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Aided phytostabilisation over two years using iron sulphate and organic amendments: effects on soil quality and rye production

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Abstract

An outdoor macrocosm experiment using Fe-based and organic amendments over 2 years was set up to evaluate the effectiveness of aided-phytostabilisation. For that, a soil contaminated with As- and Cu-rich waste material (~13000 mg As kg^{-1} and ~500 mg Cu kg^{-1}) was treated with combinations of iron sulphate (Fe) with lime, paper mill sludge (PS), holm-oak biochar (BC), olive mill waste compost (OMWC) or green waste compost (GWC). Rye (*Secale cereale* L.) was grown in the treated and non-treated soils 16 months after addition of the amendments. Arsenic and Cu dynamics in soil were assessed throughout the experiment and soil quality parameters (soil nutrients, organic matter and soil biology) were measured almost two years after addition of the amendments. All treatments resulted in a reduction of soluble and extractable Cu during the experiment and, despite the increase in soil pH (from 5 to 68) and DOC (from 10 up to 50 mg DOC L^{-1}) provoked by the amendments, As was not significantly mobilised in the treated soils. Treatments combining Fe sulphate with the organic materials, especially biochar and both composts, resulted in an increase in soil available nutrients

and enhanced rye growth. In this semi-field scale experiment, the combination of Fe sulphate with holm-oak biochar showed the most promising results in terms of soil fertility (nutrient availability) , plant As and Cu uptake and soil C sequestration. Further research should focus on monitoring long-term effects of the soil amendments on crops, following repeated applications.

Keywords: phytostabilisation***;*** Secale cereale; iron; biochar; compost; soil functions

1. Introduction

Mining and smelting activities often result in multi-element contamination of surrounding areas, which provokes detrimental effects on soil quality and poses a risk for the environment and for human health (Panagos et al., 2013; Bes et al., 2014; Burges et al., 2015). So-called gentle remediation options (GRO), including the use of plants and amendments (aided-phytostabilisation) have been further developed and investigated in the last decades, as they are considered minimally invasive remediation techniques that may help to simultaneously stabilise the contaminants and improve soil functions and services (Cundy et al., 2013; Kumpiene et al., 2014; Quintela-Sabarís et al., 2017).

Restoration of soil services also involves the recovery of the soil´s capacity to provide, for example, raw materials that might be used for human or livestock feeding, or for biomass production (Volchko et al., 2013). Therefore, using plant species that can be employed for the above-mentioned aims might broaden the benefits of a phytostabilisation strategy. For instance, rye (*Secale cereale* L.) straw and grains can be used either for animal and human feeding or to produce biogas and bioethanol. Additionally, due to its high resistance and tolerance to a wide range of climatic conditions, this species can potentially be grown throughout the majority of Europe (Petersson et al., 2007; Tuck et al., 2006). Furthermore, rye has shown low As translocation to shoots and grains, which makes it a suitable species for its use in phytostabilisation of As-contaminated sites (Álvarez-Ayuso et al., 2016).

The presence of contaminants with different chemical properties, such as As and metals, hinders the selection of adequate soil amendments in aided-phytostabilisation strategies. Given their high sorption capacity, addition of iron oxides or their precursors, might stabilise (i.e. immobilise) As and metals in soils even in the long-term (Hartley et al., 2004; Doherty et al., 2017; Moreno-Jiménez et al., 2017). On the other hand, organic amendments can increase the mobility and bioavailability of As even though metals are generally stabilised (Udovic et al., 2012; Beesley et al., 2014). Therefore, with aided-phytostabilisation the aim is to facilitate the development of a plant cover (by using selected amendments) that minimises dispersion of contaminants through wind erosion or leaching while improving soil functions. Organic amendments have shown to enhance soil functions, especially by correcting soil acidity and improving soil fertility, allowing the establishment of a plant cover (Alvarenga et al., 2009a; Touceda-González et al., 2017). Moreover, the use of organic residues as amendments enhances the sustainability of the phytostabilisation strategy. Nevertheless, possible adverse effects effects of these materials and their effectiveness in the long-term need to be evaluated prior application to real scenarios (Alvarenga et al., 2009a). In general, since the sustainability of a soil remediation strategy entails little maintenance, if not none, long-term studies should always be carried out to evaluate the durability of the stabilising effects (Mench et al., 2006; Bidar et al., 2016).

Other authors have reported the combination of iron-based amendments and organic materials, such as biochar and compost, as a promising approach to deal with co-contamination issues (Sneath et al., 2013; Garau et al., 2019, 2017). Moreover, previous pot experiments showed promising results when iron sulphate and organic materials were applied to an As- and Cu-contaminated soil, because sometimes this combination can immobilise metals and increased plant growth (Fresno et al., 2016; 2017). These previous studies attributed the metal immobilisation to sorption in organic materials and to rises on pH and the As immobilisation to binding to solid iron phases, so the combination of both amendments may cause a synergistic effect. However, most of these experiments have been done at a small, short scales and larger scale and longer-term effects have not been evaluated yet. The present work aims to assess the

efficiency of combining iron-based and organic amendments to stabilise As and Cu in a contaminated soil, improve soil quality and establish a plant cover over 20 months. We evaluated the contaminants mobility in soil and its relationship with other soil parameters upon addition of the amendments. Several soil quality indicators, such as soil chemistry (soil C, pH and conductivity), soil fertility (nutrient availability) and the effects on rye growth, were also assessed at the end of the experiment.

2. Materials and methods

2.1. Soil, amendments and experimental set-up

A waste material with high pseudo-total contents of As and Cu was collected from the spoil heaps of an old smelting factory in the north of Madrid (Spain) and was used to contaminate a sandy-loam soil collected from a nearby area. Further details of the waste material and the area can be found in Recio-Vazquez et al. (2010) and Gómez-González et al. (2014). An outdoor macrocosm experiment was set up at the *Universidad Autónoma de Madrid* campus. PVC containers (120 cm long × 100 cm wide × 20 cm high) were filled with a ~5-cm layer of gravel; the soil and the contaminated material (~120 kg in total) were then mixed in a 90:10 (v:v) ratio, placed in the containers, thoroughly homogenised and left to react for 9 months. The experiment aimed at mimicking the process in the real world, where mine wastes are dumped on top of adjacent native soils and the remediation would involve mixing the upper layer of soil with the wastes and amendment and subsequent cropping.

Iron sulphate ($FeSO_4 \cdot 7H_2O$, PRS grade, Panreac) was used as an iron-based soil amendment and was mixed either with $CaCO_3$ (PRS grade, Panreac) or with one of the following materials: (1) paper mill sludge (PS), obtained from the company Holmen Paper S.L. (Madrid, Spain); (2) holm oak biochar (BC), produced by the pyrolysis of holm oak woodchips at 600 ºC Fresno et al., 2016); (3) olive mill waste compost (OMWC), prepared from solid olive mill waste (alperujo) and cow manure at CEBAS-CSIC (Murcia, Spain) or (4) green waste compost (GWC), made from green wastes from public gardens at the composting facility Migas

