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Abstract

A batch experiment was conducted to assess the impact of chemical oxidation using modified Fenton reaction on PAH content and on physico-chemical and biological parameters of an industrial PAH contaminated soil in unsaturated condition. Two levels of oxidant (H_2O_2, 6 and 65 g kg^{-1}) and $FeSO_4$ were applied. Agronomic parameters, bacterial and fungal density, microbial activity, seed germination and ryegrass growth were assessed. Partial removal of PAHs (14 and 22%) was obtained with the addition of oxidant. The impact of chemical oxidation on PAH removal and soil physico-chemical and biological parameters differed depending on the level of reagent. The treatment with the highest concentration of oxidant decreased soil pH, cation exchange capacity and extractable phosphorus content. Bacterial, fungal, and PAH degrading bacteria densities were also lower in oxidized soil. However a rebound of microbial populations and an increased microbial activity in oxidized soil were measured after 5 weeks of incubation. Plant growth on soil treated by the highest level of oxidant was negatively affected.

Keywords: Fenton, oxidation, PAH, soil, fertility, biological activity

1. Introduction

The presence of pollutants in soils can affect soil functions (e.g. filter and exchange, support for plant growth and biodiversity) (Smith et al., 2006; Maxim and Spangenberg, 2009). Although remediation treatments reduce or eliminate pollutant concentration, their potential effect on soil physical, chemical and biological properties, and soil functions is rarely considered. Polycyclic aromatic hydrocarbons (PAHs) are widespread contaminants in soil.

41 Their effects on human health and their carcinogenic potential is largely studied (Bispo et al.,
42 1999; Joe et al., 2004; White and Claxton, 2004). Different remediation technologies were
43 developed in the last decades to remove PAHs from contaminated soils (Wang et al., 1990;
44 Cajthaml et al., 2002; Gan et al., 2009). *In situ* chemical oxidation (ISCO) is a technology
45 generally used to remediate organic contaminants in groundwater and saturated soil (Rivas,
46 2006; Huling and Pivetz, 2006). This remediation technique based on the injection of
47 different oxidant reagents does not require soil excavation and represents an alternative to
48 conventional treatment (*e.g.* thermal desorption, venting). Fenton reagents (hydrogen
49 peroxide and iron) are among the most commonly used and studied oxidizing agents (Rivas,
50 2006) for pollutants such as petroleum hydrocarbons, BTEX, chlorinated ethenes and PAHs
51 (Huling and Pivetz, 2006). In Fenton reaction, Fe (II) catalyzes the decomposition of
52 hydrogen peroxide to generate hydroxyl radicals ($OH^{\cdot}$) which are strong and non specific
53 oxidants. However, some authors showed that $OH^{\cdot}$ radicals preferentially attack the aromatic
54 compounds (Westerhoff et al., 1999; Mikutta et al., 2005) and can also react with sorbed and
55 aqueous PAHs. Fenton oxidation generally includes acid addition since a low pH (2-3) is
56 required for optimum reaction, but modified Fenton reaction at near neutral pH using ferric
57 ions and chelating agents was also performed (Nam et al., 2001).
58 Fenton reaction was mainly studied in saturated conditions (Jonnson et al., 2006; Sirguey et
59 al., 2008; Sun and Yan, 2008; Valderrama et al., 2009) with large liquid/solid ratios. Further,
60 the amounts of oxidant used in many studies (Nam et al., 2001; Palmroth et al., 2006a;
61 Valderrama et al., 2009) were very high for field treatment. Chemical oxidation in unsaturated
62 conditions was also applied, although fewer studies were reported (Palmroth et al. 2006a,
63 Huling and Pivetz, 2006). The addition of chemical oxidants could impact other soil
64 characteristics than organic pollutant concentration and for surface soils this could lead to a
65 global loss of fertility. Chemical oxidation of PAHs was shown to induce a decrease of

organic carbon, total nitrogen and phosphorus contents in soils (Leifeld and Kögel-Knabner, 2001; Mikutta et al., 2005; Sirguey et al., 2008). Microorganism populations were negatively affected following ISCO but the impact seemed to be temporary with a rebound of cell numbers after a few weeks (Sahl and Munakata-Marr, 2006). After oxidation treatment, PAH biodegradation was enhanced, suggesting the possible combination of both chemical oxidation and biodegradation of organic pollutants (Lee and Hosomi, 2001; Nam et al., 2001; Palmroth et al., 2006a; 2006b; Valderrama et al., 2009)

