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Abstract: Among remediation approaches, the Fenton-like treatment (FLT) is an ISCO technique relying on the iron-induced H2O2 activation in the presence of additives aimed at increasing the oxidant lifetime and maximizing iron solubility under natural soil pH conditions. The efficacy of FLT in the clean-up of hydrocarbon-contaminated soils is well established at the field-scale. However, a deeper understanding on the impact of the FLT on density, diversity and activity of the indigenous soil microbiota, might provide further insights into an optimal combination between FLT and in-situ bioremediation (ISB). The aim of this work was to assess the impacts of FLT on the microbial community of a diesel-contaminated soil collected nearby a gasoline station. Different FLT conditions were tested by varying either the H2O2 concentrations (2 and 6%) or the oxidant application mode (single or double dosage). The impact of these treatments on the indigenous microbial community was assessed immediately downstream of the FLT endpoints and after 30, 60 and 90 d and compared with enhanced natural attenuation (ENA). After FLT, a dramatic decrease in bacterial density, diversity and functionality was evident. Although in 2+2% microcosms a delayed recovery of the indigenous microbiota was observed as compared to those subjected to single oxidant dose, after 60 d incubation, 7.1-fold increases in respiration rates were observed with respect to those detected at the respective FLT endpoint. Irrespective of the oxidant dose, best degradation results after 90 d incubation (around 80%) were observed with combined FLT, relying on double oxidant addition, and bioremediation.

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To the Editor of
Chemosphere

Dear Editor,

please find hereby attached the paper titled "*Impact of the Fenton-like treatment on the microbial community of a diesel-contaminated soil*" by Flavia Polli, Daniela Zingaretti, Silvia Crognale, Lorena Pesciaroli, Alessandro D'Annibale, Maurizio Petruccioli and myself.

In this work we investigated the response of the bacterial community of a diesel-contaminated soil to the disturbance of oxidative stress caused by the application of the Fenton-like treatment (FLT). This treatment was tested under different operating conditions by varying either the H_2O_2 concentrations or the oxidant application mode (single or double dosage). The impact of these treatments on the indigenous microbial community was assessed immediately downstream of the FLT endpoints and after 30, 60 and 90 d of bioremediation and compared with enhanced natural attenuation (ENA). As demonstrated by the results reported in the paper, Fenton-like treatment was equally effective operating either with a single H_2O_2 injection at higher concentration or applying a double injection at lower concentration. Despite the negative impacts of the oxidation step, the analysis performed in this study allowed to observe a delayed recovery of the indigenous microbiota with time.

Although the manuscript length exceed the word limits indicated in the Guide for authors, we believe that all the information provided in this paper are useful for better understand the effects of the Fenton-like treatment on the microbial community and hence we would prefer not to shorten the manuscript. If deemed necessary, we will be anyhow available to move some information in the supplemental files.

We believe that these results could be of interest for the readers of Chemosphere since others papers on these subjects have been already published in this journal.

I look forward to hearing from you about the outcome of the review.

Best Regards,

(Prof. Renato Baciocchi)

***Highlights (3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

IMPACT OF THE FENTON-LIKE TREATMENT ON THE MICROBIAL COMMUNITY OF A DIESEL-CONTAMINATED SOIL

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Highlights

- The effect of a Fenton-like treatment on the soil microbial community was assessed
- Adding H_2O_2 twice at lower amount is effective as adding it once at higher amount
- Higher H_2O_2 concentration entails more severe impact on microbial community
- Bioattenuation occurred after Fenton-like step despite the observed negative impacts
- Fenton-like treatment can significantly shorten the bioremediation process

IMPACT OF THE FENTON-LIKE TREATMENT ON THE MICROBIAL COMMUNITY OF A DIESEL-CONTAMINATED SOIL

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Abstract

Among remediation approaches, the Fenton-like treatment (FLT) is an ISCO technique relying on the iron-induced H_2O_2 activation in the presence of additives aimed at increasing the oxidant lifetime and maximizing iron solubility under natural soil pH conditions. The efficacy of FLT in the clean-up of hydrocarbon-contaminated soils is well established at the field-scale. However, a deeper understanding on the impact of the FLT on density, diversity and activity of the indigenous soil microbiota, might provide further insights into an optimal combination between FLT and in-situ bioremediation (ISB). The aim of this work was to assess the impacts of FLT on the microbial community of a diesel-contaminated soil collected nearby a gasoline station. Different FLT conditions were tested by varying either the H_2O_2 concentrations (2 and 6%) or the oxidant application mode (single or double dosage). The impact of these treatments on the indigenous microbial community was assessed immediately downstream of the FLT endpoints and after 30, 60 and 90 d and compared with enhanced natural attenuation (ENA). After FLT, a dramatic decrease in bacterial density, diversity and functionality was evident. Although in 2+2% microcosms a delayed recovery of the indigenous microbiota was observed as compared to those subjected to single oxidant dose, after 60 d incubation, 7.1-fold increases in respiration rates were observed with respect to those detected at the respective FLT endpoint. Irrespective of the oxidant dose, best degradation results after 90 d incubation (around 80%) were observed with combined FLT, relying on double oxidant addition, and bioremediation.

1 Introduction

In-Situ Chemical Oxidation (ISCO) is an effective technology for the remediation of soil and groundwater contaminated by a wide range of organic pollutants (Pignatello et al., 2006; Baciocchi et al., 2014). An ISCO treatment involves the injection of an oxidant, such as permanganate (Chen et al., 2016), hydrogen peroxide (Laurent et al., 2012), persulfate (Sutton et al., 2014b) or ozone (Yu et al., 2007), into the subsurface to mineralize the contaminants of concern or, at least,

39 transform them in less toxic products. Among the different formulations proposed so far, the Fenton
40 process involves the reaction between H_2O_2 and ferrous iron yielding the hydroxyl radical (·OH)
41 ferric iron and hydroxyl ions (OH-) (Huling and Pivetz, 2006). Several approaches and methods
42 involving the use of H_2O_2 and iron were investigated so far. Among these, the so called Fenton-like
43 treatment (FLT) is a process relying on the iron-induced activation of hydrogen peroxide exerted by
44 minerals naturally occurring in soils, in the presence of proper amendments (stabilizing and
45 chelating agents) aimed at increasing both oxidant lifetime and iron solubility at natural soil pH
46 conditions; its efficacy in the clean-up of soils contaminated by petroleum hydrocarbons has been
47 already established (e.g. Watts and Dilly, 1996). The acceptance of Fenton treatments by regulators
48 is often constrained by concerns on the effect of the process on soil microbial community. The
49 Fenton process indeed affects negatively activity and vitality of indigenous microflora (Waddell and
50 Mayer, 2003). Actually, hydrogen peroxide is reportedly an antiseptic reagent (Chapelle et al.,
51 2005) able to either inhibit or kill microorganisms even at lower concentrations than those typically
52 applied for other oxidants used in ISCO treatments (Waddell and Mayer, 2003; Huling and Pivetz,
53 2006; Pardieck et al., 1992, U.S. EPA, 2004). Hydroxyl radicals arising from Fenton reactions have
54 been shown to enhance mutagenesis, cell death, cell membrane damage, and to reduce both
55 microbial activity and acclimation ability of microbial populations (Waddell and Mayer, 2003; Sahl
56 and Munakata-Marr, 2006; Sutton et al., 2011). The harmful effects of H_2O_2 on microbial
57 community are concentration-dependent; as a result, the U.S. EPA recommends the use of aqueous
58 solution of H_2O_2 with a concentration lower than 500 mg L^{-1} (U.S. EPA, 2004) to limit these
59 phenomena.

