



Combined bioaugmentation and biostimulation techniques in bioremediation of pentachlorophenol contaminated forest soil

Rim Werheni Ammeri ^{a,b}, Giuseppe Di Rauso Simeone ^{c,*}, Yassine Hidri ^d, Mohamed Salah Abassi ^e, Ines Mehri ^b, Sara Costa ^c, Abdennaceur Hassen ^b, Maria A. Rao ^c

^a University of Mathematical, Physical and Natural Sciences of Tunis el Manar, Faculty of Sciences of Tunis (FST), Tunisia

^b Laboratory Wastewater Treatment and Research Center of Water Technologies, Technopark Borj-Cédria, PO Box 273, Soliman, 8020, Tunisia

^c Dipartimento di Agraria Università degli Studi di Napoli Federico II, 80055, Portici, Italy

^d Integrated Olive Oil Production Laboratory (LR 16IO3), Cité Mahrajène, BP. 208, 1082, Tunis, Tunisia

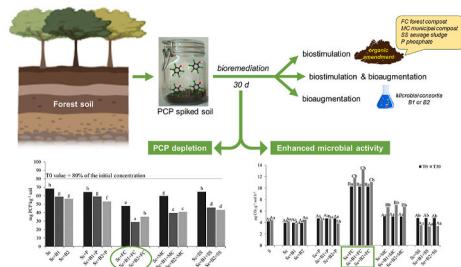
^e Institute Tunis El Manar, Institute of Veterinary Research of Tunisia, 20 Street Jebel Lakhdhar, Bab Saadoun, Tunis, 1006, Tunisia



HIGHLIGHTS

- PCP reduction by 32% in soil after 30 days occurred due to natural attenuation.
- PCP contamination depressed soil microbial biomass and enzymatic activities.
- Microbial consortia and organic amendments further reduced the PCP content.
- Combined biostimulation with FC and bioaugmentation with B1 achieved the best result.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: T Cutright

Keywords:

Bioaugmentation
Biostimulation
Microbial biomass carbon
Bacterial consortium
Respirometry
PCP

ABSTRACT

Pentachlorophenol (PCP) is quite persistent in the environment and severely affects different ecosystems including forest soil. The main objective of this work was to study different bioremediation processes of artificially PCP (100 mg kg^{-1}) contaminated forest soil (Sc). In fact, we used bioaugmentation by adding two different bacterial consortia B1 and B2, biostimulation procedures by amendments based on forest compost (FC), municipal solid waste compost (MC), sewage sludge (SS), and phosphate, and their combined treatments. Soil physical and chemical properties, residual PCP, soil microbial biomass carbon, soil respiration and some enzymatic activities at zero time and after 30 d of incubation, were evaluated. A net reduction of PCP, 71% of the initial concentration, after 30 d-incubation occurred in the sample Sc+B1+FC, as the best performance among all treatments, due to natural attenuation, immobilization of PCP molecules in the forest soil through organic amendments, and the action of the exogenous microbial consortium B1. The single application of FC or B1 led to a depletion of PCP concentration of 52% and 41%, respectively. Soil microbial biomass carbon decreased in PCP contaminated soil but it increased when organic amendment also in combination with microbial consortia was carried out as bioremediation action. Soil respiration underwent no changes in contaminated soil and increased under FC based bioremediation treatment. These results demonstrate that the combined treatments of biostimulation and bioaugmentation might be a promising process for remediation of PCP contaminated soil.

* Corresponding author.

E-mail address: giuseppe.dirausosimeone@unina.it (G. Di Rauso Simeone).

1. Introduction

Cork stoppers are created from the bark of the cork oak *Quercus suber*, an evergreen oak tree found in Portugal, Spain, France, and Italy, with smaller forests in Tunisia, Morocco and Algeria. *Quercus suber* forests, also used for browsing animals, crop rising and honey production, are the main reserve of cork and their management and survival involve around 10% of the Tunisian people (McLellan et al., 2007).

Pentachlorophenol (PCP) was used as efficient pesticide and anti-microbial compound to control wood diseases, and as bactericide and preservative in the tannery, textile, pulp industry and some other direct industrial waste discharges (Lv et al., 2014). PCP is recalcitrant to degradation and persists in various environments causing consequentially serious pollution concerns to the surface and subsurface environment (Logakanthi et al., 2013). Due to stable aromatic ring and high chloride content, PCP toxicity to human health is expected to last for a long time (Lin et al., 2016).

For counteracting PCP pollution, bioremediation is demonstrating to achieve encouraging results also compared to the available physical and chemical removal methods (Yang et al., 2006). PCP microbial degraders are ubiquitous at contaminated sites with widespread PCP contamination, but their degradation efficiency is relatively low in soil due to low solubility/bioavailability of PCP, poor nutrient level, and inappropriate soil redox conditions (Lin et al., 2016). Unlike physical and chemical methods based techniques that are expensive and producing environmental side effects, biological methods could be useful for cost-effective and environmentally friendly approaches (Hosokawa et al., 2009). Among microorganism-based methods, the inoculum of microbial consortia exhibits several advantages over that of pure cultures, such as greater flexibility, stability and metabolic capabilities (Andrabi et al., 2010). The approach could involve specific microorganisms being active and competitive to enhance a definite biological activity (bio-augmentation) in environmental cleanup (Cea et al., 2010; D'Annibale et al., 2006; Di Toro et al., 2006; Pepper et al., 2002; Vítková et al., 2011). Conversely, biostimulation techniques, addressed generally to autochthonous microorganisms, provide organic matter or supplemental nutrients such as nitrogen or phosphorous (as phosphate) in order to increase the rate of xenobiotic compound degradation such PCP (Chen et al., 2012, 2016; Tong et al., 2014). However, the relationship between nutrient supplementation and microbial degradation of organic contaminants does not appear to be completely straightforward. Thus, nutrient supply in poor environment is required to stimulate the growth of autochthonous microorganisms and enhance bioremediation process. Nevertheless, excessive nutrient availability could also inhibit the aforementioned process (Ghosal et al., 2016). Hence, bioremediation of PCP contaminated soil has become a very important issue in environmental protection over the last two decades (Hechmi et al., 2016; Li et al., 2020), mainly because of the possibility of using microbial strains, which significantly reduce costs of soil cleanup technologies. The microbial consortia could represent very efficient bio-systems specialized in bioremediation exploiting synergistic effects among different microbial species. Hassen et al. (2021) found that microbial consortium removed PCP better than a single microbial strain using PCP as sole carbon and energy source. Also Jabeen et al. (2015) describe some pesticide-degrading bacterial consortia able to improve the removal of various recalcitrant contaminants.

Microbial consortia have been shown to be more suitable for the removal of recalcitrant compounds as their biodiversity supports environmental survival and increases the number of catabolic pathways involved in contaminant biodegradation (Fuentes et al., 2013; Smith et al., 2006).

The effectiveness of the bioremediation process cannot be evaluated only by monitoring analytically the contaminant removal. It is useful to assess the soil health and quality in terms of safety and regain of the original properties by evaluating specific indicators. Among these, soil enzymes represent efficient biological indicators of soil quality because

capable of revealing the biological and biochemical changes of soil caused by anthropogenic and environmental factors and providing a fast-measurable response to such changes (Gianfreda and Rao, 2019). Hence the biological impact of pollutants on soil and the reduction of this impact attributable to bioremediation process could be evaluated through the activity of soil enzymes thus becoming an additional step of the bioremediation process (Aparicio et al., 2019; Gianfreda and Rao, 2019).

The main objective of this study was to determine the effects of combined biostimulation and bioaugmentation techniques applied on an artificial PCP contaminated forest soil. The bioremediation treatments supplied phosphate or three different organic amendments, i.e. forest compost, municipal solid waste compost or sewage sludge, in combination with two different microbial consortia isolated from contaminated sites. The clean-up process was monitored by measuring the residual PCP and the change in various soil chemical and biochemical properties over 30 d incubation.

2. Materials and methods

2.1. Chemicals

Reagent-grade PCP (>99% purity, m.w. 266.34, H_2O solubility 20 mg L^{-1}) was purchased from Sigma Aldrich (Germany). HPLC-grade solvents and all other chemicals were supplied by Carlo Erba Reagents.

2.2. Soil sampling and chemical analysis

Soil was collected in Tabarka forest (Tunisia) at 0–20 cm depth. Samples were stored in sealed sterile bags at –20 °C. Physical and chemical analyses were performed on air-dried and 2 mm sieved soil samples according to standard methods (Sparks, 1996). The texture was evaluated according to USDA Textural Soil Classification (Kellogg Soil Survey Laboratory, 2014). Electrical conductivity and pH were measured in 1:5 and 1:2.5 soil-water suspensions, respectively; cation exchange capacity (CEC) was measured with barium chloride and tri-ethanolamine solution at pH 8.1. The exchangeable bases (Ca, Mg, K, and Na) were processed by atomic absorption spectrometry (PerkinElmer AAnalyst 700). Organic C (OC) content was assayed by dichromate oxidation titration method, while total N was determined by the Kjeldahl method. Available phosphorous was determined following the bicarbonate extraction process.

