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Brassica napus has a key role in the recovery of the health of soils contaminated with metals and diesel by rhizoremediation

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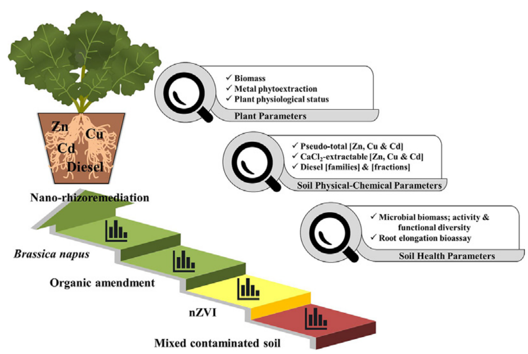
HIGHLIGHTS

- *Brassica napus* had a key role in the recovery of soil health.
- Microorganisms preferably degraded diesel hydrocarbons of biological origin.
- The organic amendment was essential for diesel degradation and plant growth.
- Soil carbonate content was a crucial factor for metal immobilization.
- The application of nZVI nanoparticles was not effective for soil remediation.

GRAPHICAL ABSTRACT



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ABSTRACT

Contaminated soils are frequently characterized by the simultaneous presence of organic and inorganic contaminants, as well as a poor biological and nutritional status. Rhizoremediation, the combined use of phytoremediation and bioremediation, has been proposed as a Gentle Remediation Option to rehabilitate multi-contaminated soils. Recently, newer techniques, such as the application of metallic nanoparticles, are being deployed in an attempt to improve traditional remediation options. In order to implement a phytomanagement strategy on calcareous alkaline peri-urban soils simultaneously contaminated with several metals and diesel, we evaluated the effectiveness of *Brassica napus* L., a profitable crop species, assisted with organic amendment and zero-valent iron nanoparticles (nZVI). A two-month phytotron experiment was carried out using two soils, i.e. amended and unamended with organic matter. Soils were artificially contaminated with Zn, Cu and Cd (1500, 500 and 50 mg kg^{-1}, respectively) and diesel (6000 mg kg^{-1}). After one month of stabilization, soils were treated with nZVI and/or planted with *B. napus*. The experiment was conducted with 16 treatments resulting from the combination of the following factors: amended/unamended, contaminated/non-contaminated, planted/unplanted and nZVI/no-nZVI. Soil physicochemical characteristics and biological indicators (plant performance and soil microbial properties) were determined at several time points along the experiment. Carbonate content of soils was the crucial factor for metal immobilization and, concomitantly, reduction of metal toxicity. Organic amendment was essential to promote diesel degradation and to improve the health and biomass of *B. napus*. Soil microorganisms degraded preferably diesel hydrocarbons of biological origin (biodiesel). Plants had a remarkable positive impact on the activity and functional diversity of soil microbial communities. The nZVI were ineffective as soil remediation tools, but did not cause any

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toxicity. We concluded that rhizoremediation with *B. napus* combined with an organic amendment is promising for the phytomanagement of calcareous soils with mixed (metals and diesel) contamination.

1. Introduction

In an increasingly industrialized world, soil contamination resulting from the intensification and expansion of human activities entails a serious threat to human and ecosystem health. A recent European report estimates a total of 2.5 million potentially contaminated sites in Europe, of which 340,000 are likely to require remediation (Van Liedekerke et al., 2014).

Metal(oid)s (e.g., zinc –Zn–, copper –Cu– and cadmium –Cd–) and mineral oils (e.g., diesel fuel) are among the most widely spread contaminants, currently affecting 35 and 24% of European topsoils, respectively (Van Liedekerke et al., 2014). Activities such as mining, metallurgy, agriculture and the use of fossil fuels discharge a considerable amount of metal contaminants into soils, whilst accidental spills of petroleum-based products used for transportation (typically diesel-type fuels) are the principal cause of contamination with organic compounds (Barrutia et al., 2011). Furthermore, these contaminants frequently appear together in contaminated soils (Khan and Kathi, 2014), rendering new challenges for their remediation (Agnello et al., 2016). Until now, few studies have focused on the remediation of mixed contaminated soils (Agnello et al., 2016; Batty and Dolan, 2013), due to the higher experimental complexity and the difficulty of selecting a suitable remediation technology for the simultaneous immobilization and/or removal of metals and (bio)degradation of the organic contaminants.

Soil contamination with metals and diesel can induce unpredictable adverse effects on soils microorganisms and plants and, therefore, compromise soil health (Vamerali et al., 2010). The mobility and bioavailability of metals are, to a great extent, responsible for metal uptake and toxicity (Vamerali et al., 2010), which are in turn conditioned by soil physicochemical characteristics such as pH, redox potential, moisture content, organic matter, and clay content, etc. (Vangronsveld and Cunningham, 1998). Metal bioavailability, in combination with null biodegradability and high persistence in soils, promotes metal bioaccumulation and biomagnification along the food chain (Dar et al., 2017). Diesel fuel, unlike metals, can be biodegraded by microorganisms and, in particular, rhizosphere microbial communities (Barrutia et al., 2011). However, its strong hydrophobic character, resulting from a low solubility and a high vapor pressure, makes diesel rapidly associate with organic matter and minerals present in soils, rendering it less bioavailable and more recalcitrant (Megharaj et al., 2011).

In contrast to conventional physical and chemical technologies for soil remediation, which often involve high economic costs, irreversible changes in soil structure, formulation of secondary contaminants, and critical damage to soil macro- and microbiota (Gil-Díaz et al., 2016), in situ Gentle Remediation Options (GROs), such as phytoremediation, can provide a cost-effective, environmentally-friendly solution to soil contamination (Agnello et al., 2016). Several studies have evidenced that phytoremediation can promote not only metal phytostabilization (reduction of mobility and bioavailability) (Epelde et al., 2009; Galende et al., 2014a, 2014b) and phytoextraction (accumulation in shoots) (Barrutia et al., 2010; Epelde et al., 2010), but also the rhizoremediation of organic contaminants (Agnello et al., 2016; Liu et al., 2017; Montpetit and Lachapelle, 2017). Plants exudate organic compounds to the rhizosphere, creating a nutrient-rich environment which influences the behavior of nutrients and metals (Kidd et al., 2009) and stimulates microbial biomass and activity, and, hence, enhances the degradation of organic contaminants (Kuiper et al., 2004) and improves soil health (Galende et al., 2014b).

The selection of plant species is a crucial aspect for phytoremediation success. There are two key criteria, often mutually exclusive, to be considered during plant selection: plant resistance to high concentrations of contaminants and high biomass production (Surriya et al., 2015). *Brassica napus* L. meets both criteria and has been recognized as suitable candidate for metal phytoremediation (Belouchrani et al., 2016). In addition, in the last decade, *B. napus* has attracted scientific and commercial attention due to its use for oil production (Cundy et al., 2016; Dhiman et al., 2016). The combination of the phytoremediation and economic potential of *B. napus* might be decisive for successful remediation of diffusely contaminated areas (Croes et al., 2013). From this perspective, the idea of "phytomanagement" arose (Cundy et al., 2016). Phytomanagement involves the use of profitable plants and the manipulation of the soil-plant system in order to control the bioavailable pool of soil contaminants, maximize economic and/or ecological revenues, and minimize environmental risks (Evangelou et al., 2015). However, the adequacy of *B. napus* to phytomanage soils simultaneously contaminated with metals and organic compounds is still largely unknown.