104 Calientes (Madrid, Spain). The main characteristics of PS, BC, OMWC and GWC are shown in
105 Table SM1.
106 The amendments were applied on a dry soil weight basis (w:w), considering bulk soil density
107 and soil volume in each container, and consisted of: (i) 1% $FeSO_4$ + 0.4% $CaCO_3$ (treatment
108 Fe+lime); (ii) 1% $FeSO_4$ + 2% PS (Fe+PS); (iii) 1% $FeSO_4$ + 5% BC; (iv) 1% $FeSO_4$ + 5%
109 OMWC (Fe+OMWC); (v) 1% $FeSO_4$ (w:w) + 5% GWC (Fe+GWC). A control consisting of the
110 non-treated soil was included. One container was used per treatment and the different soil,
111 porewater and plant samples collected were considered as replicates. Table SM2 shows soil pH,
112 electric conductivity (EC) and the pseudo-total concentration of As, Cu and Fe in each container
113 13 months after the addition of the amendments.
114 For a better understanding of the experimentation, a summary chronogram can be found in
115 Figure SM1, reporting the main sampling and analyses done in each sample. Five soil samples
116 were collected from each container 7, 13, 14 and 20 months after addition of amendments. All
117 soil samples were sieved to <2 mm; sub-samples from all sampling times were air-dried for
118 physico-chemical analysis and sub-samples from the ultimate sampling (20 months) were stored
119 at 4 ºC for the analysis of enzymatic activities.
120 Sixteen months after addition of the amendments, in late fall, the containers were sown with
121 *Secale cereale* L. (8 g seeds m^{-2}). Porewater samples (5 per treatment) were collected from each
122 container 15, 17, 18, 19 and 20 months after the addition of the amendments (*i.e.*1 month before
123 and 1, 2, 3 and 4 months after sowing, respectively). Porewater pH was immediately measured
124 and samples were stored at 4 ºC for further analysis (DOC, nutrients and metals).
125 Rye was harvested in two phases corresponding to two phenological stages, in order to analyse
126 As and Cu concentration both in shoots and grains: 4 and 6 months after sowing, before
127 flowering and at maturity, respectively. At the first harvest (4 months after sowing and 20
128 months after the start of the experiment), the soil surface in each container was divided into 8
129 subplots avoiding non-vegetated borders. Aboveground parts of plants grown in 4 non-adjacent
130 out of the 8 subplots were collected by cutting the stems at ~2 cm above the soil surface. At
131 maturity (second harvest) shoots and ears of remaining plants were collected and grains were

132 separated from the ears for further analysis. Plants were dried at 65ºC for 3 days and ground
133 with a ball mill for mineral analysis.

134 ***2.2. Soil and plant analysis***

135 Soil pH was measured in soil-water extracts (1:2.5 w:v) of samples collected at 7, 13, 14 and 20
136 months and directly in all porewater samples collected by suction with Rhizon samplers
137 (Rhizosphere Research Products, Wageningen, Netherlands). The electric conductivity was
138 measured in soil-water extracts (1:5 w:v) of the soil samples collected at 14 months and in soil
139 porewater samples.

140 The labile fraction of As and Cu was determined by extraction of soil samples collected at 7, 14
141 and 20 months with 0.1 M $(NH_4)_2SO_4$ in a 1:10 ratio soil:solution ratio, shaken at 180 rpm for 4
142 h) and subsequently filtrated with Whatman #40 filter. The pseudo-total element concentration
143 was measured in soil samples collected at 13 months after digestion of 0.5 g sub-samples with
144 HNO_3 (6 mL) + H_2O_2 (4 mL) in an autoclave (Moreno-Jiménez et al., 2010), filtrated with a
145 Whatman #40 and diluted to 50 mL with miliQ water. Arsenic fractionation was determined in
146 soil samples collected after 13 and 20 months by a sequential extraction modified from Larios et
147 al. (2012). Briefly, the fractions extracted in each step were: F1, readily soluble (H_2O, 24 h); F2,
148 strongly adsorbed onto mineral surfaces (0.5 M Na_2HPO_4, pH=8, 8 h); F3, associated with Al
149 oxyhydroxides (0.5 M NH_4F, pH=8.2, 15 h); F4, bound to organic matter (0.1 M $Na_4P_2O_7$, 16
150 h); F5 incorporated into amorphous Fe oxyhydroxides (0.2 M ammonium oxalate–oxalic acid,
151 pH 3, darkness, 2+2 h);; F6, incorporated into amorphous Fe oxyhydroxides (0.2 M sodium
152 citrate + 0.6 M $NaHCO_3$ + 0.4 M ascorbic acid pH=8, 21 h ×2); FR, residual fraction (acid
153 digestion). The concentration of available nutrients was analysed after *S. cereale* first harvest,
154 i.e. after 20 months. Exchangeable K, Mg, and Ca were determined by soil extraction with 1 M
155 ammonium acetate (pH 7) (Simard, 1993) and P-Olsen by extraction with 0.5 M $NaHCO_3$
156 (Olsen, 1954).

157 Ground rye biomass (shoots) was acid digested with 5 mL of HNO_3 (65% v/v) and 1 mL of
158 H_2O_2 (30% v/v) at 125 ºC under a pressure of 1.5 kg cm^{-2} for 30 min (modified from Lozano-

159 Rodríguez et al. (1995)). Rye grains, previously ground into a fine powder, were left overnight
160 with 5 mL of HNO_3 (65% v/v) and 1 mL of H_2O_2 (30% v/v) and then digested as indicated
161 above.
162 Arsenic was measured in the $(NH_4)_2SO_4$-extracts by HG-AFS (PS Analytica 10.055, Millenium
163 Excallibur) and Cu by atomic absorption spectroscopy (AAS) (Analyist 800, Perkin Elmer). Cu
164 and As concentration in porewater samples and plant digests was analysed by ICP-MS (Elan
165 9000 DRCe, Perkin Elmer). The concentration of Ca, Mg, K and P in soil extracts and plant
166 digests was measured by ICP-OES (ICAP 6500 DUO, Thermo Scientific). Dissolved organic
167 carbon (DOC) concentration in porewater samples collected at 15 and 20 months was analysed
168 with a TOC analyser (Shimadzu TOC-V CSH).

169 ***2.3. Soil enzymatic activities and ecotoxicity bioassay***

170 Soil enzymatic activities (dehydrogenase, β-glucosidase and acid phosphatase) were analysed
171 after *S. cereale* harvest (20 months after the start of the experiment) in the control and the
172 treated soils. For that, soil samples were sieved at <2 mm and stored at 4 ºC. For the analysis of
173 dehydrogenase activity, soil samples were incubated with 2,3,5-triphenyltetrazolium chloride
174 for 16 h at 25 ºC in the dark. The resulting product, triphenylformazan, was measured
175 spectrophotometrically at 546 nm (Tabatabai, 1994). Acid phosphatase and β-glucosidase
176 activities were determined by their reaction with the substrates p-nitrophenylphosphate and p-
177 nitrophenyl-β-glucopiranoside, respectively (Moreno-Jiménez et al., 2012). Soil samples were
178 incubated with the corresponding substrate for 1 h at 37 ºC and the product, p-nitrophenol, was
179 measured spectrophotometrically at 410 nm.
180 The inhibition of the luminescence of the bacteria *Vibrio fischeri* was used as an indirect
181 exposure bioassay to evaluate the potential toxicity of soil leachates to this organism. Soil
182 samples collected at 14 and 20 months were used for this assay. To obtain soil leachates, all
183 replicates from each treatment were merged into a composite sample, mixed with deionized
184 water in a 1:10 (w:v) ratio and shaken for 24 h (DIN 38 414-S4). The freeze-dried luminescent
185 bacteria (Biotox^{TM} Kit, Aboatox Oy, Finland) were reconstituted by suspension of a few

186 milligrams of the bacteria in 2% NaCl (w/v). Soil leachates were diluted with 2% NaCl (w/v) to
187 achieve concentrations of 0, 12.5, 25, 50 and 100% (v/v). Then, a given volume of each diluted
188 solution was put in contact with the bacteria at 15 ºC and the decrease in the luminescence was
189 measured after 15 and 30 minutes using a luminometer (Optocom I, MGM Instruments).

190 ***2.4. Statistical analysis***

191 The statistical analysis of data was carried out using the software IBM SPSS 21.0. One-way
192 ANOVA analyses were performed after homogeneity of variances was confirmed. Post hoc
193 analyses were carried out to establish differences among treatments; Tukey´s HSD test was used
194 for homoscedastic data and Games-Howell´s for heteroscedastic data. Bivariate correlations and
195 multiple linear regression analyses were used to evaluate relationships between variables.

196

3. Results

3.1. Soil pH and As and Cu mobility along the experiment

The pH of the control soil was acidic and did not change significantly over the experiment (Fig. 1). The treatments Fe+lime, Fe+PS and Fe+BC showed the greatest liming effect, since their addition provoked a significant increase ($P < 0.05$) in soil pH compared to the control all throughout the experiment. At the last sampling time (20 months) soil pH was higher ($P < 0.05$) in all treated soils than in the control.