The present study was conducted to assess the impact of chemical oxidation to surface soil using modified Fenton reaction on PAH removal and on soil physico-chemical and biological parameters. Since surface soils are mostly unsaturated, we performed the oxidation in unsaturated condition. An industrial PAH contaminated soil was treated with a volume of reagents calculated to keep the soil at water holding capacity. We used a modified Fenton reaction without acid addition to the soil to avoid a strong pH decrease that may drastically affect biological activity. Two concentrations of reagents were tested. Analyses of PAH concentration, soil characteristics (pH, cation exchange capacity, organic carbon, nitrogen, phosphorus), microbial density and activity and seed germination and plant growth using ryegrass (*Lolium perenne)* were performed with oxidized and control soil.

2. Materials and methods

2.1. Soil sampling and analysis

An industrial soil sample was collected from a former coking plant site (Neuves-Maisons, France) (Biache et al., 2008; Monsérié et al., 2009). The soil is a sandy loamy soil (11.9% clay, 21.5% silt and 66.6% sand). The soil presents a high content of organic carbon (50g kg^{-1}), a neutral pH (7.3) and the contamination by the 16 US-EPA priority PAHs reaches

1200 mg kg^{-1}. Large amount of soil was excavated and homogenized by quartering. Sub-samples were sieved at 2 mm and air dried at 25°C. Soil analyses were performed by INRA laboratory (Arras, France) according to international standard methods: water holding capacity (WHC) (ISO 11464), pH, total organic carbon (ISO 10694), total nitrogen (ISO 13878), extractable phosphorus (ISO 11263), cation exchange capacity (CEC, NF X 31-130).

2.2. Oxidation experiment

The modified Fenton reaction was performed with two levels of reagents corresponding to 6 g kg^{-1} (low, L) and 65 g kg^{-1} (high, H) of H_2O_2. Three glass containers were filled with 1500 g of 2 mm sieved soil at 5 % humidity. In two of them, the soil sample was mixed with 7 (L) or 82 (H) g of solid ferrous sulfate ($FeSO_4$, $7H_2O$, 99.5% purity) to catalyse the decomposition of H_2O_2 without increasing the volume of solution added. Then, 450 mL of a solution of H_2O_2 at either 0.54 (L) or 6.3 M (H) were added gradually to reach water holding capacity. The third one was a control with addition of 450 mL of water and no reagent addition. After 48 h, the soil sample in each container was homogenized and sub-sampled for further analysis. The soil was then air dried and sieved to 2 mm again. For microbial analysis, sub-samples were directly used for quantification of culturable heterotroph bacteria and incubation. For DNA extraction sub-samples were stored at -20°C.

2.3. Measurement of microbial activity (carbon mineralization) in soil after oxidation

Fenton oxidized and control soil samples (20 g), with five replicates each, were incubated at 100% WHC in 150 mL serum bottles with a Teflon septum for 5 weeks at 24°C to measure microbial activity by CO_2 production. To avoid anaerobic conditions during incubation, bottles were aired out every week. Carbon dioxide (CO_2) release was measured every day by

115 infrared spectrophotometer (Binos, absorption at 2325.6 cm^{-1}) (Quantin et al., 2005). After 5
116 weeks, five replicates were taken for microbial and PAH analysis as described hereafter. An
117 abiotic control of each modality (autoclaved twice at an interval of 48 h) was performed for a
118 week in order to evaluate the abiotic release of CO_2.
119
120 *2.4. MPN counts of heterotroph microorganisms*
121 Soil (1 g fresh) was suspended in 10 mL sterile ultra-pure water with 6 g sterile glass-beads
122 and soil suspension was shaken during 1 h. Serial dilution of soil suspension (10^{-2} to 10^{-7}) was
123 prepared in NaCl (0.85%). The most-probable-number (MPN) was counted in 96-well
124 microplates. A volume (25 µL) of dilution was added with 200 µL nutrient broth liquid
125 medium (1 g L^{-1}) in each well with 40 replicates by dilution. Microplates were incubated for
126 48 h at 28°C then absorbance was measured at 620 nm and MPN counts calculated by a
127 computer program using McCrady table (Binet et al., 2000).
128
129 *2.5. Quantification of bacteria, fungi and PAH-degrading bacteria*
130 DNA extractions were performed independently for each biological replicate. DNA was
131 extracted using the FastDNA© Spin kit for soil according to manufacturers recommendations
132 (MP Biomedicals) from 1 g of soil. Bacteria, fungi and PAH-degrading bacteria were
133 quantified by targeting 16S rDNA, 18S rDNA and PAH-RHD_α genes using real-time PCR
134 quantification of gene copy number, with the primer sets 968F/1401R (Felske et al., 1998),
135 FF390R/Fung5F (Lueders et al., 2004), PAH-RHD_α GN F/R and PAH-RHD_α GP F/R (Cébron
136 et al., 2008), respectively. Real-Time PCR experiments were conducted in triplicate with an
137 iCycler iQ (Bio-Rad), associated with iCycler Optical System Interface software (version
138 2.3). The final volume used (20 µL) contained 10 µL of iQ SYBR GREEN SuperMix
139 (Biorad), 0.4 µM each primer, 0.06% bovine serum albumine (BSA), 0.2 µL DMSO, 0.08 µL

