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Microcosm Experiment to Assess the Capacity of a Poplar Clone to Grow in a PCB-Contaminated Soil

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Abstract: Polychlorinated byphenyls (PCBs) are a class of Persistent Organic Pollutants extremely hard to remove from soil. The use of plants to promote the degradation of PCBs, thanks to synergic interactions between roots and the natural soil microorganisms in the rhizosphere, has been proved to constitute an effective and environmentally friendly remediation technique. Preliminary microcosm experiments were conducted in a greenhouse for 12 months to evaluate the capacity of the Monviso hybrid poplar clone, a model plant for phytoremediation, to grow in a low quality and PCB-contaminated soil in order to assess if this clone could be subsequently used in a field experiment. For this purpose, three different soil conditions (Microbiologically Active, Pre-sterilized and Hypoxic soils) were set up in order to assess the capacity of this clone to grow in the polluted soil in these different conditions and support the soil microbial community activity. The growth and physiology (chlorophyll content, chlorophyll fluorescence, ascorbate, phenolic compounds and flavonoid contents) of the poplar were determined. Moreover, chemical analyses were performed to assess the concentrations of PCB indicators in soil and plant roots. Finally, the microbial community was evaluated in terms of total abundance and activity under the different experimental conditions. Results showed that the poplar clone was able to grow efficiently in the contaminated soil and to promote microbial transformations of PCBs. Plants grown in the hypoxic condition promoted the formation of a higher number of higher-chlorinated PCBs and accumulated lower PCBs in their roots. However, plants in this condition showed a higher stress level than the other microcosms, producing higher amounts of phenolic, flavonoid and ascorbate contents, as a defence mechanism.

Keywords: natural-based remediation strategies; Monviso clone; plant physiology; antioxidant defence; soil microbial communities

1. Introduction

Polychlorinated byphenyls (PCBs) are a class of Persistent Organic Pollutants (POPs) differing in the number of chlorine atoms attached to their biphenyl rings. Their characteristics (high molecular stability, low solubility in water and high tendency to adsorb to the particulate phase) make PCBs particularly hard to eliminate from different matrices like soils and sediments. Owing to their widespread use in industry in the past and their persistence, and although they have been banned in

several countries since 1979, there is still much environmental contamination. Their elimination from contaminated areas is therefore a challenge [1].

Natural restoration strategies are preferred since they use existing flows of energy and matter, take advantage of local solutions and follow seasonal and climatic changes in ecosystems [2,3]. Among natural-based remediation strategies, the use of plants to promote PCB degradation in the rhizosphere (plant assisted bioremediation, [4]) can be an effective, cost-competitive and environmentally friendly alternative to the most traditional remediation techniques [5].

Despite the high chemical stability and low bioavailability of PCBs, they can potentially undergo biological degradation. The latter involves bacteria, fungi and plants, and can occur differently in aerobic and anaerobic conditions. Anaerobic reductive dechlorination happens when PCBs serve as electron acceptors, thus being turned into less chlorinated congeners, and aerobic transformation involves the lower-halogenated congeners (<5 Cl) and leads to the breakdown of the biphenyl structure [6]. Numerous studies have in this context shown an increase in degradation of PCBs, involving mostly the low-halogenated congeners in vegetated soil as compared with non-vegetated soil [1,7–9]; this is the so-called plant-assisted bioremediation [4]. In the rhizosphere, the plant-microorganism association can increase the degradation of PCBs due to synergic exchanges between the natural soil microbial community and roots [9–12]. In fact, plant roots provide a large surface on which microbial cells can increase in number and be helped to spread through the soil. Some secondary plant compounds exudated by roots can have several functions [13], including acting as growth substrates and/or chemical signals, helping the bacterial enzymes involved in the degradation of PCBs [7,14,15] and promoting the growth of PCB-degrading bacteria [16,17]. Roots can also favour degradation of these contaminants by increasing the permeability of soil and oxygen transfer [18].

