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# Assessment of biochar and zero-valent iron for *in-situ* remediation of chromated copper arsenate contaminated soil

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## Highlights

- Chromate copper arsenate (CCA) contaminated soils pose risks to the environment
- *In-situ* stabilization of CCA contaminated soil was tested with biochar and ZVI
- Soil remediation was evaluated based on chemical and microbiological techniques
- Biochar reduced bioavailable ( $\text{Cu}_{\text{bio}}$ ), but not water-extractable Cu ( $\text{Cu}_{\text{water}}$ )
- Combination of biochar and ZVI most effectively reduced toxic effects of CCA

## 1 **Abstract**

2 Chromated copper arsenates (CCA) have been extensively used as wood impregnation agents in  
3 Europe and North America. Today, CCA contaminated sites remain abundant and pose environmental  
4 risks that need to be properly managed. Using a TRIAD approach that combined chemical,  
5 ecotoxicological and ecological assessment of soil quality, we investigated the abilities of biochar and  
6 zero-valent iron (ZVI) to remediate CCA contaminated soil in a microcosm experiment. Soil samples  
7 from a highly contaminated CCA site (1364, 1662 and 540  $\mu\text{g g}^{-1}$  of As, Cu and Cr, respectively) were  
8 treated with two different biochars (fine and coarse particle size; 1 % w w<sup>-1</sup>) and ZVI (5 % w w<sup>-1</sup>), both  
9 as sole and as combined treatments, and incubated for 56 days at 15 °C. In general, bioavailable As  
10 ( $\text{As}_{\text{bio}}$ ) and Cu ( $\text{Cu}_{\text{bio}}$ ) determined by whole-cell bacterial bioreporters corresponded well to water-  
11 extractable As and Cu ( $\text{As}_{\text{water}}$  and  $\text{Cu}_{\text{water}}$ ). However, in biochar treatments, only  $\text{Cu}_{\text{bio}}$  and not  $\text{Cu}_{\text{water}}$   
12 was significantly reduced. In contrast, under ZVI treatments only  $\text{Cu}_{\text{water}}$  and not  $\text{Cu}_{\text{bio}}$  was reduced,  
13 demonstrating the value of complementing analytical with bacterial bioreporter measurements to  
14 infer bioavailability of elements to soil microorganisms. The combined fine particle size biochar and  
15 ZVI treatment effectively reduced water extractable concentrations of Cr, Cu, and As on site by 45%,  
16 45% and 43 % respectively, and led to the highest ecological recovery of the soil bacterial community,  
17 as measured using the [<sup>3</sup>H]leucine incorporation technique. We conclude that the combined  
18 application of biochar and ZVI as soil amendments holds promise for *in-situ* stabilization of CCA  
19 contaminated sites.

## 20 **Keywords**

21 metal-contaminated soils, soil remediation, biochar, zero-valent iron, bioavailability, whole-cell  
22 biosensors

## 23        **1. Introduction**

24        Chromated copper arsenate (CCA) constitutes a mixture of Cr and Cu salts ( $\text{CrO}_3$  and  $\text{CuO}$ ) and arsenic  
25        acid ( $\text{H}_3\text{AsO}_4$ ) and has been extensively used as a wood preservative since the 1940s. In 2003, legislation  
26        in Europe and North America was passed which severely limited CCA application (European  
27        Commission, 2003; Humphrey, 2002; U.S. EPA, 2002), but a large number of CCA legacy contaminated  
28        sites remain (Bhattacharya et al., 2002; Hopp et al., 2006), with more than 100 sites in Denmark alone  
29        (Amternes Videntcenter for Jordforurening, 1997; Nielsen et al., 2010).

30        CCA contaminated sites are of environmental concern as Cr, Cu and As pose severe risks to both human  
31        and environmental health. Arsenic is of particular concern due to its toxicity and mobility, by which it  
32        may also threaten surface and groundwater drinking water resources (Nielsen, 2013). In soils, As  
33        occurs most commonly as the inorganic oxyanions arsenite (As(III)) and arsenate As(V)) (Masscheleyn  
34        et al., 1991), with As(III) generally the most toxic and mobile form (Peters et al., 1996). However, Cu is  
35        less mobile, but may adversely affect ecosystem services provided by soil biota (Arthur et al., 2012;  
36        Nunes et al., 2016) and co-select for antibiotic resistance (Ashbolt et al., 2013; Song et al., 2017). Cr  
37        occurs in soil mostly as Cr(0), Cr(II), Cr(III) and Cr(VI), with Cr(III) the most stable and prevalent Cr redox  
38        species in most soils (Namiesnik and Rabajczyk, 2012), but the Cr(VI) species used in CCA formulations  
39        is considered substantially more toxic than reduced forms of Cr (Nielsen et al., 2010).

40        CCA and other multi-element contaminated soils can be remediated in various ways.  
41        Full remediation is often only possible by excavation, but due to the sheer number and size of  
42        contaminated sites worldwide, this approach is seldom feasible (Nielsen et al., 2016). Hence, *in-*  
43        *situ* stabilization by application of different soil amendments has been proposed as a more  
44        economical approach for reducing the mobility and toxicity of trace element contaminants (Bolan et  
45        al., 2014; Maurice et al., 2007), but the challenge remains to identify suitable soil amendments for

46 multi-element contaminated sites, as the individual elements may respond differently to soil  
47 treatments (Kumpiene et al., 2008; Qiao et al., 2018; Silveti et al., 2014; Zhou and Haynes, 2010).

48 Biochar, a solid produced by pyrolysis of organic material, has gained particular interest as a cheap and  
49 effective amendment for remediation of cationic pollutants such as Cu, mainly by increasing pH and  
50 providing additional sorption sites (Beesley et al., 2011; Buss et al., 2012; Ippolito et al., 2012; Lehmann  
51 and Joseph, 2015). However, biochar may not work equally well for anionic trace element  
52 contaminants, as previous studies have observed an increase in bioavailability and mobility of As  
53 (Beesley et al., 2010; Hartley et al., 2009; Kim et al., 2018; Wang et al., 2017). Rather, iron-bearing  
54 compounds such as zero-valent iron (ZVI) have proven more useful for *in-situ* stabilization of As  
55 (Kumpiene et al., 2006; Miretzky and Cirelli, 2010; Nielsen et al., 2011). Sneath et al. (2013) proposed  
56 a combination of biochar and ZVI as a promising amendment for *in situ* stabilization of soil  
57 contaminated with complex mixtures of metals, arsenic and organics. Several recent studies  
58 investigated the synthesis and characterization of iron-coated biochar for metal(loid) removal from  
59 aqueous media and found enhanced sorption of Cr(VI) (e.g. Zhu et al., 2018; B. Wu et al., 2018; Diao  
60 et al., 2018), Cu (e.g. Kolodynska and Bak, 2018; Yang et al., 2018b) as well as As (e.g. Bakshi et al.,  
61 2018; He et al., 2018; Zhou et al., 2014). However, the combination of biochar and iron as a treatment  
62 for remediation of multi-element contamination of soils and other media is less well studied (Lu et al.,  
63 2018).

64 Consequently, the aim of the present study was to perform a soil microcosm study to understand both  
65 the independent and combined efficiency of biochar and ZVI for stabilizing CCA contaminated  
66 soil. Remediation treatment effects were assessed after 1, 7, 28 and 56 days using a soil quality TRIAD  
67 approach. The TRIAD approach combines investigations of soil chemistry (exposure and  
68 bioavailability), (eco)toxicology, and ecology as recently recommended for site-specific risk assessment  
69 of contaminated soils (ISO 19204, 2017). Exposure and bioavailability were assessed by a combination  
70 of chemical analyses (ICP-OES, trace element speciation analysis etc.) and of bioluminescent whole-

71 cell bacterial bioreporters responding specifically to bioavailable Cu and As, respectively. Soil toxicity  
72 was assessed in a laboratory bioluminescence inhibition assay with a whole-cell bacterial bioreporter.  
73 Soil ecology was studied by following the recovery of growth in indigenous soil bacteria as measured  
74 by the [<sup>3</sup>H]leucine incorporation technique, providing a proxy for secondary bacterial productivity  
75 (Brandt et al., 2015). We hypothesized that a combination of ZVI and biochar would be more efficient  
76 in reducing environmental risks posed by Cr, Cu and As in CCA contaminated soil than independent  
77 applications of ZVI and biochar. In addition, we hypothesized that the high surface area of fine biochar  
78 would stabilize multiple elements more efficiently than coarse biochar.