140 T4gp32 (MP Biomedicals) and 1 µL of DNA as described in Cébron et al. (2008).
141 Quantitative calibrations of Real-Time PCR assays were performed with 10 times dilution
142 series (from 10^8 to 10^3 target gene copies μL^{-1}) of standard linearized plasmids (pCR2.1
143 vector from Invitrogen) with target gene fragment inserted (Cébron et al., 2008).
144
145 *2.6. Germination and plant growth test*
146 Fenton oxidized and control soil samples (100 g) with five replicates were placed in 200 mL
147 pots. Soil was watered with deionised water to 70% WHC and soil moisture was daily
148 adjusted. Twenty seeds were sown on each pot, thinned to 5 plants after germination. The pots
149 were placed in a growth chamber at 24°C/20°C, 16 h day light, 250 µmol m^{-2} s^{-1} intensity.
150 The germination rate was recorded every day and conducted over a period of 2 weeks. After 4
151 weeks, plants were harvested, and roots gently rinsed in water. Shoot and root dry weights
152 were measured after drying at 105°C for 24 h.
153
154 *2.7. PAH analysis*
155 PAHs were extracted from soil samples in triplicates using ASE 200 (Accelerated Solvent
156 Extraction Dionex). Anhydrous sodium sulfate (1 g) and 1 g of Florisil® was added to each
157 sample (1 g) in the extraction cell to purify the extract. The extraction solvent was composed
158 of 50% acetone and 50% methylene chloride and extraction was run at 100°C and 130 bars
159 for 5 min (Ouvrard et al., 2011*)*. After evaporation of most of the solvent, samples were
160 diluted in 5 mL of acetonitrile and evaporated to obtain exclusive acetonitrile extracts.
161 Analysis of 16 US-EPA priority PAHs concentrations was performed by high-performance
162 liquid chromatography (Varian) (Monsérié et al., 2009). Results were reported for dry soil for
163 comparison between treatments.
164

165 *2.8. Statistical analysis*

166 Statistical analyses were performed using XLSTAT© Software. Comparisons were made by
167 ANOVA after verification of applicability conditions. A multiple post hoc comparison
168 (Tukey) test was applied to determine the difference per pair. Gene quantification data were
169 log transformed for statistical comparisons. To test time effect of bacteria recolonization a
170 Student t-test was applied. For seed germination a Fisher test was applied to calculate LSD
171 (least significant difference).

172

173 **3. Results**

174

175 *3.1. Effect of Fenton oxidation on soil PAH concentration and physico-chemical*
176 *characteristics*

177 Total PAH concentration was significantly lower in Fenton oxidized than in control soil with
178 22% and 14% decrease with H and L oxidant concentration, respectively (Table 1). The same
179 result was found for the individual PAHs except for the three low molecular weight PAHs
180 naphthalene, acenaphtylene and acenapthene where the concentration decrease was not
181 significant (Table 1). No clear difference was observed for the degradation rate between the 3
182 (18 to 36% decrease with H oxidant concentration), 4 (20.3 to 24.3%), 5 (16 to 22.1%) and 6
183 (24.3 to 24.6 %) ring PAHs. No further significant PAH concentration decrease occurred
184 during the 5 weeks incubation (data not shown). Although no replicate analysis was
185 performed for organic carbon and nitrogen content, the carbon content tended to be lower in
186 oxidized than control soil, while nitrogen content was not affected. After Fenton H treatment,
187 several parameters were strongly affected: a CEC decrease of 2 units, a pH decrease of 2.5
188 units and a decrease of available phosphorus (77% of the initial content) were measured.

