirred (150 rpm) at 20 °C ± 1 and 16:8 h (light/dark) photoperiod. The algae were transferred to fresh culture medium weekly to ensure exponential growth at the start of the assay. The test was carried out according to the corresponding OECD guideline (OECD 201: 2006) adapted to 24 multi-well plates (Geis et al. 2000) at an actinic light intensity of 100 μE m^2 s^{-1}. Extract concentrations of 6.25, 12.5, 25, 50 and 100 % were selected for testing (dilution factor of 0.5). The algae were exposed to S, B or SB extracts for 72 h at 20 ± 1 °C under continuous light exposure, during which cell re-suspension was done twice a day. The test concentration representing 100 % extract was supplied with proportional amount of nutrients, in order to ensure unlimited growth (OECD 201: 2006). Woods Hole nutritive MBL culture medium was the negative control (blank) in each bioassay. Three replicates

Table 2 Characterisation of the undiluted aqueous extracts (1:2 w w^{-1}) of Lufa 2.2 soil (S), biochar (B) and Lufa soil-biochar (SB) mixture (at 80 t ha^{-1}), based on triplicate measurements. When available, standard errors of means are given as ± SE. Analytical detection limit is given by d.l. (0.75 < d.l. < 1.94 ng l^{-1})

Parameters	Aqueous extracts		
	S[a]	B	SB
pH (H_2O; 1:5)	6.32 ± 0.1	9.50 ± 0.3	7.28 ± 0.1
DOC (mg l^{-1})	88.5	106	111
Metals (μg l^{-1})			
Cr	1.60	3.30	1.70
Mn	117	43.0	66.0
Co	0.49	0.31	0.54
Ni	5.90	7.20	5.30
Cu	6.50	1.70	7.80
Zn	44.0	12.0	12.0
As	<1.00	2.20	2.60
Cd	0.27	<0.1	<0.1
Pb	0.93	0.47	0.32
Hg	0.17	0.09	0.08
∑metals[b]	178 ± 6.18	70.4 ± 3.05	96.3 ± 3.88
PAHs (ng l^{-1})			
NAP	27.5 ± 3.59	9.43 ± 1.02	29.6 ± 3.1
ACY	4.13 ± 0.33	10.2 ± 1.02	6.43 ± 0.14
ACE	5.48 ± 0.10	3.82 ± 0.44	3.87 ± 0.5
FLU	<d.l.	1.95 ± 0.01	1.18 ± 0.09
PHE	10.9 ± 0.23	23.3 ± 1.76	23.3 ± 2.32
ANT	5.67 ± 0.42	6.35 ± 0.25	6.70 ± 0.40
FLT	6.58 ± 0.94	6.71 ± 1.09	8.91 ± 0.72
PYR	1.97 ± 0.04	8.87 ± 1.15	7.49 ± 1.49
CHR	<d.l.	<d.l.	<d.l.
BaA	<d.l.	<d.l.	3.61 ± 0.33
BbF	<d.l.	4.98 ± 0.70	<d.l.
BkF	4.84 ± 0.61	7.25 ± 1.12	9.10 ± 0.3
BaP	<d.l.	<d.l.	5.29 ± 0.87
DBA	<d.l.	<d.l.	<d.l.
BgP	2.33 ± 0.41	7.25 ± 0.74	6.18 ± 0.88
IND	2.65 ± 0.13	3.40 ± 0.07	4.73 ± 0.88
$\sum_{16}$PAHs[b]	72.1 ± 1.27	93.5 ± 9.64	106 ± 10.3

[a] DOC and concentrations of metals and PAHs in Lufa 2.2 soil extracts as reported by Bastos et al. (2014)

[b] Sum of mass concentrations of metals correspond to 3.07, 1.22 and 1.70 mol l^{-1} (S, B and SB, respectively); Sum of mass concentrations of PAHs correspond to 0.45, 0.51 and 0.59 mol l^{-1} (S, B and SB, respectively)

NAP naphthalene, *ACY* acenaphthylene, *ACE* acenaphthene, *FLU* fluorene, *PHE* phenanthrene, *ANT* anthracene, *FLT* fluoranthene, *PYR* pyrene, *CHR* chrysene, *BaA* benz(a)anthracene, *BbF* benzo(b)fluoranthene, *BkF* benzo(k)fluoranthene, *BaP* benzo(a)pyrene, *DBA* dibenz(a,h)anthracene, *BgP* benzo(g,h,i)perylene, *IND* indeno(1,2,3-cd)pyrene

were used per test concentration, as well as six negative controls. Algal growth rate (expressed as a change in algal cell numbers/day) was determined through microscopic (Olympus CKX41) cell count in a Neubauer chamber. The pH was measured at the beginning and at the end of the test, with no required adjustments.