The concentration of $(NH_4)_2SO_4$-extractable As was measured at three time points: 7, 14 and 20 months after addition of the amendments (Fig. 1). After 7 months, treatments Fe+lime, Fe+PS and Fe+GWC resulted in a significant decrease ($P < 0.05$) in the concentration of extractable As compared to the control, whilst Fe+OMWC and Fe+BC increased or did not affect As mobility, respectively ($P < 0.05$). A slight decrease in the concentration of extractable As was observed from 7 to 14 months in most treatments. At 14 months extractable As was lower ($P < 0.05$) in Fe+lime and higher ($P < 0.05$) in Fe+PS than in the control, and no differences were found between Fe+BC, Fe+OMWC, Fe+GWC and the control. Between 14 and 20 months all treatments except Fe+PS showed a significant increase ($P < 0.05$) in extractable As. No statistical differences were found between the control and the treated soils at 20 months, likely due to the high variability of the data within each treatment, but extractable As was slightly higher in Fe+PS, Fe+BC, Fe+OMWC and Fe+GWC than in the control (Fig. 1).

Extractable Cu was reduced by 55-94% ($P < 0.05$) by all treatments at the first sampling time; the greatest reduction was observed in Fe+BC (94%) and Fe+PS (91%). In general, the concentration of $(NH_4)_2SO_4$-extractable Cu remained similar over the experiment, but in treatments combining iron and both composts (Fe+OMWC and Fe+GWC) it was even lower at 20 than at 7 months ($P < 0.05$). At the last sampling time, a reduction of 92-99% was observed in all treated soils (Fig. 1).

223 Figure 2 shows the distribution of As in different soil fractions extracted in a seven-step
224 sequential extraction applied to samples collected at 13 and 20 months. Due to the heterogeneity
225 of the pseudo-total As concentration between treatments (Table SM2), results of the sequential
226 extraction are shown as the percentage of As extracted in each step with respect to pseudo-total
227 concentration. The recovery of the sequential extraction procedure was in all cases > 90%. In
228 soil samples collected at 13 months, the concentration of As extracted in F1 (readily soluble As)
229 was similar in the control and the treated soils. This fraction accounted for up to 0.12% of the
230 pseudo-total As, but As concentration leached in this step was high in all treatments (7.6-21.2
231 mg kg^{-1}). Readily soluble As increased ($P < 0.05$) between 13 and 20 months (from 0.05-0.12%
232 to 0.16-0.35%) and was higher in all treated soils than in the control. Arsenic strongly adsorbed
233 onto mineral surfaces (F2) represented ~1% of the pseudo-total As after 13 months and was
234 significantly increased ($P < 0.05$) by Fe+lime, Fe+PS, Fe+BC and Fe+GWC, with respect to the
235 control. A significant increase ($P < 0.05$) in this fraction was observed in Fe+lime, Fe+PS,
236 Fe+BC between 13 and 20 months. The concentration of As released in F3 (associated with Al
237 oxyhydroxides), was also low compared to the total As in the soil (less than 2%). This As
238 fraction was not significantly affected by any treatment within the same sampling time, but a
239 significant increase ($P < 0.05$) was observed in Fe+PS between 13 and 20 months. Arsenic
240 concentration extracted in F4 (bound to organic matter) was similar in the control and the
241 treated soils at both sampling times, but increased ($P < 0.05$) between 13 and 20 months in all
242 cases. Arsenic was primarily incorporated into Fe (hydr)oxides; the sum of F5 and F6 accounted
243 for more than 70% in all cases. This As fraction was not greatly affected by the amendments, as
244 no significant differences were found between the control and the treated soils. Amorphous iron
245 (hydr)oxides (F5) was the most abundant As-associated fraction and decreased ($P < 0.01$) from
246 60-69% to 43-49% between 13 and 20 months in the control and most treatments, except in
247 Fe+OMWC, where remained similar (54-59%). On the other hand, As extracted in F6
248 (associated with poorly crystalline Fe (hydr)oxides) increased over time in all cases, although
249 differences were significant ($P < 0.05$) only in Fe+lime (from 14% to 24%), Fe+BC (from 20 to
250 28%) and Fe+GWC (from 21% to 31%). The residual As fraction, FR, accounted less than 20%

in all cases and was significantly higher only in Fe+PS and Fe+BC than in the control in the last sampling time.

3.2. Soil porewater chemistry during Secale cereale cultivation

Soil porewater was collected 1 month before and 1, 2, 3 and 4 months after sowing *S. cereale* seeds, corresponding to 15, 17, 18, 19 and 20 months after amendments addition. Figure 3 shows porewater pH values and the concentration of As and Cu throughout the experiment, and DOC concentration at 15 and 20 months (first and last sampling times).

The addition of all treatments promoted an increase in porewater pH along the experiment. pH values were between 0.8 and 1.6 units higher in Fe+lime, Fe+OMWC and Fe+GWC than in the control soil and ~2 pH units higher in Fe+PS and Fe+BC. Arsenic concentration in porewater was not affected by most of the treatments, and only Fe+PS provoked a significant increase ($P < 0.05$) with respect to the control (by up to 9.7-fold) and the other treatments. Despite no statistical differences were found in As solubility along the experiment ($P < 0.05$), an increasing tendency was observed in most cases. Copper concentration in porewater was reduced ($P < 0.05$) by all treatments at all sampling times. Within treatments combining iron and organic materials (i.e. Fe+PS, Fe+BC, Fe+OMWC and Fe+GWC), soluble Cu was generally lower in Fe+BC, especially in the last sampling times, when concentrations in Fe+BC were lower ($P < 0.05$) than in Fe+OMWC and Fe+GWC (19 months) and Fe+PS (20 months). After 15 months, the addition of Fe+PS, Fe+BC, Fe+OMWC and Fe+GWC increased ($P < 0.05$) DOC concentration in porewater, which increased over time in the control and Fe+BC. At the last sampling, DOC was higher ($P < 0.05$) in Fe+PS, Fe+OMWC and Fe+GWC than in the control and Fe+lime, whereas F+BC did not show statistical differences with any treatment.

3.3 Effects on soil nutrients and S. cereale growth and nutritive status

The addition of iron sulphate and both composts (Fe+OMWC and Fe+GWC) increased ($P < 0.05$) exchangeable K and Mg, P-Olsen and TOC in soil with respect to the control, Fe+lime and Fe+PS after 20 months (Table 1). Moreover, both treatments enhanced TN content by ~20 fold with respect to the control. Addition of biochar (Fe+BC) also promoted a significant

278 increase ($P < 0.05$) in exchangeable K, P-Olsen and TOC and resulted in ~30 fold higher TN
279 (not statistical difference) over the control soil. On the other hand, the effect of Fe+lime and
280 Fe+PS on soil nutrients was negligible and only showed a significant effect on exchangeable
281 Ca, which was higher ($P < 0.05$) in all treated soils than in the control (Table 1).
282 Plant growth was evaluated 4 and 6 months after sowing by means of rye shoots dry weight
283 (DW) (Fig. 4). In agreement to effects observed on soil nutrients, addition of Fe+BC,
284 Fe+OMWC and Fe+GWC improved rye growth, increasing shoots DW by 5.1-, 2.6- and 4.0-
285 fold with respect to the control after 4 months (Fig. 4). Treatment Fe+PS did not affect plant
286 growth after 4 months, but between 4 and 6 months, plants in this treatment increased their
287 biomass by >8-fold, resulting in significant differences with respect to the control at the last
288 sampling. After 6 months, rye DW were higher ($P < 0.05$) in Fe+PS, Fe+BC and Fe+OMWC
289 than in the control, slightly higher in Fe+GWC and similar in Fe+lime. In terms of plant cover,
290 evaluated 4 months after sowing, treatments Fe+BC, Fe+OMWC and Fe+GWC showed the
291 most promising results, as rye colonised almost the entire soil surface (Fig. SM2). On the other
292 hand, Fe+lime and Fe+PS slightly enhanced seed germination, as shown by a poor plant cover
293 in these treated soils.
294 The concentration of several macro and micronutrients was analysed in shoots of plants
295 collected after 4 months (Table 2). In general, the treatments did not positively affect nutrients
296 concentration in plant tissues, as none of them led to a significant increase in the elements
297 analysed. Due to the high variability in plant growth between treatments and thus possible
298 dilution effects, nutrients content in shoots was calculated (not shown). The greatest effect was
299 observed for treatments combining iron and compost or biochar. Contents of K, Ca, Mg, P and
300 Mo in plant tissues were enhanced by Fe+BC and Fe+OMWC with respect to the control ($P <$
301 0.05), whereas Fe+GWC significantly increased K, P, Ni accumulation in rye shoots ($P < 0.05$),
302 and slightly increased Mg and Mo content. Generally, Fe+lime and Fe+PS had little effect on
303 the accumulation of nutrients in rye shoots.