189

190 *3.2. Effect of Fenton reaction on microbial density and activity*

191 No production of CO_2 was observed in the abiotic controls (data not shown). The Fenton H
192 treatment significantly and rapidly increased carbon mineralization rate, while mineralization
193 rate was not significantly different with Fenton L treated and control soil (Figure 1). After
194 oxidation (T0), the number of culturable heterotrophs, the 16S, 18S rDNA and PAH
195 degrading (PAH-RHD_{α}) gene copy numbers were significantly lower in Fenton H treated than
196 in control soil (Figure 2). After 5 weeks of incubation (T5), these numbers were significantly
197 higher than at T0 in Fenton H treated soil (Figure 2), while they did not significantly differ
198 between T0 and T5 for control and Fenton L treated soils. After 5 weeks the number of
199 culturable heterotrophs and of 18S rDNA gene copies were even significantly higher in
200 Fenton H treated than in control soil. However, in Fenton L treated soil there was no
201 significant difference in culturable heterotrophs, 16S, 18S and PAH-RHD gene copy number
202 with the control soil at T0 and five weeks after incubation (T5).

203

204 *3.3. Effect of treatment on germination and growth*

205 Ryegrass germination rate reached 75-80% in Fenton treated and control soil after 14 days.
206 However, a 3-days significant delay in seed germination was observed for the Fenton H
207 treated soil (Figure 3). Root and shoot biomass was significantly lower in Fenton H treated
208 soil (35% and 43% decrease) than in the control soil, but it was not affected in Fenton L
209 treated soil (Figure 4).

210

211

212 **4. Discussion**

213

The modified Fenton reaction applied in unsaturated conditions significantly reduced the amount of PAHs in soil. The degradation concerned the different PAHs except the lower molecular weight ones. Similar degradation rates of PAHs regardless of the number of aromatic rings or lower degradation rates of higher molecular weight PAHs were previously reported depending on the H_2O_2/Fe^{2+} ratio (Tran et al., 2010). However, our degradation rate, obtained with 5-56 g oxidant added for 1 g PAHs, was lower than observed in other studies where higher concentrations of oxidants were applied in saturated conditions (100 to 4000 g oxidant for 1 g PAHs) for spiked (Nam et al., 2001; Kulik et al., 2006) or aged contaminated soils (Nam et al., 2001; Jonsson et al., 2006, 2007). In soil, the non specific action of hydroxyl radicals could oxidize the targeted molecules as well as the organic matter, which increases the quantity of oxidant needed to degrade PAHs. The soil contains more than 50 g kg^{-1} of organic carbon and PAHs represent only 2% of it (Monsérié et al., 2009). The apparent lower C_{org} content in both oxidized treatments than in the control soil, could be due to the action of hydroxyl radicals, but this should be confirmed by further investigations. Moreover, the soil used in this study had a high carbonate content which can limit the efficiency of Fenton reaction. At the same time, this reaction lead to a decrease of pH, probably due to the injection of an acidic H_2O_2 solution and to the reaction between Fe (III) and H_2O_2 (Huling and Pivetz, 2006). Carbonates can also buffer the environment of hydroxyl radicals which present a higher efficiency in acidic conditions (Huling and Pivetz, 2006). Another explanation could be the low accessibility of aged pollutants in this historically contaminated soil (Katayama et al., 2010; Ouvrard et al., 2011). Contrary to other studies, the contact between PAHs and the reagent was not enhanced by the use of a high volume of solution. The fact that hydroxyl radicals have a short half life (Sies, 1993) added to the difficulty to access PAHs located in newly formed aggregates specific to the industrial soil (Monsérié et al., 2009) can be another limitation for the degradation by such chemical processes.