Daphnia magna acute immobilization assay

Prior to the test, *D. magna* cultures were prepared with newborn female individuals (clone K6, 3rd–5th broods) maintained within 800 ml glass bottles with ASTM hard water medium (ASTM 1998) and seaweed (*Ascophilum nodosum*) extract (Baird et al. 1989), at 20 °C ± 1 and 16:8 h (light/dark) photoperiod. The daphnids were fed daily with *P. subcapitata* (3.0×10^5 cells/ml/daphnia) and transferred to fresh-culture media every 2 days. The acute immobilization test was performed according to the OECD guideline (OECD 202: 2004). ASTM solution (ASTM 1998) was used both as the eluent and the negative control (blank). A range of S, B and SB extract concentrations of 6.25, 12.5, 25, 50 and 100 % were prepared. Four replicates, each with 5 neonates (<24 h) per test concentration were used, including for the negative controls. Following a 48 h exposure at 20 ± 1 °C and 16:8 h (light/dark) photoperiod, during which the organisms were not fed, the number of immobilized/dead individuals was recorded. pH and DOC were measured both at the beginning and at the end of the assay, with no required adjustments prior to the test.

Statistical analysis and calculation of parameters

For the Microtox®, EC_{50}, EC_{20} and EC_{10} (corresponding to 50, 20 and 10 % bioluminescence reduction in *V. fischeri*) and the corresponding 95 % confidence intervals (CI) were obtained using Microtox Data Collection and Reduction software (Microbics) and IBM SPSS.19, respectively. For *P. subcapitata*, the lowest observed effect concentration (LOEC) and no-observed effect concentration (NOEC) were estimated by one-way ANOVA, followed by the Dunnett's multiple comparison test (Zar 1999) using IBM SPSS.19. For *D. magna* immobilization, EC_{20} and EC_{10} values (extract concentrations for which a 20 and 10 % mobility reduction was observed) alongside the corresponding 95 % CI were estimated using Probit regression analysis and IBM SPSS.19.

Results

Table 1 summarises the individual and total contents of selected metals, metalloids as well as the 16 priority PAHs in Lufa soil and biochar. The sum of potentially toxic compounds in biochar compared to that in Lufa soil, were approximately 6 times higher in respect to metals ($\sum$metals 281178 vs 46378 μg kg^{-1}) and nearly double for the 16 PAHs ($\sum_{16}$PAHs 712 vs 366 μg kg^{-1}). The main physical–chemical characteristics of the undiluted aqueous extracts are indicated in Table 2. Comparisons between S and SB extracts showed approximately half the total content of extracted metals for the SB treatment ($\sum$metals 96.3 μg l^{-1}) in relation to Lufa soil ($\sum$metals 178 μg l^{-1}) and a 30 % higher total PAH concentration ($\sum_{16}$PAHs 106 vs 72.1 ng l^{-1}).

V. fischeri bioluminescence inhibition test

The Microtox® basic test (BT) was conducted in order to evaluate the bioluminescence response of the bacteria to the test extracts after 5 or 15 min of exposure. Table 3 summarizes the estimated EC_{50}, EC_{20} and EC_{10} values and associated 95 % CI, while the luminescence inhibition (%) as a function of extract concentration is shown in Fig. 1. Significantly lower toxicity was induced by the amended soil (SB) extract (EC_{50} of 75.8 and 90.7 % extract, for 5 and 15 min), compared to that of biochar alone (B) (EC_{50} of 30.9 and 33.1 %, for 5 and 15 min), irrespective of exposure time. The highest observed inhibition for SB did not exceed 44.7 % for the highest extract concentration. The extent of inhibition generally increased with extract concentration and surpassed that for the control for concentrations >41 %. For both exposure times, a similar response pattern was found between un-amended and biochar-amended soil extracts.

P. subcapitata growth inhibition assay

Inhibitory effects on the growth of the microalgae *P. subcapitata* exposed for 72 h to aqueous extracts were generally low regardless of treatment. Further, growth rate and inhibition response patterns between un-amended and biochar-amended Lufa soil were similar ($p > 0.05$), with a statistically significant ($p < 0.05$) 10 % growth inhibition being observed upon exposure to the highest extract concentration (LOEC of 100 % and NOEC of 50 % extract).