Poplar is a model plant for phytoremediation. In fact, it has a fast growth rate and a root system that is able to grow in a wide area, and it is capable of growing in nutrient-poor soil and resisting high concentrations of metal in soil [19,20]. It is well known that plant root exudates facilitate soil microbe activity by providing carbon and nitrogen sources and promoting the growth of PCB-degrading bacteria [16,17]. Various studies involving PCBs and poplar have been undertaken in microcosm studies [11,21–23] and very few have been performed as field studies of historically contaminated soils with PCBs. In the studies, little attention has been paid to poplar growth, physiology and biochemistry during the phytoremediation process. The selection of plant species for remediation purposes has to take into consideration not only the success of previous studies about the same clone, but also the site-specific conditions, which can influence the effectiveness of the strategy. Plant species can adapt to a specific environment and/or respond to some soil threats by using different strategies, including ecophysiological, structural and biochemical responses Moreover, some soil threats such as contamination, nutrient deficiencies, flooding and warming may alter plant morphology, physiology and biochemistry [24–26]. For instance, it is known that hypoxic conditions may lead to a decrease in photosynthetic performance [27–30] and that the extent of this decrease depends on a species' tolerance to soil hypoxia. Trees with a high level of tolerance can maintain photosynthetic rates at relatively high levels. On the other hand, the CO_2 assimilation rates of less tolerant species are strongly reduced [30–32].

On the other hand, though each of the various stress conditions raises different physiological and biochemical plant responses such as stomatal closure (drought) [33], photo-inhibition (high light) [34] or induction of ethanolic fermentation (hypoxia) [35], all of them can lead to an accumulation of Reactive Oxygen Species (ROS) [36]. ROS act normally as signalling molecules [37,38] involved in growth regulation, development and responses to environmental stimuli. Indeed, ROS signalling can lead to plant adaptation to stress through the activation of acclimation pathways [39–42]. However, ROS can also damage cellular components when they overwhelm antioxidant defence mechanisms [43]. The redox equilibrium and its capacity to scavenge ROS thus have a key role in the normal development of a plant and for perception, signalling and acclimation to stress [42]. To maintain balanced ROS levels under stress, a common response of plants is the activation of the enzymatic and non-enzymatic

antioxidant system. Non-enzymatic antioxidants comprise ascorbic acid, reduced glutathione, alpha-tocopherol, carotenoids, phenolic compounds and flavonoids, a particular group of phenolic compounds widely distributed in plants [44,45].

This study aimed at evaluating the capacity of the Monviso poplar hybrid clone (*Populus generosa* A. Henry × *P. nigra* L.) [46] to grow in a low-quality soil sampled from a site where different kinds of waste, including dielectric fluids (containing PCBs), were present. Three different soil conditions (microbiologically active, pre-sterilized and hypoxic) were set up in a greenhouse experiment in order to assess the poplar capacity to sustain degradative microbial activity under these soil conditions. The results of this experiment were useful for the subsequent application of a phytoremediation strategy using the Monviso clone in an area chronically contaminated by PCBs.

2. Material and Methods

2.1. Soil Collection from the Historically Contaminated Area and Characterization

The soil was sampled from an area close to the city of Taranto (southern Italy). The sampling site was used for several decades (about 40 years) as an improper dump for dielectric fluids (oil containing polychlorinated biphenyls) and different kind of waste. The latter have accumulated above the original limestone soil with the result that the soil consisted of inhomogeneous materials and was unsuitable for plant growth. A previous analysis by the local environmental agency found a heterogeneous contamination by PCBs and their concentrations exceeded the national legal limits (60 ng/g) for garden, parks and residential areas in numerous soil samples [47].

Equal aliquots of surface soil (0–20 cm) were collected from three different contaminated places in order to obtain a composite sample. After the removal of stones and other residues, the soil samples were air-dried (room temperature) and sieved (2 mm). The soil was classified under the USDA soil classification system as a sandy loam (sand 58%, silt 27%, clay 15%). It had a mildly alkaline pH (about 8), with a total organic carbon content of 14.94 g/Kg, and total nitrogen content of 0.2 g/Kg.

2.2. Microcosm Experimental Design

Aliquots of the composite soil were used to fill 16 microcosms (pots, 3 L capacity). The experimental set comprised three different conditions:

- Microbiologically active soil (MA): Historically polluted soil where a poplar cutting was planted.
- Pre-sterilized soil (Pre-sterilized): Historically polluted soil previously sterilized by autoclaving it (at 121 °C, 20 min), where a poplar cutting was subsequently planted;
- Microbiologically active soil under hypoxic conditions (Hypoxic): Historically polluted soil where a poplar cutting was planted; then each pot was submerged in water for all the experimental period. This treatment was intended to limit the oxygen concentration in the soil in order to reproduce a hypoxic environment for promoting the transformation of higher-chlorinated PCBs.

Un-planted soil microcosms were used as controls (Control).