3.4. Arsenic and Cu concentrations in S. cereale shoots and grains

Arsenic and Cu concentrations in shoots were analysed in rye shoots collected after 4 and 6 months and in grains collected after 6 months (Table 3). In the first harvest, only Fe+PS increased ($P < 0.05$) As concentration in shoots with respect to the control, although As content was also slightly higher in Fe+GWC. After 6 months, shoots As concentration was lower ($P < 0.05$) in Fe+BC, Fe+OMWC and Fe+GWC, however, this was likely due to a dilution effect, since As content in shoots was similar in the control and all treated soils. Arsenic concentration in rye grains varied between 0.32 and 0.71 mg kg^{-1} and was slightly lower in Fe+lime and Fe+PS than in the control, whereas the reduction ($P < 0.05$) in treatments Fe+BC, Fe+OMWC and Fe+GWC accounted for 52%, 41% and 55%, respectively (Table 3).

Only Fe+BC and Fe+OMWC significantly reduced shoots Cu concentration in plants collected after 4 months, but similar to As, this seemed to be a dilution effect, since an increase in Cu content was found in plants grown in all treated soils (Table 3). After 6 months, no statistical differences were found in shoots Cu concentration in the control and the treated soils, and total Cu uptake was ∼2.5 fold higher in Fe+PS, Fe+OMWC and Fe+GWC (not significant) and ∼3 fold higher ($P < 0.05$) in Fe+PS than in the control. Grain Cu concentration was significantly reduced ($P < 0.05$) by Fe+BC, Fe+OMWC and Fe+GWC.

3.5. Effects on soil enzymatic activities and toxicity towards Vibrio fischeri

Results of dehydrogenase, acid phosphatase and β-glucosidase activities in the control and the treated soils are shown in Figure 6. Dehydrogenase activity was higher ($P < 0.05$) in Fe+lime, Fe+PS, Fe+OMWC and Fe+GWC treated soils, the two latter showing the greatest effect. No significant differences were found between the control and Fe+BC-treated soil. Acid phosphatase activity was lower ($P < 0.05$) in Fe+PS and Fe+BC and significantly higher ($P < 0.05$) in Fe+GWC than in the control, whereas Fe+lime and Fe+OMWC had little effect on this enzymatic activity. Only Fe+GWC and Fe+BC significantly affected ($P < 0.05$) β-glucosidase activity; an increase was observed in the former while the latter provoked a decrease. The addition of the other treatments did not greatly affect this enzyme activity.

The effect of soil leachates on the luminescent bacteria *Vibrio fischeri* was assessed for the control and the treated soils sampled 14 and 20 months after addition of amendments, the last one corresponding to the first *S. cereale* harvest. Results obtained are shown as the percentage of soil leachates that caused a reduction of 50% on the luminescence of the bacteria (EC_{50}, Table SM3). At the first sampling time (14 months), leachates from control soil resulted to be more toxic for *V. fischeri* than those from all treated soils, as shown by the lowest EC_{50} values. Treatments Fe+lime, Fe+PS and Fe+BC had similar effect on the luminescent bacteria and the the greatest EC_{50} values were obtained for Fe+OMWC, 2.4 and 2.9 fold higher than in the control after 15 and 30 minutes of exposure, respectively. At the last sampling time (20 months), EC_{50} values obtained for control, Fe+lime and Fe+PS soils were higher than at 14 months, whereas those for Fe+BC and Fe+GWC remained similar and even decreased for Fe+OMWC.

4. Discussion

4.1 Effect of soil amendments on As and Cu mobility and their transfer to rye above-ground tissues

In this semi-field scale experiment we observed that addition of Fe sulphate to the contaminated soil, combined with other materials, stabilised Cu and did not substantially increase extractable As over two years (Fig. 1). In spite of the significant reduction (Fe+lime; Fe+PS and Fe+GWC) or increase (Fe+OMWC) that treatments provoked on the concentration of the $(NH_4)_2SO_4$-extractable As seven months after addition of the amendments, no statistical differences between treatments were found after 20 months, although As seemed to be slightly mobilised in Fe+PS, Fe+BC and Fe+OMWC.

A linear regression analysis was performed with data from the last sampling time (20 months) in order to evaluate the influence of several known factors on extractable As. We selected total As, soil pH, available P (P-Olsen) and DOC in porewater as variables, which have been reported to be critical factors affecting As mobility in soils (Smith et al., 1998; Fitz and Wenzel, 2002; Tao et al., 2006; Beesley et al., 2010; Moreno-Jiménez et al., 2013).

$$[As]_{ext,\ 20m} = 6.101 + 0.136\ [DOC]_{PW,\ 20m} \quad R^2 = 0.169 \quad F_{1,29} = 5.7 \quad P < 0.05 \quad \text{(Eq. 1)}$$

Equation 1 shows that, among all the variables tested, DOC was the factor that better explained variations in extractable As between treatments 20 months after treating the soils. This is not surprising considering the addition of soluble organic carbon might provoke the release of labile As forms, even if the soil presents high As adsorption capacity (Bauer and Blodau, 2006; Arco-Lázaro et al., 2016).

Results of soluble As somehow mirrored those observed for extractable As, as most treatments had little effect on As concentration in porewater between 15 and 20 months. Only Fe+PS provoked a significant increase in As solubility with respect to the control and the other treatments. A significant positive correlation was found between pH and As in porewater at all

sampling times (15 months: r = 0.439, $P < 0.05$; 17 months: r = 0.392, $P < 0.05$; 18 months: r = 0.651, $P < 0.001$; 19 months: r = 0.355, $P < 0.05$; 20 months: r = 0.565, $P < 0.01$), which could partly explain the effect observed for Fe+PS. This treatment provoked a significant increase in porewater pH relative to the other treatments (similar to Fe+BC), besides a significant increase in DOC, suggesting that the increase in porewater pH in this treatment may have decreased the metal oxides positively-charged surface area, thus competition of soluble organic anions with arsenate for sorption sites could have been enhanced, resulting in As mobilisation (Bauer and Blodau, 2006). Our results confirm that increasing the pH is critical to recover acid mine sites and immobilise metals, but a careful control of soil pH is needed to avoid As mobilisation in cases of co-contamination with metals.

One interesting finding regarding As mobility was the increase in extractable As observed in all treatments, including the control, between months 14 and 20 (Fig. 1).

Kumpiene et al. (2007), observed that pH as a single factor had little influence on As mobility over time in a zero valent iron-stabilised soil, and our findings are concurrent. Since generally little changes were observed in soil pH along the experiment, this did not seem to be a determining factor on the mobilisation of As found between 14 and 20 months, in agreement with Kumpiene et al. (2007), who observed that pH as a single factor had little influence on As mobility over time in a zero valent iron-stabilised soil. Similarly, DOC did not seem related to the increase in As mobilisation, since extractable As increased over time in all treatments, regardless of the DOC dynamics (Fig. 3). The sequential extraction performed in samples collected at 13 and 20 months revealed that most of the As was associated to amorphous Fe oxides in the control and all treated soils (F5; Fig. 2). Moreover, the percentage of As extracted from poorly crystalline Fe oxides and from the most labile fractions (F1 and F2) was always higher at 20 than at 13 months (although not always significantly). However, between the sample points of 13 and 20 months, a decrease in the percentage of As associated to amorphous Fe oxides was observed. This suggests that ageing resulted in the transformation of amorphous iron oxides into more crystalline phases, which might result in a decrease in the density of

sorption sites, lowering the binding capacity of Fe oxides and leading to As mobilisation (Dixit and Hering, 2003; Kumpiene et al., 2012). Additional to this effect, plants could also have played a role in soil As immobilisation in less reactive fractions over the experiment (i.e. before and after cropping), considering that plants were shown to induce changes in rhizosphere As fractionation and/or availability that can lead to an immobilisation of As (Moreno-Jiménez et al., 2012; Obeidy et al., 2016).