The success of phytoremediation also involves the recovery of soil health, defined as the ability of the soil to perform its functions (Pardo et al., 2014). Soil microbial properties can be used as ecologically relevant biological indicators of soil health, owing to their quick response, high sensitivity, and capacity to provide information that integrates many environmental factors (Gómez-Sagasti et al., 2012). Besides, biostimulation, through the application of organic and/or inorganic amendments, is a well-known strategy to enhance the success of biological remediation methods. Thus, the application of organic amendments can improve the physicochemical properties of the contaminated soil, by supplying organic matter and nutrients, affecting metal bioavailability, and stimulating the microbial degradation of organic contaminants (Galende et al., 2014b; Sandrin and Maier, 2003).

Finally, nanomaterials (diameter < 100 nm) and, specifically, Zero-Valent Iron nanoparticles (nZVI), have emerged as promising tools to remediate contaminated soils and waters (Patil et al., 2016) via a strategy known as nanoremediation. nZVI particles have been used for the remediation of soils contaminated with metals (Gil-Díaz et al., 2017) and organic contaminants (Li et al., 2016). Nonetheless, there is a paucity of information on the effectiveness of nZVI for the remediation of soils simultaneously contaminated with several inorganic and organic contaminants, despite this being the most real scenario. Moreover, soil physicochemical properties can strongly influence the effectiveness and possible toxicity of the applied nanoparticles (Fujioka et al., 2016; Vítková et al., 2017). The application of nZVI to actual contaminated soil is likely to represent a beneficial practice for remediation, but, at the moment, it appears too expensive to be deployed at a large scale in contaminated field sites. Besides, their use is still surrounded by many uncertainties, including potential interference with other remediation phytotechnologies and potential risk for both human and environmental health (Patil et al., 2016).

Here, under microcosm conditions, we studied the effectiveness of nano-rhizoremediation assisted with an organic amendment for the recovery of mixed contaminated soils with organic (diesel) and inorganic contaminants (Zn, Cu, Cd). The specific objectives were as follows: (i) to evaluate the effectiveness of *B. napus* plants, and/or an organic amendment and/or nZVI, to accumulate and/or immobilize Zn, Cd, Cu and to degrade diesel; (ii) to assess the potential of these technologies for the recovery of soil health determined by microbial and plant indicators; and, finally, (iii) to analyze the ecotoxicity of nZVI.

2. Materials and methods

2.1. Experimental design

In September 2015, a soil from a peri-urban area near the city of Vitoria-Gasteiz (42°50′N; 2°40′W, northern Spain) was amended with 100 t ha^{-1} of an organic amendment produced from the recycling of urban organic wastes. Soil from the same peri-urban area remained unamended. In July 2016, topsoil (0–15 cm) was collected from both the unamended and amended area, sieved to <6 mm, air-dried and subjected to physicochemical characterization (Table 1). In the laboratory, half of each soil was artificially contaminated with a mixture of metals and commercial diesel fuel purchased from a petrol station. Final metal concentrations were (in mg kg^{-1} DW soil): Zn (1500), Cu (500) and Cd (50). As metals were added as nitrate salts, KNO_3 was added to non-contaminated soils (control) in order to compensate the additional content of nitrate in contaminated soils. Immediately after, diesel (6000 mg kg^{-1} DW soil) was added to already metal contaminated soils, following ISO 15952 (2006). Then, 700 g DW of contaminated or non-contaminated soil were placed in 1 L pots to complete a total of 64 pots: 16 treatments and 4 replicates per treatment (Table 2). In order to allow contaminant stabilization, pots were kept for one month in a phytotron under the following controlled conditions: photoperiod 14/10 h day/night, temperature 25/18 °C day/night, relative humidity 60/80% day/night, and a photosynthetic photon flux density of 200 μmol photon m^{-2} s^{-1}.

After the 1-month stabilization period (August 2016), nZVI (NanoFer Star, Nanoiron s.r.o) were activated following the manufacturer's instructions with Milli-Q water for 24 h and then applied in aqueous solution to half of the pots (contaminated and non-contaminated) at a concentration of 1 g nZVI kg^{-1} DW soil. The nZVI treatment was identified as "n".

Soil samples for chemical analysis and biological assays were taken immediately before and after nZVI application and then stored at 4 °C. Three days after nZVI application, half of the pots were sowed with *Brassica napus* L. (30 seeds pot^{-1}). After plant emergence (6 days), seedling number per pot was reduced to 3, by manually removing extra seedlings with their roots, and photosynthetic photon flux density was increased to 250 μmol photon m^{-2} s^{-1} to enhance plant growth. The experiment was conducted under the above-mentioned phytotron controlled conditions and pots were bottom watered periodically as needed. One month after sowing (September 2016), plants and soils were collected for chemical analysis and biological assays.

Considering the presence of amendments (A-amended/U-unamended), the presence of contaminants (C-control/M-mixed contamination), the presence of plants (P-planted/N-non-planted) and the treatment with nZVI (n), the experiment was conducted with 64 pots belonging to 16 treatments, with 4 replicates each (Table 2).

Table 1
Soil physicochemical characteristics.

	Unamended soil	Amended soil
Total clay (%)	23.4	15.7
Coarse sand (%)	17.9	14.5
Fine sand (%)	21.3	25.1
Total silt (%)	37.5	44.0
Texture class (USDA)	Loam	Loam
pH (1:2.5)	7.9	8.0
Carbonates (%)	54.7	44.0
Organic matter (%)	1.0	19.5
C organic/N organic	6.7	8.6
Total N (% DW)	0.1	0.9
Total C organic (% DW)	0.6	7.3
[Zn] Tot/Bio mg kg^{-1}	41.4/0.0	127.8/0.0
[Cu] Tot/Bio mg kg^{-1}	6.9/<0.1	73.3/<0.1
[Cd] Tot/Bio mg kg^{-1}	0.3/0.0	0.5/0.0

Tot: Pseudo-total metal concentration; Bio: $CaCl_2$-extractable metal concentration; USDA: United States Department of Agriculture.

2.2. Soil physicochemical characterization

Amended and unamended soils were collected at: (i) T0 (September 2015), just after the addition of the organic amendment to the soil, (ii) T1 (July 2016), immediately after the addition of the contaminants; (iii) T2 (August 2016), just after the 1-month stabilization period; and (iv) T3 (September 2016), at harvest time. Immediately after sampling, soils were kept at 4 °C prior to the determination of soil microbial properties (see Section 2.3).