Each condition was performed in four replicates. Poplars were planted as 20 cm long unrooted cuttings of the Monviso clone (*Populus generosa* A. Henry × *P. nigra* L.), supplied by Alasia Franco Vivai (Savigliano, CN, Italy). All microcosms were maintained in a greenhouse under natural light and at an environmental temperature for more than 12 months (364 days).

The MA, Pre-sterilized and Control microcosms were regularly watered, and the soil water content was maintained at approximately 65% of its field capacity throughout the experiment.

2.3. Sampling of Soil and Plant for Various Analysis

The microcosms were sampled at 6 and 12 months after the experimental setup. At each sampling time, two replicates were sacrificed for each condition (MA, Pre-sterilized and Hypoxic).

From each microcosm, the soil sampled was homogenized and divided into two portions. One was immediately used for the microbial analysis (total microbial abundance and dehydrogenase activity), and the other one was stored at −20 °C for the subsequent PCB analysis.

Roots were also sampled and lyophilized for PCB analysis at 6 and 12 months. A preliminary step was performed by washing the roots to eliminate soil particles attached to them. Firstly, each root was manually shaken down for 10 min, washed (0.9% NaCl) and finally rinsed quickly under running water in a sieve [48].

Plant biomass (roots, leaves, branches) was also assessed in the various conditions at 6 and 12 months. Finally, total phenolic compounds, flavonoids and ascorbate were analysed in the leaves (when present), stems and roots of each poplar tree at the end of the experiment.

Each chemical, biochemical or microbiological analysis was performed in at least three replicates from the same microcosm. Each datum presented is the average of six values.

2.3.1. PCB Markers in Soil and Roots

The PCB congeners analysed were those of the PCB markers (28, 52, 101, 153, 138 and 180). The latter are commonly analysed in environmental studies because they are the most frequently found and are PCB pattern indicators in various sample types [49]. Each congener was named in accordance with the IUPAC numbering system. The PCB analysis was performed using the EPA method 1668 [50].

The PCB extraction from soil and roots was achieved using an ASE 200 (accelerated solvent extraction), as reported in Technical Note 210 (Thermo Fisher Scientific, MA USA). This technique makes it possible to remove interferences due to the sample matrix using adsorbents, joining extraction and purification in a single stage. The extracts were analysed with the Finnigan TRACE GC ultra-chromatograph (Thermo Fisher Scientific, MA USA), coupled with a mass spectrometer in accordance with Muir and Sverko [51]. The detection limit for each PCB congener analysed was 0.5 µg/Kg dry soil. The quantification of the individual PCB congeners was performed with a $^{13}C_{12}$ internal standard multi-point calibration using six calibration standard solutions (P48-M, Wellington Laboratories) in three replicates, from 0.1 pg/µL to 5 ng/µL. The compounds were quantified using the ratio of the analyte and internal standard response (peak area). The instrument limit of quantification (LOQ, the concentration at which quantitative results can be reported with a high degree of confidence), was determined with an approach based on parameters from the analytical curve [52]. LOQ values for each indicator PCB congener are reported in Table 1.

Table 1. Limits of quantification for PCB marker congeners.

PCB Congener (IUPAC Number)	LOQ (pg)
28 (7012-37-5)	43.73
52 (35693-99-3)	25.46
101 (37680-73-2)	11.00
138 (35065-28-2)	29.65
153 (35065-27-1)	26.59
180 (35065-29-3)	27.97

2.3.2. Microbial Abundance and Dehydrogenase Activity

Soil samples from the various microcosms were analysed in order to assess the abundance and activity of the microbial community, evaluating their changing over time in the presence of poplar. Microbial abundances (No. cells/g soil) were determined by the epifluorescence direct count method, with DAPI (4′,6-diamidino-2-phenylindole) as the fluorescent dye [53]. For each analysis, 1 g of soil was put in a test tube with a filter-sterilized fixing solution, as previously described [54]. To detach microbial cells from soil particles, the test tube was shaken for 15 min (400 rpm), and the suspension was then left for 24 h. An aliquot of supernatant (100 µL) was put in contact (20–30 min) with a DAPI solution. The supernatant was then filtered through a 0.2 µm Nuclepore Polycarbonate Black Membrane Filter

(Whatman, Maidston, UK) which was subsequently mounted on a glass slides, and the microbial cells were counted with a Leica epifluorescence microscope (DM 4000B, Leica Microsystems GmbH, Wetzlar, Germany).