Additionally, a heterogeneous distribution of the newly formed iron oxides in the soil may explain the lack of As stabilisation found in this experiment, in contrast to that found in previous pot experiments (Fresno et al., 2016; 2018). Whereas in pot experiments the amendments can be thoroughly mixed with the soil, this is difficult to achieve with such a large amount of soil in this macrocosm experiment (~120 kg), and is possibly more representative of field conditions for large scale amendments.

Concentrations of both extractable and soluble Cu were effectively reduced by all treatments. Based on the significant negative correlation between soil pH and extractable Cu (7 months: $r = -0.729$, $P < 0.001$; 14 months: $r = -0.565$, $P < 0.01$) and between porewater pH and Cu concentration ($r \geq -0.520$, $P < 0.01$ at all samplings), the increase in pH provoked by the treatments seemed to have strongly affected Cu mobility. Copper mobility in soils is highly dependent on variation of soil pH and, generally, an increase in soil and porewater pH may result in precipitation of Cu hydroxides, besides increasing the negatively-charged surface area of metal oxides, which can enhance the adsorption of free and complexed Cu (Soler-Rovira et al., 2010). Soil organic matter may also control Cu mobility through the formation of stable complexes with functional groups (Zhou and Wong, 2001; Soler-Rovira et al., 2010). Since metal (Fe, Al, Mn) oxides are generally good sinks for Cu (Kumpiene et al., 2008), the formation of supplemental iron (hydr)oxides upon addition of iron sulphate, in addition to the increase in soil pH, could have mitigated the effects of DOC by enhancing the adsorption of Cu-OM complexes onto iron (hydr)oxides surface (Tiberg et al. 2016).

Reflecting our results on extractable As, the treatments did not have a great effect on As uptake in rye (Table 3). After 4 months, only Fe+PS increased As concentration in shoots, likely related to the highest As solubility in this treatment. Nevertheless, the effect of this treatment was mitigated over time, due to plant growth and thus to a dilution effect (Fig. 4). Although addition of Fe+BC, Fe+OMWC and Fe+GWC resulted in lower As concentrations in shoots after 6 months from sowing compared to the control soil, this effect seemed to be driven by the effect of the amendments on plant growth. These treatments also lowered As concentration in grains (Table 3). Rye straw can be used for animal feed and its grains are used for breadmaking, thus it is important to evaluate the risks associated with its consumption. In all cases, the concentration of As in shoots exceeded the tolerable limit for animal feed established at 2 mg kg^{-1} (Directive 2002/32/EC). Arsenic concentration in grains was also above the limit for children feed recently established at 0.25 mg kg^{-1} in rice (Commission Regulation EU 2015/1006). Therefore, no treatment was able to reduce As levels in rye above-ground organs sufficiently to meet the current legal limits and in this case rye biomass should be only used for obtaining bioenergy or for other non-feeding purposes and this limitation should be seriously considered (e.g. physically confining the rye in As-polluted soils so that the animals cannot feed in it). Our study showed that Cu concentration in rye was within usual ranges (Marschner, 2012) despite the high levels of Cu in the soil.

4.2. Evaluation of soil quality parameters over two years

The best results in terms of nutrient availability were obtained in soils treated with Fe+BC, Fe+OMWC and Fe+GWC. These amendments enhanced available K, Ca, Mg and P and TOC and TN concentrations in soil (Table 1), whereas Fe+lime- and Fe+PS-treated soils did not improve in terms of nutrient availability. Organic amendments generally improve soil fertility due not only to the supply of available nutrients, but also to the improvement of soil physio-chemical properties such as soil CEC, porosity and water-holding capacity (Walker and Bernal, 2008; Chan et al., 2007; Biederman and Stanley Harpole, 2013; Cellier et al., 2014).

450 The nutritional status and the enhancement of above-ground biomass of rye plants grown in
451 Fe+BC, Fe+OMWC and Fe+GWC was in accordance with the effects of these treatments on
452 nutrient availability (Table 2, Figs. 4 and 5). Treatments Fe+BC and Fe+OMWC, increased K,
453 Ca, Mg and P contents in shoots and slightly increased some micronutrients uptake such as Zn,
454 Ni and Mo (not shown). Improvement in plant growth upon addition of biochar and compost to
455 contaminated soils has been previously reported (Álvarez-López et al., 2016; Gil-Loaiza et al.,
456 2016; Jones et al., 2016; Fresno et al., 2018). Nutritional status of plants grown in Fe+lime and
457 Fe+PS reflected the effects on soil nutrients availability, as little differences were found with
458 control plants. Because rye yield was not related to soluble or extractable As, the plant growth
459 might rather be related to the effects of treatments on soil fertility.
460 Soil enzymatic activities are helpful tools to evaluate a soil remediation process (Alkorta et al.,
461 2003; Alvarenga et al., 2009b; Pardo et al., 2014). Dehydrogenases are intracellular
462 oxidoreductases that reflect the oxidative activities of soil microbial communities and hence
463 their activity can be used as an indicator of viable microbial activity and the benefits of soil
464 amendments on soil health (García-Gil et al., 2000; Alkorta et al., 2003; Manzano et al., 2014;
465 Pardo et al., 2014). In this work we found an increase in dehydrogenase activity in most of the
466 treated soils (Fig. 5). This activity is positively correlated with C mineralisation (Ouyang et al.,
467 2014) and an increase generally occurs when organic amendments are applied to soils (García-
468 Gil et al., 2000; Crecchio et al., 2001; Pardo et al., 2014), which might explain the greatest
469 effect provoked n Fe+OMWC and Fe+GWC. BC was not as efficient as OMWC or GWC
470 enhancing dehydrogenase activity despite a similar C input to the soil (Table SM1). Elzobair et
471 al. (2016) observed no microbial activity in response to biochar addition, in contrast to the
472 enhancement observed when manure was applied and suggested that either organic C supplied
473 by biochar was not available for microbial degradation or the labile biochar C source was
474 degraded prior to sampling. Our results support the idea that organic C supplied by biochar
475 would be more stable than that supplied by compost. Yet, further research is needed to confirm
476 and give light to such findings. Phosphatases are hydrolases involved in the P cycle that can be
477 used as indicators of soil health and organic matter quality (Alkorta et al., 2003; Pardo et al.,

2014). However, phosphatases might not be so efficient for soil quality assessment in As-contaminated soils, as they can be stimulated by the presence of arsenate (Lyubun et al., 2013). In this work, acid phosphatase activity was only increased by Fe+GWC. Similar to that observed by Pardo et al. (2014), a significant negative correlation was found between soil pH and phosphatase activity at 20 months ($r = -0.588$, $P < 0.01$), suggesting that the increase in soil pH provoked by most of the treatments could have impaired its activity (Alvarenga et al., 2009b), together with an increase in the available P in Fe+BC and Fe+OMWC (Epelde et al., 2009). The test with *Vibrio fischeri* was not well related to other approaches (extractability, plant growth, soil enzymes), in agreement with previous observations, which suggests that this organism seems more sensitive to other parameters than to trace element mobility (Fresno et al., 2016; Pardo et al., 2016).

5. Conclusions

The addition of organic materials did not provoke over two years of experimentation a significant As mobilization similar to that observed in many previous studies when iron sulphate was not co-applied and an increase in soluble As was observed only for Fe+PS. Additionally, all the treatments resulted in a reduction in Cu mobility, which remained lower than in the control soil over time.

The combination of iron sulfate with biochar or compost in a rate 1:5% (w:w) improved soil properties, improved rye growth and nutrition, and reduced As concentration in shoots and grains. The best results in terms of soil and crop quality were obtained for Fe+BC, which seems to provide a stable source of organic C.

Future research should deal with upscaling (field) and longer-term experimentation (decades) and refining the amendments application rates (e.g. proportions of BC and iron sulphate to optimize As immobilization).

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Figures captions

Figure 1. Soil pH and concentration of extractable As and Cu in samples collected 7, 14 and 20 months after addition of the amendments. Mean (n = 5) ± SE. Different lower case letters above bars are used to differentiate between treatments for each sampling time ($P < 0.05$) and upper case letters (italics) differentiate between samplings within each treatment ($P < 0.05$).