For the determination of soil physicochemical properties and for the root elongation phytotoxicity bioassay (see Section 2.4), soil samples were oven-dried at 35 °C for 48 h. Soil pH was determined (1:2.5 *w/v* soil:water) using 10 g of 2-mm sieved dried soil and 25 mL of deionized water. Physicochemical parameters, i.e. particle size distribution, % organic matter, total organic carbon (TOC; detection of CO_2 by infrared after oxidation), total nitrogen (Kjeldhal method), and % carbonates were determined following official methods (MAPA, 1994). Dry soil samples were ground and sieved at 0.125 mm prior to the analysis of pseudo-total and $CaCl_2$-extractable fraction of metals by Inductively-Coupled Plasma-Mass Spectrometry (ICP-MS) (Agilent 7700). In order to determine total concentration of Zn, Cu and Cd in soil, samples were subjected to acid digestions with HCl and $HNO_3 + H_2O_2$ according to US-EPA Method 3051A (2007). The $CaCl_2$-extractable fraction, an indicator of metal bioavailability, was determined according to Houba et al. (2000). Aliphatic hydrocarbon concentration in soil was determined as described by Bartolomé et al. (2005). Shortly, 15 mL of acetone (extraction solvent) was added to 0.5 g of dried soil. Extraction was performed using a MDS-2000 closed microwave solvent extraction system (CEM, Matthews, NC, USA). The filtered (0.45 μm) extract was cleaned by performing a solid phase extraction (SPE) with Florisil® cartridges. All the compounds were analyzed by Gas Chromatography–Mass Spectrometry (GC–MS). The profile of n-alkanes in the commercial diesel contained hydrocarbons from n-C9 to n-C30. In order to monitor preferential degradation of total petroleum hydrocarbons (TPH) of diesel, we identified three main groups; (i) n-Alkanes (n-Alk); (ii) polycyclic aromatic hydrocarbons (PAHs); and (iii) fatty acid methyl esters (FAMEs). We also determined n-alkane degradation according to the length of the carbon chain, i.e. the following four fractions: (i) n-C9–C12; (ii) n-C13–C16; (iii) n-C17–C21; and (iv) n-C22–C30.

2.3. Soil microbial properties

Soil samples were used to determine the following microbial properties, as detailed in Galende et al. (2014a): (i) microbial activity was determined by basal respiration (BR) following ISO 16072 (2002); (ii) potentially active microbial biomass was determined by substrate-induced respiration (SIR) following ISO 17155 (2002); (iii) average well color development (AWCD) and (iv) number of metabolized substrates (NUS) were determined from Biolog EcoPlates™.

2.4. Root elongation phytotoxicity bioassay

A root elongation bioassay with *Cucumis sativus* was performed to evaluate soil phytotoxicity. Seeds of *C. sativus* (c.v. Marketmore) were pre-germinated on 8.5 cm diameter Petri dishes, containing wet filter paper, for 3 days under controlled conditions (14/10 h day/night; 25/18 °C day/night; and full darkness). Concurrently, 10 g of dried soil were placed on Petri dishes, hydrated with deionized water, mixed vigorously, and covered with filter paper. After pre-germination, seven seeds of *C. sativus* showing a radicle length of 5–10 mm were placed over the filter paper of soil-containing Petri dishes. Afterwards, dishes were placed at an angle of 45° and incubated for 72 h under the following conditions: photoperiod 14/10 h day/night, temperature 25/18 °C day/night, relative humidity 60/80% day/night and photosynthetic photon flux density of 100 μmol photon m^{-2} s^{-1}. Three technical replicates were analyzed for each biological replicate. Images of the seedlings

Table 2
Experimental treatments.

	Without nZVI				With nZVI			
	Unamended		Amended		Unamended		Amended	
	Control	Mixed C	Control	Mixed C	Control	Mixed C	Control	Mixed C
Not planted	UCN	UMN	ACN	AMN	nUCN	nUMN	nACN	nAMN
Planted	UCP	UMP	ACP	AMP	nUCP	nUMP	nACP	nAMP

Control: non-contaminated soil; Mixed C: mixed contaminated soil with several metals and diesel.

were taken at the beginning and after 72 h of incubation with the soil. Images were processed by ImageJ software. Root Elongation (RE) ($RE = RE_{T2} - RE_{T1}$) was calculated for each seedling. The percentage of RE was calculated considering "amended control soil, non-treated with nZVI (ACN)" as reference state (i.e., 100%).

2.5. Plant growth, physiological status and metal concentrations

Prior to harvest, maximal photochemical efficiency of PSII (Fv/Fm) was determined in leaves of plants at predawn state using a portable modulated fluorimeter (FluorPen FP 100. Photon System Instruments) as described in García-Plazaola and Becerril (2000). Subsequently, 5 leaf discs of 6 mm diameter (40 mg FW) were collected, frozen in liquid N, and stored at −80 °C until pigment analysis. Photosynthetic and photoprotective pigments (chlorophylls and carotenoids) as well as lipophilic antioxidants (α-, β- and γ-tocopherol), were determined according to García-Plazaola and Becerril (2001). Finally, 1-month-old *B. napus* whole-plants were harvested. Leaves, stems and roots were manually separated, weighted (fresh weight, FW) and washed with deionized water. Roots were also soaked with 0.01 M $CaCl_2$ for 30 min to remove adsorbed metals. Plant samples were then oven-dried at 80 °C for 48 h and their dry weights (DW) recorded and used for metal determination according to Zhao et al. (1994). Metal phytoextraction (shoot metal concentration × shoot biomass) was also calculated.

2.6. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 23. Normality was checked performing a Kolmogorov-Smirnov test. Normal data were tested using Student's *t*-test and ANOVA, using Duncan post hoc when there was homocedasticity (checked with Levene test) and Games-Howell when not. Non-normal data were analyzed by applying non-parametric tests as Mann-Whitney's *U* test and Kruskal-Wallis test.

3. Results

3.1. Effect of treatments on contaminant concentrations

The soils collected for this study have a loam texture, alkaline pH (8.0) and a very high content of carbonates (55 and 44% for unamended and amended soils, respectively) (Table 1). The main difference between both soils lays in the higher values of metal (Zn, Cu and Cd) concentration (because of the concentration of these metals in the amendment, as shown in Table 1 in (Lacalle et al., 2017), C and N contents (both 10-fold higher), and organic matter content (19.5%) shown by the amended soil, as compared with the unamended soil.

Pseudo-total Cd, Cu and Zn concentrations in soil were not lower at harvest time (T3) than immediately after the 1-month stabilization period (T2) (Fig. 1A–C). In fact, higher metal concentrations in soil were found for all metal treatments, especially in amended soils, as compared with the initial spiked concentrations of Zn, Cu and Cd (1500, 500 and 50 mg kg^{-1} DW soil, respectively). By contrast, $CaCl_2$-extractable metal fractions decreased to a great extent as soon as metals were added to the soil (T1) (Fig. 1D-F). Immediately after metal addition (T1), $CaCl_2$-extractable metal fractions represented as low as 0.25% of the pseudo-total concentrations for all metals and treatments. Interestingly, the presence of the organic amendment increased the bioavailability of Zn and Cu (Fig. 1D, E), but reduced Cd bioavailability (Fig. 1F). Metal bioavailability progressively decreased, to a lesser extent, throughout the experiment, particularly during the stabilization period (T2). At harvest time (T3), Zn and Cu bioavailability still remained higher in the presence of the organic amendment than in its absence, while Cd bioavailability was lower. Values of pseudo-total and bioavailable metal concentrations were not significantly affected by the presence of plants (Fig. 1, Table 2 in (Lacalle et al., 2017)) nor by the presence of nZVI (Table 2 in (Lacalle et al., 2017)).