Soil dehydrogenase activity was used as a microbiological indicator for the overall activity of the microbial community and how it was influenced by the presence of poplar. Soil dehydrogenase activity was determined using the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) solution to triphenylformazan (TPF), measured in two replicates. 6 g of soil were collected and analysed as reported in Grenni et al. [55]. Soil dehydrogenase activity was expressed as μg TPF/g dry soil.

2.4. Analysis for Growth Monitoring, Plant Physiology and Plant Antioxidants

2.4.1. Growth Monitoring Measurements

During the vegetative growth, plant biomass was recorded and reported on a dry weight basis. Plants were carefully removed from the pots and washed with distilled water to remove any particles attached. Plant organs (roots, shoots, leaves) were then separated and dried at 60 °C in an air-forced oven and 72 h later their dry weights were determined [56]. The root biomass and the branch biomass of each plant were measured at 6 months (autumn) and at 12 months (spring) after planting. Leaf biomass was measured only at 12 months, due to a lack of leaves on the trees in late autumn (six months).

2.4.2. Plant Physiology Measurements

The physiological status of the poplar plants was assessed through their leaf chlorophyll content and fluorescence measurements. A Minolta chlorophyll meter (SPAD) was used to estimate the leaf chlorophyll content as previously described [57]. The following equation was used to convert the SPAD measurement into chlorophyll content.

$$Chlorophyll\ content\left[\frac{\mu g}{cm^{-2}}\right] = \frac{99 \times SPAD}{144 - SPAD}$$

On the same leaves chosen for SPAD readings, the chlorophyll fluorescence transient (OJIP transients) was measured using a plant efficiency analyser (PEA, Hansatech Instruments Ltd., King's Lynn, UK) as reported in Pietrini et al. [58].

The chlorophyll content and fluorescence were measured on five leaves per poplar all over the tree at 4 and 12 months, when there were leaves.

2.4.3. Plant Antioxidants

Phenolics, flavonoids and the ascorbate content were used as indicators of plant antioxidant status for evaluating any stress caused by treatments. Total phenolic compounds, flavonoids and ascorbate were analysed (three replicates) in leaves, stems and roots for each poplar tree at six months and the end of the experiment.

The extraction of total phenolic compounds and flavonoids was performed from 200 mg of plant material with 80% methanol (1.5 mL) for 3 min in an ultrasonic bath. The extraction was repeated twice. The amount of extracted total phenolic compounds was determined with the Folin–Ciocalteu reagent [59]. Each analysis was performed in duplicate for each extract. The gallic acid was used as the standard and the total phenolic compounds were expressed as mg of gallic acid equivalents (GAE) per g of fresh weight.

Total flavonoid content was measured using the aluminium chloride method described by Chang et al. [60]. The absorbance was read at a 415 nm wavelength. Analysis was done in triplicate for each extract. Standard solutions of quercetin were used to obtain a standard curve. The total flavonoid content was reported as mg of total quercetin equivalents per g of fresh weight.

Ascorbate was extracted from the plant tissue (about 100 mg fresh weight) in 1.5 mL 3% perchloric acid, and the mixture was centrifuged (5000 rpm, for 20 min) at 4 °C. The reduced ascorbate (ASC) and

oxidized ascorbate (DHA) measurements are based on the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid in acidic solution. Fe^{2+} forms complexes with bipyridyl that absorb at 525 nm. Sample pre-incubation with dithiothreitol (DTT) reduced DHA to ASC. The excess DTT was removed with N-ethylmaleimide, and the total ascorbate was determined. The amount of DHA was calculated by subtracting ASC from total ascorbate. The contents were calculated using a standard curve [61]. The ascorbate ratio was then calculated as the proportion between reduced ascorbate and total ascorbate and expressed as [reduced-/total-ascorbate].

2.5. Statistical Analysis

Analysis of variance (one-way analysis of variance) was used to assess the significant differences among treatments in PCB concentration, dehydrogenase activity, total microbial abundance in soil samples and antioxidant content in root, leaf and branch samples. The PC Program used was SIGMASTAT 3.1 software (Systat Software Inc., Point Richmond, CA, USA). The significance level of 0.05 was utilized to indicate whether the treatments were significantly different from each other and from the control. A multiple comparison procedure (Dunn's method) was used in order to isolate the group or groups that differed from the others. Finally, the Post-hoc test was performed on plant antioxidant results.

3. Results

3.1. Soil PCB Concentration

At the start of the experiment (experimental set-up), the sum of the PCB markers analysed (PCB 28, 52, 101, 153, 138, 180) was 47.6 ± 2.5 µg/Kg.