2.3. Compost and sludge

A six months matured forest compost (FC) was collected from forest plants of North of Tunisia while municipal solid waste compost (MC), eight months aged, was collected from composting station of Beja (North Tunisia). Urban secondary sewage sludge (SS) was obtained from the activated sludge wastewater treatment of El Manzeh Tunis, Tunisia. Composts and sludge were characterized for the main physical and chemical properties according to Hassen et al. (2001).

2.4. Microbial consortia

Eight different strains (pure cultures) were isolated from various polluted sites such as petroleum refineries, forest soil, industrial wastewater and sludge.

In order to obtain a standard inoculum, bacteria were grown individually for 16 h on tryptic soy broth (TSB) medium at 28 °C in an orbital shaker at 150 rpm. All selected strains were screened for their ability to grow and tolerate PCP as sole carbon source. The degradation tests were carried out for each of the eight strains in liquid mineral medium (MSM) prepared in accordance with Ammeri et al. (2021) to which the PCP solution (100 mg L^{-1}) was added.

Two consortium B1 and B2 with eight pure cultures were set up in

MSM. After that, the ability of both consortia to grow and/or remove PCP was also tested.

Antagonism test among bacterial strains was also realized using toothpick method for the strains forming bacterial consortia B1 and B2 (Hassan et al., 2015). This method assesses the ability of strains to inhibit each other's growth in Tryptic Soy Agar (TSA). TSA plates were freshly inoculated with single strain (10^8 UFC mL $^{-1}$) for 24 h. A sterile toothpick was used to place some young colonies of the tested strain in spot form and all plates were incubated at 30 °C for 48 h. A positive result of the antagonism was indicated by the clear area around the toothpick strain spot. This antimicrobial test was performed in triplicate.

2.5. Forest soil spiking

The forest soil was rewet to 30% of water holding capacity (Alef and Nannipieri, 1995) before spiking procedures according to Rao et al. (2017). Suitable amount of PCP stock solution (10 g L $^{-1}$ in acetone) was added to 4.5 kg of soil in order to reach a final concentration of 100 mg kg $^{-1}$. The spiked soil was placed in glass jars and mixed overnight for inversion (Rao et al., 2017). After that, contaminated soil was incubated at 25 °C for 21 d (Rao et al., 2017; Scelza et al., 2007, 2010).

2.6. Soil microcosms under biostimulation and bioaugmentation

To 60 g of 100 mg kg $^{-1}$ PCP spiked soil, placed in 1 L-glass jars, 175 mg kg $^{-1}$ phosphate as salt crystal of K₂HPO₄ or 0.6 g of FC, MC, or SS corresponding to 30 t ha $^{-1}$ were added as biostimulation technique. In other similar glass jars PCP spiked soil was inoculated with bacterial consortia B1 or B2 as bioaugmentation technique. The combined technique providing the consortium inoculum simultaneously to the addition of phosphorous or organic amendment was also set up (Ambujom, 2001). An uncontaminated non-amended soil (S) and a PCP contaminated non-amended soil (Sc) were also prepared as controls. More details for the different microcosms are given in Table S1. All jars were incubated at 25 °C for 30 d in the dark. All the microcosms were performed in triplicate.

2.7. PCP extraction and analysis

PCP concentration from soil samples was evaluated according to Rao et al. (2017). Contaminated soil samples (0.6 g of dry weight) were treated with 12 mL water-ethanol mixture (50:50, v:v). The extract was concentrated by evaporation under vacuum (LABOROTA 4000, Heidolph) and then dissolved in 2 mL of methanol. This latter was analysed by high-performance liquid chromatography (HPLC) (Agilent® Serie 1100) using a Phenomenex C-18 RP column (250 mm × 4.6 mm, 4 µm) and a diode-array detector. Methanol and buffered water (1% acetic acid) were the mobile phase (90:10, v:v) and the flow rate was set on 1.0 mL min $^{-1}$. The retention time for PCP at 220 nm was about 5 min. The PCP concentration was calculated on calibration curve by using PCP in methanol as external standard at different concentration (1, 5, 10, 15, and 25 mg L $^{-1}$).

2.8. Microbial biomass carbon determination

Microbial biomass carbon (MBC) was measured as soon as possible after the incubation time (T0 and T30) in according to the fumigation-extraction method (Vance et al., 1987). Briefly, 10 g of moist soil were exposed to CHCl₃ for 24 h at 25 °C and then treated with 0.5 M K₂SO₄ for 30 min under mechanical shaking at 200 rpm; the suspension was filtered through Whatman 42 filter paper. A non-fumigated control underwent the same procedures described above without the CHCl₃ exposure. Organic carbon in the extracts was determined after oxidation with 0.033 M K₂Cr₂O₇ at 110 °C for 1.5 h by titration with 0.1 M Mohr salt solution. Results were expressed in mg C 100 g $^{-1}$ dried soil. All determinations were in triplicate.

2.9. Soil respiration

The CO₂ released from contaminated soil samples, differently treated by biostimulation and/or bioaugmentation, during the incubation in optimal controlled conditions of temperature and humidity, was measured by gas chromatography analysis. Briefly, 4 g of soil were placed in 30 mL bottles and distilled water was added to reach 55% of the water holding capacity of the soil. Subsequently, the bottles were closed hermetically, ventilated by injecting compressed air, and then placed in an incubator for 1 h at 25 °C. CO₂ present in the headspace was drawn with a 10 mL syringe and injected into a gas chromatograph Trace GC Ultra (Thermo Fisher, Bremen, Germany) under a stream of nitrogen (carrier gas) equal to 40 mL min $^{-1}$. Standard CO₂ at concentrations ranging between 1000 and 10,000 ppm was used for the calibration curve (Castaldi et al., 2013).

2.10. Microbial indices

Microbial quotient, which represents the fraction of soil organic C occurring as microbial biomass (Haynes, 2000), expressed as mg microbial C g $^{-1}$ OC, was also calculated as well as metabolic quotient (qCO₂) that is CO₂ evolved per unit of microbial biomass C, expressed as mg CO₂ g $^{-1}$ C mic h $^{-1}$ (Anderson and Domsch, 1990).

2.11. Enzymatic activity assay

Enzyme activities were determined within 15–20 d from the collection of the samples on fresh soil stored at 4 °C. Dehydrogenase (DH) was determined with tetrazolium salts (TTC) solution (Alef and Nannipieri, 1995). The fluorescein diacetate hydrolysis (FDA) was assessed as described by Green et al. (2006). Alkaline phosphatase (PHO) was determined in according to Tabatabai and Bremner (1969). The laccase (LAC) activity was measured in according to the method of Diez et al. (2006) based on the spectrophotometer measurement of the green compound developed by the enzymatic oxidation of ABTS (2,2'-Azino-bis (3-ethylbenzotiazolin-6-sulfonic acid). Triplicates were analysed for each activity assay.

2.12. Statistical analyses

Results of the bioremediation experiment on PCP contaminated soil were subjected to two-way (time of incubation, type of treatment) analysis of variance (ANOVA). The significance between treatments at the same sampling time ($p < 0.05$) was determined by using the Duncan test. The difference between the two sampling times was assessed by paired *t*-test ($p < 0.05$). The statistical analysis of data was performed using the SPSS program package (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

Principal component analysis (PCA) was performed in order to evaluate differences among all variables at two different sampling times (0 and 30 d, named T0 and T30, respectively). The PCA results were shown as a biplot to highlight the interaction between samples and variables. The PCA was performed by SPSS program package (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

3. Results and discussion

3.1. Microbial consortia characteristics

Molecular analyses of the different bacterial isolates revealed four bacterial strains of *Klebsiella* species isolated from the various sampling sites, and the other bacterial strains belonged to *Citrobacter*, *Staphylococcus*, *Micrococcus* and *Enterobacter* (Table S2). All these strains were screened for their ability to grow with PCP as the only carbon source (Table S2). These eight selected strains (*Leclercia adecarboxylata* isolate

TPY2, bacterium isolate RLF11, *Klebsiella* sp. *Klebsiella oxytoca*, *Citrobacter freundii*, *Klebsiella variicola* strain RCB1013, *Staphylococcus equorum* isolate YXY-2, *Micrococcus lylae* strain ES142) grown separately in MSM, showed a PCP removal from 65.31% of *Staphylococcus equorum* isolate YXY-2 to 88.21% of *Klebsiella variicola* strain RCB1013 when the pollutant was initially added at 100 mg L⁻¹. Antagonism test proved that strains used in the same consortium B1 or B2 can be associated having negative response. Thus, two microbial consortia B1 (*Leclercia adecarboxylata* isolate TPY2, bacterium isolate RLF11, *Klebsiella* sp. *Klebsiella oxytoca*) and B2 (*Citrobacter freundii*, *Klebsiella variicola* strain RCB1013, *Staphylococcus equorum* isolate YXY-2, *Micrococcus lylae* strain ES142con) were formulated by mixing equal proportions (10⁷ CFU mL⁻¹) of four bacterial strains in MSM (Table S2). As reported in Table S2, consortium B1 included strains all more tolerant to PCP (800 mg L⁻¹) and more performant in PCP removal than those belonging to consortium B2. The PCP removal (%) of consortia B1 and B2 were 81.5 and 63.5%, respectively (data not shown).