60 Nevertheless, there is also evidence that the negative effect exerted by Fenton processes does not
61 hinder a recovery of biological activity over time. For instance Sutton et al. (2014c), who
62 investigated the effect of Fenton and modified Fenton processes on microbial diversity and activity
63 during 8 weeks of incubation in two diesel-contaminated soils, observed that although microbial

communities was adversely affected in the first 2 weeks, a rebound of microbial abundance and biodegradation activity was observed after 4 weeks. This behaviour opened the way for the development of a treatment train based on the sequential application of ISCO and in situ bioremediation (ISB), which was investigated in several studies. For instance, Chen et al. (2016) investigated the efficiency of diesel removal by in situ chemical oxidation evaluating the effects of different oxidants on indigenous microorganisms; they observed that the contaminant removal was highest in ISCO-treated microcosms enabling a rapid recovery of the microbiota. These studies indicated that the combination of chemical oxidation and in situ bioremediation could be more efficient than ISCO alone in achieving the clean-up goals, since chemical oxidation was capable of reducing the contaminant's concentration to a level suitable for the further biological degradation of the residual contamination. The optimal coupling of ISCO and ISB requires a careful design of the chemical oxidation step, aimed at limiting its negative effects on the microbial community, as also shown by Chen et al. (2016).

Despite the available literature, there is still need of further investigation in view of ISCO integration with ISB. This regards in particular Fenton-like processes since, although previous studies have analysed the combination of bioremediation and other ISCO treatment, such as Fenton, modified Fenton, persulfate and permanganate and other oxidants (e.g. Chen et al., 2016; Sutton et al., 2014b, 2014c), the combination of ISB with Fenton-like processes has not been fully investigated yet. Thus, this study was aimed to gain more insights on this issue and to assess specifically the response of the bacterial community of a diesel-contaminated soil to the disturbance of oxidative stress caused by the application of FLT treatment. In particular, the soil underwent different treatment conditions, differing for either H_2O_2 concentrations (2 and 6%) or application mode (single injection or double after depletion of the first dose). Short-term and long-term effects on the bacterial community of the different tested treatments were evaluated using different molecular tools (i.e., Real Time PCR and DGGE) and biochemical tests (respirometry and dehydrogenase activity). The obtained results were then compared, both in terms of degradation

outcomes and impact on the microbial community with those obtained by enhanced natural attenuation (ENA).

2 Materials and methods

2.1 Reagents

Hydrogen peroxide (H_2O_2, 30% w/w), titanium (IV) oxysulfate, sulphuric acid (96%), monobasic potassium phosphate (KH_2PO_4), used as stabilizing agent, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (99%), used as chelating agent, were purchased by Sigma Aldrich. 5-alfa-androstane (99.9%, 2.00 μg mL^{-1} in methylene chloride, Sigma Aldrich) and 1-chlorooctadecane (99.6%, Sigma Aldrich) were used as internal standard and the surrogate, respectively. Anhydrous sodium sulphate (Sigma Aldrich > 99%), previously thermally activated at 500 °C for 4 h, was used for drying the soil samples during hydrocarbons extraction. All aqueous solutions employed were prepared with deionized water produced by reverse osmosis (Zeneer Power System).

2.2 Soil Characterization

All experiments were performed using a Diesel-contaminated soil collected in a gasoline station site and sampled from the capillary fringe at a depth of 1-2 m. Before use, the contaminated soil was sieved to remove the coarser fraction (d > 1.0 cm), mechanically homogenized in a stainless steel vessel and its main chemical and biological properties were evaluated. The particle size distribution was determined applying the ASTM D422 (2007) procedure. The total organic carbon (TOC) content was determined as difference between the total carbon (TC) and inorganic carbon (IC) data measured using a Shimadzu TOC VCPH analyser equipped with a SSM-5000A solid sampler (EN 15936:2012). The soil oxidant demand (SOD) and total oxidant demand (TOD) were measured by potassium permanganate consumption (Crimi et al., 2003). The pH was measured in the supernatant of a suspension made by 10 g of soil and 25 mL of a $CaCl_2$ solution 0.01 M. The total Fe and Mn content was determined by acid extraction (US EPA 3050B, 1996) followed by ICP-OES analysis (Agilent 710-ES) of the solution (ISO 11885:2011). The initial Diesel Range Organics (DRO)

content of the soil was also analysed measuring the concentration of hydrocarbons C8-C28 by gas chromatography (see subsection 2.4).

2.3 Experimental set up

Two types of static batch tests were performed using the diesel-contaminated soil, i.e. Fenton-like treatment (FLT) coupled with bioremediation (coded as FLT-BR) and enhanced natural attenuation (coded as ENA) alone. Table 1 reports a synoptic scheme of the remediation microcosms and main parameters of both the chemical oxidation and the biological attenuation step (i.e., liquid/solid ratio, oxidant dose and application mode, endpoint for each Fenton-like treatment, mineral solution, water content and bioremediation treatment times).

Table 1. Synoptic scheme of experimental design and main related conditions.

	Chemical oxidation step						Biological Attenuation Step		
Test	H_2O_2 (% wt)	KH_2PO_4:H_2O_2 (mol/mol)	EDTA (mmol kg^{-1})	N° solution injection	L/S ratio (l kg^{-1})	Treatment end-point (d)	Mineral solution (ml)	Water content (%)	Time (d)
Enhanced Natural Attenuation (ENA)									
ENA	-	-	-	-	-	-	2	30	30, 60, 90
Fenton-like treatment (FLT) and bioremediation (BR)									
FLT 2% H_2O_2	2	1:30	10	1	2	8	2	30	30, 60, 90
FLT 2+2% H_2O_2	2	1:30	10	2	2	13	2	30	30, 60, 90
FLT 6% H_2O_2	6	1:30	10	1	2	16	2	30	30, 60, 90

In the FLT-BR tests, the chemical oxidation step was followed by bioremediation; the ENA tests were incubated in parallel with the beginning of the bioremediation phase of FLT-BR microcosms, which was scheduled at the respective endpoints of each chemical oxidation treatment. This was done in order to assess the possible recovery of the indigenous microbiota in FLT-treated microcosms and to compare the clean-up efficiency of the combined FLT-BR treatments with those attained by ENA.