239 Soil fertility was affected by the Fenton oxidation with the higher concentration of oxidant
240 added. The main effect was a decrease of soil pH, a negative effect on plant growth, as
241 previously observed by Sirguey et al. (2008) and a decrease of CEC and available P. The
242 decrease in CEC could be explained by the consequence of the pH drop on the charge of the
243 organic matter carboxyl groups, which partly turned to COOH instead of COO^- (Sirguey et
244 al., 2008). Moreover, it can result from the ionisation in acidic conditions of mineral OH
245 groups. Cation exchange capacity reflects the mobility and the availability of ions which are
246 important components of soil fertility. The decrease of available phosphorus content could
247 result from the formation of ferric phosphate in acid condition which is an insoluble form of
248 phosphorus. The pH decrease, the low available phosphorus content and the decrease of
249 cation exchange capacity could explain the decrease of biomass and root elongation. Soil
250 fertility and plant growth could possibly be restored by addition of ameliorants (lime,
251 fertilizer) (Séré et al., 2008).
252 Besides decreasing soil fertility, the oxidation treatment affected microbial density and
253 activity. The decrease in bacterial, fungal, and PAH degrading bacteria population densities
254 could be explained by the toxicity of hydroxyl radicals strongly reacting with biological
255 membranes (Slater, 1984). Moreover, the heat produced by the exothermic Fenton reaction
256 and the decrease of pH could contribute to the decreased microbial number and activity just
257 after chemical oxidation. The decrease of the number of microorganisms after Fenton reaction
258 as well as the rebound have been previously observed (Ferguson et al., 2004; Palmroth et al.,
259 2006b; Sahl and Munakata-Marr, 2006). The increased microbial activity, estimated by the
260 increased CO_2 release, and the increased number of cultivable and total bacteria and fungi
261 during incubation after oxidation indicated that recolonization occurred. Cultivable bacteria
262 which are heterotrophic preferentially use the most available organic carbon. The dead
263 bacterial cells (Palmroth et al., 2006b) as well as the organic carbon affected by the oxidation

264 treatment (Leifeld and Kögel-Knabner, 2001; Mikutta et al., 2005) could potentially provide a
265 supplementary available amount of organic carbon. Contrary to other studies, no further
266 degradation of PAHs occurred in our experiment following oxidation, and no variations in
267 PAH-degrading populations were observed. However, it was previously shown that PAH
268 availability in this historically contaminated soil was low and limited natural attenuation
269 (Ouvrard et al. 2011).
270

271 **5. Conclusion**

272 Partial removal of PAHs (14 and 22%) in a historically contaminated soil was possible in
273 unsaturated conditions using Fenton reagents with the low and high doses of H_2O_2 (6 and 65 g
274 kg^{-1}). The impact of chemical oxidation on PAH concentration and soil physico-chemical and
275 biological parameters differed with the level of reagent used. PAH degradation was not
276 proportional to the quantity of reagent added. With the highest oxidant level applied, soil
277 parameters were highly impacted, especially pH value and biological parameters such as the
278 quantity of microorganisms, seed germination and plant growth. The impact on
279 microbiological parameters was temporary and disappeared rapidly. Specific microbial
280 functions could be investigated to further check whether soil microbial functioning was
281 affected. Reduction of soil pH was a major limiting factor for plant growth at high level of
282 oxidant. Further investigations could be performed with different concentrations of reagents
283 to optimize PAH degradation without impact on vegetation. Otherwise, chemical oxidation
284 could be combined with soil restoration strategies.
285

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287

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291
292
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Figure captions

Figure 1: Cumulated production of CO_2 over time of incubation with oxidized (Fenton L and H) and control soil (Control). Mean ± SE (n=5). Arrows represent days of oxigenation.

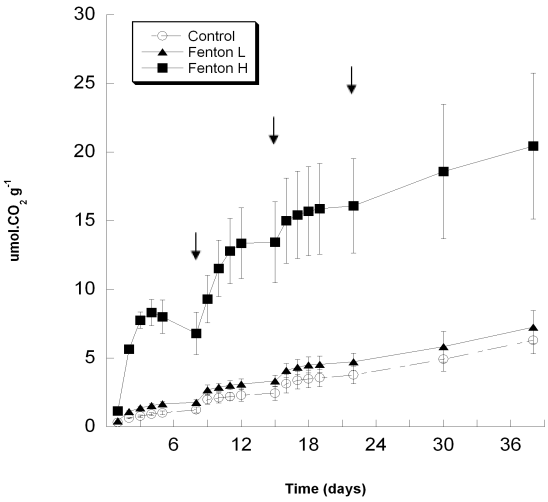
Figure 2: Density of cultivable bacteria, 16S rDNA, PAH-RDHα and 18s rDNA gene copy numbers in oxidized (Fenton L and H) and control soil (Control) at the start (T0) and after 5 weeks (T5) of incubation. As the evolution of PAH-RHD_α GN and GP populations was not significantly different, the two populations were added to represent the global population of degraders. Mean ± SE (n=3). LD: detection limit. Different letters within category data indicate significant differences between treatments; stars indicate significant differences between T_0 and T_6 by Mann and Whitney test.