3.2. Main physicochemical characteristics of organic amendments

Physical and chemical characteristics of the three organic amendments FC, MC and SS are summarized in Table 1. FC was found to be the richest in organic carbon, total nitrogen and phosphorus (206.9 g kg⁻¹, 32.6 g kg⁻¹ and 620 mg kg⁻¹, respectively) compared to MC and SS. By adding organic matter and so increasing the C stock in soil, the sorption of PCP to the soil is expected to be favored and enhanced (Chien et al., 2018; Miller et al., 2004; Scelza, 2008). Indeed, compost generally provides several microbial species and nutrients that enable and enhance natural degradation process when applied to contaminated soils. Compost based strategies have been successfully applied to PCP-contaminated soil, especially because compost accelerates the PCP removal (Chien et al., 2018; Semple et al., 2003).

3.3. Main physical and chemical characteristics of forest soil

The soil used for this study was characterized for physical and chemical properties before biostimulation and/or bioaugmentation treatments according to the standard methods (Sparks, 1996) (Table S3). According to USDA the soil was classified as a loamy sand soil (clay 69 ± 0.4 g kg⁻¹, sand 806 ± 22 g kg⁻¹, lime 125 ± 22 g kg⁻¹). The pH value was 7.01 ± 0.01. According to Chien et al. (2018), soil pH is an important factor that affects PCP adsorption: by decreasing the pH value the adsorption process is favored (Chien et al., 2018). Tam et al. (2002) and Park and Bielefeldt (2003) showed the adsorption of PCP molecules to soil particles was a combination of hydrophobic and electrostatic soil pH-dependent interactions.

Electrical conductivity (EC) that is the indirect measure of the total concentration of dissolved salts related to both the porosity and the cation exchange capacity, was about 0.035 ± 0.140 dS m⁻¹, indicating

low soil salinity of the forest soil. The cation exchange capacity (CEC) was on average 17.1 ± 1 cmol₍₊₎ kg⁻¹ due to the low clay content of the forest soil (69 g kg⁻¹).

The organic carbon and total nitrogen content of the forest soil were 11.4 g kg⁻¹ and 1.8 g kg⁻¹, respectively (Table S3), with an organic matter content of 19.6 g kg⁻¹. The C/N ratio was 6.3 thus indicating that with C/N ratio < 9 a dominance of oxidation reactions and also a release of the nitrogen stock could be observed (Park and Bielefeldt, 2003). Different studies have shown that degradation of PCP molecules in soil might be associated with the presence of a significant amount of organic matter (Banerji et al., 1993; Chien et al., 2018; Park and Bielefeldt, 2003).

The available phosphorus content (P₂O₅) was 8.5 mg kg⁻¹ indicating a scarce availability of this element (Table S3).

After incubation time some chemical properties of the contaminated soil following various biostimulation and/or the bioaugmentation procedures at the beginning (T0) and at the end of the incubation (T30) were measured (Fig. 1).

The value of organic carbon increased in all treatments providing organic amendment at the initial T0; a little further enhancement at T30 was also observed (Fig. 1a). The total nitrogen content remained unchanged (Fig. 1b) around 1.8 g kg⁻¹ and did not change after 30 d of incubation for any of the studied treatments. Available phosphorus strongly increased until 140 mg kg⁻¹ in soil samples biostimulated with phosphate, whereas it doubled on average in all samples under organic amendment especially when FC and bacterial consortia were added (Fig. 1c). The inoculum of the two bacterial consortia improved also the amount of available phosphorus combined with FC, MC or SS after 30 d incubation (Fig. 1c) playing an important role in making organic phosphorous available in form of phosphate from these organic amendments.

The CEC values (data not shown) of soil differently treated did not show significant differences. In fact, these values ranged from 15 to 20 cmol₍₊₎ kg⁻¹ of dry soil for PCP-uncontaminated soil (S) and treated PCP-contaminated soil.

3.4. PCP residual concentration in forest soil

Residual PCP was evaluated at zero time and 30 d after biostimulation/bioaugmentation actions. PCP concentration was 82 mg kg⁻¹ at T0 and no significant difference among treatments was observed (line in Fig. 2). After 30 d the residual PCP significantly decreased in the control sample (Sc) (Fig. 2). In accordance to Hansen et al. (2004) and Rao et al. (2017) these reductions were essentially due to an intrinsic soil property called "natural attenuation", related to soil properties. Among these, organic matter into soil is known as the most important sorbent for organic pollutants. In particular, soil organic matter may enhance PCP sorption (Cea et al., 2010; Scelza, 2008) thus explaining the strong reduction of PCP (32%) in Sc sample at T30. The relevant role of indigenous microorganisms could also contribute to PCP removal (Scelza, 2008). Although natural attenuation can produce very encouraging results in bioremediation of polluted soil, further scenarios could arise as the production of toxic metabolite(s) of a biodegraded compound or persistence of compounds emphasizing the need to exploit metabolic processes of microorganisms to clean the soil from anthropogenic substances (Hussain et al., 2009).

No significant difference in the treatments Sc+P and Sc+SS respect to Sc was observed (Fig. 2). Conversely, the PCP removal efficiency of the microbial consortia in the treatment of the PCP-contaminated soil was ascertained. By adding microbial consortia to the contaminated soil having already indigenous microorganisms capable of degrading PCP, a further significant decrease of the residual PCP was observed (Sc+B1 and Sc+B2, 59 and 56 mg kg⁻¹, respectively).

A marked decrease of the PCP amount occurred in the microcosms where combined biostimulation/bioaugmentation actions were adopted. The PCP contaminated soil treated with phosphorus based

Table 1
Main physical and chemical properties (means ± standard deviation) of forest compost, municipal solid waste compost and urban sewage sludge.

Parameters	FC	MC	SS
pH, in H ₂ O	5.7 ± 0.3	7.8 ± 0.1	3.5 ± 0.2
EC, dS m ⁻¹	3.0 ± 0.5	5.3 ± 0.1	8.0 ± 0.5
Dry matter, %	64.2 ± 3.5	70.0 ± 5.0	8.3 ± 0.2
OC, g kg ⁻¹	206.9 ± 5.5	116.9 ± 1.6	82.7 ± 0.8
OM, %	20.2 ± 0.4	16.8 ± 0.2	6.1 ± 0.2
TN, g kg ⁻¹	32.6 ± 0.2	14.1 ± 0.2	12.2 ± 0.1
C/N	6	8	7
P, mg kg ⁻¹	620 ± 30	350 ± 20	50 ± 1
Ca, mg kg ⁻¹	1130 ± 68	496 ± 64	270 ± 8
Mg, mg kg ⁻¹	4.9 ± 0.1	3.6 ± 0.4	4.9 ± 0.2
Na, mg kg ⁻¹	104 ± 2.3	2645 ± 462	327 ± 69
K, mg kg ⁻¹	42.9 ± 1.6	3.9 ± 0.4	2.0 ± 0.4

FC: forest compost; MC: municipal solid waste compost; SS: sewage sludge.