2.3.1 Fenton-like treatments

In the FLT-BR tests, a 50 mL plastic vial was loaded with the contaminated soil (12 g) and the hydrogen peroxide solution (24 mL). Prior to the oxidation step, the soil was saturated with a

135 solution of EDTA for 24 h; then, the H_2O_2 solution (either 2 or 6%, w/w) stabilized with KH_2PO_4
136 was added. The concentrations of the additives used in the FLT-BR tests were based on previous
137 ISCO studies (Innocenti et al., 2014; Piscitelli et al., 2015). In particular, the stabilizing agent was
138 added in order to achieve a KH_2PO_4:H_2O_2 molar ratio equal to 1:30 and EDTA was dosed at a
139 concentration of 10 mmol kg^{-1} soil. The oxidation reaction was allowed to proceed until the residual
140 hydrogen peroxide concentration was around 10% of its initial value. Tests were also performed
141 with a sequential addition of a 2% hydrogen peroxide solution (2+2% tests) and the second dose
142 was applied as the residual H_2O_2 concentration had dropped by 90%. Based on this criterion, the
143 end-points of each Fenton-like treatment was as follows: 8 d for 2% H_2O_2 test, 13 d for 6% H_2O_2
144 test and 16 d for 2+2% H_2O_2 test. At the end of the oxidation phase, the slurry samples were
145 centrifuged at 4 °C (8000 rpm, 15 min) and, after discarding the supernatant, the recovered soil
146 sample used for the subsequent bioremediation step.

147 **2.3.2 Bioremediation treatments**

148 Microcosms containing the FLT-treated soil (12 g) were mixed with 2 mL mineral solution ($CaCl_2$,
149 0.05 g L^{-1}; $MgSO_4$*$7H_2O$, 0.1 g L^{-1}; $FeCl_3$, 0.12 mg L^{-1} and NH_4Cl, 0.5 g L^{-1}) and incubated in the
150 dark at 20 °C for 3 months in 50 mL plastic vials with screw caps. The soil moisture was
151 maintained at 30% in order to simulate typical conditions found in the capillary fringe, from which
152 the soil samples were collected. In the ENA tests, the same procedure was applied to a sample of
153 untreated soil. Each FLT-BR and ENA microcosm was performed in triplicate for each time point
154 (i.e, 0, 30, 60 and 90 d).

155 **2.4 Chemical analyses**

156 H_2O_2 concentration was measured using an UV–VIS spectrophotometer TU-1880S Double Beam
157 after colour development with titanium sulphate (Schumb et al., 1955). The DRO residual
158 concentrations in the different soil samples were determined according to the U.S. EPA 3550C,
159 (2007) procedure. In particular, the soil (5.0 g) was mixed in a 50 mL glass vial with 5.0 g of

anhydrous sodium sulphate, 5.0 mL of n-hexane, 10 mL of acetone (ISPRA, 2011) and 1 mL of 1-chlorooctadecane solution (MADEP-EPH-04, 2004) and incubated for 30 min in an ultrasonic bath (Fisher Scientific FB 15053). After ultrasonic extraction, the supernatant was transferred to a funnel with 50 mL of DI water in order to remove acetone by liquid-liquid extraction, thus allowing to obtain an extract with only n-hexane as solvent. The extract was then purified with 0.4 g of previously activated Florisil and 0.4 g of anhydrous sodium sulphate (ISPRA, 2011) and 5-alfa-androstane was added as internal standard (MADEP-EPH-04, 2004).

DRO concentration was measured by GC/MS (direct injection, split ratio 1:20, 1μL-injection volume) with a Shimadzu GC QP2010SE instrument equipped with a TR-5MS column (30 m × 0.25 mm ID × 0.25 μm film thickness) (Thermo Scientific), using helium as carrier gas. The initial oven temperature set at 50°C, held for 1 min, ramped up to 110 °C (10 °C min^{-1}) and then ramped to 320 °C (3°C min^{-1}) and, finally, held at 320 °C for 3 min (adapted from Xie et al., 1999). DRO concentrations are referred to unit mass of soil (EN 15934:2012).

2.5 Biological analyses

2.5.1 Molecular analyses

Microbial abundance and diversity were assessed by quantitative polymerase chain reaction (q-PCR) and denaturant gradient gel electrophoresis (DGGE) respectively.

Total soil DNA was extracted using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc.) and q-PCR experiments were performed with LightCycler® 480 Real-Time PCR System (Roche) using the fluorescent molecule SYBR Green I. The 16S rDNA was amplified using the primers 331F (TCCTACGGGAGGCAGCAGT) and 797R (GGACTACCAGGGTATCTAATCCTGTT) (Nadkarni et al., 2002). For each reaction, 10 μL SYBR Green PCR Master mix (Roche), 1 μL of each primer 1 μL DNA and 3 μL H_2O were used. Polymerase chain reaction (PCR) amplification was conducted with an initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation, annealing (60 °C) and extension at 72 °C for 1 min and a final melting step. Bacterial

concentrations were calculated based on a standard curve obtained using triplicate 10-fold serial dilutions of known concentration of plasmid pGem T-easy cloning vector containing the bacterial target as the insert.

In DGGE analysis, the hypervariable V3 region of the 16S rDNA was amplified using the primers 341F (ACG GGG GGC CTA CGG GAG GCA GCAG) and 534R (ATT ACC GCG GCT GCT GG) (Muyzer et al., 1993). Reaction mix used for each sample was composed by 2μL template DNA, 1 μL of each primer, 25 μL Nzytaq MasterMix (NZYtech,Lisbon, Portugal) and 21 μL H_2O. PCR amplification was conducted with an initial denaturation at 95 °C for 2 min followed by the annealing phase in which the temperature was decreased by 0.5 °C in each cycle from 65 °C till 55 °C and then further ten cycles were performed. Each amplification product was checked on 1.2% agarose gel prior to denaturing gradient gel electrophoresis (DGGE) analysis, which was performed using INGENY phorU-2 system (Ingeny International BV, Goes, NL). A 6% polyacrylamide gel on TAE 10X (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA, pH 7.8) with a 40-60% urea-formamide linear gradient was used and electrophoresis was performed at 60 °C and 100 V for 16 h. After electrophoresis, the gel was stained using Gel Star (Invitrogen) in TAE 1X for 45 min and the image was detected using a UV trans-illuminator with camera (Chemidoc, Biorad).