## 79 **2. Materials and Methods**

### 80 *2.1. CCA contaminated soil and experimental soil amendments*

81 Contaminated soil was collected from the upper 20 cm of a former wood impregnation site north of  
82 Copenhagen, Denmark (sampling point: 55°57'19.7"N 12°21'18.1"E, station L4), highly contaminated  
83 with Cr, Cu, and As (see Nielsen et al., 2011, for detailed site description and Tardif et al., 2019, for  
84 detailed physico-chemical characterization). The texture was classified as loamy sand with 39.9 %  
85 coarse sand (200-2000 µm), 44.6 % fine sand (20-200 µm), 11.0 % silt (2-20 µm), and 4.5 % clay (< 2  
86 µm) and had an initial gravimetric moisture content of 17.0 %. After sampling, soil was placed under a  
87 fume hood for air-drying, sieved (2 mm mesh) and stored in plastic buckets until setup of soil  
88 microcosm experiments. General soil characteristics are summarized in Table 1.

89 Biochar in two different particle sizes was produced from *Miscanthus x giganteus* (charring at 850°C  
90 for 30 min; PYREG, Dörth, Germany): coarse biochar, pieces of max. 0.3 mm diameter and 20 mm  
91 length, and fine biochar, milled to a particle size of < 2 mm. Biochar characteristics can be found in  
92 Table 1; see Bamminger et al. (2016) for full details. Microscale zero-valent iron (ZVI) (Ferox-Flow,  
93 Hepure, New Jersey, USA) had a particle size ranging from 45 – 150 µm and contained up to 2.5 %

94 carbon and 2 % silicon. The ZVI powder had been stored for about 1.5 years previous to use and  
95 therefore its reactivity may have been somewhat reduced compared to fresh ZVI.

## 96 *2.2. Chemical characterization of soil and biochars*

97 The elemental composition of both soil and biochars were analyzed by inductively coupled plasma  
98 optical emission spectroscopy (Agilent Technologies 5100 ICP-OES 16/6-16, Agilent Technologies Inc.,  
99 Santa Clara, USA) following microwave digestion of 0.2 g pulverized dried material with 5 ml conc.  
100 HNO<sub>3</sub> and 1 ml of 15 % H<sub>2</sub>O<sub>2</sub> in a Milestone Ultrawave microwave (Single Reaction Chamber Microwave  
101 Digestion System). pH of soil and biochar was measured in deionized water suspension, the former in  
102 a soil:water ratio of 1:2.5 (w v<sup>-1</sup>) and the latter in a biochar:water ratio of 1:12.5 (w v<sup>-1</sup>) due to its low  
103 material density. Measurement was performed with a 913 pH meter (Metrohm, Herisau, Switzerland)  
104 after manual shaking and sedimentation for at least 30 min. Soil texture was analyzed by a combined  
105 sedimentation and sieving method as described by Borggaard et al. (2011).

## 106 *2.3. Experimental design*

107 Replicate soil microcosms ( $n = 4$ , except  $n = 6$  for control treatment) were each prepared with 200 g  
108 air dried soil in polypropylene centrifuge bottles (250 mL). The following experimental treatments  
109 were established by mixing the soil homogeneously with biochar and ZVI, in the following  
110 combinations: control (CCA contaminated soil only), BC<sub>coarse</sub> (CCA contaminated soil + 1 % w w<sup>-1</sup> coarse  
111 biochar), BC<sub>fine</sub> (CCA contaminated soil + 1 % w w<sup>-1</sup> fine biochar), ZVI (CCA contaminated soil + 5 % w  
112 w<sup>-1</sup> ZVI), BC<sub>coarse</sub> + ZVI (CCA contaminated soil + 1 % w w<sup>-1</sup> coarse biochar + 5 % w w<sup>-1</sup> ZVI), and BC<sub>fine</sub> +  
113 ZVI (CCA contaminated soil + 1 % w w<sup>-1</sup> fine biochar + 5 % w w<sup>-1</sup> ZVI). Subsequently, the soils were  
114 rewetted with 20 mL Milli-Q water (Time 0), resulting in gravimetric water content of 10.0, 9.9, 9.5,  
115 and 9.4 % for control, biochar only, ZVI only, and the combined treatments respectively, based on dry  
116 weight of soil plus amendments. Microcosms were sealed with perforated parafilm and incubated at  
117 15 °C in the dark for up to 56 days. Soil moisture was adjusted every two to four days to ensure a



118 maximum deviation from initial moisture content of  $\pm 0.9\%$  (absolute). Soil subsamples (7 g) were  
119 collected from each microcosm after 1, 7, 28, and 56 days and immediately extracted and analyzed for  
120 chemical and microbiological properties (see subsequent sections below).

#### 121 *2.4. Soil extraction for chemical and whole-cell bioreporter analyses*

122 Each microcosm soil sample was extracted with 35 mL Milli-Q water (i.e. soil:water-ratio 1:5) in 50 mL  
123 Falcon tubes by shaking for 2 hours at 200 rpm in a horizontal position followed by centrifugation at  
124 10000 g for 20 minutes. The resulting supernatant was analyzed for total water-extractable As ( $As_{\text{water}}$ ),  
125 bioavailable Cu ( $Cu_{\text{bio}}$ ), bioavailable As ( $As_{\text{bio}}$ ), As redox speciation (As(V) and As(III)), pH, and dissolved  
126 organic carbon (DOC). Samples from the last sampling day were additionally analyzed for water-  
127 extractable total Al, As, Ca, Cr, Cu, Fe, K, Mg, P, and Zn as described below.

#### 128 *2.5. Chemical characterization of the soil-water extracts*

129  $As_{\text{water}}$  was determined by GF-AAS (PinAAcle 900Z Atomic Absorption Spectrometer, PerkinElmer,  
130 Waltham, Massachusetts, USA) on acidified subsamples of the extract (0.2 %  $HNO_3$ ). DOC  
131 concentrations were measured with a Shimadzu TOC-VPN-analyser (Shimadzu Corp., Kyoto, Japan) on  
132 a subsample of the supernatant that had been passed through a 0.45  $\mu\text{m}$  cellulose acetate filter (Q-  
133 Max RR syringe filters, Frisette, Denmark) that had been stored at 4 °C for a maximum of 4 weeks.  
134 pH was analyzed on all extracts as described for the general soil characterization (2.2).

135 For samples collected at the end of the experiment (Day 56), water-extractable total Al, As, Ca, Cr, Cu,  
136 Fe, K, Mg, P and Zn were analyzed in the water-extract (3.5 %  $HNO_3$  acid concentration, stored at room  
137 temperature) by ICP-OES.

138 2.6. Whole-cell bacterial bioreporter assays for bioavailable Cu, As and soil toxicity

139  $Cu_{bio}$  was measured with *Pseudomonas fluorescens* DF57-Cu15 while *P. fluorescens* DF57-Cu40E7 was  
140 used as a constitutive control strain for taking potential sample matrix effects (e.g. masking of emitted  
141 light) into account (Brandt et al., 2008; Tom-Petersen et al., 2001). Bioavailable Cu was operationally  
142 defined as Cu species that were able to induce expression of Cu-regulated *luxAB* genes in the employed  
143 *P. fluorescens* DF57-Cu15 bioreporter within a 1.5 h incubation period. Both analysis and subsequent  
144 calculations were performed according to Brandt et al. (2008), except for final re-suspension of the  
145 biosensor cells, which was performed in a MOPS-buffered minimal medium with a low capacity for Cu-  
146 complexation (see Supporting Information **Error! Reference source not found.**).

147 Analogously,  $As_{bio}$  was determined with *Escherichia coli* pJAMA *arsR* (Stocker et al., 2003), and *E. coli*  
148 pUCD 607 HB101 (Rattray et al., 1990) as a constitutive control strain. Thereby, As bioavailability was  
149 operationally defined as As species that were able to induce expression of arsenite-regulated *luxAB*  
150 genes in the utilized *E. coli* pJAMA *arsR* bioreporter within a 2 h incubation period. The bioreporter  
151 strain possesses an arsenate reductase and responds to both inorganic As(V) and As(III). Therefore, the  
152 bioreporter analysis was carried out immediately after the extraction in order to minimize possible  
153 changes in arsenic speciation. Details on the bioassay as well as calibration by As-speciation data can  
154 be found in Supporting Information B, C and Figure S1.

155 Although  $As_{bio}$  and  $As_{water}$  gave, on average, similar results, in this paper we focus our data presentation  
156 and analysis on the  $As_{water}$  data due to the comparably higher precision of this method (Supporting  
157 Information, Figure S2). Likewise,  $Cu_{bio}$  and  $Cu_{water}$  yielded results in the same range, with about 92 %  
158 of  $Cu_{water}$  actually being bioavailable (Figure 2B), but in this case the bioreporter data was of sufficient  
159 quality; we thus used both  $Cu_{bio}$  and  $Cu_{water}$  for data presentation and analysis.

160 *E. coli* pUCD 607 HB101 was also used as a stand-alone toxicity assay in the soil-water extracts in order  
161 to assess the ecotoxicological effects on an introduced test organism. For all bio-assays,

162 bioluminescence was recorded on a plate reader (FLUOStarOptima, BMG Labtech, Ortenberg,  
163 Germany) after addition of decanal as described previously (Nybroe et al., 2008).