D. magna acute immobilisation assay

Overall, there were mild inhibitory effects on the invertebrate *D. magna* exposed to the test extracts for 48 h. Exposure to SB extracts yielded an EC_{20} and EC_{10} of 79.3 % (CI 48.9–101.2) and 54.6 % (CI 27.7–92.0) extract respectively, while no effects were observed upon exposure to extracts of both soil and biochar alone. Immobilization reached 20 % for 50 % of SB extract and 25 % for the undiluted (100 %) extract.

Table 3 Estimated effect concentrations, EC_{50}, EC_{20} and EC_{10} (based on extract concentration, %) with 95 % confidence intervals (in brackets) for the Microtox® Basic Test, with *Vibrio fischeri* exposed to aqueous extracts of Lufa 2.2 soil (S), pine-wood chips biochar (B) and of Lufa 2.2 amended with the biochar at 80 t ha^{-1}(SB), for 5 or 15 min

	Effect concentration					
	5 min			15 min		
	EC_{50}	EC_{20}	EC_{10}	EC_{50}	EC_{20}	EC_{10}
S	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B	30.9	8.73	5.32	33.1	8.76	4.65
	(27.4–38.6)	(6.51–10.9)	(2.51–6.20)	(24.8–47.4)	(5.50–11.6)	(3.07–7.11)
SB	75.8	20.5	11.2	90.7	21.4	11.3
	(47.3–108)	(14.6–31.5)	(5.84–16.3)	(67.2–128)	(16.9–28.5)	(8.89–15.1)

n.d. indicates 'not determined' due to low toxicity (>100 % of extract)

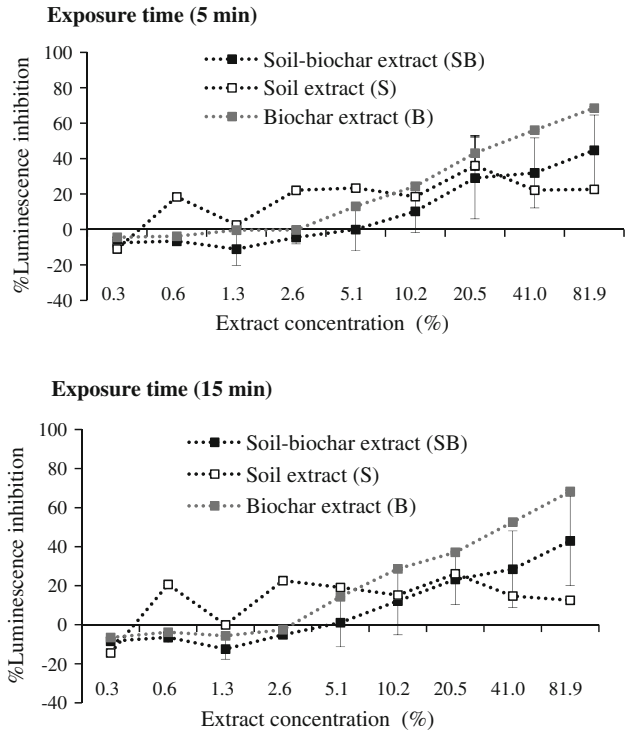

Fig. 1 Bioluminescence inhibition (%) for the marine bacteria *V. fischeri* using the Microtox® Basic Test (BT) for 5 or 15 min exposure to aqueous extracts of Lufa 2.2 soil (S, control), biochar (B) and the soil-biochar (SB) mixture, at 80 t ha^{-1}. *Vertical bars* represent ± standard deviations of means of three replicated tests

Discussion

Metal and PAHs concentrations in biochar were in agreement with earlier studies using slow pyrolysis biochars from woody feedstocks (Fabbri et al. 2013; Hale et al. 2012; Marks et al. 2014a,b), with contents of most individual compounds falling in the range of background soils (Chen et al. 2001; Chen and Yuan 2011; Zhao et al. 2007; Fabbri et al. 2013), as previously suggested (e.g. Freddo et al. 2012). Chemical characterisation of aqueous biochar and amended soil extracts revealed that a fraction of such priority contaminants is water-extractable, and therefore, potentially bioavailable to biota. The initial short (24 h) equilibration time of the SB mixture and the water-extraction procedure used aimed at simulating a worst-case scenario of enhanced extraction (Kelsey et al. 1997), being therefore in line with traditional approaches for ecotoxicological assessment of soil and soil materials (Eisentraeger et al. 2004).