The obtained DGGE images were then analysed using Quantity-one image analysis software (v. 4.2.5 Bio-Rad Laboratories, Hercules, CA) to assess the biodiversity of the bacterial species present by calculating diversity indices Richness (R), Shannon-Weaver (H') and Evenness (E). In particular R was the number of detected bands. H' index was calculated as $-\sum p_i \ln p_i$, where p_i was the ratio between the single band intensity and the sum of bands intensity of each lane and Evenness (E) was calculated as $H' \ln R^{-1}$ (García-Delgado et al., 2015). Pareto Lorenz curves were calculated according to Marzorati et al. (2008).

2.5.2 Biochemical analyses

Community level physiological profile (CLPP) of the untreated soil was determined by the Biolog EcoPlate TM system (Biolog Inc., CA, USA). Each 96-well plate consists of three replicates, each one comprising 31 sole carbon sources and a water blank. Aliquots of soil elutriate (100 μl), obtained by suspending soil in the physiological solution (1:10, w/v) and incubating in an unltrasonic bath for 15min, followed by 15 min orbital shaking, were inoculated into the microplate. The plates were incubated at 20 °C, and colour development in each well was recorded as optical density (OD) at 590 nm with a plate reader at regular 12 h-intervals. Absorbance values for each of the 31 substrates were corrected by subtraction of the blank control value.

Soil dehydrogenase activity was determined according to the method of Trevors et al. (1984). In particular, the soil (1.0 g), the moisture of which had been adjusted to 60% of the water-holding capacity, was incubated at 37 °C for 36 h prior to the addition of a 2,3,5-triphenyltetrazolium chloride (TTC, 1.5%, w/v) solution in Tris-HCl buffer 0.1 M pH 7.6. At the end of incubation, the triphenylformazan (TPF) was extracted with 8 ml of acetone, the extract was filtered and the absorbance of the filtrate read at 546 nm (ε= 15.4 mM^{-1} cm^{-1}).

Microbial respiration was determined using the MicroResp^{TM} soil respiration system (Micro-Resp^{TM}, Macaulay Scientific Consulting Ltd, Aberdeen, UK) according to the method of Campbell et al. (2003). In particular, approx. 0.5 g from each soil microcosms, the moisture of which had been previously adjusted to 60% of the WHC, were placed in each one of the 96 deep-well plates and two replicates were used for each microcosm. To determine the evolved CO_2, a colorimetric method relying on the change in the pH of a gel-based solution of bicarbonate containing cresol red was used. The absorbance of the detection plate was read at 595 nm at start and after 6 h of incubation at 25 °C by using a Zenyth multi-mode reader (Anthos, Wals, Austria). The absorbance after 6 h was normalized for any differences recorded at time zero and converted to the headspace

232 CO_2 concentration by using a calibration curve obtained as described by the manufacturer.
233 Respiration data were expressed in terms of µg C-CO_2 g^{-1} soil h^{-1}.

234 **2.6 Statistical analysis**

235 One-way analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) post-
236 hoc tests were performed by using the SigmaStat 3.5 software package (Jandel Scientific,
237 Germany).

238

239 **3 Results and Discussion**

240 **3.1 Soil Characterization**

241 The main chemical and biological properties of the soil under study, which was a backfill sand, are
242 shown in Table 2. The analysis of the particle size distribution confirmed that the soil had a coarse
243 texture and could be defined as a sand with gravel. Its TOC content was around 0.83% and was
244 considered suitable for ISCO application since a limited unproductive oxidant consumption by soil
245 organic matter is expected (Baciocchi et al., 2014). Furthermore, the obtained SOD and TOD values
246 were equal to 2.1 and 10.9 g_{ox} kg_{soil}^{-1}, respectively, suggesting that the unproductive consumption of
247 the oxidant through reaction with natural soil reductants for this soil is lower than the suggested
248 maximum value of 30 g_{ox} kg_{soil}^{-1} (Baciocchi et al., 2014). The pH of the soil (i.e., 7.6) was also
249 suitable for Fenton-like treatments (Sigriest et al., 2011). The total Fe content was equal to 41.9 ±
250 4.0 mg kg^{-1} while the DRO content was 1847±378 mg kg^{-1} soil.

251 The microbial abundance in the soil under study was evaluated through both cultivation-dependent
252 approaches (viable counts) and (q-PCR) experiments targeting the 16S rRNA gene. The untreated
253 soil had a bacterial density of 3.1× 10^6 CFU g^{-1} corresponding to a 16S copy number of 6.54 ×10^8 g^{-1}.
254 Such a microbial density was deemed to be compatible with an enhanced natural attenuation.
255 CLLP analysis evidenced the indigenous microbial community capacity to metabolize the following
256 substrates (data not shown): pyruvic acid methyl ester, D-xylose, D-galacturonic acid, L-asparagine,

257 Tween 40, Tween 80, D-mannitol, itaconic acid, glycyl-L-glutamic acid. The microbial activity
258 against Tweens could indicate the hydrocarbon-degrading potential of the indigenous microbial
259 community.

260

261

262

263 **Table 2.** Main chemical and biological properties of the contaminated soil sample.

Parameter	Units.	Mean value
Particle Size Distribution		
d > 2 mm	%	34.3
1.7 < d < 2 mm	%	2.3
0.84 < d < 1.7 mm	%	15.3
0.425 < d < 0.84 mm	%	20.3
0.125 < d < 0.425mm	%	25.7
d < 0.125 mm	%	2.1
Water content	%	4.3-5
TC	%	0.94
IC	%	0.11
TOC	%	0.83
pH	-	7.6
Fe	mg kg^{-1}_{soil}	41.9 ± 3.97
Mn	mg kg^{-1}_{soil}	0.87 ± 0.02
Soil Oxidant Demand (SOD)	g_{ox} kg^{-1}_{soil}	2.1
Total Oxidant Demand (TOD)	g_{ox} kg^{-1}_{soil}	10.9
DRO (C_{12}-C_{28})	mg kg^{-1}_{soil}	1847 ± 378
Bacterial abundance	CFU $g^{-1} \times 10^6$	3.1±0.45
	16S rRNA gene copy number g^{-1}_{soil}	6.54 ± 0.64 $\times 10^8$
Respiration rate	µg $_{C\text{-}CO2}$ g^{-1} h^{-1}	0.15 ± 0.01
Dehydrogenase activity	nmol $_{TPF}$ g^{-1} h^{-1}	3.05 ± 0.33

264

265 **3.2 Effects of treatments on the microbial abundance**

266 Figure 1 shows that in the ENA microcosm, the 16S rRNA gene copy number remained stable
267 throughout the incubation, ranging from 10^8 to 10^9 and reaching a value that did not significantly
268 differ from that found in the soil at start.