Figure 2. Fractionation of As (% of the pseudo-total concentration) determined by a sequential extraction done on samples collected 13 and 20 months after addition of the amendments. Means (n = 5). **F1**: readily soluble; **F2**: strongly adsorbed onto mineral surfaces; **F3**: associated with Al oxyhydroxides; **F4**: bound to organic matter; **F5**: incorporated into amorphous Fe oxyhydroxides; **FR**: residual fraction.

Figure 3. Soil porewater pH and As and Cu concentration at 15, 17, 18, 19 and 20 months; DOC concentration in soil porewater at 15 and 20 months after treatments application. Means (n = 5) ± SE. Different letters above DOC bars indicate significant differences between treatments within each sampling time ($P < 0.05$).

Figure 4. Dry weights (DW) of the shoots of *S. cereale* grown for 4 or 6 months in the control and the treated soils. Mean (n = 4) ± SE. Different letters indicate significant differences among treatments ($P < 0.05$); lower case letters correspond to the first harvest and upper case letters to the second harvest. The asterisks show where significant differences were found between harvests ($P < 0.05$).

Figure 5. Dehydrogenase, acid phosphatase and β-glucosidase activities in the control and the treated soils 20 months after addition of amendments. Enzyme activities are represented as the amount of product (TPF or PNP) formed after 1 hour of soil and substrate incubation. Mean (n = 5) ± SE. Different letters above bars indicate significant differences between treatments ($P < 0.05$).

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Tables

Table 1. Soil pH, porewater EC (dS m^{-1}), concentration of exchangeable K, Ca and Mg, P-Olsen (mg kg^{-1}**)**, total organic carbon (TOC) and total nitrogen (TN; g kg^{-1}) in the control and the treated soils at 20 months, at first *S. Cereale* harvest. Mean (n = 5) ± SE. Different letters in the same column indicate significant differences between treatments ($P < 0.05$).

Treatment	pH	$EC_{porewater}$	K	Ca	Mg	P	TOC	TN
Control	4.8 ± 0.1^{a}	1.4 ± 0.6^{a}	48.0 ± 1.9^{a}	1187 ± 116^{a}	13.9 ± 2.1^{ab}	10.6 ± 0.5^{a}	3.7 ± 0.2^{a}	0.09 ± 0.03^{a}
Fe+lime	6.5 ± 0.3^{bc}	1.9 ± 0.2^{a}	46.4 ± 3.0^{a}	3069 ± 247^{b}	5.1 ± 0.3^{a}	8.7 ± 0.0^{a}	4.1 ± 0.6^{a}	0.13 ± 0.01^{a}
Fe+PS	6.8 ± 0.1^{c}	2.2 ± 0.3^{a}	45.7 ± 2.1^{a}	4738 ± 295^{c}	16.4 ± 1.6^{b}	8.6 ± 0.8^{a}	10.1 ± 1.0^{a}	0.26 ± 0.04^{a}
Fe+BC	7.1 ± 0.1^{c}	1.77 ± 0.03^{a}	116.0 ± 4.8^{b}	2646 ± 204^{b}	16.2 ± 1.5^{ab}	18.1 ± 1.0^{b}	36.4 ± 1.9^{c}	2.78 ± 1.10^{ab}
Fe+OMWC	5.9 ± 0.1^{b}	1.5 ± 0.6^{a}	243.9 ± 17.5^{c}	2646 ± 43^{b}	50.1 ± 4.8^{c}	29.1 ± 1.2^{c}	36.2 ± 4.3^{c}	2.19 ± 0.42^{b}
Fe+GWC	6.0 ± 0.1^{b}	2.4 ± 0.4^{a}	96.2 ± 3.4^{b}	2918 ± 180^{b}	21.0 ± 2.7^{b}	36.1 ± 3.0^{d}	25.3 ± 3.3^{b}	1.89 ± 0.36^{ab}

Table 2. Nutrients concentration (mg g^{-1}) in *S. cereale* shoots grown in the control and the treated soils for 4 months. Mean (n = 4) ± SE.

Different letters in the same column indicate significant differences between treatments ($P < 0.05$).

Treatment	K	Ca	Mg	P	Fe	Zn	Ni	Mo
Control	26.2 ± 5.5^{a}	3.23 ± 0.72^{ab}	0.90 ± 0.29^{a}	2.20 ± 1.24^{a}	161.7 ± 64.4^{bc}	38.9 ± 4.2^{d}	787.3 ± 96.5^{c}	1.16 ± 0.12^{a}
Fe+lime	24.4 ± 0.6^{a}	3.17 ± 0.30^{ab}	0.71 ± 0.08^{a}	1.78 ± 0.18^{a}	82.3 ± 24.4^{abc}	17.7 ± 1.4^{bc}	196.4 ± 6.8^{a}	0.87 ± 0.09^{a}
Fe+PS	26.8 ± 0.4^{a}	4.81 ± 0.30^{b}	1.11 ± 0.22^{a}	1.91 ± 0.52^{a}	190.8 ± 41.4^{c}	21.2 ± 1.5^{c}	366.0 ± 44.4^{ab}	1.02 ± 0.21^{a}
Fe+BC	25.0 ± 1.2^{a}	2.77 ± 0.22^{a}	0.74 ± 0.06^{a}	2.40 ± 0.27^{a}	35.5 ± 12.5^{a}	10.0 ± 0.7^{a}	306.8 ± 51.1^{a}	0.78 ± 0.09^{a}
Fe+OMWC	22.7 ± 1.2^{a}	2.27 ± 0.12^{a}	0.79 ± 0.05^{a}	2.48 ± 0.30^{a}	35.9 ± 5.1^{ab}	12.3 ± 0.9^{ab}	978.2 ± 184.2^{c}	0.98 ± 0.09^{a}
Fe+GWC	24.4 ± 1.8^{a}	2.27 ± 0.17^{a}	0.77 ± 0.19^{a}	2.68 ± 0.31^{a}	120.2 ± 47.2^{abc}	12.6 ± 1.2^{ab}	674.8 ± 150.3^{bc}	0.94 ± 0.22^{a}

Table 3. Arsenic and Cu concentration in shoots and grains (mg kg^{-1}) and their accumulation in shoots (µg) of *S. cereale* grown for 4 or 6 months in the control and the treated soils. Mean (n = 4) ± SE.

Treatment	4 months				6 months					
	$[As]_{shoots}$ (mg kg^{-1})	As_{shoots} (µg)	$[Cu]_{shoots}$ (mg kg^{-1})	Cu_{shoots} (µg)	$[As]_{shoots}$ (mg kg^{-1})	As_{shoots} (µg)	$[As]_{grain}$ (mg kg^{-1})	$[Cu]_{shoots}$ (mg kg^{-1})	Cu_{shoots} (µg)	$[Cu]_{grain}$ (mg kg^{-1})
Control	13.0 ± 2.0 ^{a}	28.9 ± 7.2 ^{a}	10.8 ± 1.3 ^{cd}	34.4 ± 9.3 ^{a}	12.9 ± 1.4 ^{b}	75.5 ± 14.4	0.71 ± 0.13 ^{b}	7.4 ± 0.9	30.3 ± 2.3 ^{a}	7.3 ± 0.1 ^{c}
Fe+lime	16.7 ± 6.1 ^{ab}	24.0 ± 4.1 ^{a}	8.5 ± 0.3 ^{bcd}	88.4 ± 16.8 ^{b}	11.6 ± 1.6 ^{b}	70.2 ± 9.3	0.46 ± 0.04 ^{ab}	5.0 ± 0.4	30.5 ± 4.0 ^{a}	6.5 ± 0.3 ^{bc}
Fe+PS	41.7 ± 8.6 ^{b}	65.2 ± 9.3 ^{ab}	12.3 ± 1.7 ^{d}	82.9 ± 21.2 ^{ab}	8.5 ± 1.9 ^{ab}	92.2 ± 21.9	0.44 ± 0.06 ^{ab}	6.1 ± 1.1	79.4 ± 8.6 ^{ab}	7.1 ± 0.2 ^{c}
Fe+BC	6.3 ± 1.0 ^{a}	48.9 ± 9.2 ^{ab}	4.6 ± 0.2 ^{a}	175.0 ± 40.1 ^{b}	5.2 ± 0.9 ^{a}	117.6 ± 19.1	0.34 ± 0.01 ^{a}	4.0 ± 0.6	95.8 ± 20.5 ^{b}	5.2 ± 0.3 ^{a}
Fe+OMWC	8.1 ± 1.1 ^{ab}	40.0 ± 9.2 ^{a}	5.8 ± 0.8 ^{ab}	162.8 ± 28.4 ^{b}	6.3 ± 0.8 ^{a}	74.2 ± 21.3	0.42 ± 0.4 ^{a}	5.3 ± 1.2	81.8 ± 17.0 ^{ab}	6.0 ± 0.2 ^{ab}
Fe+GWC	19.4 ± 5.5 ^{ab}	135.8 ± 32.0 ^{b}	7.1 ± 0.4 ^{abc}	129.0 ± 17.0 ^{b}	4.8 ± 0.4 ^{a}	56.8 ± 13.7	0.32 ± 0.02 ^{a}	6.6 ± 0.1	74.7 ± 13.6 ^{ab}	5.9 ± 0.2 ^{ab}

Different letters indicate significant differences between treatments ($P < 0.05$); where there are no letters, no statistical differences were found.