Immediately after diesel addition (T1), TPH concentrations in soil were 2900 and 2400 mg kg^{-1} DW soil for unamended (UMN) and amended (AMN) soils, respectively (Fig. 2A). At this time, the main TPH family corresponded to n-Alk (92%), followed by FAMEs (7%). The concentrations of n-Alk and FAMEs were initially lower in amended soils; soil concentration values for both families rapidly decreased after one month of stabilization (T2). This was particularly relevant for FAMEs, which almost disappeared at T2, in both amended and unamended soils. At harvest time (T3), a concentration of 900 mg TPH kg^{-1} DW soil of TPH was detected in all contaminated soils (UMN, UMP, AMN, AMP) (Fig. 2A). Neither plants (Fig. 2A) nor nZVI (Fig. 2D) affected the degradation of these compounds (Table 3 in (Lacalle et al., 2017)).

When different n-alkanes fractions were separately analyzed (Fig. 2B), the most abundant fractions in our commercial diesel were long chain n-alkanes (n-C17–n-C21, followed by n-C22–n-C30 and n-C13–n-C16). Very low levels of n-C9–n-C12 were observed (data not shown). The degradation pattern of these longer chain n-alkanes (n-C13–to n-C30) was similar to that previously described for total n-Alk (i.e., lower concentration in amended soils and progressive degradation throughout the experiment). Concentration values detected here for the shortest n-alkanes (n-C9–n-C12) and PAHs correspond to hydrocarbons and aromatic compounds of biological origin, already present in the non-contaminated soil and in the amendment itself (data not shown).

Globally considered, at the end of the study, diesel concentration values for the main families and fractions were similar among treatments, and neither plants (Fig. 2A–C) nor nZVI (Fig. 2D–E) stimulated a preferential degradation of any of them.

3.2. Effect of treatments on plant growth, physiological status and metal concentrations

As shown in Table 3, biomass of *B. napus* plants significantly increased in the presence of the organic amendment, both in control plants (ACP > 4-fold UCP) and, remarkably, in plants grown in contaminated soils (AMP > 17-fold UMP). The presence of the amendment alleviated contaminant phytotoxicity, as we found no significant differences in biomass between controls (ACP) and plants grown in contaminated soils (AMP). Other plant parameters, such as photochemical efficiency, total chlorophyll, total carotenoids and VAZ/Chl, were not affected by treatments (Table 3). However, the tocopherol/Chl index increased in plants grown in contaminated soils and in unamended soils. nZVI treatment had no significant effect on the plant parameters studied here.

Metal concentrations in plant shoots were markedly influenced by the applied metal dose, metal mobility, and the presence of the organic amendment. Plants grown in amended soils showed lower metal

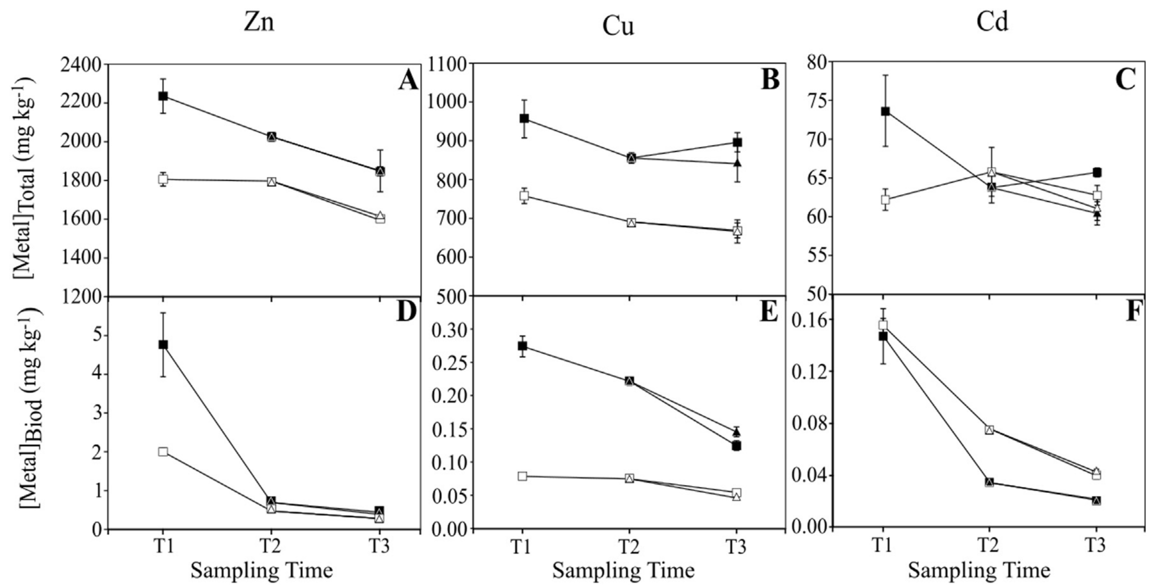

Fig. 1. Pseudo-total and $CaCl_2$-extractable concentrations of Zn (A, D), Cu (B, E) and Cd (C, F) in soils. White icons represent unamended soils and black icons refer to amended soils. Squares: mix contaminated, non-planted. Triangles: mix contaminated, planted. T1: Time immediately after the artificial contamination of the soil. T2: Time one month after soil contamination (sowing time). T3: Time two months after soil contamination (harvesting time).