Figure 1 shows photos of the Monviso clone at three and six months. The concentrations of PCB markers in the various soil microcosms at 6 months and compared to the control soil are reported in Figure 2A. A general decrease in PCB concentration, with the exception of PCB 180, was observed in all the plant-treated microcosms. This reduction was significant ($p < 0.05$) for PCBs 101 (2,2′,4,5,5′-Pentachlorobiphenyl), 138 (2,2′,3,4,4′,5′-Hexachlorobiphenyl) and 153 (2,2′,4,4′,5,5′-Hexachlorobiphenyl) in the microbiologically active conditions (MA), with decrease percentages ranging from 20 to 64%.

Figure 1. Photos of the Monviso clone plants. (**A**): Poplars at three months. (**B**,**C**): Pictures of the roots at six months.

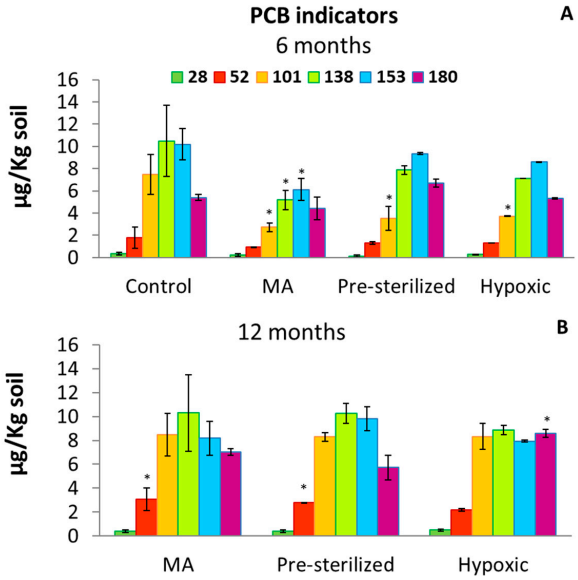

Figure 2. PCB markers (28, 52, 101, 138, 153 and 180) in the various soil microcosms at 6 months (**A**) and at 12 months (**B**). MA: Microbiologically active soil; pre-sterilized: Soil previously sterilized by autoclaving it; hypoxic: Microbiologically active soil microcosms immerged in water; control: Microbiologically active soil, un-planted. Significant differences ($p < 0.01$) among the different sampling times are indicated with an asterisk.

The overall concentrations of PCBs analysed at 12 months increased in all conditions as compared to 6 months, with differences in some congeners (Figure 2B). The low-chlorinated PCB 52 was higher in the MA and Pre-sterilized and the high-chlorinated congener 180 in the hypoxic microcosms ($p < 0.05$).

3.2. PCB Concentrations in Roots

The PCB markers detected in the roots of the planted poplars (MA, Pre-sterilized and Hypoxic) at 6 and 12 months are shown in Figure 3. At month 6, PCB concentrations in roots ranged from 1.31 µg/Kg (PCB 28, Hypoxic) to 89.5 µg/Kg (PCB 153, MA), (Figure 3A). The average values of PCBs in the MA microcosms were significantly higher ($p < 0.05$) than in the other conditions.

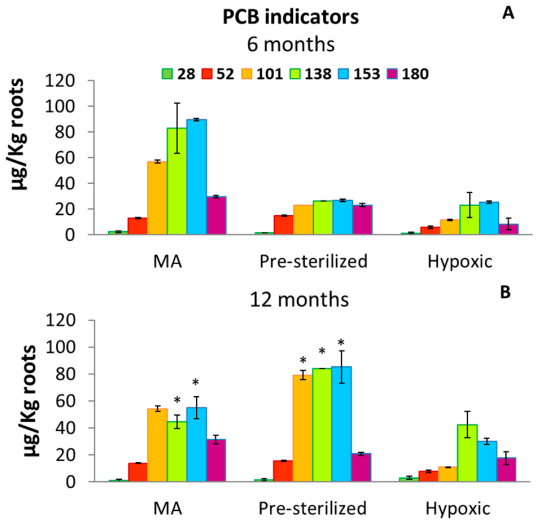

Figure 3. PCB markers in plant roots at 6 months (**A**) and at 12 months (**B**). MA: Microbiologically active soil; hypoxic: Microbiologically active soil under hypoxic conditions; pre-sterilized: Soil previously sterilized by autoclaving it. Significant differences ($p < 0.01$) between 6 and 12 months for the same PCB congener are indicated with an asterisk (*).