Figure

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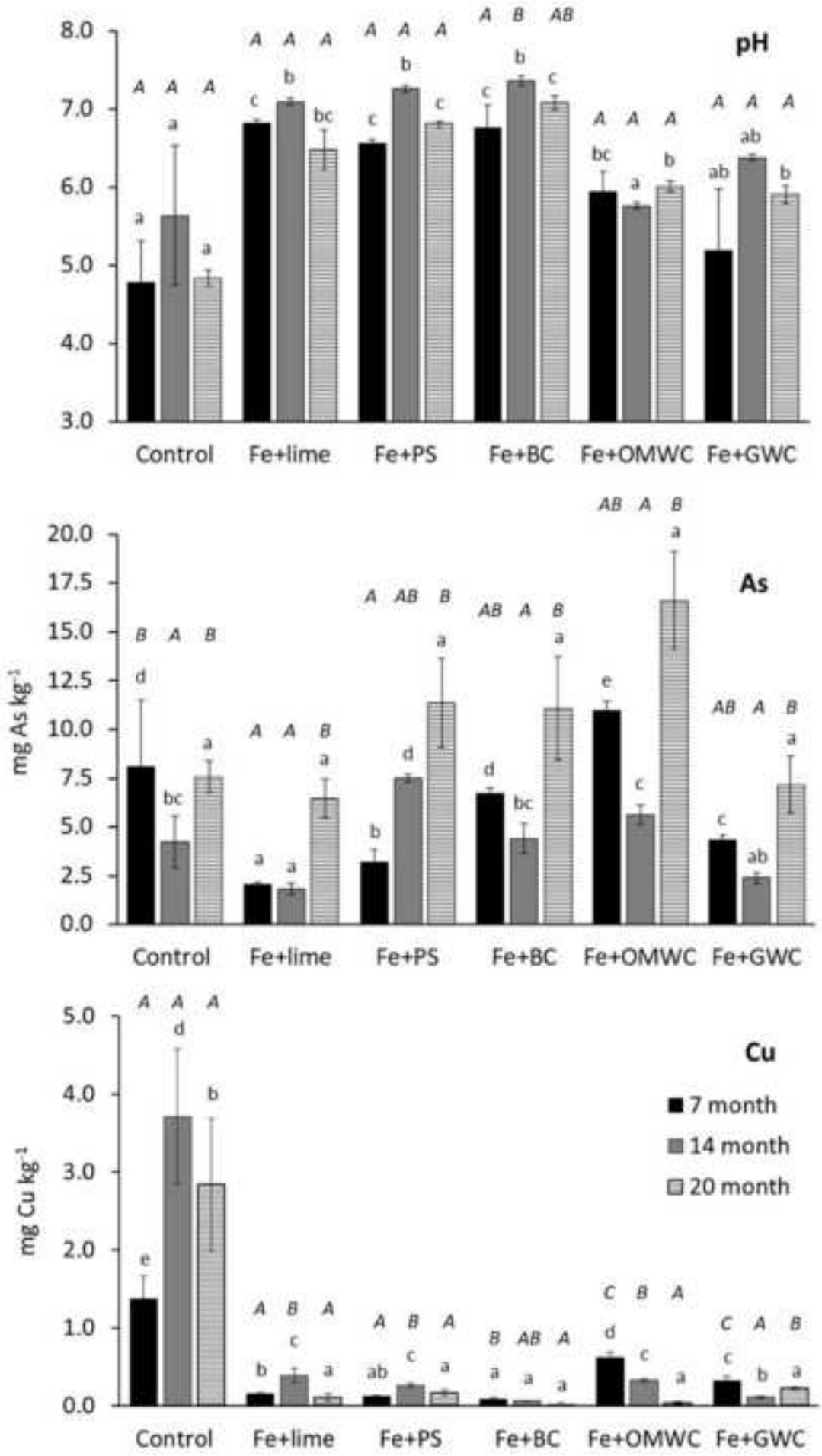

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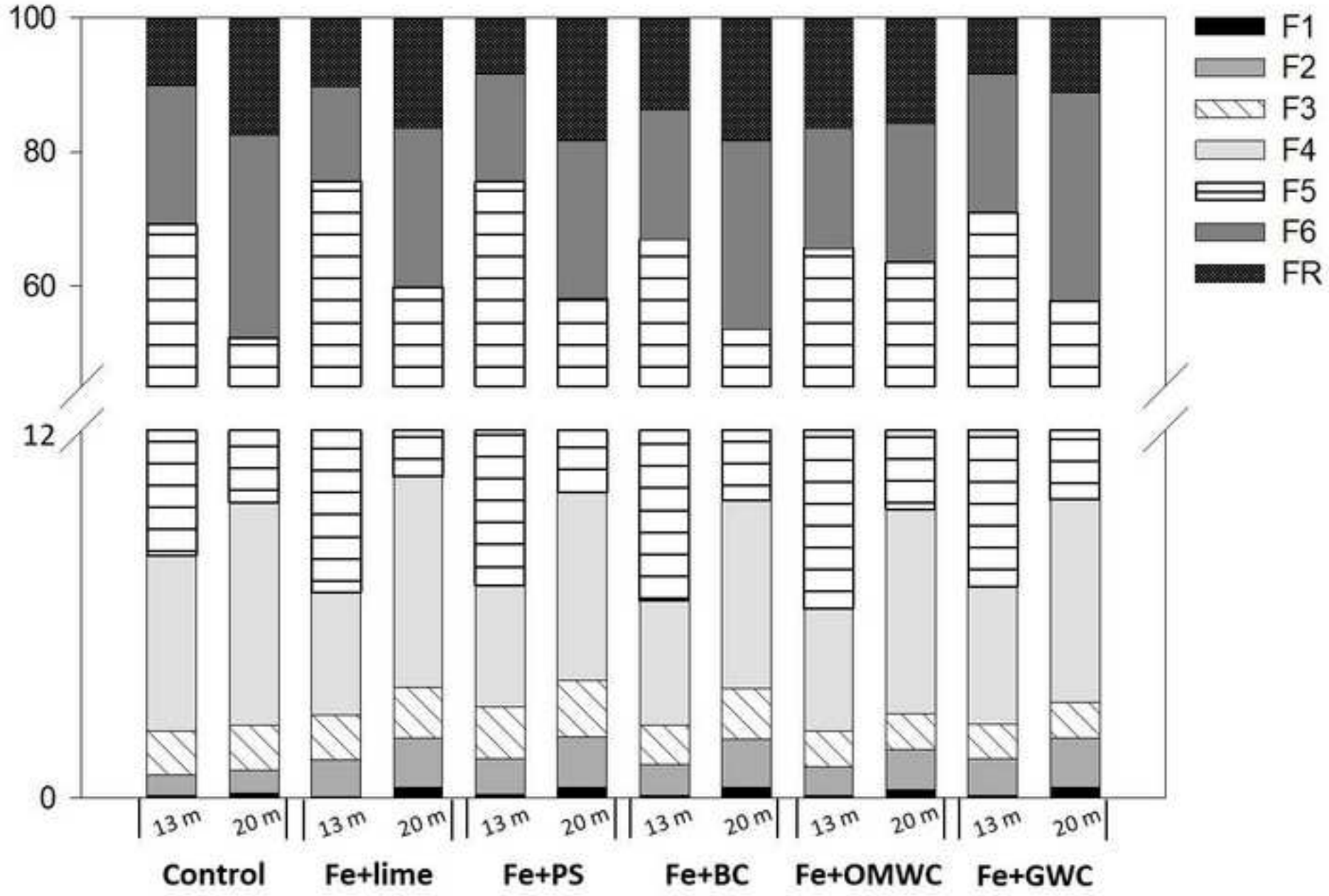