As expected, the application of the FLT exerted a marked suppressive effect on the indigenous bacterial community immediately after the end-point of each treatment. In fact, after Fenton oxidation, no growth was detected by the cultivation-dependent method (data not shown) and q-PCR detected a low bacterial abundance (10^5 16S copies g^{-1}_{soil}) regardless of both the dose and mode of hydrogen peroxide injection (single or double). The known biocide effect of H_2O_2 on microbial growth is exerted by oxidative stress targeting lipids, proteins and, above all, DNA which may be significantly affected by nicking (Linley et al., 2012). However, microorganisms may cope with oxidative stress through defence mechanisms based on enzymatic reactions (catalase and/or superoxide dismutase) and/or genetic repair tools enabling the indigenous bacterial community to withstand and/or recover after the cessation of the stress factor.

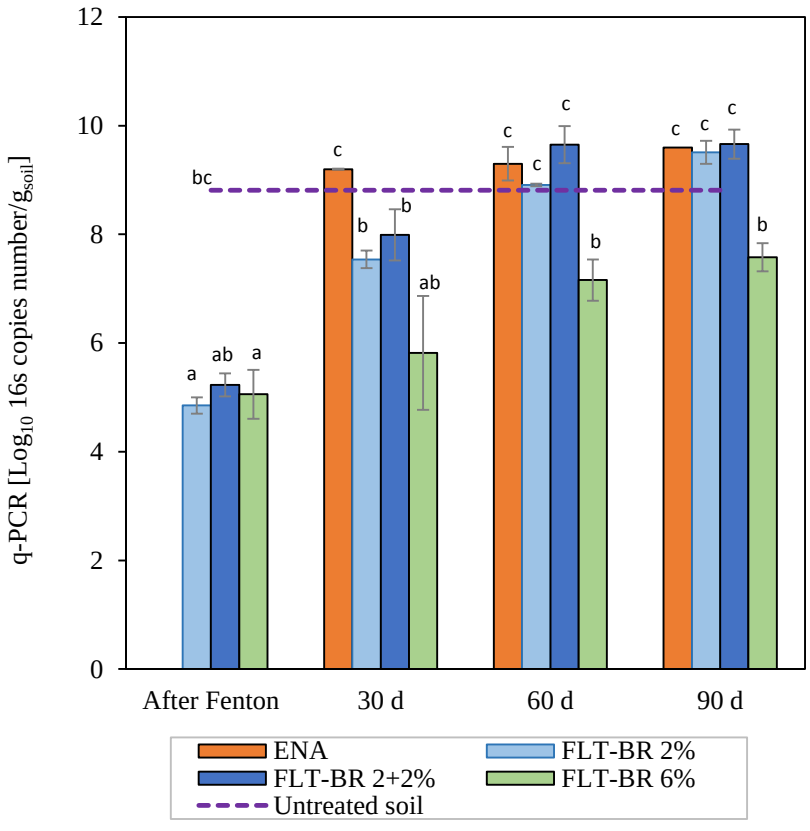


Figure 1: 16S rRNA gene copy number in Enhanced Natural Attenuation (ENA) microcosm and in microcosms subjected to sequential Fenton-like treatment and bioremediation (FLT-BR) at different times of incubation (0, 30, 60 and 90 d). Statistical pairwise multiple comparison of data was carried out by the least significant difference (LSD) test and the same letters above bars indicate the absence of significant differences ($P \leq 0.05$). The dashed line represents the 16S copy number in the initial contaminated soil.

The time-dependent response of the bacterial community to the chemical oxidation treatments was studied over three months starting from the end-point of each Fenton-like treatment. Fig. 1 shows that after 30 d, the community responded differently in dependence on the dose of the oxidant. At that time, a partial recovery of the community abundance (from 10^5 to 10^7 16S RNA gene copy number g^{-1}) was observed in microcosms subjected to 2% oxidant dose, irrespective of the single or double injection; conversely, no rebound was observed when the applied dose was 6%. After 60 d, the microcosms subjected to 2% H_2O_2, either with single or double injection, reached similar values to those observed in both untreated soil and in coeval ENA microcosms, showing a total rebound capacity of the community, at least from the quantitative point of view. A slower recovery was observed in microcosms that had undergone 6% oxidant treatment where even after three months the bacterial abundance remained two orders of magnitude lower than that observed in the untreated soil.

3.2.1 Effects on the microbial community structure

Since chemical oxidation treatments are known to affect also the community structure, microbial biodiversity and evenness distribution of the resilient communities were assessed by the analysis of DGGE profiles, the PCR-amplified 16S rDNA (Fig. S1) which enabled the calculation of diversity indices (Table 3) and Pareto Lorenz curves (Fig. 2).

Table 3: Bacterial community diversity indices calculated from DGGE profiles obtained immediately downstream of the chemical oxidation treatment endpoint and after 30, 60, 90, in Fenton-like-treated microcosms and in parallel enhanced natural attenuation (ENA) microcosms. Statistical pairwise multiple comparison of column data was carried out by the least significant difference (LSD) test and the same letters above bars indicate the absence of significant differences ($P \leq 0.05$).

Diversity indices				
	Incubation Time	**Shannon-Weaver (H')**	**Richness (R)**	**Eveness (E)**
Untreated soil		2.32 ± 0.16^{b}	15 ± 1^{bc}	0.86 ± 0.02^{ab}
ENA	0	2.39 ± 0.13^{b}	$16 \pm 1a^{bc}$	0.85 ± 0.03^{ab}
	30 days	2.56 ± 0.15^{bc}	19 ± 2^{c}	0.87 ± 0.02^{ab}
	60 days	2.51 ± 0.11^{bc}	13 ± 2^{bc}	0.87 ± 0.03^{a}
	90 days	2.53 ± 0.16^{bc}	12 ± 2^{c}	0.86 ± 0.02^{ab}

Fenton-Bioremediation				
2%	0	2.38 ± 0.18^{b}	14 ± 2^{b}	0.90 ± 0.03^{ab}
	30 days	2.89 ± 0.12^{c}	23 ± 2^{d}	0.92 ± 0.02^{b}
	60 days	2.49 ± 0.15^{bc}	13 ± 1^{ab}	0.96 ± 0.03^{c}
	90 days	2.63 ± 0.11^{bc}	18 ± 1^{c}	0.91 ± 0.03^{b}
2+2%	0	2.47 ± 0.15^{b}	15 ± 1^{bc}	0.93 ± 0.02^{b}
	30 days	2.92 ± 0.17^{c}	19 ± 2^{c}	0.99 ± 0.03^{c}
	60 days	3.16 ± 0.16^{c}	25 ± 1^{d}	0.98 ± 0.03^{bc}
	90 days	3.08 ± 0.13^{c}	23 ± 1^{d}	0.98 ± 0.03^{bc}
6%	0	2.30 ± 0.13^{b}	14 ± 1^{b}	0.90 ± 0.02^{b}
	30 days	1.99 ± 0.12^{a}	8 ± 2^{a}	0.96 ± 0.03^{bc}
	60 days	2.30 ± 0.15^{b}	10 ± 1^{a}	0.99 ± 0.02^{bc}
	90 days	2.18 ± 0.14^{ab}	9 ± 1^{a}	0.99 ± 0.02^{b}