At 12 months (Figure 3B), PCBs were found in poplar roots even in the Pre-sterilized condition. In the Hypoxic microcosms, PCBs remained relatively low, with the highest values for the higher-chlorinated congeners (PCB 138, 153 and 180).

3.3. Microbiological Analysis

A significant increase ($p < 0.01$) in microbial abundance (No. cells/g soil) was observed from day 0 to 6 months in the plant presence (MA, Pre-sterilized and Hypoxic soils); at the end of the experiment, although the average values in all planted microcosms were higher than in the control ones, the number of cells decreased (Figure 4).

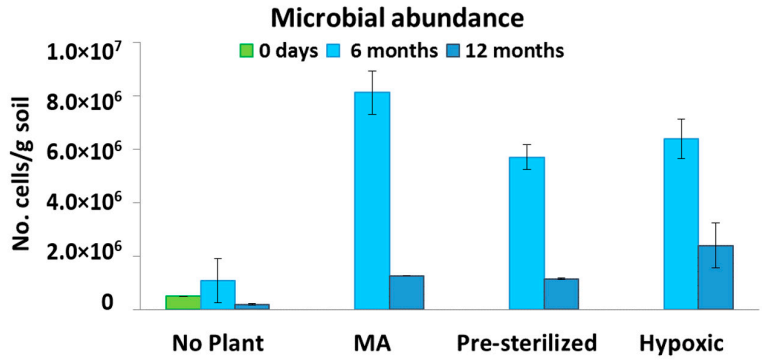

Figure 4. Microbial abundance (No. cells/g soil) in the various conditions. Control: Microbiologically active soil, un-planted; MA: Microbiologically active soil; pre-sterilized: Soil previously sterilized by autoclaving it; hypoxic: Microbiologically active soil under hypoxic conditions.

The average values of dehydrogenase activity were higher ($p < 0.01$) in the planted soils than in the Control at 6 months; the highest value of DHA was observed in the Pre-sterilized soil (Figure 5). At the end of the experiment, the lowest value was found in the hypoxic condition.

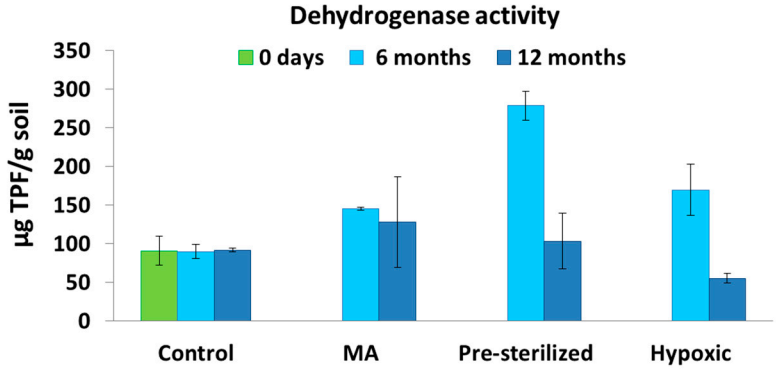

Figure 5. Dehydrogenase activity (µg TPF/g soil) in the various conditions in the three sample times. Control: Microbiologically active soil, un-planted; MA: Microbiologically active soil; pre-sterilized: Soil previously sterilized by autoclaving it; hypoxic: Microbiologically active soil under hypoxic conditions.

3.4. Plant Growth and Physiology

The plant biomass (root, leaves and branches) in the various conditions (MA, Hypoxic, Pre-sterilized) at 6 and 12 months is reported in Table 2.

At 6 months, the lowest root and branch biomass was observed in the MA condition; the differences in root and branch biomass were significant ($p < 0.05$) between the MA condition and the Pre-sterilized one. The branch biomass was significantly lower ($p < 0.05$) in plants from MA than in those from the hypoxic condition. However, these differences were never observed in the subsequent sampling (12 months).

Table 2. Plant biomass (roots, leaves, branches, ±standard deviations) in the various conditions at 6 and 12 months. MA: Microbiologically active soil; pre-sterilized: Soil previously sterilized by autoclaving it; hypoxic: Microbiologically active soil under hypoxic conditions. Letters (a, b) indicate significant differences ($p < 0.05$).