#### 164 2.7. Bacterial growth

165 Bacterial growth (i.e. heterotrophic productivity of indigenous soils bacteria) was measured using the  
166 [<sup>3</sup>H]leucine incorporation microcentrifugation technique (Bååth et al., 2001). Briefly, bacteria were  
167 extracted from soil (1 g fresh wt) with 10 mL Milli-Q water on a multi-shaker at highest speed for three  
168 minutes. Following centrifugation (1000 × g, 10 min), 1.5 mL aliquots of the resulting soil bacterial  
169 suspensions (supernatants) were amended with 50 µl of [<sup>3</sup>H]-labelled leucine (6.4 kBq per 50 µL) to  
170 yield a final leucine concentration of 200 nM. Incubations were terminated after either 1 or 2 hours by  
171 adding 50 % trichloroacetic acid (TCA). Bacteria in dead controls were killed with 50 % TCA prior to  
172 addition of [<sup>3</sup>H]leucine. Finally, the incorporated [<sup>3</sup>H]leucine was physically separated from non-  
173 incorporated [<sup>3</sup>H]leucine via a series of centrifugation and washing steps (Bååth et al., 2001) and  
174 radioactivity was measured by scintillation counting (Tri-Carb 2910 TR, Perkin-Elmer, USA). Results  
175 were normalized using the mean growth rate of the control at Day 1.

#### 176 2.8. Statistical analysis

177 Statistical analyses were performed using R (R version 3.3.1, The R Foundation for statistical  
178 computing, 2016) and Microsoft Office Excel (Version 14.0.7166.5000, Microsoft Office Home and  
179 Student, 2010). The *lmer*-function was used to perform regression analysis on  $A_{S_{water}}$ ,  $A_{S_{bio}}$ ,  $Cu_{bio}$ ,  
180 toxicity, and [<sup>3</sup>H]leucine incorporation data. The general model included the interaction between the  
181 two treatments (biochar and ZVI), considering time as fixed effect and the microcosms as random  
182 effects. Model validation was performed by qq-plotting and Shapiro-Wilk-Normality test. Except for  
183  $A_{S_{water}}$ , all statistical analyses were performed on log-transformed data. Non-significant factors and  
184 interactions were excluded from the model by *step*-function. *Contrast*-function within the *lsmeans*-

185 package was used for deriving p-values for pairwise comparisons. P-value adjustment for multiple  
186 comparisons was performed according to the Holm-Bonferroni-method, which controls family-wise  
187 error rate (Holm, 1979). For the data from the ICP-OES analysis (water-extractable total metals at the  
188 last sampling time), two-way ANOVA (*aov*-function) was performed, since no time effect had to be  
189 considered. As for the other analyses, the Shapiro-Wilk Test was performed to check for normality.  
190 Only analysis of Cr-data was performed on log-transformed data, while raw data was used for all other  
191 elements. P-values for statistically significant differences between the treatments were derived using  
192 TukeyHSD test. Throughout, a significance level of  $p < 0.05$  was applied.

### 193 **3. Results**

#### 194 *3.1 Treatment effects on soil chemical properties and bioavailability of As and Cu*

195 In general, the combined treatment of ZVI with BC<sub>fine</sub> was most effective in reducing As<sub>water</sub>, Cu<sub>water</sub>, and  
196 Cr<sub>water</sub> by 43 %, 45 %, and 45 %, respectively, compared to the control and measured after 56 days  
197 (Figure 1). Looking into the effects of the separate treatments, ZVI alone also reduced the three  
198 elements, but to a lesser extent: As<sub>water</sub> was reduced by 27 % while Cu<sub>water</sub> was reduced by 29 %  
199 compared to the control ( $p < 0.001$ ). In addition, Cr was significantly reduced by ZVI alone by 39 %,  
200 resulting in a concentration of  $0.40 \mu\text{g g}^{-1}$  dry soil ( $p < 0.05$ ). Biochar alone did not have a significant  
201 effect on the extractability of any of the elements. Nevertheless, biochar affected the bioavailability of  
202 Cu (Figure 2). At Day 1 and Day 7, Cu<sub>bio</sub> was significantly reduced by BC<sub>fine</sub>, ( $p = 0.014$  and  $p = 0.004$ ,  
203 respectively) (Figure 2A). This was also observed at the end of the incubation period, but the effect  
204 was not statistically significant. In contrast to Cu<sub>water</sub>, which was reduced by ZVI as mentioned above,  
205 Cu<sub>bio</sub> was not affected by ZVI alone (Figure 2B).

206 Soil amendments also affected other chemical soil properties. On average, treatment with biochar  
207 increased pH from  $6.4 \pm 0.04$  to about  $6.7 \pm 0.07$  both with and without ZVI (Day 1). DOC decreased  
208 after the first sampling and generally was very low ( $< 8 \text{ mg L}^{-1}$ ) (data shown in supporting information,

209 Figure S3). As there was an analytical problem with some of the blanks, interpretation of the data must  
210 be treated cautiously; however, it seems that both biochar and ZVI slightly reduced DOC (except for  
211 sampling at Day 56).

212 Treatments with biochar and/or ZVI also affected other elements, as summarized in Table 2. Biochar  
213 significantly increased water extractable fractions of P and K ( $p < 0.01$  and  $p < 0.001$ ), and significantly  
214 reduced possible toxic elements such as Al and Zn ( $p < 0.001$ ). ZVI enhanced the reductions in Al and  
215 Zn content in the extract; however, it also reduced P. In addition, ZVI strongly reduced extractable Fe  
216 and Mg.

217 In general, soil amendment effects on soil chemistry were remarkably stable over time. Hence,  $As_{\text{water}}$   
218 (Supporting Information, Figure S2),  $Cu_{\text{bio}}$  (Figure 2A), pH (data not shown) and As speciation  
219 (Supporting Information, Figure S1) did not exhibit any statistically significant changes during the  
220 experimental period of 56 days.

### 221 *3.2. Effects of soil amendments on soil toxicity*

222 Treatments did not have any consistent effect on toxicity of the soil-water extracts as assessed by the  
223 bioreporter assay with *E. coli* pUCD 607 HB101 (Supporting Information, Figure S4). ZVI increased the  
224 relative response of the bioreporter; however, the effect was only statistically significant at Day 7. No  
225 general trends over time could be observed.

### 226 *3.3. Treatment effects on bacterial growth rate*

227 [ $^3\text{H}$ ]leucine incorporation increased markedly from Day 1 to the subsequent sampling days (Figure 3).  
228 At the last two sampling times (Day 28 and Day 56), biochar significantly increased bacterial growth  
229 rate irrespective of whether or not ZVI was present ( $p < 0.001$  at Day 28,  $p < 0.005$  at Day 56). ZVI  
230 slightly increased [ $^3\text{H}$ ]leucine incorporation as well, but the effect was not statistically significant.  
231 These effects could not be seen at the earlier samplings; instead, the opposite was observed: At Day

232 1, biochar significantly reduced bacterial growth rate compared to the treatments without biochar (p  
233 < 0.001).

## 234 **4. Discussion**

### 235 *4.1. Effects of soil amendments on soil quality*

236 To the best of our knowledge, our study is the first to investigate *in-situ* stabilization with both biochar  
237 and ZVI for remediation of a CCA-contaminated soil and to assess soil quality recovery using a TRIAD  
238 approach with three lines of evidence: chemistry, (eco)toxicology, and ecology. In our study, the  
239 (eco)toxicology line of evidence (bioluminescence inhibition assay) was insensitive to changes imposed  
240 by the experimental treatments and soil quality was best assessed by the other two lines of evidence.  
241 Our study thus demonstrates the value of using the [<sup>3</sup>H]leucine incorporation assay as a sensitive  
242 ecological indicator of soil quality recovery and argues against sole reliance on classical  
243 ecotoxicological short-term assays with introduced test organisms. A pronounced increase in bacterial  
244 growth during the first weeks of the study was observed for all treatments, including the control, and  
245 was certainly a rewetting effect (Meisner et al., 2015), as the soil had been air-dried prior to setting up  
246 the microcosm experiment.