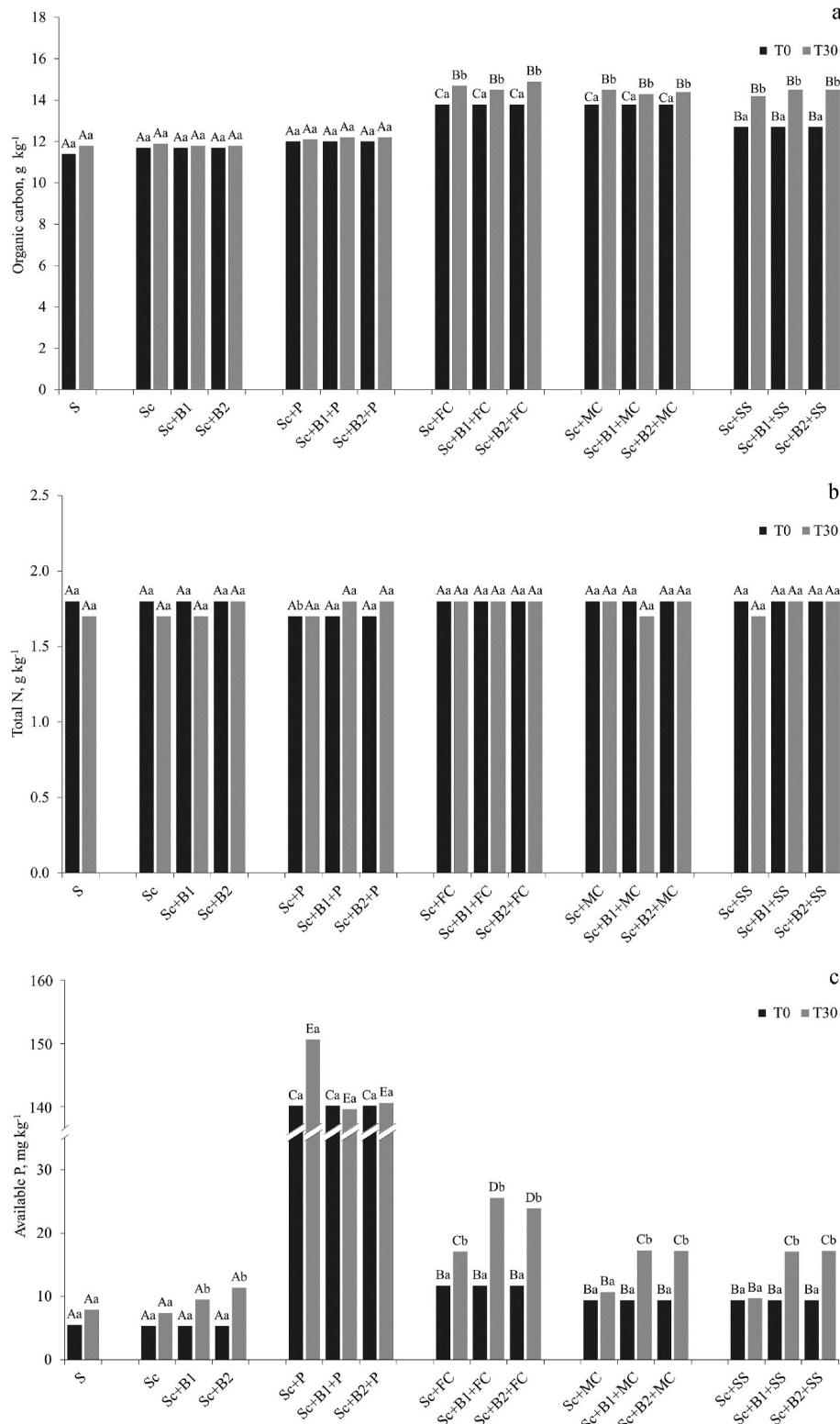


Fig. 1. Organic carbon (a), total nitrogen (b) and available P (c) of PCP contaminated forest soil microcosms during biostimulation and bioaugmentation processes at 0 time and after 30 d incubation. Different capital letters indicate significant differences among treatments, in the same sampling time at Duncan post-hoc test ($p < 0.05$), whereas different lower case letters indicate significant differences among sampling times in the same treatment assessed by paired t-test ($p < 0.05$). S: soil; Sc: PCP contaminated soil; FC: forest compost; MC: municipal solid compost; SS: Sewage sludge; B1 and B2: microbial consortia.

biostimulation and bioaugmentation (microbial inoculum) showed a little reduced PCP concentration (Fig. 2). A more encouraging effect arose by using MC in organic amendment also combined with consortium B1 and B2 reducing the PCP concentration to 40 mg kg^{-1} on average. The most relevant result was observed upon the treatment with FC and the microbial consortium B1 (Sc+B1+FC), where residual PCP dropped down to 28.9 mg kg^{-1} (Fig. 2) corresponding to a reduction of 71%. The

treatment providing only FC (Sc+FC) already determined a significant reduction in the residual PCP, approximatively 50 mg kg^{-1} (Fig. 2). These results are in according to Taccari et al. (2012) who found the combined action of biostimulation through compost addition and bioaugmentation by inoculating a selected and well adapted microbial consortium. This treatment provided the best results for total petroleum hydrocarbon removal from contaminated soil. The role of organic

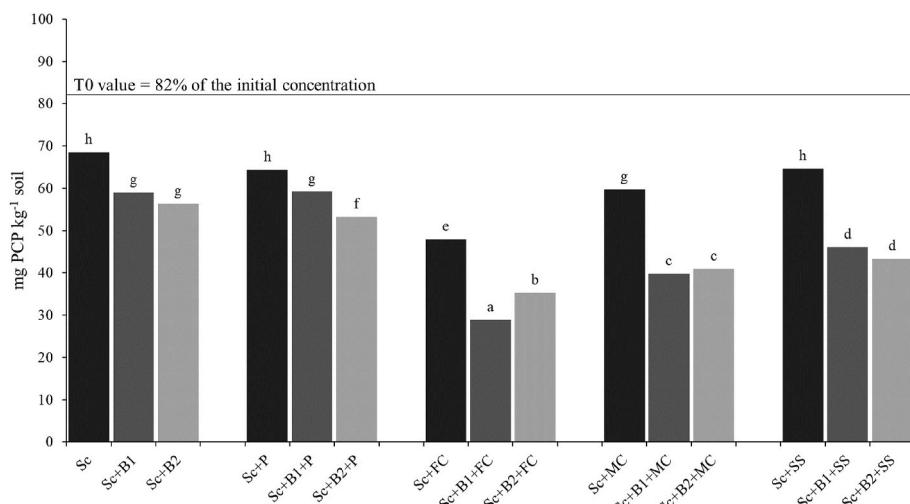


Fig. 2. Extractable PCP in differently treated microcosms after 30 d incubation. Different lower case letters indicate significant differences among treatments at Duncan post-hoc test ($p < 0.05$). S: soil; Sc: PCP contaminated soil; FC: forest compost; MC: municipal solid compost; SS: Sewage sludge; B1 and B2: microbial consortia.