Metal concentrations in SB extracts were lower than or comparable to that in extracts of the reference soil, with individual metal concentrations falling in the range of the regulated targets for inland and other surface waters (2008/105/EC) and of the reviewed toxicological benchmarks for aquatic biota (Suter and Tsao 1996). Also, metal extractability from soil decreased upon biochar application, corroborating previous studies (Fellet et al. 2014; Rees et al. 2014). Nonetheless, the sum of extracted metals from the amended soil significantly exceeded that from biochar alone, possibly as a result of the lower pH (Chuan et al. 1996) and/or interactions with soil organic matter (Uchimiya et al. 2010). In turn, the sum of extracted PAHs ($\sum_{16}$PAHs 106 ng l^{-1}) in biochar-amended soil was only 32 % higher than in extracts of the reference soil. Individual contents of the seven regulated PAHs (ANT, FLU; BaP, BbF, BkF, BgP, IND) for protection of surface waters (2008/105/EC) remained a factor of 10 (at least) below the defined Environmental Quality Standards (EQS) and maximum allowed concentrations and met the corresponding aquatic toxicological benchmarks, when available (Suter and Tsao 1996). The exception was the sum of BgP and IND (10.9 ng l^{-1}), being five times higher than the annual average EQS for inland and other surface waters (2 ng l^{-1}; 2008/105/EC), with no defined maximum allowable concentrations for the individual compounds. Overall, analytical characterization of the SB extracts in respect to potentially bioavailable metals and PAHs,

suggest that aqueous fractions of soil amended with a representative biochar up to 80 t ha^{-1} are unlikely to pose a significant risk to aquatic organisms in the short term.

Acute biological responses to soil-biochar aqueous extracts

Acute exposure to the test extracts by a range of representative aquatic organisms, however, revealed species-specific response patterns, explained by their individual physiological and functional traits. We have used conventional statistically estimated ecotoxicological criteria (such as EC_{50}, LOEC and NOEC) to provide the likelihood of a significant effect of aqueous extracts of biochar-amended soil, as a heterogeneous matrix, on aquatic biota. By comparing the EC_{50} values among the tested organisms, *V. fischeri* was the most sensitive species to biochar aqueous solutions, although extracts of biochar-amended soil yielded lower toxicity than that of biochar alone, independent of contact time (5 or 15 min). This possibly reflects a pH effect or reduced bioavailability of biochar components that can exert toxicity to the bacteria, when mixed with soil. In contrast, biochar application to soil at 80 t ha^{-1} did not affect the growth of *P. subcapitata* and had a relatively mild effect on the mobility of *D. magna*, with up to 25 % reduction upon exposure to the undiluted biochar-amended soil extract.

Contextualization with the relevant literature is limited by the lack of ecotoxicological data for soil-biochar mixtures and of established procedures for assessing their bioavailable components and the effects of interactions between them. Further, the heterogeneity of the water-extracted soil-biochar fractions confounds the assessment as to which factor, or combination herein, might explain the observed biological responses. The heterogeneous nature of aqueous extracts of biochar derived from eucalyptus wood, olive pomace and greenhouse wastes has already been addressed in the relevant literature (Graber et al. 2014). For instance, while pH could partially explain the reduced toxicity of SB extracts to *V. fischeri*, compared to biochar alone (light output peaks at pH 6.8 and 7.3, for 5 and 15 min of exposure; Berglind et al. 2010), it is unlikely to be the main explanatory factor for *D. magna*'s immobilization (Rendal et al. 2012).

There was also a significant increase in Mn (66.0 μg l^{-1}) and Cu (7.80 μg l^{-1}) concentrations in amended soil extract, compared to that of biochar-alone (43.0 and 1.70 μg l^{-1} respectively) but values were still comparable to those extracted from un-amended soil. Similarly, As contents in SB extract surpassed that in the other treatments (<1 μg l^{-1} in S vs 2.20 μg l^{-1} in B vs 2.60 μg l^{-1} in SB), although it remained (at least) one order of magnitude below the respective conventional acute and chronic benchmarks for freshwater organisms (Suter and Tsao 1996). While potentially bioavailable metals and metalloids have been previously linked to toxicity to biota exposed via the aqueous phase (Loureiro et al. 2005; Gomez-Eyles et al. 2011; Rocha et al. 2011), the same applies to PAHs, both individually and in mixtures (Neff 1979; Olmstead and LeBlanc 2005; Bellas et al. 2008). For example, NAP, BaA and BaP individual concentrations in SB extracts exceeded those in the remaining treatments, but remained a factor of 10-100 below their reported median lethal concentrations for daphnids and other aquatic invertebrates (Lampi et al. 2006; Bellas et al. 2008), suggesting no ecotoxicological concern. Therefore, it is likely that the observed responses to the heterogeneous SB aqueous matrices can be explained by a combination of factors, as they reflect their toxic potential, as a whole. These may include species-specific synergistic and/or antagonistic effects arising from interactions between multiple stressors, as described for a range of aqueous solutions or extracts from contaminated soils (Loibner et al. 2004; Olmstead and LeBlanc 2005; Xie et al. 2006; Eom et al. 2007; Li et al. 2009; Berglind et al. 2010). In the context of biochar application, a study by Smith et al. (2013) supports this discussion. Although there were inhibitory effects on aquatic photosynthetic microorganisms exposed to water extracts of pinewood biochar (with no correlation to extract pH or DOC levels), the specific inhibitory compound(s) could not be determined (Smith et al. 2013). Further, authors attributed the observed toxicity to a water-extractable organic molecule containing at least one carboxyl group, which may interact with co-occurring compounds in the aquatic environment (e.g. forming organic acid—metal cation complexes) upon deprotonation (Smith et al. 2013). This underlines the need to integrate physical–chemical and ecotoxicological characterization of potentially bioavailable fractions of biochar-amended soil to better understand and predict their potential risk to exposed aquatic ecosystems.