247 As hypothesized, we found that fine biochar in combination with ZVI was the most effective treatment  
248 to significantly decrease the risks posed by As, Cu and Cr. Hence, chemical and ecological lines of  
249 evidence consistently indicated that the combination of BC<sub>fine</sub> and ZVI was the most effective treatment  
250 for reducing exposure and bioavailability of metals (Figures 1 and 2) and restoring the ecological  
251 functionality of the soil (Figure 3). The latter claim is based on the assumption that an increase in  
252 secondary bacterial productivity as measured by the [<sup>3</sup>H]leucine incorporation technique is indicative  
253 of ecological recovery. Generally, treatments with milled biochar (BC<sub>fine</sub>) have invoked a greater effect  
254 as compared with coarse biochar, likely due to its provision of additional sorption sites due to smaller

255 particle size and increased surface area. Nevertheless, the observed treatment effects were moderate  
256 and As and Cu concentrations in the water extract (Figure 1) still exceeded Danish groundwater quality  
257 criteria, by 185-fold and approximately 4-fold, respectively (Danish EPA, 2002). These were chosen as  
258 reference criteria because no general guidelines for water-extractable concentrations exist. Other  
259 studies that have explored the use of novel iron-modified biochars have found them to be comparable,  
260 or even more effective at reducing extractable fractions of As (Qiao et al., 2018; C. Wu et al., 2018), Cu  
261 (Yang et al., 2018a), or Cr (Lyu et al., 2018; Mandal et al., 2017; Zhang et al., 2017); however, none of  
262 them looked into the combined effect on CCA-contaminated soil.

263 Regarding the isolated effect of ZVI, our study is consistent with previous soil studies demonstrating  
264 the ability of ZVI to stabilize Cr, Cu and As (Kumpiene et al., 2006; Nielsen et al., 2016, 2011; Sneath et  
265 al., 2013). With respect to As specifically, our reported two-fold decrease in water-extractable As is  
266 consistent with Sneath et al. (2013), but much lower than the 93 and 98 % reductions in leachable As  
267 reported by other aforementioned studies. These findings suggest that the efficacy of the remediation  
268 treatment is highly dependent on the quality and specific properties of the amendment. We also found  
269 that while water-extractable Cu decreased after treatment with ZVI, albeit less strongly than seen in  
270 the above studies, bioavailable Cu did not. Similarly, Kumpiene et al. (2006) reported a decrease in  
271 pore water concentrations of Cu after treatment with ZVI, but pointed out that ZVI did not reduce  
272 bioaccessible Cu (determined by sequential extraction) and in fact, even increased plant uptake.  
273 Generally, we observed a low water-extractability of Cr, likely because Cr at this site was reported to  
274 be present almost exclusively in the form of Cr(III) (Nielsen et al., 2016) and primarily associated with  
275 hard-to-extract Fe oxides in the oxic top soil (Tardif et al., 2019). In accordance with others, we also  
276 observed a significant reduction in water-extractable Cr upon addition of ZVI (Nielsen et al., 2011;  
277 Sneath et al., 2013; Zhang et al., 2018), probably due to provision of additional sorption sites on Fe-  
278 (hydr)oxide surfaces for Cr(III) (Fendorf, 1995; Kumpiene et al., 2006). Concomitant with the effects of

279 ZVI on soil chemistry, ZVI amendment reduced toxicity only slightly as indicated by the *E. coli* pUCD  
280 607 HB101 biosensor assay and did not significantly increase [<sup>3</sup>H]leucine incorporation rates.

281 Biochar amendments alone did not substantially reduce concentrations of water-extractable elements,  
282 in contrast to earlier findings (Beesley et al., 2014; Mitchell et al., 2018; Sneath et al., 2013; Uchimiya  
283 et al., 2011a), but as expected, biochar did significantly reduce Cu bioavailability. Likewise, a previous  
284 study found that biochar addition to a sandy soil did not alter water-extractable Cu although it did  
285 reduce Cu phytoavailability (Namgay et al., 2010). Our study thus demonstrated that although biochar  
286 was generally less effective than ZVI in reducing water-extractable concentrations of the contaminants,  
287 it was more effective in reducing bioavailable Cu. This may possibly explain why bacterial growth was  
288 more effectively restored with biochar than under ZVI treatment (Figure 3). Hence, Cu most likely  
289 represented the most toxic element in the studied CCA contaminated soil (Tardif et al., unpublished  
290 results) and bioavailable Cu determined with the *P. fluorescens* bioreporter used here has previously  
291 been shown to constitute an excellent predictor of Cu toxicity effects in soil bacterial communities  
292 (Nunes et al., 2016; Song et al., 2017). Our findings are also in accordance with a recent study showing  
293 that biochar amendment restored microbial activity in Cu contaminated soil (Moore et al., 2018). A  
294 number of factors, such as soil properties (e.g. pH, texture, or CEC (Uchimiya et al., 2011a)), biochar  
295 properties (e.g. pyrolysis temperature (Uchimiya et al., 2011b)), redox conditions (El-Naggar et al.,  
296 2018), and/or Cu-concentrations in soil (Ippolito et al., 2012; Lu et al., 2017; Mackie et al., 2015)  
297 influence Cu water extractability and bioavailability in soil after biochar addition. As soil DOC was very  
298 low and soil pH was near neutral in our study, it is plausible that biochar influences were relatively  
299 small and not as pronounced as seen in studies with more acidic soils (Beesley et al., 2014; Uchimiya  
300 et al., 2011a). In contrast to previous literature (Choppala et al., 2015), we did not see any effect of  
301 biochar on Cr<sub>water</sub>. This is likely to have been due to a high fraction of the total Cr having already been  
302 reduced and stabilized in soil oxide minerals (Nielsen et al., 2016, 2011; Tardif et al., 2019) (see above  
303 discussion on effects of ZVI) and therefore, no additional effects of the treatment could be observed.



304 Although our study cannot provide clear predictions of long-term field applicability of ZVI and biochar  
305 amendments for remediation of CCA contaminated soils, it demonstrated that treatment effects on  
306 soil chemical parameters remained quite stable over time. The effects of remediation treatments on  
307 both Cu and As bioavailability were detectable after only one day, indicating that oxidation and  
308 sorption reactions must have occurred during the first 24 hours, as previously shown (Jain et al., 1999;  
309 Nielsen et al., 2011). Long-term efficacy of ZVI has been previously suggested by Tiberg et al. (2016)  
310 who argued that the effect of ZVI on As leaching and mobility should remain stable over at least up to  
311 15 years. In contrast, the efficacy of biochar may be more short-lived, as shown in our study by the fact  
312 that significant reduction in Cu bioavailability ( $Cu_{bio}$ ) was only observed during the first week. This  
313 raises the question of whether or not the observed biochar effect would diminish over time. Also,  
314 organic acids released from plants grown at a site could potentially lead to a release of Cu from biochar  
315 (Oustriere et al., 2017), which would be problematic, since long-term stability of the amendments is  
316 crucial for remediation. Furthermore, flooding, which was frequently observed at this study site, could  
317 be problematic; under anaerobic conditions biochar has been shown to enhance As mobilization,  
318 possibly by acting as an electron shuttle (Kappler et al., 2014; Wang et al., 2017). This was shown to be  
319 of particular concern for biochar produced at high pyrolysis temperatures (Beiyuan et al., 2017).

#### 320 *4.2. Comparison of chemical and microbiological measurements of bioavailability of Cu and As*

321 Assessment of bioavailable element fractions depends on the underlying definition of “bioavailability”  
322 (Semple et al., 2004) and should not be assessed solely by chemical analysis (Kumpiene et al., 2017;  
323 Touceda-Gonzalez et al., 2017). Since *in-situ* stabilization of contaminated sites relies on reducing the  
324 bioavailability of contaminants, we complemented soil chemical analysis with whole-cell biosensors  
325 specific for both As and Cu, but not for Cr, as no biosensor was available. In our study, biosensor  
326 analysis generally yielded concentrations in the same range as chemical analysis, implying that all  
327 dissolved Cu and As species remained bioavailable (Supporting Information, Figure S2 and Figure 2).  
328 This suggests that under the given conditions, water-extractable fractions of both, As and Cu roughly

329 reflected the bioavailable, and thus toxicologically most relevant fractions. Nevertheless, the deviation  
330 between  $As_{water}$  and  $As_{bio}$  appeared rather random and could not be explained by any systematic effect,  
331 but instead likely revealed the sensitivity of the bioassay to other soil properties. Specific interferences  
332 with the As biosensor could be changes in As redox speciation, presence of other elements such as Fe  
333 or P (e.g. Kuppardt, 2010; Trang et al., 2005), influence of co-contaminants, and other general matrix  
334 effects such as changes in pH. Hence, we suggest that further optimization of the As biosensor assay  
335 for contaminated soils is needed. On the other hand,  $Cu_{bio}$  measured with the Cu biosensor did not  
336 significantly exceed  $Cu_{water}$ , but instead showed a consistent trend in the effects of the treatments on  
337  $Cu_{bio}$ . These findings suggest that the Cu biosensor reliably assessed Cu bioavailability even in the  
338 studied multi-element contaminated soil (Figure 2). Consistent with previous studies, which showed  
339 even larger deviations between  $Cu_{bio}$  and  $Cu_{water}$  especially for samples with higher DOC content  
340 (Brandt et al., 2008; Maderova et al., 2011; Nybroe et al., 2008), findings from our study further  
341 confirm the usefulness of complementing chemical measurements with bacterial biosensor data to  
342 infer Cu bioavailability in contaminated soils.