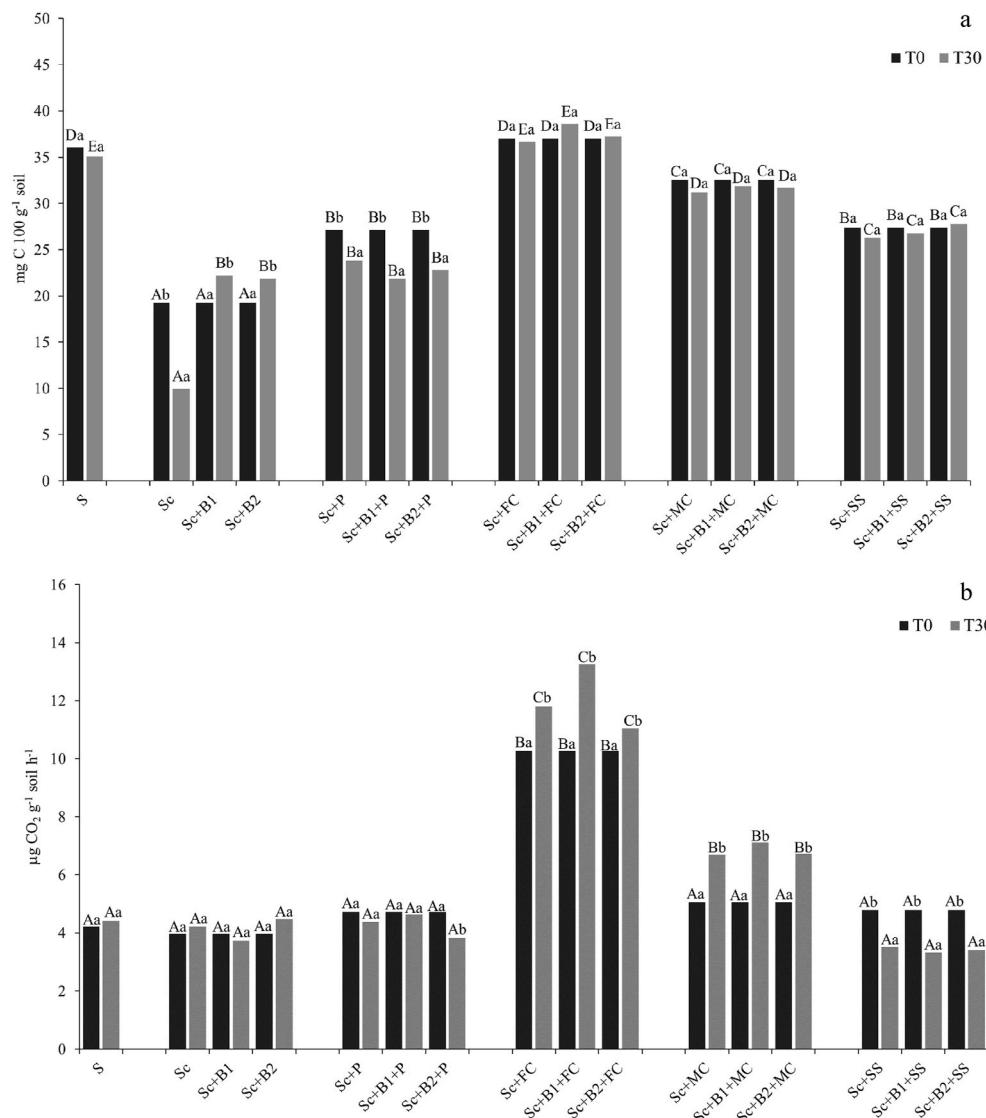


Fig. 3. Soil microbial biomass C (a) and soil respiration (b) of PCP contaminated forest soil microcosms during biostimulation and bioaugmentation processes at 0 time and after 30 d incubation. Different capital letters indicate significant differences among treatments, at the same sampling time at Duncan post-hoc test ($p < 0.05$), whereas different lower case letters indicate significant differences among sampling times in the same treatment assessed by paired *t*-test ($p < 0.05$). S: soil; Sc: PCP contaminated soil; FC: forest compost; MC: municipal solid compost; SS: Sewage sludge; B1 and B2: microbial consortia.

matter can be however relevant as Bosso et al. (2015) reached almost 95% of the PCP removal from 25 mg kg⁻¹ spiked soil after 28 d of incubation by using only compost. On the other hand, the microorganism contribution could not be diminished as also their action, especially in mixed culture, could be efficient. Pu and Cutright (2007) obtained 63% PCP degradation rate in a bioaugmentation experiment on a PCP contaminated soil based on mixed culture of *Arthrobacter* sp. and *Flavobacterium* sp. It seems clear that a real synergistic effect arises when biostimulation and bioaugmentation are combined making more effective the microbial metabolism and amplified the interaction of organic matter with pollutants.

3.5. Microbial biomass C changes in the incubated forest soil

Microbial biomass is defined as the fraction of living organic matter in soil (Jenkinson and Ladd, 1981). The carbon associated with microbial biomass is the carbon contained in cellular and microbial structures. As it is very sensitive to changes induced by natural and anthropic activity microbial biomass C acts as an “early indicator” of disturbances in the soil, thus assuming the role of bio-indicator of the soil quality (Huera-Lucero et al., 2020).

A macroscopic downfall of microbial biomass C (47%) occurred immediately (T0) in PCP contaminated soil (Sc) and no recovery was registered even if inoculated with bacterial consortia (Fig. 3a). All remediation treatments enhanced microbial biomass C regardless microbial inoculum at T0 in comparison with contaminated soil (Sc), but particularly FC-based soil amendment (Fig. 3a). In fact, after biostimulation or biostimulation combined with bioaugmentation treatments the microbial biomass C significantly raised. Also phosphorous addition (Sc+P) with or without bacterial inoculum led to an increase of microbial biomass C (compared to Sc+B1 and Sc+B2) but not as much as reached with the organic amendments (FC and MC) combined or not with consortia B1 or B2. Phosphorus fertilization increased strongly available P in these microcosms reaching 140.1 mg kg⁻¹ able to stimulate the growth of autochthonous microorganisms (Hu et al., 2011) rather than those supplied through inoculum (consortia B1 or B2) at T0 time. In fact, the value of microbial biomass C of Sc+B1+P and Sc+B2+P at T0 remained unchanged respect to Sc+P (Fig. 3a).

The treatments Sc+FC, Sc+B1+FC and Sc+B2+FC showed the same microbial biomass C content as S (37 mg 100 g⁻¹ dry soil) at time T0 and T30. The common treatment based on FC compost seems to be responsible for this result. The OC content of this compost was two-fold greater than that measured in MC and even two-fold and half greater than that of SS (Table 1). The larger amount of organic matter allowed a more intense interaction with PCP molecules (Fig. 2) due to simultaneous processes of degradation, sequestration, adsorption, etc. thus limiting or neutralizing its toxic effect on the microbial biomass growth. Soil organic matter is the most important sorbent for phenolic compounds thus improving the sorption of the PCP molecules into various soil aggregates (Cea et al., 2010; Scelza, 2008).

MC and SS did not perform as FC because both led to a reduction of microbial biomass C respect to S following the order FC > MC > SS (Fig. 3a) corresponding to their relative OC content (Table 1).

Also for organic amendments, the role of microbial consortia would seem to be negligible as a similar microbial biomass C content in amended samples with or without microbial inoculum at T0 was observed. At this time no difference of PCP concentration was registered (Fig. 2). Conversely, at T30, when a clear decrease in the microbial biomass carbon in Sc occurred, both the microbial consortia B1 and B2 in Sc+B1 and Sc+B2 seem developing as highlighted by the greater microbial biomass C in contaminated soil (Fig. 3a). Different behavior arose in soil samples treated with P fertilizer: at the end of incubation a reduction of microbial biomass C occurred (on average 16%) also in the presence of microbial inoculum.

The sample Sc+B1+FC reached the most beneficial effects in terms of reduction of PCP amount and microbial biomass C in the contaminated

soil highlighting a synergistic outcome from combined biostimulation and bioaugmentation technique (Figs. 2 and 3). This positive effect seems to be related to the quality of the FC amendment able to stimulate degradative metabolisms of microorganisms belonging to consortium B1 (Fig. 2).

Among the three organic amendments SS seems to have produced less encouraging results. Besides the lacking OC, the sludge concentrates potential toxic and inhibitory molecules as well as electrolytes that could affect seriously the microbial growth (Kończak and Oleszczuk, 2018).

Bouzaiane et al. (2007) observed a net increase of the microbial biomass carbon after the application of municipal solid compost in agricultural soil. Soil microbial biomass is influenced by changes in soil carbon stock (Schnürer et al., 1985) and the nature of the applied organic fertilizer (Hasebe et al., 1984).

3.6. Respiration study of treated forest soil

The respiratory activity of the forest soil appeared not sensitive to PCP contamination, microbial inoculum or phosphate fertilization during throughout the incubation period (Fig. 3b). In contrast, the FC based treatments Sc+FC, Sc+B1+FC and Sc+B2+FC stimulated strongly soil respiration activity at T0 and still more at T30 reaching values of 11.8, 13.2 and 11.0 µg CO₂ g⁻¹ h⁻¹ soil, respectively (Fig. 3b). A similar but less marked effect was also observed in contaminated soil amended with MC able to enhance soil respiration mainly after 30 d incubation (Fig. 3b). Besides the greater soil respiration these soil samples had the PCP contamination level reduced more than in other samples. This behavior seems to be attributable mainly to the effect of compost. FC as well as MC brought great amount of organic matter, the main responsible of this positive response (Fig. 3b). Therefore, biostimulation processes seems to be predominant in the complex soil systems.

In a previous work Ouattara et al. (2010) found that the released CO₂ content was greater in the soil fertilized by compost as compared to one fertilized by the mineral compounds. The increase in respiratory activity would be not due to the number of microorganisms, but mainly to the intensification of their activity of degradation of the various organic compounds available into the soil. However, a high respiratory activity into soils enriched with a high organic matter is generally observed (Chaussod et al., 1992; Ouattara et al., 2010). In addition, the stimulation of the soil microbial activity can be enhanced by the richness in easily metabolizable organic compounds (Annabi et al., 2007). Also Scelza (2008) and Bosso et al. (2015) found a significant increase in the respiratory activity in agricultural soil contaminated with PCP after the application of municipal solid waste compost.

3.7. Microbial indices

The microbial quotient decreased for Sc differently treated respect to the uncontaminated soil S already at T0 (Fig. S1a). The value of this index remained closer to that of S when biostimulation and bioaugmentation (organic amendments, phosphate fertilization and microbial inoculum) were applied. Then the value continued further to fall down at T30 also in the control Sc, but not in Sc+B1 and Sc+B2 where inoculated microorganisms, selected just for their ability to grow in the presence of PCP, grew significantly within 30 days. The addition of phosphate fertilizer (Sc+P) stimulated autochthonous microbial biomass already at T0 increasing the microbial index even more than simple microbial inoculum (Sc+B1 and Sc+B2) which needed more time for adaptation and growth without nutrient stimulation (Hu et al., 2011; Huang et al., 2006). However, the organic amendments, in particular that based on FC, allowed achieving better performance of microbial index than other microcosms with or without B1 or B2.