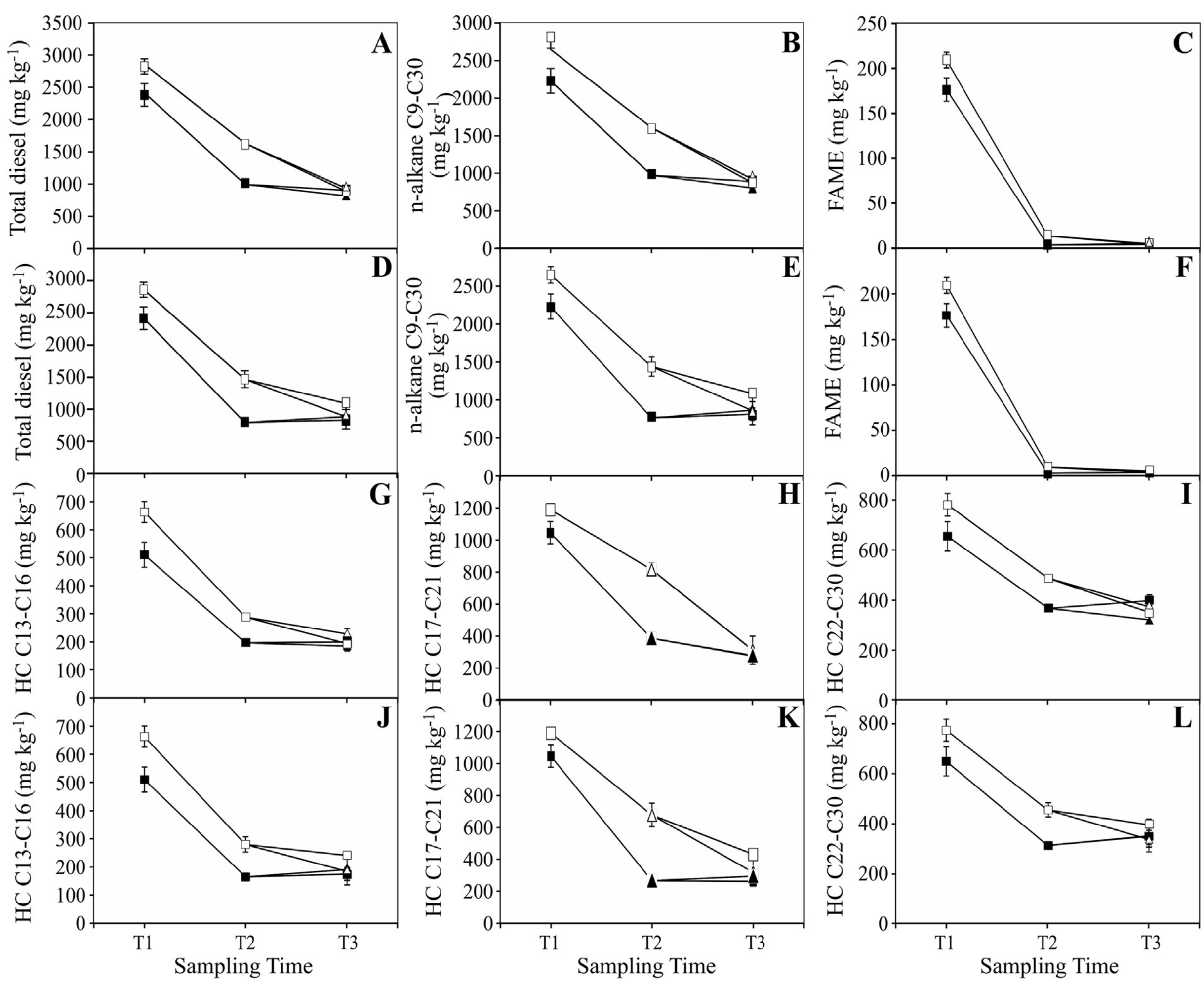

Fig. 2. Total and fractioned hydrocarbon concentration in soil (without, with nZVI). Total Petroleum Hydrocarbon (TPH) (A, D); n-alkanes (n-Alk) (B, E); FAMEs (C, F); n-alkane fractions C13–C16 (G, J); C17–C21 (H, K); and C22–C30 (I, L). White icons represent unamended soils and black icons refer to amended soils. Squares: mix contaminated, non-planted. Triangles: mix contaminated, planted. T1: Time immediately after the artificial contamination of the soil. T2: Time one month after soil contamination (sowing time). T3: Time two months after soil contamination (harvesting time).

Table 3
Plant parameters in *Brassica napus* at harvest time (T3). Biomass (g); $[Metal]_{Shoot}$ (mg kg^{-1}); Phytoextraction (μg); Fv/Fm; Chl a + b (pmol mm^{-2}). CAR_T, VAZ and TOC_T were expressed in pmol pmol Chl^{-1}.

	UCP	UMP	ACP	AMP	nUCP	nUMP	nACP	nAMP
B_{Shoot}	1.1 ± 0.2 b	0.3 ± <0.1 c	4.8 ± 0.8 a	5.3 ± 0.5 a	1.2 ± 0.2 b	0.4 ± <0.1 c	4.3 ± 0.8 a	5.9 ± 0.4 a
Zn_{Shoot}	–	1305.2 ± 85.6 a	–	497.3 ± 54.8 b	–	1162.7 ± 137.3 a	–	361.3 ± 8.2 c
Cu_{Shoot}	–	51.9 ± 3.5 a	–	27.77 ± 2.88 a	–	41.4 ± 7.1 a	–	19.0 ± 0.4 b
Cd_{Shoot}	–	29.7 ± 4.8 a	–	14.05 ± 1.09 b	–	18.7 ± 2.1 b	–	8.7 ± 0.2 c
P_{Zn}	–	383.0 ± 36.3 b	–	2549.4 ± 144.6 a	–	512.3 ± 86.8 b	–	2129.0 ± 165.1 a
P_{Cu}	–	15.2 ± 1.4 c	–	143.1 ± 10.8 a	–	18.5 ± 4.1 c	–	112.0 ± 9.1 b
P_{Cd}	–	8.5 ± 0.8 c	–	72.6 ± 4.5 a	–	8.2 ± 1.2 c	–	51.19 ± 4.1 b
Fv/Fm	0.78 ± 0.01 ab	0.78 ± <0.1 ab	0.79 ± 0.1 ab	0.76 ± <0.1 b	0.77 ± <0.1 ab	0.78 ± <0.1 ab	0.79 ± <0.1 a	0.78 ± <0.1 a
Chl a + b	548.4 ± 27.0 a	515.1 ± 9.4 a	598.7 ± 26.9 a	539.8 ± 47.5 a	601.8 ± 31.3 a	600.4 ± 47.3 a	514.8 ± 20.3 a	477.6 ± 18.3 a
CAR_T	319.6 ± 6.6 a	306.8 ± 5.5 a	306.2 ± 3.5 a	314.4 ± 4.9 a	317.9 ± 7.0 a	312.9 ± 8.0 a	301.9 ± 2.7 a	308.6 ± 4.1 a
VAZ	66.4 ± 5.1 a	62.1 ± 1.1 ab	57.7 ± 1.3 ab	60.6 ± 3.1 ab	66.5 ± 3.4 a	68.1 ± 3.0 a	55.5 ± 2.3 b	52.2 ± 1.9 b
TOC_T	32.5 ± 4.9 b	28.8 ± 1.6 bc	18.7 ± 1.5 cd	38.2 ± 3.3 b	29.3 ± 4.7 bc	32.8 ± 2.7 b	16.6 ± 1.0 d	52.4 ± 5.4 a

B: Biomass; P: Phytoextraction; CAR_T: Total Carotenoids; TOC_T: Total Tocopherols.

concentrations in their shoots than plants from unamended soils (Table 3). The highest values of metal shoot accumulation were detected for Zn, followed by Cu and Cd (Table 3). The addition of nZVI appeared to decrease shoot metal concentration, but this effect was only statistically significant for Zn and Cu in plants grown in amended soil, and for Cd in plants grown in both unamended and amended soils. Although shoot metal concentrations in plants grown in amended soils (AMP, nAMN) were lower than those in plants grown in unamended soils (UMP, nUMP), the total amount of phytoextracted metal was higher in the former, due to a higher plant biomass (Table 3). Highest values of phytoextraction were found for Zn, followed by Cu and Cd. As indicated for metal concentrations in shoots, nZVI treatment significantly decreased metal phytoextraction in contaminated amended soils (AMP vs. nAMP, Table 3). In any case, phytoextraction values were very low for all treatments, most likely due to the low values of metal bioavailability.