Figure 3: Germination rate (%) of *Lolium perenne* seeds in oxidized (Fenton L and H) and control soil. Mean ± SD (n=5). Stars indicate a significant difference between Fenton H treated and Control soil

Figure 4: Shoot and root dry weight of *Lolium perenne* after 4 weeks on oxidized (Fenton L and H) and control soil. (Mean ± SE. n=5). Values followed by the same letter are not significantly different at the 5% level (two-way ANOVA, Tukey test)

Table 1: Physico-chemical characteristics of the soil after Fenton treatment at low (L) and high (H) concentration of oxidant. Concentrations of PAHs, given as mean, followed by the same letter are not significantly different at the 5% level (one-way ANOVA, Tukey test)

		Control	Fenton L	Fenton H
	Units			
Org C	*(g kg^{-1})*	61,7	57.7	57.1
N	*(g kg^{-1})*	2.4	2.4	2.3
Olsen P	*(g kg^{-1})*	0.049	0.068	0.011
CEC	*(cmol kg^{-1})*	10.3	10.5	8.7
pH		7.3	7.4	4.9
Naphthalene		24.8^{a}	26.4^{a}	22.0^{a}
Acenapthtylene		0.7^{a}	0.6^{a}	0.8^{a}
Acenaphthene		57.9^{a}	54.8^{a}	52.7^{a}
Fluorene		25.9^{a}	22.9^{b}	20.4^{b}
Phenanthrene		89.1^{a}	80.9^{ab}	72.9^{b}
Anthracene		36.8^{a}	26.2^{b}	23.6^{b}
Fluoranthene		235.5^{a}	206.1^{b}	186.9^{c}
Pyrene		128.8^{a}	111.4^{b}	102.6^{c}
Benzo(a)Anthracene	*(µg kg^{-1})*	101.2^{a}	87.0^{b}	78.4^{c}
Chrysene		56.7^{a}	47.4^{b}	42.9^{c}
Benzo(b)Fluoranthene		96.7^{a}	83.5^{b}	77.3^{b}
Benzo(k)Fluoranthene		30.8^{a}	26.6^{b}	24.5^{b}
Benzo(a)Pyrene		108.8^{a}	91.5^{b}	84.7^{b}
Dibenzo(a.h)Anthracene		13.1^{a}	11.3^{b}	11.0^{b}
Benzo(g.h.i)Perylene		64.2^{a}	52.7^{b}	48.4^{b}
Indeno(1.2.3-cd)Pyrene		90.9^{a}	75.4^{b}	68.8^{b}
Total PAHs		1161.9^{a}	1004.9^{b}	917.9^{c}