Conditions	Roots (g)	Leaves (g)	Branches (g)
	6 months		
MA	1.31 a ± 1.11	—	5.42 a ± 1.95
Pre-sterilized	4.55 b ± 1.30	—	11.98 b ± 1.84
Hypoxic	3.35 ab ± 2.46	—	11.00 b ± 0.27
	12 months		
MA	5.14 a ± 0.03	5.77 a ± 2.26	8.35 a ± 0.03
Pre-sterilized	7.64 b ± 2.06	8.28 a ± 1.25	8.02 a ± 0.37
Hypoxic	5.40 ab ± 1.34	7.53 a ± 1.00	10.34 a ± 1.61

Maximum quantum yield of PSII (Fv/Fm) and leaf chlorophyll content were measured at 4 and 12 months (Table 3). Fluorescence analysis showed Fv/Fm indices ranging between 0.76 and 0.82. The highest values were observed in the Hypoxic and Pre-sterilized conditions although with no significant differences among treatments and sampling times (Table 3).

Table 3. Maximum quantum yield of PSII (Fv/Fm) and leaf chlorophyll content (±s.d.). MA: Microbiologically active soil; hypoxic: Microbiologically active soil under hypoxic condition; pre-sterilized: Soil previously sterilized by autoclaving it. Letters (a, b) indicate significant differences ($p < 0.05$).

Treatment	Fv/Fm	Chlorophyll (μg/cm^2)
	4 months	
MA	0.76 a ± 0.11	37.40 a ± 5.76
Hypoxic	0.80 a ± 0.04	28.93 b ± 2.98
Pre-sterilized	0.79 a ± 0.08	27.97 b ± 3.96
	12 months	
MA	0.80 a ± 0.06	31.88 a ± 2.41
Hypoxic	0.82 a ± 0.05	32.90 a ± 4.70
Pre-sterilized	0.82 a ± 0.11	31.59 a ± 5.68

The average values of leaf chlorophyll ranged between 27.97 and 37.40 μg/cm^2 (Table 3). The highest value ($p \leq 0.001$) was found for the MA condition four months after planting. However, at 12 months after planting, no significant differences were found for chlorophyll contents among the different treatments.

3.5. Plant Antioxidants

In general, the phenolic compounds, flavonoids and total ascorbate were significantly higher in leaves than in roots and shoots ($p < 0.001$) (Figure 6A–D).

Overall values for plant antioxidants were higher in the Hypoxic condition than in the MA and Pre-sterilized ones and these values were significantly higher ($p < 0.05$) at 12 months. It is also noticeable that whereas at 6 months the phenolics and flavonoids values were significantly ($p < 0.05$) lower in plants from the Pre-sterilized treatment than in plants from the MA and Hypoxic treatments, at 12 months these differences were never observed.

In leaves at 12 months the phenolic compound content (Figure 6A) was about 1.3-fold higher in the Hypoxic than in the MA and Pre-sterilized conditions. Leaf flavonoid content (Figure 6B) was also 1.8–2.0-fold higher in the Hypoxic than in the MA and pre-sterilized microcosms, respectively. Moreover, the total ascorbate (Figure 6C) was higher at 12 months in leaves and shoots, with significantly higher values for leaves compared to other plant parts.