As reported in Fig. S1b, the metabolic quotient (qCO₂) increased in contaminated soil (Sc) in comparison with S at T0 and still more at T30 reaching a peak of 42.4 mg CO₂ g⁻¹ C mic⁻¹ h⁻¹ when microbial

communities under stress conditions released more CO₂ per unit microbial biomass than all the other treatments. The PCP concentration in this microcosm was high and the nutritional conditions were not favorable to microbial growth and activity. This result is in accordance with Frische and Hoper (2003) who observed the metabolic quotient was higher in contaminated stressed soil than in uncontaminated non-stressed one.

The inoculum of both microbial consortia in Sc kept the qCO₂ values almost unaltered, whereas the addition of phosphate, MC or SS little reduced this index at least at T0 and then it grew up again with MC at T30. Conversely, organic amendment based on FC treatments induced a more intense metabolic activity especially at T30 when the metabolic quotient grew up significantly. These indices are useful for elucidating effects of environmental changes as PCP contamination and different treatments on microbial communities and quantifying the metabolic status of forest soils (Anderson and Domsch, 1990).

3.8. Enzymatic activities of the processed forest soil

After biostimulation and/or bioaugmentation process some enzymatic activities were also measured in forest soil artificially contaminated with PCP at 100 mg kg⁻¹ in order to determine change in the biological activity and functionality.

Dehydrogenase activity. As shown in Fig. 4a, the DH activity in

contaminated soil appeared strongly depressed unless the contaminated soil was amended with compost or sludge at T0. In addition, the smaller depletions were achieved also in soil samples receiving at the same time FC and inoculum B1 or B2 (3.7 µg TPF g⁻¹ soil h⁻¹) at T0. Phosphate also together with inoculum B1 or B2 led to further reduction at T30 similarly to untreated contaminated soil (Sc). Both the compost FC and MC contributed to maintain high the activity level but the combination of MC with microbial consortium (B1 or B2) was not able to guarantee the DH activity level as FC did. On the other side, SS combined with consortia B1 or B2 was able only to double the values of Sc. In any case a synergistic effect of organic amendment and microbial inoculum occurred. After 30 d in any bioaugmentation and/or biostimulation treatment of contaminated soil, the DH activity fell down reaching values smaller than of 0.76 µg TPF g⁻¹ h⁻¹ and uncontaminated soil underwent an halving too.

Dehydrogenases are intracellular enzymes involved in redox processes of a wide range of organic molecules whose activity depends on the survival of the microbial cell (Diez et al., 2006). Thus, DH activity is strictly correlated with soil microbial biomass and its metabolic activity (Dick, 2011). In our study the microbial biomass C in contaminated soil remained unchanged only upon the FC based amendment and gradually reduced with MC and SS (Fig. 3a). In amended soil marked oxidative processes occurred in the same order as demonstrated by respiration rate (Fig. 3b). The decrease observed in DH activity, especially after 30 d,

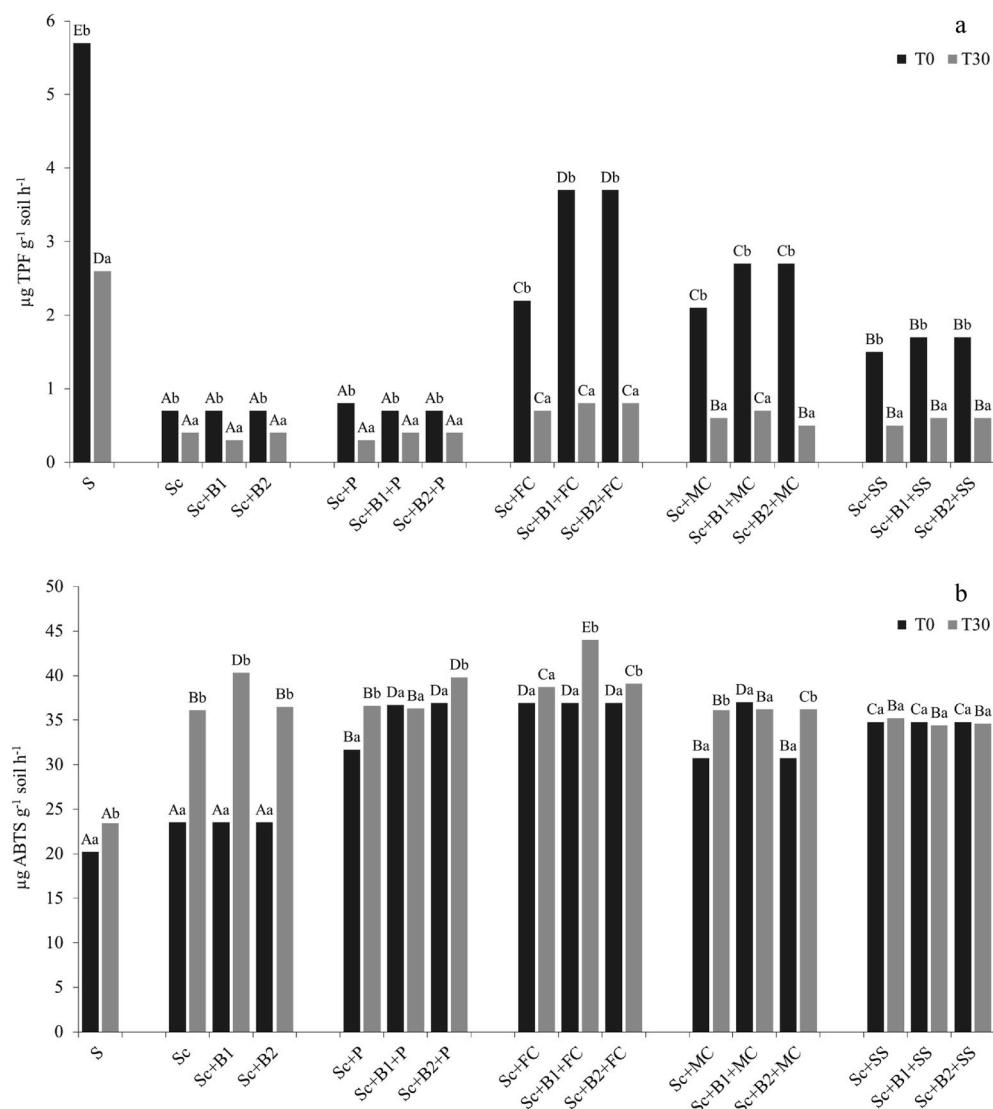


Fig. 4. The activity of a) dehydrogenase and b) laccase of PCP-contaminated forest soil microcosms during biostimulation and bioaugmentation processes at 0 time and after 30 d incubation. Different capital letters indicate significant differences among treatments at the same sampling time at Duncan post-hoc test ($p < 0.05$), whereas different lower case letters indicate significant differences among sampling times in the same treatment assessed by paired *t*-test ($p < 0.05$). S: soil; Sc: PCP contaminated soil; FC: forest compost; MC: municipal solid waste compost; SS: sewage sludge; B1 and B2: microbial consortia.

could be attributable to the toxic effects of PCP in according to Scelza (2008) who found a negative correlation ($r = -0.833$) between the PCP concentration and the DH activity. Similar studies Bosso et al. (2015) achieved a decrease in soil DH activity as response to the PCP addition (25 mg kg^{-1}). Also when the PCP concentration in soil was around 41 mg kg^{-1} (Diez et al., 2006) DH activity was negatively affected. The decrease in DH activity could be reasonably explained by a strong inhibitory and/or destructive effect of PCP on the microbial cells. As demonstrated by Yang and Chen (2016) for denitrificant bacteria, PCP can induce the ROS generation, which decreased enzymes activities related to glycolysis process, causing the disturbance of the metabolism of glucose and cell growth. As the residual PCP concentration decreased, the degradation of easy metabolic products was probably formed and PCP molecules were removed by partial or total immobilization in the soil matrix (Diez et al., 2006). Inhibition of DH activity by PCP occurred in a six-week experiment McGrath and Singleton (2000) carried out in sterile soil inoculated with a single species of fungus and in another soil inoculated with a combination of fungus species. DH activity considerably decreased in 250 mg kg^{-1} PCP contaminated soils. The simultaneous application of municipal solid waste and sewage sludge composts in the PCP contaminated soil can stimulate the DH activity, as these organic fertilizers contain and enclose both intra and extracellular enzymes (Liang et al., 2005). Scelza (2008) and McGrath and Singleton (2000) found that soil DH enzyme activity significantly decreased and did not recover throughout an experiment of 6 weeks of around 50 and 250 mg kg^{-1} PCP. Thus, the DH activity showed a negative response to PCP already after 1 day of incubation with the loss of 50% respect the non-PCP-contaminated and non-treated soil.