307

308 The addition of the oxidant at 2% in single dose led to an increase of both bacterial richness and
309 diversity. The Fenton treatment at 2% with double injection gave rise to a higher and prolonged
310 increase of both bacterial diversity and richness as compared to the microcosm undergoing single
311 dose (Table 3). This effect, already described in literature (Chen et al., 2016; Sutton et al., 2014c),
312 was suggested to be due to the release of degradable products in the soil which enhanced latent
313 microbial species. In this respect, a previous study had shown the formation of several soluble
314 compounds such as carboxylic acid, aldehydes and ketones due to the H_2O_2-induced oxidation of
315 diesel (Villa et al., 2008), the enhanced biodegradability of which was also inferred by an increase
316 in the BOD_5/COD ratio in another investigation (Mater et al., 2007). In addition, Sutton et al.,
317 (2014c) interpreted this temporary increase of biodiversity as a transient adaptation of a resilient
318 community until the attainment of a new "steady state" condition. Conversely, a severe negative
319 impact on microbial community richness in the soil that underwent 6% H_2O_2 treatment was
320 observed (Table 3).
321 In order to investigate the stability of the resilient bacterial communities that established 90 d after
322 the endpoints of the different FLTs, Pareto Lorenz (PL) distribution curves, derived from their
323 respective DGGE profiles, were plotted (Fig. 2).

In general, the more the PL curve deviates from the 45° diagonal (the theoretical perfect evenness line), the greater the shift in the evenness of the studied community (Marzorati et al., 2008). The PL curve of the untreated soil showed that 20% of the cumulative proportion of bands corresponded to a 54% of cumulative relative abundance of the bands, resembling the curve of the 90-d-old ENA microcosm. These results indicated the presence of dominant species without, however, a high degree of specialization for that habitat (Marzorati et al., 2008). The deviations of the PL curves of the Fenton-like treated microcosms with respect to the perfect evenness line (45° diagonal) tended to decrease as a function of the oxidant dose (Fig. 2). In particular, in microcosms treated with 2% H_2O_2, the 20% intercept value was still around 45%, while in the 2+2% and 6% microcosms, the intercepts amounted to 32 and 22% that indicated almost the absence of dominant species.

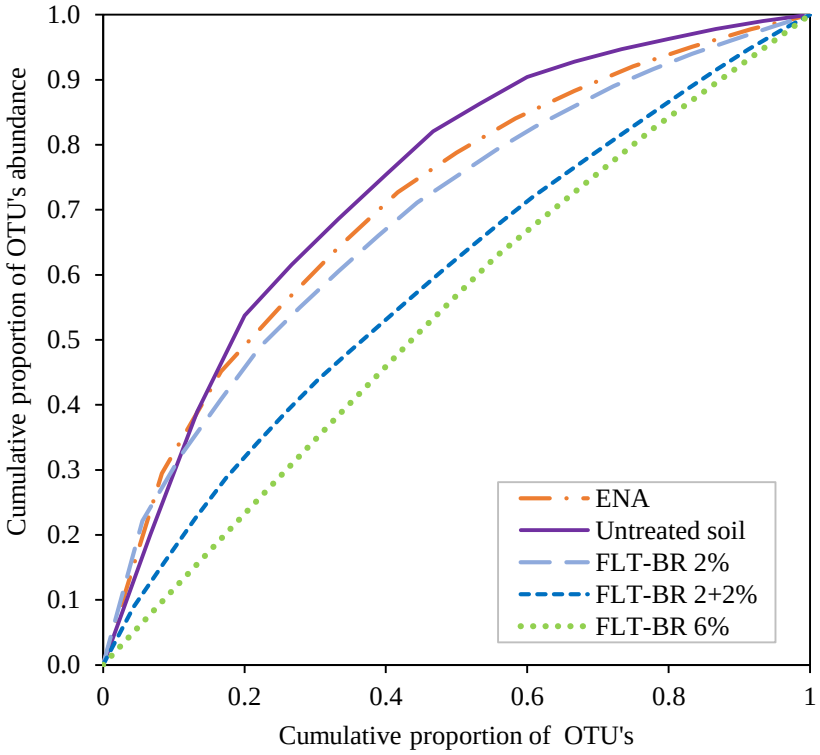


Figure 2: Pareto Lorenz curves derived from DGGE profiles of the initial soil (IS), Enhanced Natural Attenuation (ENA) microcosms and those subjected to sequential Fenton-like treatment and bioremediation (FLT-BR) after 90 d of incubation.

Marzorati et al. (2008) suggested that the absence of dominant species (such as in 2+2% and 6% H_2O_2 microcosms) might lead to a relatively long lag phase in the presence of a further stress exposure. Conversely, the community that experienced the 2% H_2O_2 treatment, having a 45% PL curve, is expected to deal effectively with changing environmental conditions and to preserve its functionality, even after a possible secondary stress disturbance.

3.2.2 Effects on Microbial function

To gain further insights into the effect of the chemical oxidation treatments on the indigenous microbiota, some indices of microbial function were also determined. To this aim, respiration rate and dehydrogenase activity in the microcosms undergoing ENA were compared with those determined after 30, 60 and 90 d from the endpoint of each FLT.

On a comparative basis, the respiration rates in both the initial soil (Table 2) and all remediation microcosms was lower by around one order of magnitude than those found in natural and agricultural soils. (Stoyan et al., 2000; Renault et al., 2013), This was somehow expected since, in addition to the contamination, the soil under study had a highly predominant sandy texture with an ensuing low organic matter content. Anyhow, as shown in Fig. 3, a time dependent rise in respiration rate was observed in ENA microcosm with a three-fold increase after 90 d with respect to the microcosm at start (0.45 vs. 0.15 μg $C\text{-}CO_2$ g^{-1} soil h^{-1}). Irrespective of both dose and application mode, respiration was markedly depressed when measured immediately after the endpoint of each FLT with values ranging between 0.006 to 0.037 μg $C\text{-}CO_2$ g^{-1} soil h^{-1}. However, a prompt recovery was observed in the microcosms that underwent single addition of H_2O_2.