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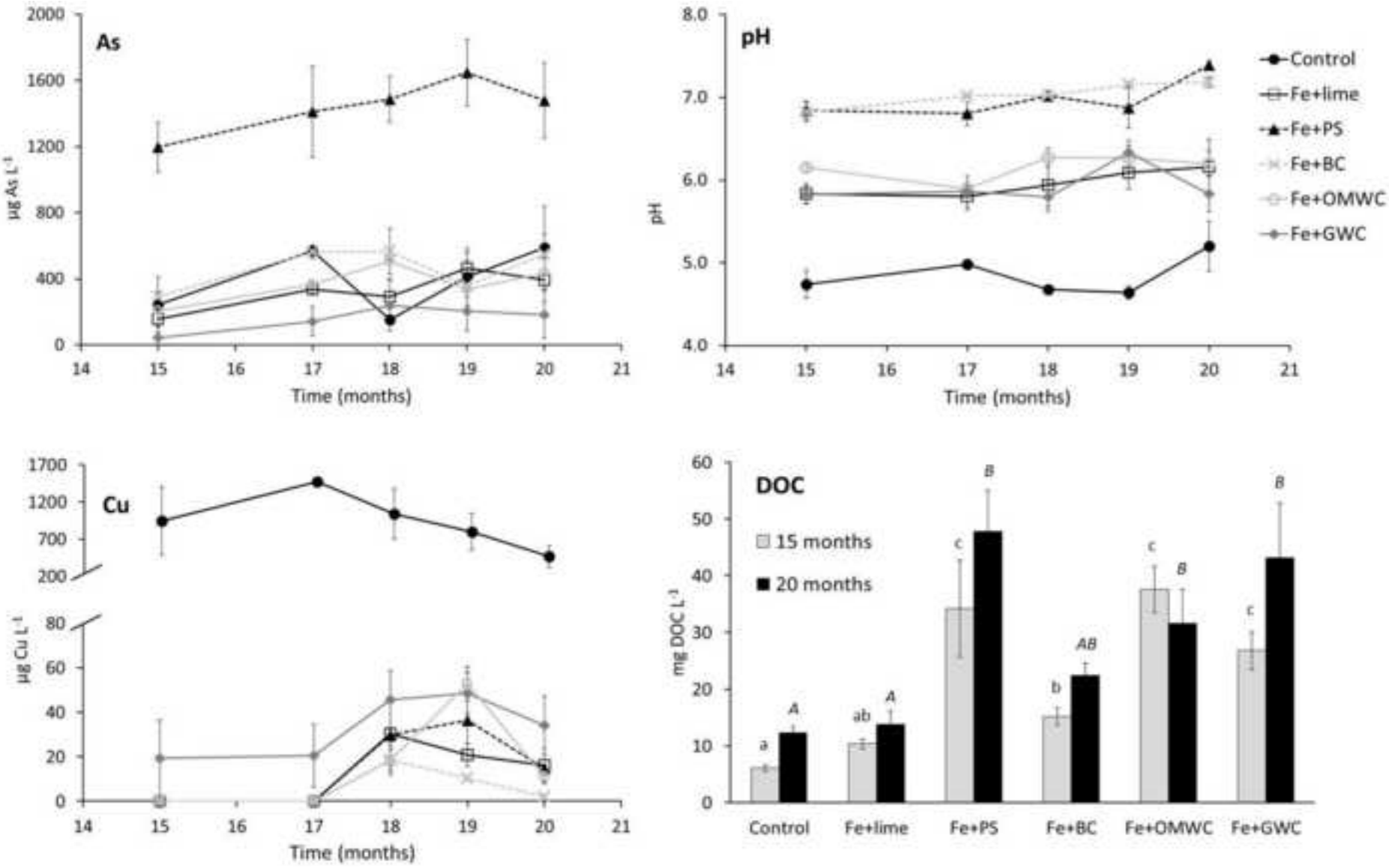

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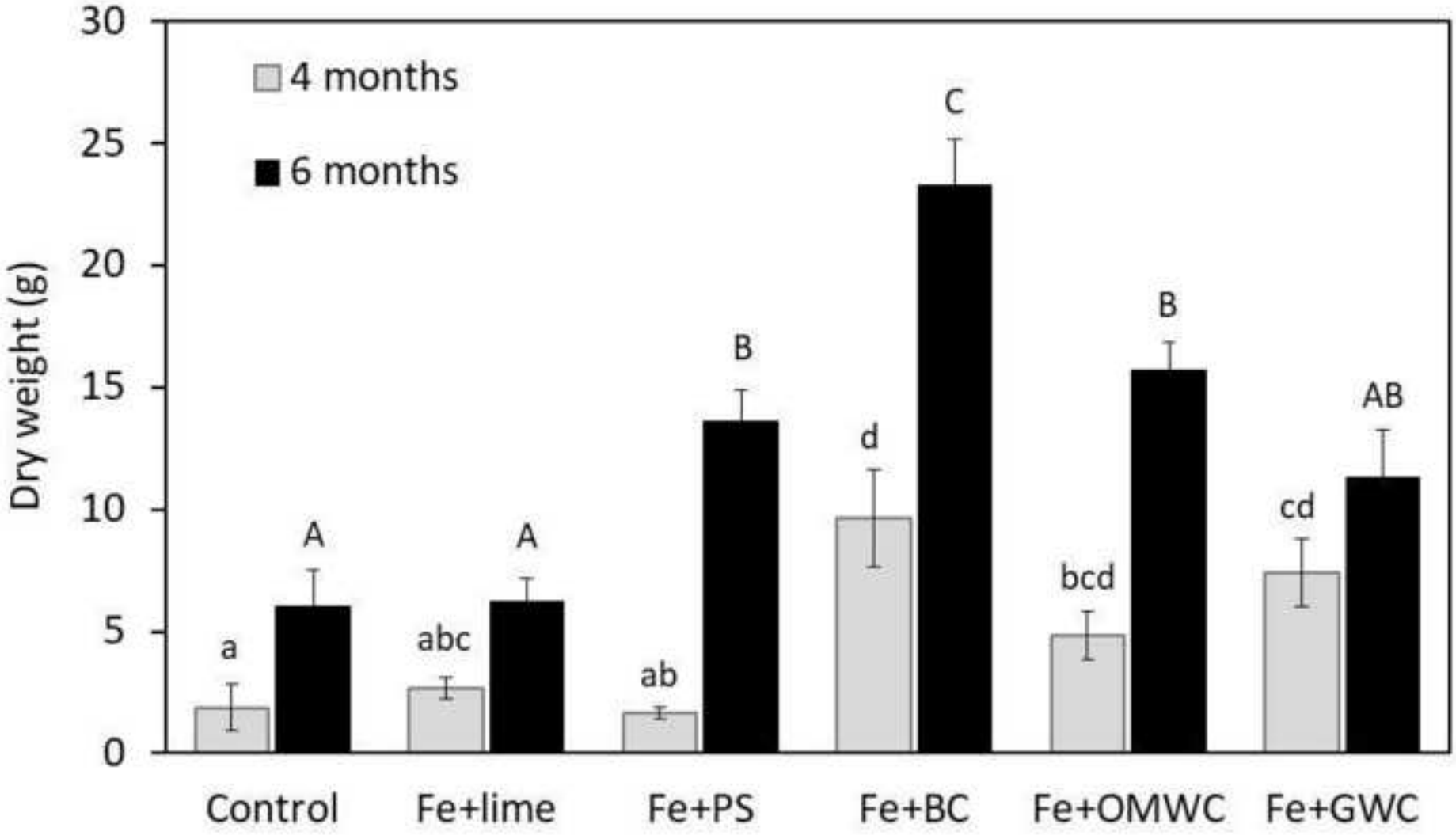

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