3.3. Effects of treatments on biological indicators of soil health

3.3.1. Soil microbial properties

At T0 and T1, amended and unamended soils showed similar SIR values (Fig. 3C, D); in contrast, higher BR values were found in amended soils (Fig. 3A, B). At the end of the experiment (T3), control (non-contaminated) amended soils presented higher values of both BR and SIR than unamended soils. The addition of contaminants (T2) had no effect on SIR values (Fig. 3C, D), but greatly increased BR values (Fig. 3A, B). Indeed, BR was the most increased microbial parameter, not only as a result of the application of the amendment and contaminants but also due to the presence of plants. Consequently, at the end of the study (T3), AMP and n-AMP treatments showed the highest BR values. This fact could be related to plant wellness, as we did not detect such an effect on unamended planted soils (UCP). At T2, AWCD and NUS values decreased in all treatments, but without significant differences among them (Fig. 4). At harvest time (T3), highest AWCD and NUS values (Fig. 4A, C) were observed for treatments with amendment, plants and contaminants (AMP, nAMP). nZVI treatment did not have any significant effect on microbial activity and biomass (Fig. 3A-D), nor on microbial functional diversity (Fig. 4A-D). The results of the statistical tests are shown in Tables 4 and 5 in (Lacalle et al., 2017).

3.3.2. Effect of treatments on soil phytotoxicity

At T2, *C. sativus* seedlings exposed to the mixed contaminated soil were notably affected by the presence of the amendment. A significant decrease of root elongation (RE) was obtained in contaminated unamended soils (Fig. 5A, Table 6 in (Lacalle et al., 2017)), whereas a significant increase in RE was observed in corresponding amended soils (AMN, nAMN; Fig. 5A). At harvest time (T3), RE % of *C. sativus* seedlings was significantly higher than in T2 in almost all treatments (Fig. 5B). The negative effect of the mixed contamination was only observed in unamended soils, while there were no differences between control (non-

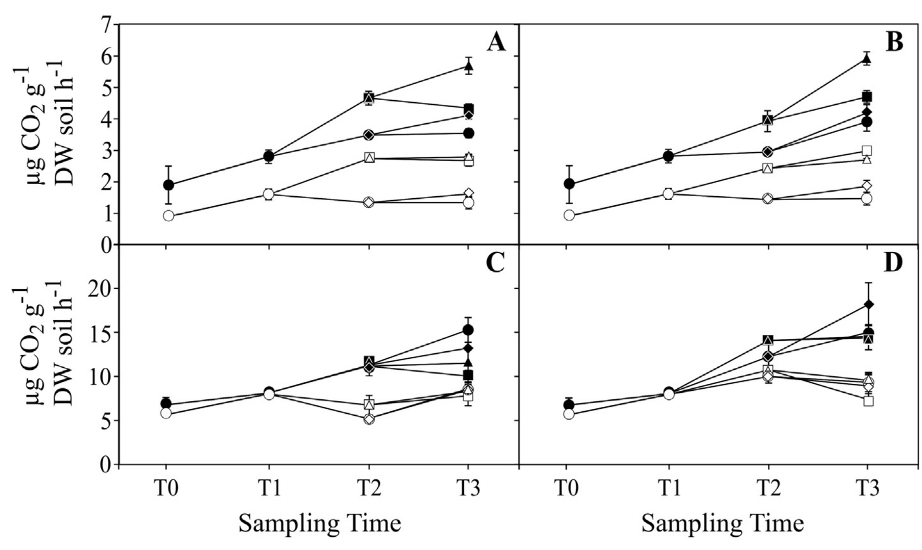

Fig. 3. Soil microbial activity determined by Basal Respiration (BR) (A, B) and microbial biomass determined by Substrate-Induced Respiration (SIR) (C, D) in soil without and with nZVI. White icons represent unamended soils and black icons refer to amended soils. Circles: non-contaminated, unplanted. Diamonds: non-contaminated, planted. Squares: mix contaminated, non-planted. Triangles: mix contaminated, planted. T0: Time of soil collection, immediately after amendment application. T1: Time immediately after the artificial contamination of the soil. T2: Time one month after soil contamination (sowing time). T3: Time two months after soil contamination (harvesting time).

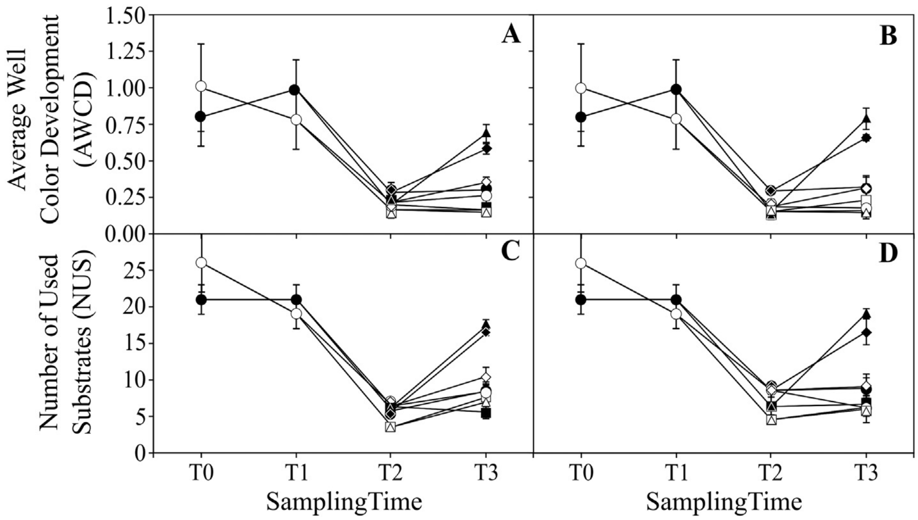

Fig. 4. Soil microbial functional diversity measured with Biolog™ Ecoplates (without, with nZVI). Average Well Color Development (AWCD) (A, B), Number of Used Substrates (C, D). White icons represent unamended soils and black icons refer to amended soils. Circles: Non-contaminated, non-planted. Diamonds: Non-contaminated, planted. Squares: mix contaminated, non-planted. Triangles: mix contaminated, planted. T0: Time of soil collection, immediately after amendment application. T1: Time immediately after the artificial contamination of the soil. T2: Time one month after soil contamination (sowing time). T3: Time two months after soil contamination (harvesting time).

contaminated) and contaminated amended soils. Neither the presence of *B. napus* plants nor the nZVI treatment had a clear impact on the RE of *C. sativus* seedlings.