#### 343 4.3. Concluding remarks

344 We conclude that combined application of biochar and ZVI as soil amendments holds promise for *in-*  
345 *situ* stabilization of CCA contaminated sites and for the ecological recovery of soil microbiota at these  
346 sites. As treatment effects were less pronounced than in other studies, we suggest that future attempts  
347 to use these types of amendments for *in-situ* stabilization of multi-element contaminated sites should  
348 focus on the quality and properties of biochar, ZVI, and novel iron-biochar composites to ensure  
349 optimization of remediation. Further research should focus on the long-term stability of these  
350 amendments, their field applicability, and on replicability of the results with other types of biochar and  
351 different soils. More studies are needed to evaluate whether the achieved improvement in soil quality  
352 is sufficient to enable plant growth, and thereby to serve, as suggested previously (e.g. de Oliveira et  
353 al., 2017; Sneath et al., 2013), as a first step towards further remediation.

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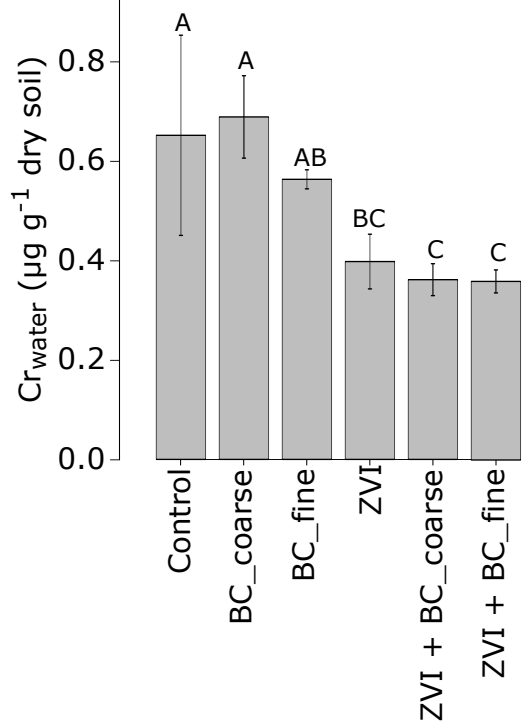
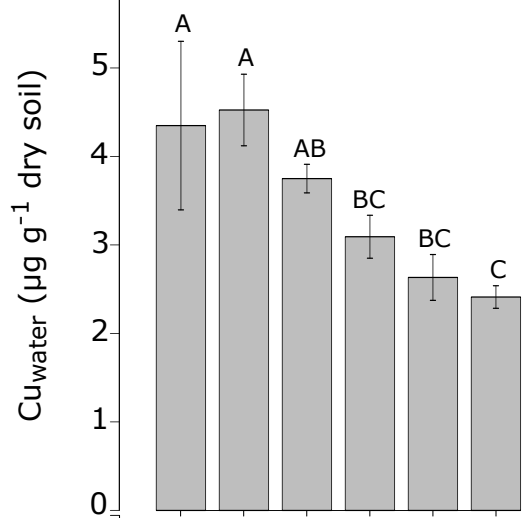
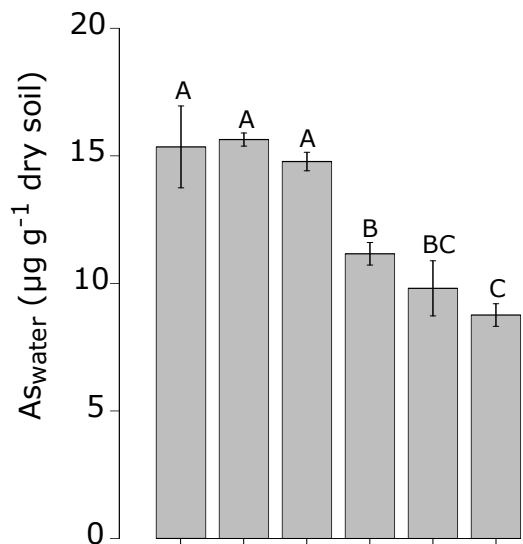
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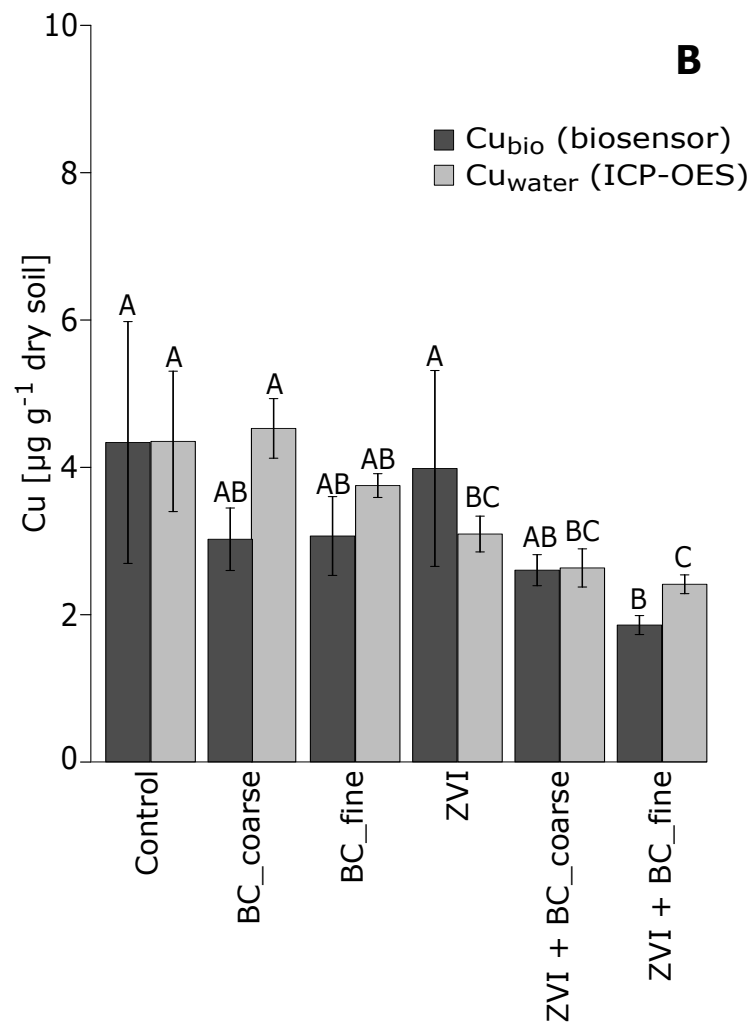
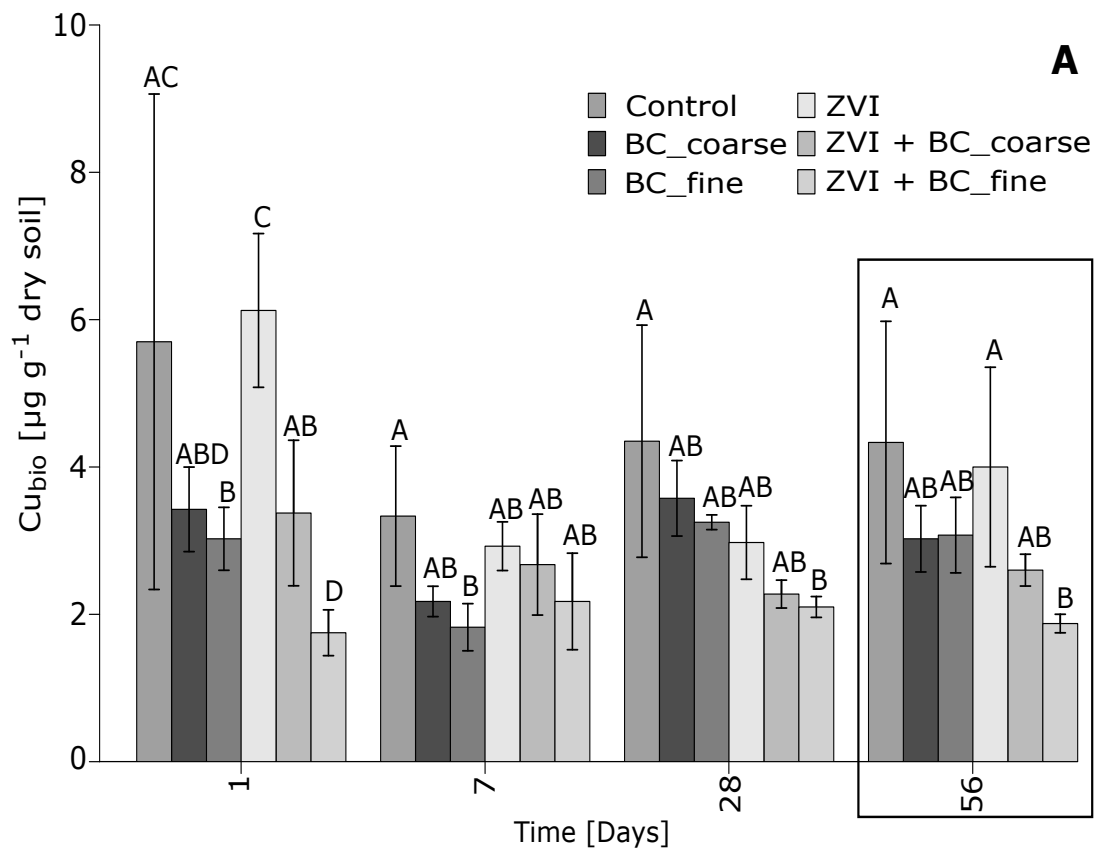
614 **Figure legends.**