Laccase activity. The LAC activity was stimulated immediately (T0) in PCP contaminated soil amended with phosphate or any organic fertilizers with or without microbial inoculum (Fig. 4b). The activity ranged from 20.2 to $44.0 \mu\text{mol ABTS g}^{-1} \text{ h}^{-1}$. Conversely, both microbial consortia as example of bioaugmentation were not able to stimulate LAC activity at T0 when the activity remained below $23.5 \mu\text{mol ABTS g}^{-1} \text{ h}^{-1}$, value not significantly different from $20.2 \mu\text{mol ABTS g}^{-1} \text{ h}^{-1}$ of S (control). It is possible to highlight a combined effect deriving from bioaugmentation and biostimulation namely when the amendment was realized with phosphate or MC whereas generalized enhancement of the LAC activity occurred with FC and SS (Fig. 3b).

After 30 d a marked increase of the LAC activity was recorded not only in Sc+B1 and Sc+B2 contaminated soil but also in Sc thus indicating no positive effect attributable to bioaugmentation approach. The incubation time produced a positive effect on the LAC activity in samples Sc+B2+P, Sc+B1+FC, Sc+B2+FC, and Sc+B2+MC equal to 8%, 19%, 6%, and 18%, respectively.

The LAC activity has an important role in the bioremediation process investigated in this work. Microorganisms deriving from both inoculum and organic amendment are able to colonize PCP-contaminated soil and determine significant increase in LAC activity during the incubation time. On the other side, also the presence of a substrate such as PCP stimulated directly the LAC activity. In general, laccases oxidize a wide range of substrates among these substituted phenols, which are transformed into free radicals able to start domino reactions, leading to complex chemical transformations (Arregui et al., 2019). When a direct oxidation is not achievable due to steric hindrance/active site penetration and/or redox potentials incompatibility, chemical mediators play an important role as redox intermediates to overcome this limitation (Bassanini et al., 2021). Natural compounds such as phenolic ones derived from lignin degradation, still present in composted materials, are capable of promoting aromatic compound degradation (Vipontnik et al., 2021). In according to Cascant et al. (2016) biowaste and sewage sludge, like MC and SS, with a low percentage of organic matter have the minimum concentration of total phenolic compounds comparing different compost. Conversely, biogenic wastes such as vegetable compost, like FC, with greater concentration of organic matter contain until to $5 \text{ mg gallic acid equivalents g}^{-1}$ dw. This difference in chemical

composition of organic fertilizers could explain the greater LAC activity measured in soil amended with FC related to possible mediators present in this compost used for the amendment of the PCP contaminated soil (Fig. 4b).

On the other side, the availability of sources of organic or mineral nutrients carried net effects of improving the biological activity into soils. In particular, lignin and phenolic compounds as well as nutritional factors such as C/N ratio, the amount of dissolved oxygen, organic salts enhanced LAC synthesis from bacteria (Chauhan et al., 2017).

As reported by Böhmer et al. (1998) and D'Annibale et al. (2006), LAC is used for the treatment of phenolic effluents, polycyclic aromatic hydrocarbons and polychlorinated biphenyls as well as for the bleaching of textile dyes (Kandelbauer and Guebitz, 2005). Also, according to Yin et al. (2010), LAC enzymes are proteins that represent a very valuable group of oxidoreductase activities that offer a high decontamination potential in polluted natural environments. For this purpose, soil microorganisms could produce several various oxidoreductases, such as laccases, peroxidases, hydroxylases, oxygenases, and reductases. LAC is known to be involved in the oxidative coupling processes of O_2 with chlorophenols (Bollag, 1992; Gianfreda and Bollag, 2002).

Fluorescein diacetate hydrolysis activity. The FDA activity appeared unchanged in PCP contaminated soil at the beginning of incubation T0, also with microbial inoculum, compared with control soil (S) (Fig. 5a). The FDA activity decreased (~20%) upon phosphate based fertilization of contaminated soil; conversely, it enhanced until $30.4 \text{ mg fluorescein g}^{-1} \text{ h}^{-1}$ dry soil with organic amendments following the order FC > MC > SS ($p < 0.05$) regardless the microbial inoculum. At the end of the incubation, T30, the FDA activity fell down in all tested treatments (Fig. 5a) but in the samples with the microbial inoculum a less marked reduction of the enzymatic activity occurred except for the samples amended with SS. In Sc, Sc+P, Sc+MC and Sc+SS FDA activity dropped below the value of the control soil S whereas Sc+FC behaved similarly to S. At this time a relevant effect of microbial inoculum appeared: where bioaugmentation was applied simultaneously, the FDA activity maintained a higher level. The microbial consortium B1 better performed with FC and MC amendment (Fig. 5a). This trend corresponds to that observed in microbial biomass C at T0 (Fig. 3a) but it did not at T30. During the incubation PCP had a broad-spectrum biocide that takes, in general, a negative impact on the main microbial biological capacity and activity. According to Cea et al. (2010), the FDA showed a good response to PCP without strong inhibition, especially during the first d of incubation. Similar results were obtained in an area contaminated with long-acting: pentachlorophenol contamination affected barely FDA activity from native soil microflora (Bosso et al., 2015). To explain the FDA trend at T30 the enzymatic catalysis should be also analysed: substrates of hydrolytic enzymes have been degraded progressively and this deficiency limited likely their activity. This latter phenomenon was balanced out by organic amendments in the order FC > MC > SS.

Alkaline phosphatase activity. The forest soil artificially contaminated with PCP showed a significant depletion, respect the control soil (S), in the PHO until $0.2 \mu\text{mol pNP g}^{-1} \text{ h}^{-1}$ regardless the microbial inoculum at T0 (Fig. 5b). After the phosphate based treatment the PHO activity recovered reaching the value of the control soil S ($0.5 \mu\text{mol pNP g}^{-1} \text{ h}^{-1}$), whereas it even enhanced once organic amendments based on FC or MC were performed regardless of whether microbial inoculum was or not in samples ($0.6 \mu\text{mol pNP g}^{-1} \text{ h}^{-1}$). Conversely, no effect was produced by the sewage sludge on the PHO of contaminated soil: it remained $0.2 \mu\text{mol pNP g}^{-1} \text{ h}^{-1}$ as it was in Sc.

The behavior of this soil enzymatic activity changed after 30 d-incubation time. The PHO activity in Sc recovered reaching values of control soil S growing up to $0.4 \mu\text{mol pNP g}^{-1} \text{ h}^{-1}$. In addition, the advantageous role of microbial inoculum arose already without any fertilizer or amendment (Sc+B1 and Sc+B2). Bioaugmentation action, in particular with B1, favored the PHO activity also in the soil samples treated with phosphate but above all combining organic amendments and microbial inoculum ($0.8 \mu\text{mol pNP g}^{-1} \text{ h}^{-1}$ in Sc+B1+FC,