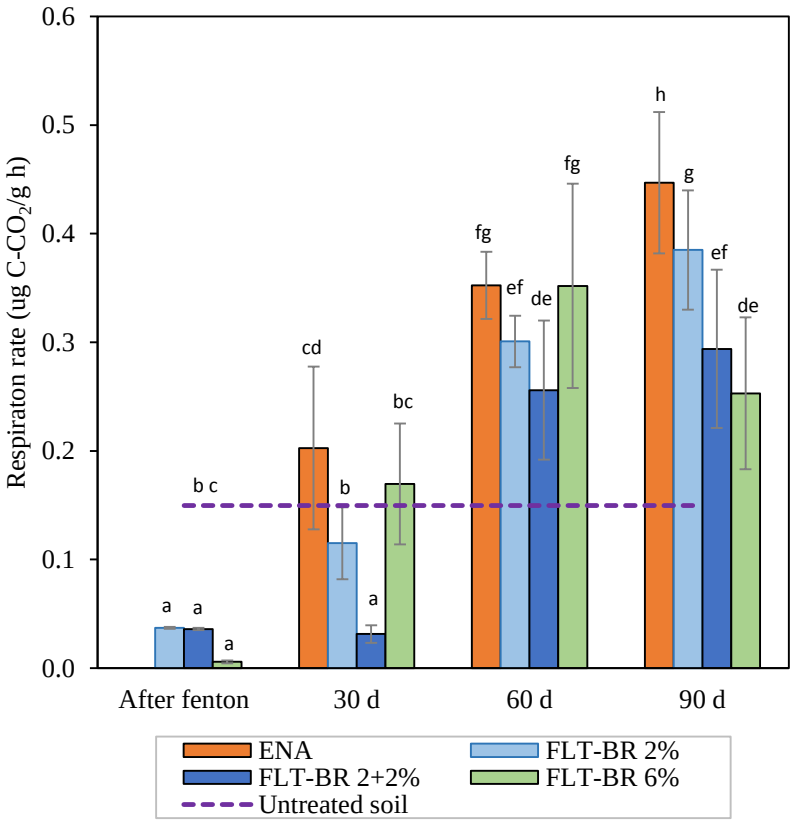


Figure 3: Respiration rates determined immediately downstream of the chemical oxidation treatment endpoints and after 30, 60, 90 d in Fenton-like-treated microcosms and in parallel enhanced natural attenuation (ENA) microcosms. Statistical pairwise multiple comparison of data was carried out by the least significant difference (LSD) test and the same letters above bars indicate the absence of significant differences ($P \leq 0.05$). The dashed line represents the basal respiration in the initial contaminated soil.

In the microcosms that had been treated with 2% oxidant, respiration tended to increase over time although its amounts were lower than those detected in the coeval ENA microcosms. In microcosm that had been treated with 6% oxidant, the extent of recovery was higher than that found in 2% microcosms and respiration did not significantly differ from that found in coeval ENA microcosms in the early 30 d of incubation. Conversely, in the microcosms that were subjected to double addition of 2% H_2O_2, the recovery was delayed as it can be inferred from the similarity of respiration values in 30-d-old microcosms and those at start. However, after 60 d incubation, 7.1-fold increases in respiration rates were observed in 2+2% microcosms, with respect to those detected in the same microcosms immediately downstream of the endpoint of the chemical

oxidation treatment. This recovery effect was also extended to the further harvest (90 days) for the 2+2% H_2O_2 microcosm.

Besides respirometry, the activity of some key enzymes in soil is recognized as an index of functionality and vitality of the indigenous microbial communities. Among them, dehydrogenase (DH-ase) activity represents an indicator of soil's redox capacity and, as such, is considered a widely accepted index of soil quality and of recovery of functionality in remediation scenarios (Dawson et al, 2007).

On a comparative basis with several soil typologies (Kumar et al., 2013), DH-ase activity was found to be very low in the initial contaminated soil (Table 2) and further depressed immediately downstream of the endpoints of FLT (Fig. 4).

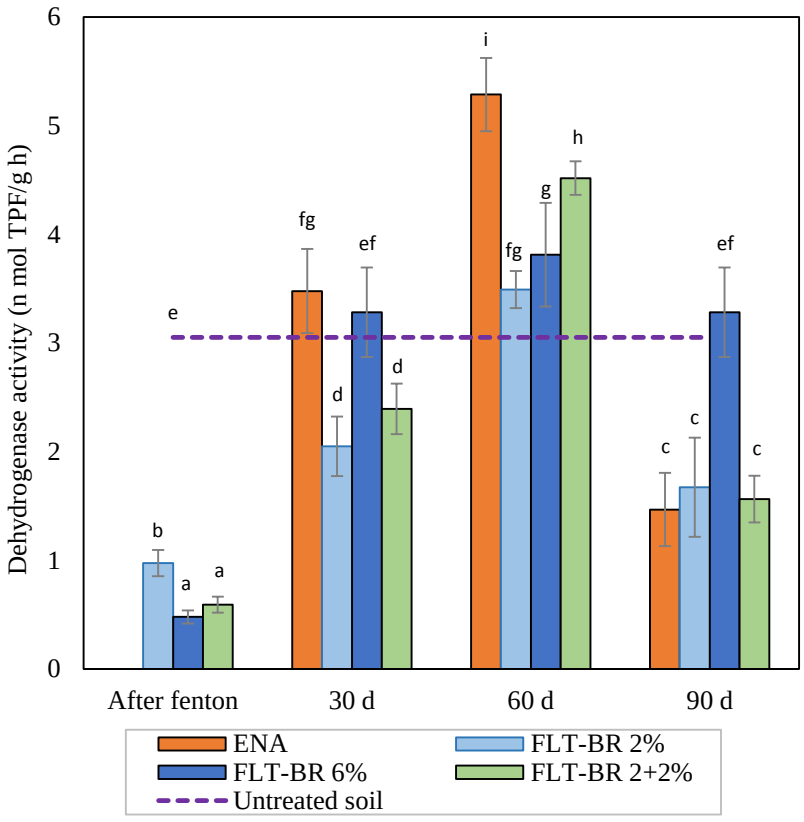
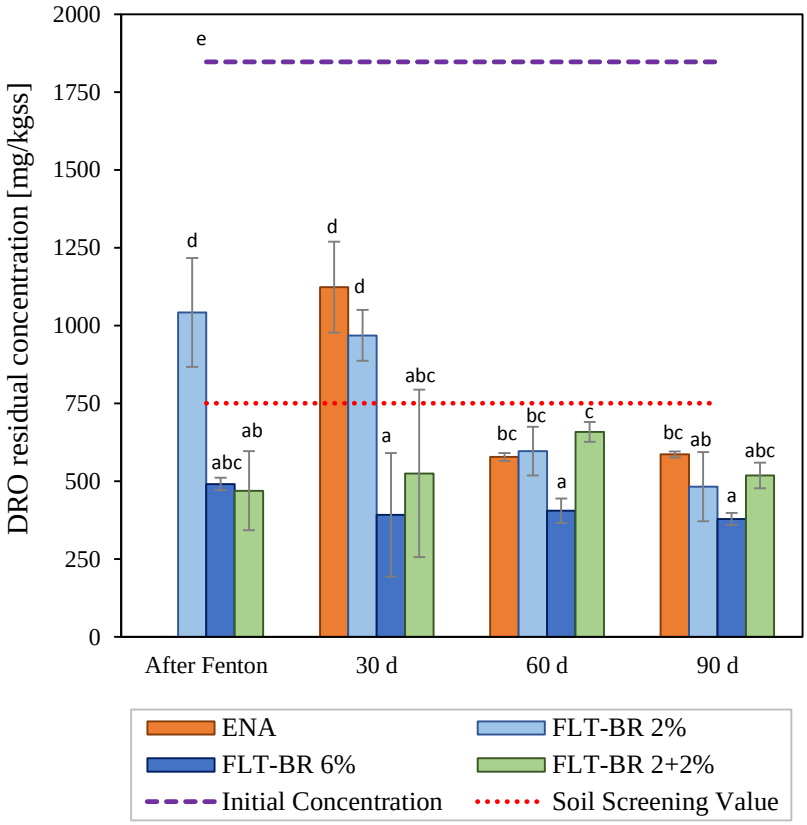