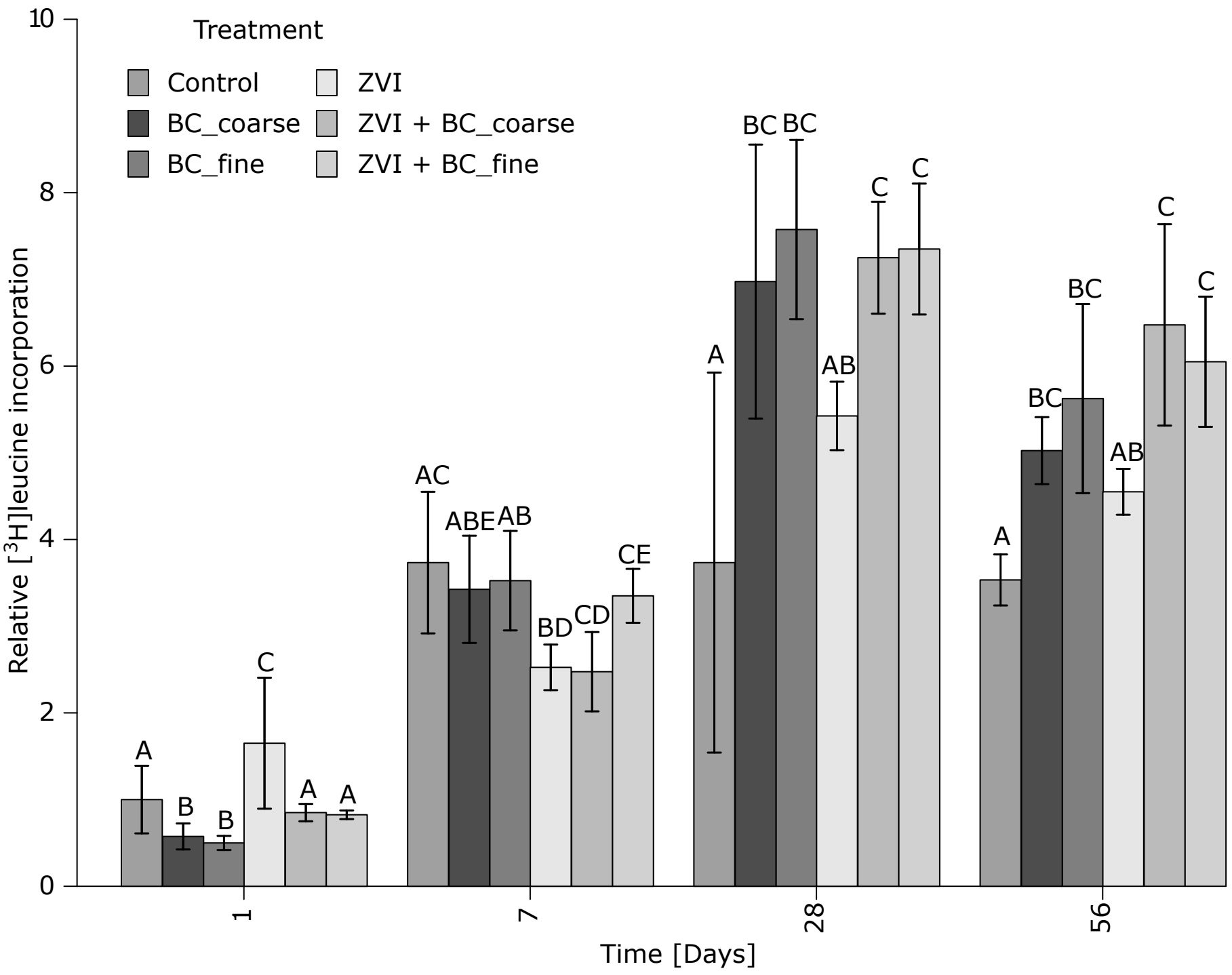
615 **Figure 1.** Total water-extractable arsenic ( $As_{water}$ ), copper ( $Cu_{water}$ ) and chromium ( $Cr_{water}$ ) after 56 days  
616 of incubation, measured with ICP-OES (n = 4 except for control, n = 6). Error bars represent standard  
617 deviations; different letters indicate statistically significant differences between treatments at  $p < 0.05$ .  
618 BC = biochar; “coarse” and “fine” refer to different particle sizes of the biochar; ZVI = zero-valent iron.

619 **Figure 2.** (A) Bioavailable copper ( $Cu_{bio}$ ) measured by Cu biosensor analysis with *Pseudomonas*  
620 *fluorescens* DF57 Cu15 (n=4 except for control, n=6). Error bars represent standard deviations;  
621 different letters indicate statistically significant differences between the treatments ( $p < 0.05$ ). BC =  
622 biochar; “coarse” and “fine” refer to different particle sizes of the biochar; ZVI = zero-valent iron. (B)  
623 Comparison of bioavailable copper ( $Cu_{bio}$ ) and total water-extractable copper ( $Cu_{water}$ ) after 56 days of  
624 incubation. (n=4 except for control, n=6). Error bars represent standard deviation; different letters  
625 indicate statistically significant differences between the treatments ( $p < 0.05$ ); statistical analysis was  
626 performed separately for  $Cu_{water}$  and  $Cu_{bio}$ , meaning that different letters cannot be used for  
627 comparison between  $Cu_{water}$  and  $Cu_{bio}$ . BC = biochar; “coarse” and “fine” refer to different particle  
628 sizes of the biochar; ZVI = zero-valent iron.

629 **Figure 3.** [ $^3H$ ]leucine incorporation, normalized by the mean of the control at Day 1 (n=4 for non-  
630 control treatments; n=6 for control treatment except for control at Day 7, n = 5). Error bars represent  
631 standard deviations; different letters indicate statistically significant differences between treatments  
632 within one sampling time at  $p < 0.05$ . BC = biochar; “coarse” and “fine” refer to different particle sizes  
633 of the biochar; ZVI = zero-valent iron.







**Table 1: Soil and biochar characteristics.**

	$C_{org}$	$N_t$	pH [H <sub>2</sub> O]	Al	As	Cr	Cu	Fe	Pb	Zn
	%	%		[ $\mu\text{g g}^{-1}$ ]						
Soil	2.3	<LOD	6.4	7974	1364	540	1662	10580	29	120
Biochar	77.44	0.58	10.1	911	9	8	50	993	5	69

Table 2: Treatment effect on water-extractable elements after 56 days of incubation. (n = 4 except for control, n=6). Results are presented as means  $\pm$  std. Different letters indicate statistically significant differences at  $p < 0.05$ ). BC = biochar; “coarse” and “fine” refer to different particle sizes of the biochar; ZVI = zero-valent iron.