4. Discussion

Phytomanagement focuses on the growth of profitable crops on contaminated vacant sites, in order to simultaneously maximize both economic profit and the provision of ecosystem services, while reducing contaminant mobility and bioavailability, and, hence, adverse ecological impact (Cundy et al., 2016). However, few studies have been conducted to establish the suitability of GROs and associated strategies for mixed contaminated (with inorganic and organic compounds) soils, such as those often found in industrial and urban brownfields. In this study, in order to explore feasible strategies to reduce environmental risk and increase soil functions, we studied soils from a peri-urban vacant area. Here, soils were artificially contaminated with Zn, Cu, Cd and diesel, as these contaminants are commonly found in mixed contaminated soils. Our experimental approach allows to (i) specifically select any combination of contaminants and contaminant concentrations, (i) remove or immobilize such contaminants during the timeframe of the experiment, and (iii) compare the values of soil health parameters between contaminated/remediated soil and uncontaminated (control) soil. However, due to some key factors such as the aging of contaminants and the complexity of natural field conditions, our results cannot be directly related to actual contaminated soils.

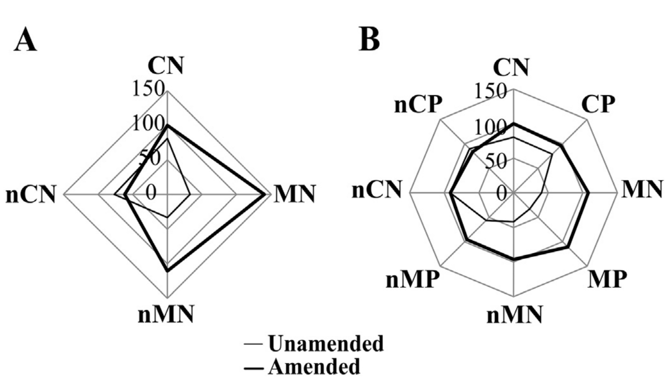

Fig. 5. Variation of Root Elongation percentage (RE %) of *Cucumis sativus* seedlings in T2 (sowing time) (A) and T3 (harvesting time) (B) time-points. The thin line represents unamended soils, whilst the bold line represents amended ones.

4.1. Contaminant concentrations and physicochemical parameters

The most relevant result obtained from the addition of metals to this peri-urban soil was the large and rapid immobilization of metals observed in both amended and unamended soils prior to the stabilization period (Fig. 1D–F). Precipitation and adsorption are two key processes affecting soil metal bioavailability, and both processes are greatly dependent on soil pH (Adriano, 2001). Alkaline pH values, such as those observed here, can enhance precipitation and adsorption processes (Adriano, 2001) and, therefore, contribute to the low values of metal bioavailability observed in this study. However, the soil factor that can better explain these low bioavailability values is most likely the large carbonate content of our soils (44 and 55% for amended and unamended soils, respectively) (Table 1). Other soil components, such as sulfates, hydroxides, phosphates, silicate clay (Adriano, 2001) and organic matter (Alvarenga et al., 2009), as well as plant growth (Galende et al., 2014b) can secondarily account for a reduction in metal bioavailability. The continuous interaction of metal contaminants with all these soil components could explain the progressive reduction of metal bioavailability observed along the study, after T1. Organic amendments can decrease metal bioavailability (Park et al., 2011), not only due to the interaction between metals and organic components, but also due to the increase in pH (Galende et al., 2014a). Conversely, Cu and Zn bioavailability was increased in amended soils. This contradictory effect could be explained by the lower carbonate content of amended soils, as well as the fact that the organic amendment itself adds Zn and Cu to the soil (Table 1).

The presence of plants can also decrease metal bioavailability through plant metal uptake and/or metal immobilization in the rhizosphere (Park et al., 2011). However, under our experimental conditions, metal bioavailability in soil was not influenced by *B. napus* growth. The low bioavailability of metals in soil (Fig. 1D–F) is probably responsible for the severely limited metal accumulation in shoots and phytoextraction (Table 3). Accordingly, Zn showed highest values of bioavailable concentration and, concomitantly, highest values of metal accumulation and amount of metal phytoextracted. Regarding shoot metal accumulation, the most relevant difference refers to the lower shoot metal concentrations found in amended soils, compared with unamended ones, owing to the growth dilution factor (Galende et al., 2014b; Hill and Larsen, 2005). Actually, AMP shoot biomass was 17-

fold higher than UMP. Due to this growth dilution factor, plants grown in amended soils extracted more Zn (6.6-fold), Cu (9.5-fold) and Cd (10-fold) than those grown in unamended soils. In any event, the total amount of metal phytoextracted was very low for all cases and, then, pseudo-total metal concentrations in soils did not decrease to a significant extent. The higher pseudo-total metal concentrations detected in our study, compared to the spiked doses, can be explained by the sample processing. Thus, soil samples were ground, sieved and, then, fine particles were collected. Metals are usually associated to the fine particle-size fractions of the soil (Xu et al., 2014). This is an important methodological issue that should be taken into account to avoid a possible overestimation or underestimation of total metal concentrations in soil studies.

Under our experimental conditions, nZVI are expected to oxidize very fast, thus forming iron oxides that might then adsorb heavy metals (Komárek et al., 2013; Tiberg et al., 2016). However, considering the high level of metal immobilization in our soil due to its physicochemical properties, together with the fact that the values of Zn, Cu and Cd bioavailability in soil were not significantly different in the absence versus the presence of nZVI, it is most likely that, under our experimental conditions, nZVI did not interact with metals. Nevertheless, a statistically significant reduction of metal concentrations in shoots was observed in plants grown in the presence of nZVI and, as a result, a lower metal phytoextraction was found. Martínez-Fernández et al. (2016) reported that nanoparticles can interfere with root hydraulic conductivity, thus affecting the uptake and translocation of some elements and nutrients, but not generating stress to plants. Similarly, in our study, nZVI nanoparticles did not affect plant physiological status and health. The low levels of tocopherol indicated that ACP and nACP treatments had the lowest levels of oxidative stress.