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457 Figure 1

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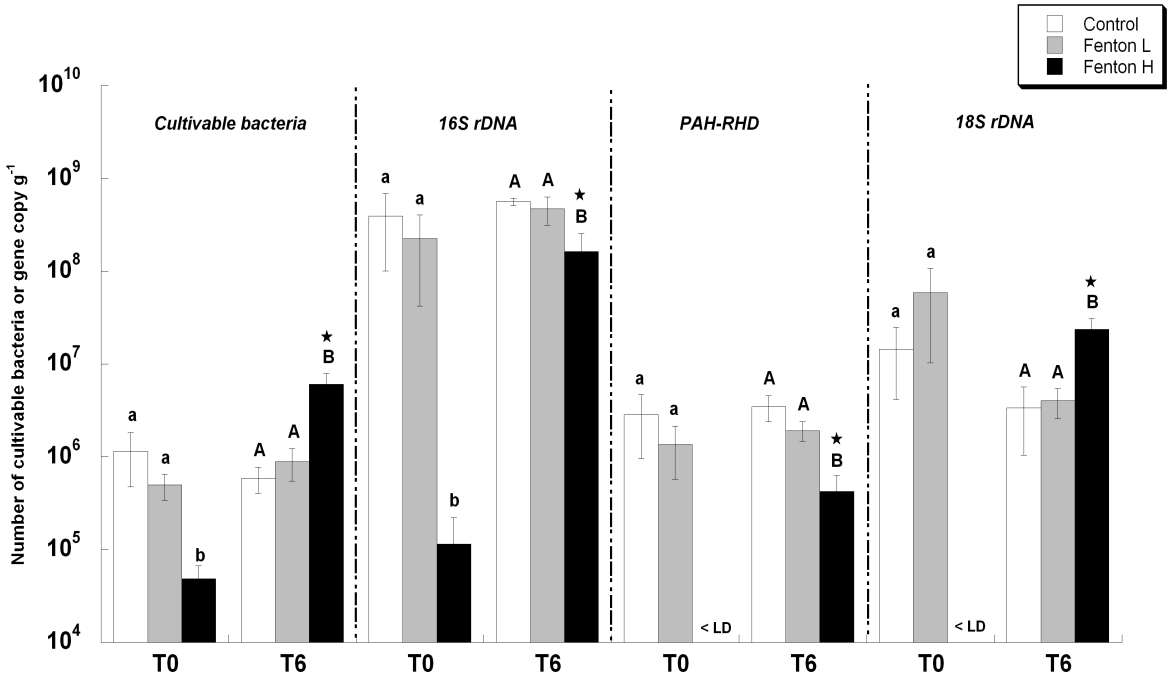

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460 Figure 2

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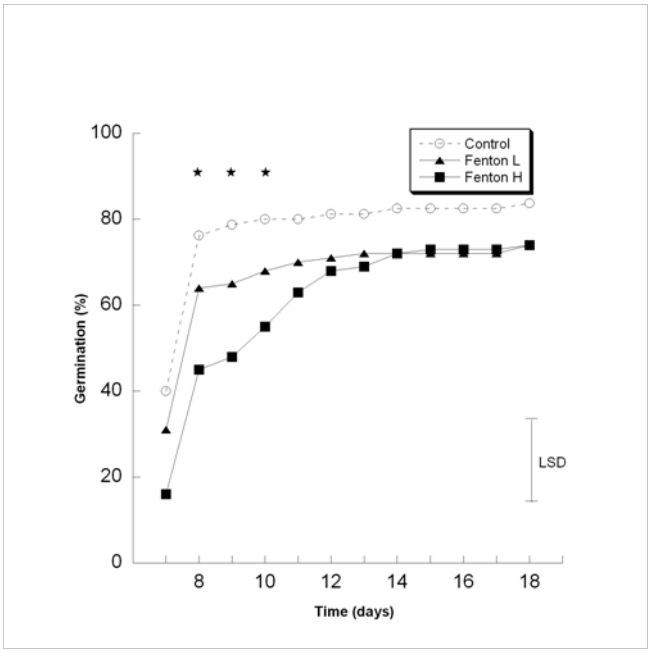

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463 Figure 3

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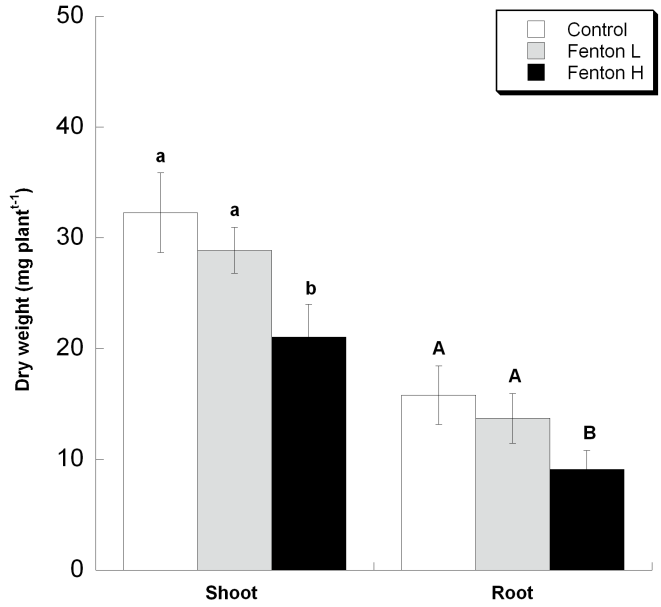

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466 Figure 4