Bioassays for evaluating risks to aquatic environments from soil amendment with biochar

A battery of simple, rapid and cost-effective aquatic bioassays was employed for evaluating the possible short-term toxicity of water-extractable fractions of biochar-amended soil, as a whole. Aqueous SB extracts were used as a proxy for run-off or leachates from soil, upon application of a representative biochar at commonly used application rates. Further, the ecotoxicological evaluation of such fractions may also provide relevant information on the retention function of amended soils, with implications for soil-dwelling organisms that are exposed via the soil pore water (e.g. Loureiro et al. 2005; van Gestel and Koolhaas 2004;

Marks et al. 2014a, b). Previously, it has been shown that exposure to water-extractable and pore-water fractions from a range of soils produced comparable effects on the springtail *Folsomia candida* (van Gestel and Koolhaas 2004), thus being in line with that suggested by Graber et al. (2014) in the context of aqueous fractions of biochar. Further, an approach that accounts for individual physiological and functional traits might be effective and ecologically relevant for routine site-specific biochar risk assessment or for complementing chemical characterization of soils, leachates and runoff from biochar amended sites. Besides fulfilling the validity criteria defined by the corresponding guidelines, the selected set of bioassays was reproducible and the results indicate species-specific response patterns.

Nonetheless, the heterogeneous nature of biochar's solid and aqueous fractions and the possible influence of soil and biochar interactions on bioavailability and toxicity of specific components, highlight the need for long-term ecotoxicological assessment using various biochar and soil characteristics, non-target species and chronic endpoints. Specifically, the usefulness of Microtox® BT tests in this context may require further testing, using a range of soil types and biochar particle sizes to account for sample turbidity and colour or help determining how bacterial adherence to suspended particles (analogous to that reported for clay; Doherty 2001) and turbidity/optical interference (Campisi et al. 2005) can influence the results.

Despite recent analytical advances, such as that for PAH quantification in biochar (Fabbri et al. 2013; Hale et al. 2012; Hilber et al. 2012), there are no established methods for characterizing biochar-amended soils or assessing the toxicity and fate of their bioavailable fractions. While a better understanding of these processes can close the gap between analytical measurements and biological effects, additional studies are required for establishing effective biochar risk assessment procedures under the current EU regulations for Soil [COM(2006) 232] and Water (2008/105/EC) protection. Further, the use of bioassays in complementing analytical characterization of potentially bioavailable components in biochar and biochar-amended soils can also provide useful information to current biochar standardization efforts that aim for its quality control and safe application. In this context, the use of a standard natural soil such as Lufa 2.2 can allow comparison and validation across analogous studies and soil and biochar types, as it combines the representativeness and ecological relevance of a natural soil for accurate risk evaluation and extrapolation.

Conclusions

Potentially toxic metals and the 16 priority PAHs were present in aqueous extracts of biochar-amended soil, at levels below the current EU regulations for surface waters and their individual toxicological benchmarks (when available) for aquatic biota. Nevertheless, short-term exposure to the test extracts of representative aquatic organisms revealed species-specific effects. While the bioluminescent bacteria *V. fischeri* was the most sensitive organism to biochar water-extractable components, there was no observed toxicity on the growth of the microalgae *P. subcapitata*. The invertebrate *D. magna* exhibited 20–25 % acute mobility impairment at reported biochar application rates. Results indicate that a battery of simple, rapid and cost-effective aquatic bioassays that account for physiological and ecological representativeness can complement analytical characterization of biochar and biochar-amended soils and/or risk assessment strategies.

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Ethical standards Authors declare that the experiments comply with the current laws of the country in which they were performed.

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