[ $\mu\text{g g}^{-1}$ ]	Control	BC <sub>coarse</sub>	BC <sub>fine</sub>	ZVI	ZVI + BC <sub>coarse</sub>	ZVI + BC <sub>fine</sub>
Al	88.27 $\pm$ 7.16 <sup>A</sup>	67.49 $\pm$ 3.86 <sup>BC</sup>	59.34 $\pm$ 2.69 <sup>BD</sup>	75.07 $\pm$ 2.16 <sup>C</sup>	51.45 $\pm$ 3.82 <sup>DE</sup>	46.94 $\pm$ 1.19 <sup>E</sup>
Ca	21.16 $\pm$ 0.91 <sup>A</sup>	18.83 $\pm$ 0.44 <sup>B</sup>	19.11 $\pm$ 0.15 <sup>B</sup>	20.58 $\pm$ 0.18 <sup>A</sup>	18.07 $\pm$ 0.36 <sup>B</sup>	18.21 $\pm$ 0.33 <sup>B</sup>
Cr	0.65 $\pm$ 0.20 <sup>A</sup>	0.69 $\pm$ 0.08 <sup>A</sup>	0.56 $\pm$ 0.02 <sup>AB</sup>	0.40 $\pm$ 0.05 <sup>BC</sup>	0.36 $\pm$ 0.03 <sup>C</sup>	0.36 $\pm$ 0.02 <sup>C</sup>
Fe	19.93 $\pm$ 6.16 <sup>AB</sup>	24.63 $\pm$ 2.38 <sup>A</sup>	21.38 $\pm$ 1.76 <sup>A</sup>	13.22 $\pm$ 0.98 <sup>B</sup>	13.90 $\pm$ 0.92 <sup>B</sup>	13.60 $\pm$ 0.85 <sup>B</sup>
K	51.10 $\pm$ 1.88 <sup>A</sup>	121.50 $\pm$ 2.26 <sup>B</sup>	123.88 $\pm$ 3.11 <sup>B</sup>	47.51 $\pm$ 0.86 <sup>A</sup>	108.79 $\pm$ 1.67 <sup>C</sup>	112.34 $\pm$ 3.12 <sup>C</sup>
Mg	4.98 $\pm$ 0.89 <sup>A</sup>	5.74 $\pm$ 0.39 <sup>A</sup>	5.41 $\pm$ 0.21 <sup>A</sup>	3.97 $\pm$ 0.16 <sup>B</sup>	4.09 $\pm$ 0.12 <sup>B</sup>	4.14 $\pm$ 0.09 <sup>B</sup>
P	0.90 $\pm$ 0.17 <sup>A</sup>	1.39 $\pm$ 0.21 <sup>B</sup>	1.43 $\pm$ 0.07 <sup>B</sup>	0.62 $\pm$ 0.07 <sup>A</sup>	0.86 $\pm$ 0.16 <sup>A</sup>	0.75 $\pm$ 0.14 <sup>A</sup>
Zn	0.49 $\pm$ 0.05 <sup>A</sup>	0.37 $\pm$ 0.03 <sup>BC</sup>	0.31 $\pm$ 0.01 <sup>B</sup>	0.40 $\pm$ 0.01 <sup>C</sup>	0.25 $\pm$ 0.02 <sup>D</sup>	0.23 $\pm$ 0.01 <sup>D</sup>

Supporting information for

# Assessment of biochar and zero-valent iron for *in-situ* remediation of chromated copper arsenate contaminated soil

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## A. MOPS-buffered minimal medium for Cu-Biosensor analysis

After harvesting the overnight culture of the Cu-biosensors, the cells were re-suspended in autoclaved MOPS-buffered minimal medium. Resuspension only took place immediately before addition to the microtiter plate. The medium consists of the following:

- 100 mM KCL
  - 20 mM MOPS (pH 7.2 buffer)
  - 7.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>
  - 4 mM glycerophosphate
- ➔ pH adjustment to 7.2 was performed with NaOH
- ➔ after autoclaving (at the day of use), glucose was added to a final concentration of 0.8 % (w v<sup>-1</sup>)



## B. Bioassay

*E. coli* bioreporter cells from both pJAMA arsR as well as pUCD 607 HB101 were revived from cryo stocks on LB agar plates with  $50 \mu\text{g L}^{-1}$  ampicillin incubated overnight at  $37^\circ\text{C}$ . One single colony was transferred to 25 ml LB +  $50 \mu\text{g L}^{-1}$  ampicillin and grown overnight at  $37^\circ\text{C}$  at 200 rpm in a horizontally positioned in 50 mL Falcon tubes. Bioreporter cells in the overnight cultures were harvested at 5000 g for 10 minutes and re-suspended in LB to a final  $\text{OD}_{600}$  of 0.02. Bioreporter cell suspension (100  $\mu\text{L}$ ) was subsequently mixed with 100  $\mu\text{L}$  of As standard solution or sample solution in white 96-well microtiter plates (NUNC™, ThermoFisher Scientific Inc., Massachusetts, USA) and incubated for 2 hours at  $28^\circ\text{C}$ . As standards were prepared with both As(V) ( $\text{Na}_2\text{HAsO}_4 \times 7 \text{H}_2\text{O}$ , 0 – 40  $\mu\text{M}$  range) and As(III) ( $\text{NaAsO}_2$ , 0 – 4  $\mu\text{M}$  range) on each microtiter plate. Finally, As(V) standard calibration data was used to calculate  $\text{As}_{\text{bio}}$ , justified by results of As redox speciation analysis revealing that on average less than 2.1 % of the total As concentration in the extracts occurred as As(III) (see supporting information SI C and Figure S 2 for both As speciation procedure and results).

## C. Arsenic speciation analysis

Arsenic speciation was performed using disposable selective cartridges (MetalSoft Center, NY, USA) which retain arsenate (As-V) while arsenite (As-III) passes through. Water extracts were diluted 10 times in order to not exceed the capacity of the cartridges and passed through the cartridge with a syringe at a speed of  $60 \pm 30 \text{ mL per minute}$ , whereby the first 5 mL were discarded (Krogsriis, 2006; Meng et al., 2001). The following 3 mL of filtrate were collected and acidified with nitric acid (0.2 % final acid concentration) and stored at  $4^\circ\text{C}$  until subsequent analysis on graphite-furnace atomic absorption spectroscopy (GF-AAS, PinAAcle 900Z Atomic Absorption Spectrometer, PerkinElmer, Waltham, Massachusetts, USA). Separation of the species by the cartridge was performed as soon as possible after extraction, at least within 5 hours. Results were compared with results for  $\text{As}_{\text{ext}}$ , giving the total As concentration before the cartridge.

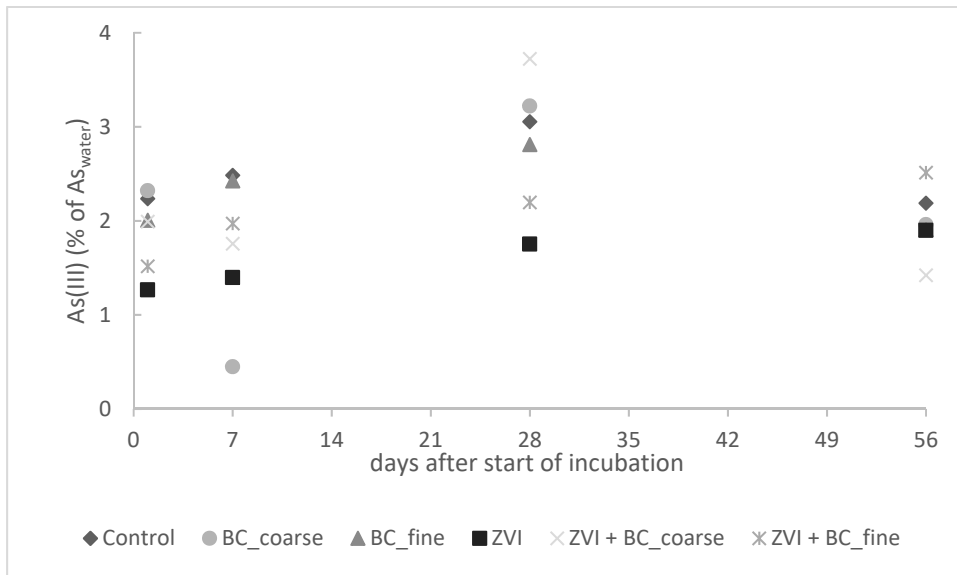


Figure S1: Arsenite (As(III)) content [%] relative to water-extractable arsenic ( $As_{ext}$ ) (GF-AAS).

Measurements were taken before and after passage through a selective speciation cartridge. ( $n = 4$ , for control  $n = 6$ ). (BC = biochar; “coarse” and “fine” refer to different particle sizes of the biochar; ZVI = zero-valent iron)