Figure 4: Dehydrogenase activities determined immediately downstream of the chemical oxidation treatment endpoints and after 30, 60, 90, in Fenton-like-treated microcosms and in parallel enhanced natural attenuation (ENA) microcosms. Statistical pairwise multiple comparison of data was carried out by the least significant difference (LSD) test and the same letters above bars indicate the absence of significant differences ($P \leq 0.05$). The dashed line represents the dehydrogenase activity in the initial contaminated soil

However, DH-ase activity tended to increase in the early two months of incubation in all the microcosms under study to decline thereafter. After 60 d of incubation, in particular, DH-ase activities were significantly higher than that found in the original untreated soil, as shown in Fig. 4. Although respiration relies on the activity of different dehydrogenase systems, the time-dependent trend of this parameter differed from that found for respiration rate. In this respect, several studies have failed to find correlation between these parameters in soil (Nohrstedt, 1985) and this has been ascribed to the inability of some microorganisms and respective dehydrogenase systems to use TTC as the electron acceptor (Tarafdar, 2003).

3.2.3 DRO degradation in microcosms

Regardless of the incubation time, in all the microcosms under study, the residual DRO concentrations were significantly lower than those found in the initial soil (1847 mg kg^{-1} soil), as shown in Fig. 5. In particular, in the early two months of incubation, the contaminant concentration markedly dropped in the ENA microcosms (1123 and 578 mg kg^{-1} after 30 and 60 d) to remain almost constant thereafter; thus, at the end of the set incubation time, this treatment yielded a contaminant removal equal to 68.3%. This was expected since CLPP showed the ability of the indigenous community to utilize hydrocarbon-related compounds, such as Tweens, which contain a significant acyl moiety in their structure; in addition, the bacterial counts in the initial soil was significantly higher than that suggested as the minimal threshold (10^3 CFU g^{-1}) to ensure the feasibility of bioremediation (Chen et al., 2016; U.S. EPA, 2004). Furthermore, respiration rate and the dehydrogenase activity in the ENA microcosm had indicated a time-dependent increase in the early two months of incubation.



420

421 **Figure 5:** DRO residual concentration determined immediately downstream of the chemical oxidation
422 treatment endpoints and after 30, 60, 90, in Fenton-like-treated microcosms and in parallel enhanced natural
423 attenuation (ENA) microcosms. Statistical pairwise multiple comparison of data was carried out by the least
424 significant difference (LSD) test and the same letters above bars indicate the absence of significant
425 differences ($P \leq 0.05$). The dotted and the dashed line indicate the DRO concentration in the initial untreated
426 soil and the regulatory limit set by the Italian legislation for industrial soil, respectively.

427

428 It can be noticed that all the FLTs, at their respective endpoints, were able to meet the soil screening
429 value (750 mg kg^{-1}) set by the Italian legislation for industrial soils with the notable exception of
430 that conducted with 2% H_2O_2 in which the onset of degradation was delayed. In fact, the residual
431 DRO concentration, determined immediately downstream of the treatment's endpoint, did not
432 significantly differ from that found after 30 d incubation (Fig. 5). Conversely, the contaminant
433 concentration dropped after 60 d to reach 482 mg kg^{-1} after 90 d of incubation. With this regard, the
434 Pareto-Lorenz curve had indicated that the evenness of the bacterial community in this microcosm
435 was similar to those of both the initial soil and 90-d-old ENA microcosm which had shown

hydrocarbon-degrading capacity. The overall clean-up efficiency of the FLT (2% H_2O_2)-BR treatment was around 74%.

In agreement with Sutton et al. (2014a), the double injection of the 2% hydrogen peroxide solution led to higher DRO removal efficiency than that obtained with single oxidant dose with a contaminant residual concentration of 491 mg kg^{-1} after the treatment endpoint. However, in this case, the impact of bioattenuation on the DRO concentration was limited as compared to that observed with the 2% microcosms, leading to a DRO residual concentration of 379 mg kg^{-1} after 90 d of incubation. In this respect, the Pareto-Lorenz curve witnessed for a loss of dominant species originally present in the initial soil. The overall clean-up efficiency of the FLT (2+2% H_2O_2)-BR treatment was 79.5%.

In the 6% H_2O_2 microcosms, the DRO residual concentration was equal to 469 mg kg^{-1} after the oxidation phase and remained fairly constant during the whole biological phase, showing a negligible effect of the bioattenuation on the DRO concentration. In this case, the Pareto-Lorenz curve was very close to the perfect evenness line (45° diagonal), indicating the absence of predominant species originally present in the initial soil.

4 Conclusions

In this study, the effect of a Fenton-like treatment (FLT) on the soil microbial community was assessed applying different oxidant concentration and single or double hydrogen peroxide dosage. The results obtained in this study showed that FLT treatment was equally effective operating either with a single H_2O_2 injection at higher concentration (6%) or applying a double injection at lower concentration (2+2%), with a DRO removal after the Fenton-like treatment around 75%. However, the analysis performed on the microcosms that underwent a FLT with higher hydrogen peroxide concentration showed a severe impact on microbial community richness and bacterial abundance. Conversely, the Fenton-like treatment proved to be less effective when operating a single injection at 2% (44% DRO removal). Nevertheless, the 2% treatment impacted less the microbial community with respect to both the 6% and 2+2%, allowing bioattenuation to occur after the chemical oxidation step, with a residual concentration after 90 days similar to the one achieved in the other tests just after the Fenton's treatment. Although the combination of chemical oxidation and bioremediation allows to overcome the limitation of biodegradation at high contaminant concentration, the results obtained in this work suggest that at the tested contaminant's concentration (1847 mg kg^{-1} DRO) enhanced natural attenuation alone was also effective, in agreement with Chen et al. (2016), but the FLT can significantly shorten the bioremediation process.

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