Brassica napus is a good candidate for phytomanagement, as it is a profitable crop currently used for biodiesel production and, besides, it can efficiently accumulate metals in its shoots (Van Ginneken et al., 2007). So far, most of the studies on metal accumulation by *B. napus* have been performed dealing with only one metal, but some problems might arise when this species is exposed to a polymetallic contamination (Mourato et al., 2015; Cocojaru et al., 2016). Likewise, the presence of organic contaminants can decrease metal phytoextraction (Batty and Dolan, 2013). To our knowledge, this is the first study that addresses the co-contamination of soils with several metals and an organic compound (diesel) through the implementation of a GRO using *B. napus*, an organic amendment and nZVI. Selection of a diesel-tolerant plant species is essential for successful rhizoremediation of this organic contaminant (Barrutia et al., 2011; Balseiro-Romero et al., 2016). *Brassica napus* has been reported as a diesel-tolerant species with potential for phytoremediation (Wojtera-Kwiczor et al., 2014). Dissipation of diesel can occur via a rapid non-biodegradative process (T0) caused by evaporation of low molecular weight compounds (Barrutia et al., 2011), followed by a second slower phase associated to biodegradative processes by indigenous or inoculated microorganisms (Balseiro-Romero et al., 2016). The faster degradation of some hydrocarbon families (i.e., n-Alk, FAMEs) in our amended soils (Fig. 2B-C) is probably due to the higher values of microbial activity present in those soils (Fig. 3). According to Balseiro-Romero et al. (2016), diesel degradation can be stimulated in soils with high organic matter content due to a better supply of nutrients and reduced toxicity by the adsorption of toxic compounds to the organic matrix. Interestingly, FAMEs (i.e., hexadecanoic acid, methyl ester; heptadecanoic acid, 16-methyl-, methyl ester; and 8-Octadecenoic acid, methyl ester) showed faster degradation rates: in fact, only 5% of their initial concentration remained after the stabilization period. FAMEs, derived from trans-esterification of animal fats or vegetable oils, are components of biodiesel and are also added to conventional diesel at low concentrations (ca. 7%). This preferential degradation of FAMEs over other diesel components has also been described for marine microorganisms (DeMello et al., 2007), and indicates that the metabolism of soil microorganisms could be more adapted for fatty acid catabolism, favouring the degradation of biodiesel components. Finally, time attenuated the differences between unamended/amended, planted/unplanted and nZVI/no-nZVI soils in terms of TPH content, thereby observing no significant differences between them at harvest time. The use of nZVI to promote the degradation of recalcitrant organic contaminants, such as polycyclic or chlorinated hydrocarbons, has been reported by several authors (Chang and Kang, 2009; San Román et al., 2013; Sunkara et al., 2010). Under our experimental conditions, however, nZVI had no clear effect on diesel degradation in soil.

4.2. Biological indicators of soil health

As pointed out above, the mitigation of potential risks to ecological receptors and the improvement of soil functions are key aspects of phytomanagement initiatives. Microbial activity, biomass and functional diversity parameters (Epelde et al., 2014), as well as soil phytotoxicity bioassays (Quintela-Sabarís et al., 2017), are frequently used as bioindicators of soil health (Galende et al., 2014b). According to our results (Fig. 4), amended soils had higher values of microbial activity and biomass, most likely due to the input of labile organic carbon easily metabolized by soil microbial communities (Galende et al., 2014a; Ros et al., 2003). After soil contamination with metals and diesel (T2), microbial activity was greatly stimulated in both amended and unamended soils. This could be understood as a consequence of: (i) a lethal effect of the added contaminants on some soil microbial populations, leading to the active growth of oportunistic populations from the labile C release associated to microbial death (Balseiro-Romero et al., 2017); (ii) the metabolic utilization of hydrocarbons present in the diesel formulation as substrates for microbial growth and activity (Siddiqui and Adams, 2002); and (iii) a requirement of more energy (microbial activity) for survival under the stressing environmental conditions characteristic of contaminated soils (Zhou et al., 2013). The fact that microbial biomass was not affected in the presence of a higher microbial activity (Fig. 3A) can be interpreted as a need to deviate energy from growth to maintain essential cell processes, in order to cope with contamination-induced stress, as microorganisms often require more energy to survive under unfavourable conditions.

Brassica napus growth also stimulated soil microbial activity (Fig. 3A). The presence of plants can help increase microbial activity in contaminated soils by releasing root exudates and creating suitable conditions for microbial growth in the rhizosphere (Balseiro-Romero et al., 2017; Barrutia et al., 2011). *Brassica napus* plants not only increased the activity of microbial communities in contaminated soils, but also its functional diversity (Fig. 4). These observations are in agreement with results by Barrutia et al. (2011), who reported a stimulatory effect of plants on microbial functional diversity, reflected by Biolog™ data and values of enzyme activities. These effects were not observed in contaminated unamended soils with *B. napus*, due to the low performance and biomass of plants under these conditions (Table 3).

Root elongation of *C. sativus* seedlings were also used to assess soil health, as this species has been described as sensitive to metal contamination (Baderna et al., 2015; Visioli et al., 2014). As shown in Fig. 5A, the organic amendment had a positive effect on root elongation at T2, in agreement with the increased wellness of *B. napus* plants and the stimulation of microbial communities observed in amended soils at T2. On the contrary, at this time-point, we detected a phytotoxic effect (decrease of root elongation) in unamended soils. This can be explained by the lower levels of diesel present at that time in the amended soils, as a result of microbial degradation. In a bioassay performed with red clover exposed to hydrocarbon contamination, Juvonen et al. (2000) found that compost addition reduced hydrocarbon-induced phytotoxicity. At the end of our study (T3, Fig. 5B), root elongation was near the optimum value (score value close to 100%) in all amended soils, without any significant differences among treatments. However, a severe inhibition of root elongation was observed in contaminated unamended soil (Fig. 5B), in agreement with the results of soil microbial parameters observed in these soils. These findings highlight the importance of amendments to stimulate plants and soil microbial communities, and to improve soil health, while reducing total and bioavailable contaminant concentrations and, hence, ecological risk.

Finally, under our experimental conditions, the application of nZVI did not decrease soil contaminant concentrations (Figs.1 and 2), but it decreased plant metal accumulation (Table 3). This can be taken into consideration to improve phytostabilization initiatives and reduce the entry of metals to the food web. Moreover, nZVI had no toxic effect on soil microbial communities (Figs. 3 and 4), although they initially caused inhibitory effects on *C. sativus* seedlings root elongation (Fig. 5A), possibly due to some interaction with organic matter. This phytotoxic effect was temporary and disappeared after one month (Fig. 5B), which could explain the contrasting observations reported by other authors (Stefaniuk et al., 2016).

5. Conclusions

This study highlights the importance of soil components (e.g., pH, carbonates and organic matter content) and organisms (microorganisms and *B. napus*) as essential tools for the design of phytomanagement strategies aimed at mixed contaminated (Zn, Cu, Cd and diesel) soils. Our calcareous soils presented low values of metal bioavailability, preventing metal entry in the food web and, thus, reducing metal ecotoxicity. Poor performance of diesel-tolerant profitable crops, such as *B. napus*, and native soil microbial populations during rhizoremediation was overcome by the application of organic amendments, which increased soil microbial activity and improved plant physiological status and growth. Under these circumstances, soil microbial communities were able to degrade diesel components, preferably fatty acids methyl esters. The presence of *B. napus* increased soil microbial activity and functional diversity. nZVI reduced shoot metal concentrations and phytoextraction performance, whilst they were not effective as remediation tools for diesel contaminated soil. At the applied doses, nZVI did not cause toxicity symptoms on soil health bioindicators, other than a reduction in root elongation possibly mediated by an indirect effect of nZVI with organic matter. To our knowledge, this is the first study that reports the usefulness of the combination of *B. napus* plants, organic amendment and nZVI for the nano-rhizoremediation of soils simultaneously contaminated with several metals and diesel, and as a suitable strategy for the phytomanagement of very poor alkaline soils.

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