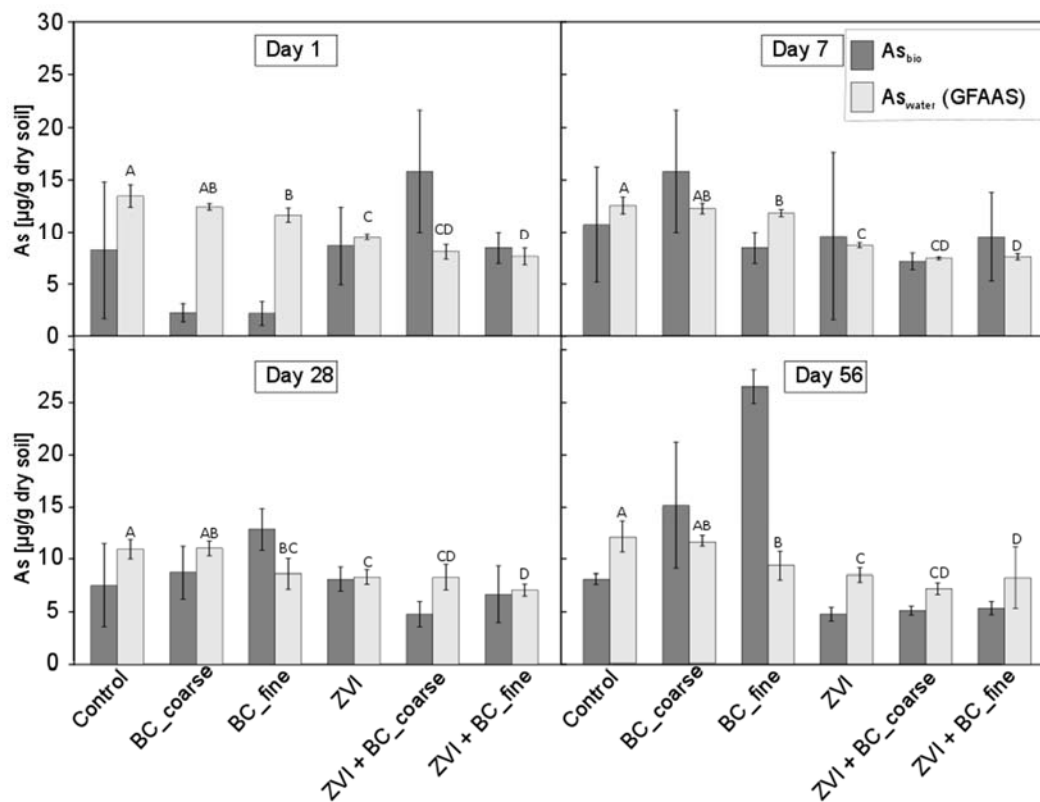


Figure S2: Comparison of  $As_{bio}$  and  $As_{ext}$  (measured with GF-AAS) at the different sampling times. ( $n=4$ , for control  $n=6$ ; error bars represent standard deviation; different letters indicate statistically significant differences between the treatments ( $p < 0.05$ ); statistical analysis was performed separately for  $As_{ext}$  and  $As_{bio}$ , meaning that different letters cannot be used for comparison between  $As_{ext}$  and  $As_{bio}$ ). (BC = biochar; “coarse” and “fine” refer to different particle sizes of the biochar; ZVI = zero-valent iron)

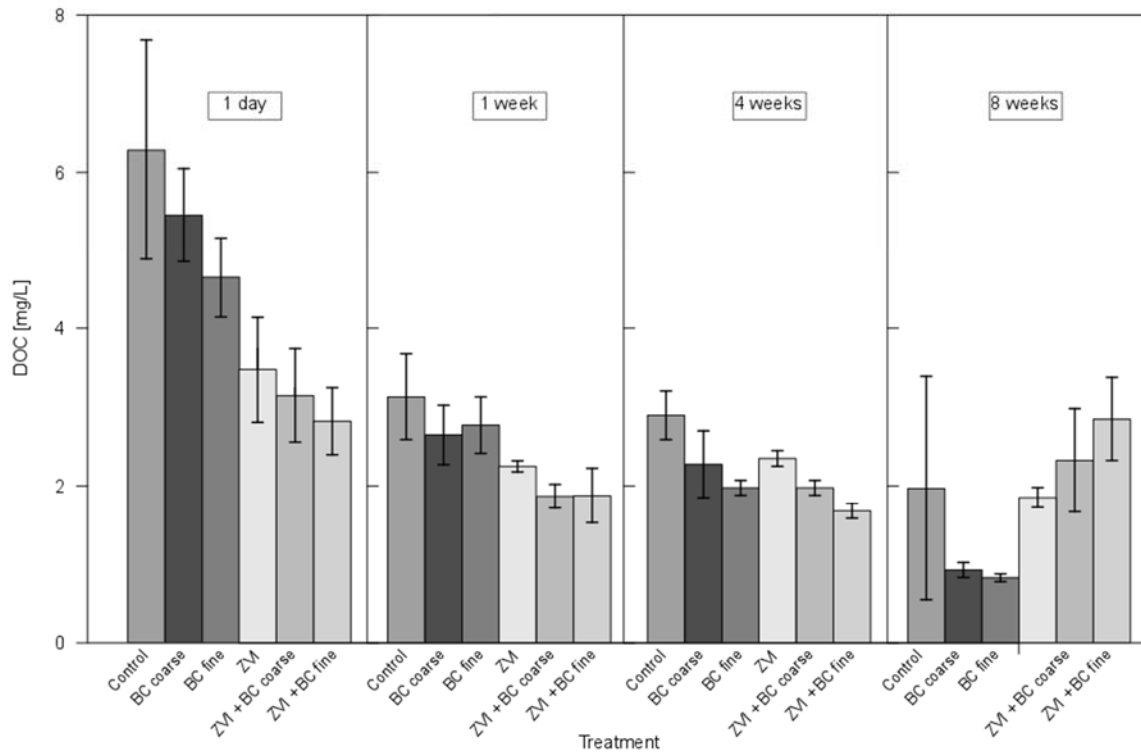


Figure S3: Dissolved organic carbon (DOC) in the water extracts (n=4, for control n=6, except ZVI (n=2) and ZVI + BC coarse (n = 3) at sampling time 1; error bars represent standard deviation) (BC = biochar; “coarse” and “fine” refer to different particle sizes of the biochar; ZVI = zero-valent iron).

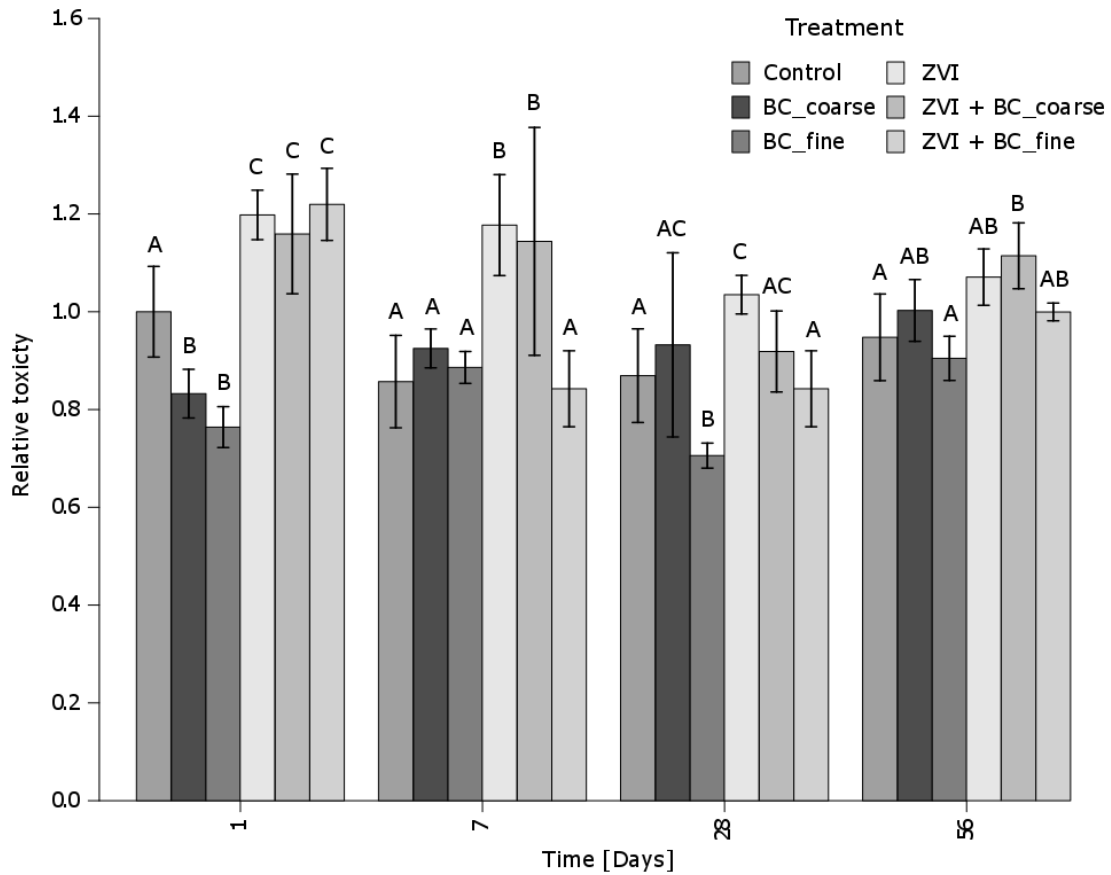


Figure S4: Relative toxicity, measured as bioluminescence from whole-cell bioreporter analysis with *E.coli* pUCD 607 HB101. Values are normalized by the mean of the control treatment at Day 1 with higher values indicating reduced toxicity (n = 4, for control n = 6; error bars represent standard deviation; different letters indicate statistically significant differences between treatments within one sampling time at p < 0.05). (BC = biochar; “coarse” and “fine” refer to different particle sizes of the biochar; ZVI = zero-valent iron)