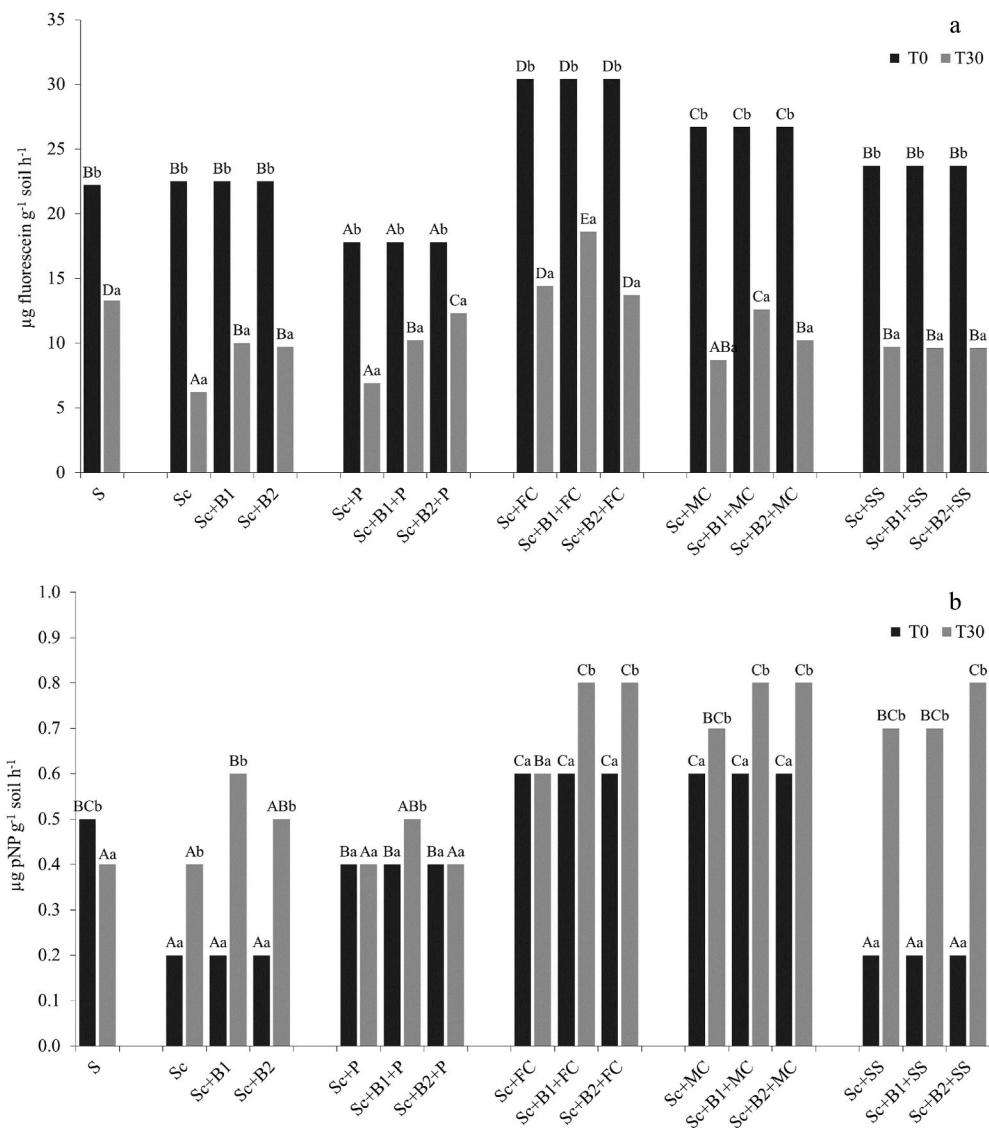


Fig. 5. The activity of a) fluorescein diacetate hydrolysis activity and b) alkaline phosphatase of PCP-contaminated forest soil microcosms during biostimulation and bioaugmentation processes at 0 time and after 30 d incubation. Different capital letters indicate significant differences among treatments at the same sampling time at Duncan post-hoc test ($p < 0.05$), whereas different lower case letters indicate significant differences among sampling times in the same treatment assessed by paired t -test ($p < 0.05$). S: soil; Sc: PCP contaminated soil; FC: forest compost; MC: municipal solid waste compost; SS: sewage sludge; B1 and B2: microbial consortia.

Sc+B2+FC, Sc+B1+MC, and Sc+B2+FC). SS stimulated the enzymatic activity also without microbial inoculum (Sc+SS) respect T0 and still more with B2 (Sc+B2+SS) (Fig. 5b).

PCP exerts depressing effects on the soil microbial activity (Bosso et al., 2015). Disturbances caused by some specific environmental factors, such as pollution, can cause a significant effect on soil microorganisms and indirectly on soil enzymatic activities (Burns, 1982). In agreement to Ammeri et al. (2020), at the PCP rate adopted in this study, soil bacteria growth may be affected. The disappearance of PCP during the incubation time due to biostimulation and bioaugmentation allowed recovering the activity levels. As reported and clarified by Munnecke et al. (1982), hydrolysis of pesticides results in a loss of biospecificity and by-products with lower stability and higher biodegradability. The role of organic amendments in boosting the PHO activity was also relevant. After all the PHO activity appears positively correlated with the organic matter content in according to Imran et al. (2020). The presence of fresh organic materials makes available substrates for enzyme molecules positively modulating their activity.

3.9. Principal component analysis

In order to summarize the variability observed in chemical and biochemical characteristics of PCP contaminated forest soil microcosms

during biostimulation and/or bioaugmentation process at 0 time and after 30 d, a discrimination of different treatments by Principal Component Analysis procedure was performed. The procedure allowed the extraction of three principal components reported as regression factor scores (PC1, PC2 and PC3) and graphically visualized in a three-dimension space (Fig. 6). The main three components accounted for 71% of the total variance. In particular, PC1 expressed the variability in organic carbon, total nitrogen, available phosphorous, microbial biomass C, respiration, microbial quotient, and PHO (Fig. 6b). PC2 expressed the variability in DH, FDA hydrolysis, and LAC (Fig. 6b). Finally, PC3 expressed the variability in metabolic quotient and PCP content (Fig. 6b). The PCA graphically discriminated soil samples in according to different treatments and sampling time (T0 and T30) (Fig. 6a). Compost based treatment enhanced the PC2 score and the better performance of FC especially with the support of microbial inoculum was confirmed. The control soil S had obviously the greatest PC1 score and any biostimulation and/or bioaugmentation treatments were able to recover the origin soil properties.

4. Conclusion

The PCP contamination of forest soil induced the depletion of the microbial biomass C and some enzymatic activities such as

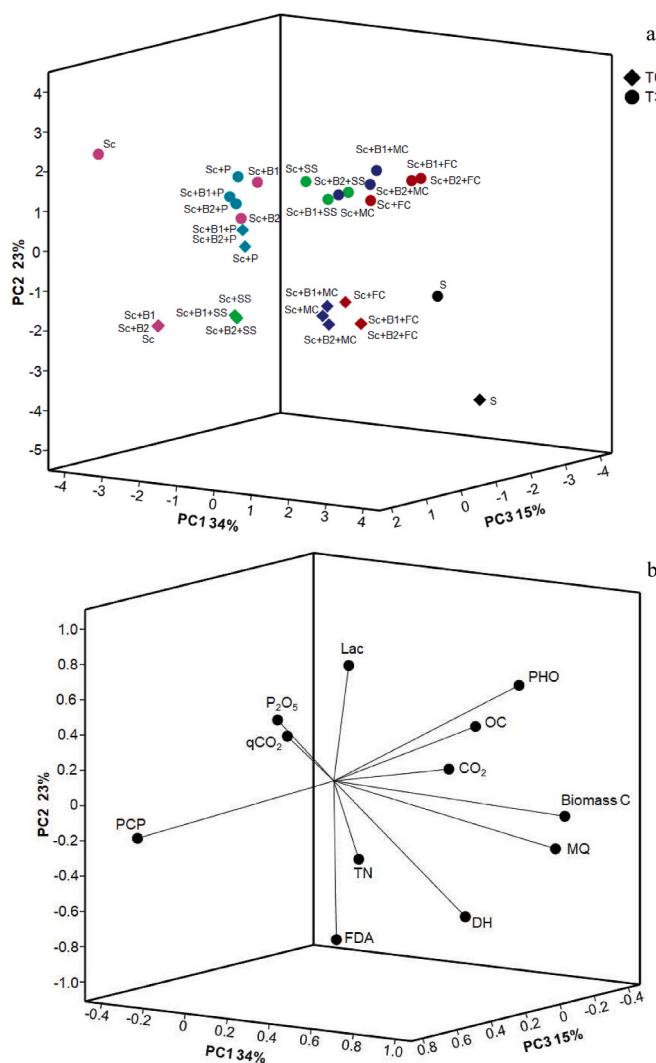


Fig. 6. Principal component analysis of PCP contaminated forest soil microcosms during biostimulation and bioaugmentation processes at T0 and T30: a) projection of the cases and b) projection of the variables.

dehydrogenase and phosphatase. Biostimulation processes based on the addition of forest and municipal waste compost as well as sewage sludge have proven to be very effective in reducing the PCP contamination in soil. Possible degradation and/or immobilization of PCP molecules occurred. Conversely, bioaugmentation process for PCP contaminated forest soil, depending on microbial consortia, seemed not sufficient to control this pollutant. Nevertheless, the simultaneous application of biostimulation and bioaugmentation revealed a better performance in the PCP removal highlighting a more effectiveness of this combined technique.

Although numerous positive effects of organic amendments are well known and they are able to achieve interestingly the remediation purpose alone, the use and effective application of bioaugmentation and biostimulation could be even more performant. Further efforts should focus on expanding the research regarding soil-microbe-amendment-contaminant interactions to understand better the capability of indigenous and exogenous microorganisms under different environmental conditions.

Author contributions statement

Rim Werheni Ammeri: Writing – original draft, Formal analysis, Methodology, Investigation, Validation. **Giuseppe Di Rauso Simeone:**

Writing – review & editing, Methodology, Formal analysis, Visualization, Investigation, Validation. **Yassine Hidri:** Formal analysis. **Mohamed Salah Abassi:** Formal analysis, Project administration. **Ines Mehri:** Formal analysis. **Sara Costa:** Investigation, Formal analysis. **Abdennaceur Hassen:** Conceptualization, Resources, Writing – review & editing, Project administration, Funding acquisition. **Maria A. Rao:** Writing – review & editing, Conceptualization, Methodology, Validation, Resources, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors would like to thank the Department of Agricultural Sciences, University of Naples Federico II, hosting Mrs. Rim Werheni. This research was funded by the Tunisian Ministry of Higher Education and Scientific Research and the Centre des Recherches et des Technologies des Eaux (CERTE).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2021.133359>.

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