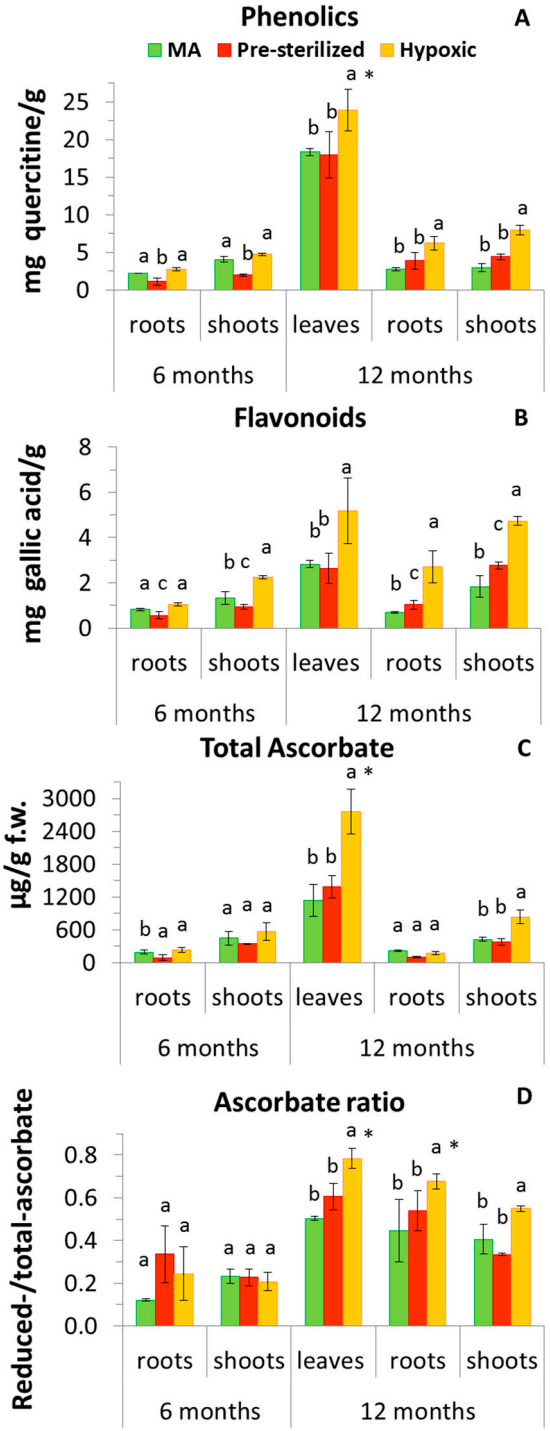

Figure 6. Plant antioxidants found in roots, shoots and leaves at 6 months and at 12 months. (**A**) Phenolics (mg gallic acid/g); (**B**) Flavonoids (mg quercetin/g biomass); (**C**) Total Ascorbate (µg/g fresh weight); (**D**) Ascorbate ratio expressed as [reduced-/total-ascorbate]; MA: Microbiologically active soil; hypoxic: Microbiologically active soil under hypoxic condition; pre-sterilized: Soil previously sterilized by autoclaving it. Letters (a, b) indicate significant differences among treatments ($p < 0.05$). Asterisks (*) indicate significant differences among plant parts ($p < 0.001$).

Finally, the ascorbate ratio (Figure 6D) was generally higher in plants grown under hypoxic soil conditions than in any other treatment in all plant materials (Figure 6D) and these differences were statistically significant for leaves and shoots ($p < 0.05$) at 12 months.

4. Discussion

Poplar trees have been found to be tolerant to several contaminants [62,63] and thanks to their high transpiration, deep root system and fast growth rate, are considered suitable for phytoremediation of both organic and inorganic contaminants [21,64]. For this reason, we tested the capacity of the Monviso clone to grow in a chronically contaminated soil, analysing its physiological response, and to stimulate microbial activity, which can improve PCB transformations.

In our experiment, only after 6 months did the plant promote a general decrease in the soil in concentrations of the PCBs analysed, and this was particularly evident in the microbiologically active condition (MA), (Figure 2A). A synergic effect between plant roots and the natural microbial community of soil was also found. In fact, an increase in the cell number and activity was observed (Figures 4 and 5). Positive effects of the plant and rhizosphere soil microbial community in improving the quality of a PCB contaminated soil were found in a recent work using the Alfalfa plant [48]. In the rhizosphere, plant metabolites can act as chemical signals for inducing the microbial enzymes involved in PCB degradation [65–67]. In return, PCB-degrading bacteria can produce plant growth stimulators [68,69]. At 12 months a general increase in PCB markers was observed in all conditions (Figure 2B). This result could be presumably ascribed to the microbial-root association effect of stimulating the desorption of some non-analysed PCBs, which were attached to the historically contaminated soil, favouring their transformation to the six PCB markers analysed in this work (i.e., 28, 52, 101, 138, 153 and 180), as also found by other authors [6,11,21,70]. Another possibility could be the transformation of higher chlorinated PCBs to the PCB markers analysed, as also found in other studies [1,9]

The quantity of PCB markers detected in the roots (Figure 3) was ascribable to the wide growth of the root system (Figure 1, Table 2), which filled the pots and acts as a strong bioconcentrator. Plant exudates can act as surfactants, forming hydrophilic complexes with PCBs and making possible their transportation into roots [71]. PCBs are not expected to enter the transpiration stream due to their high hydrophobicity and other studies have shown that they were not found in the upper part of the plant [72].

It is interesting to note that when the highest PCB concentrations were found in roots (MA at 6 months, Figure 3A), the root and branch biomasses were significantly the lowest (Table 2).

Overall, the results suggest that, in association with the natural microbial community of soil (MA), this poplar clone was well adapted to the PCBs and the microbial populations responded differently at 6 and 12 months. In fact, they initially (6 months) increased in number with the decrease of the six PCB markers; however, they maintained their general activity, but diminished in number when the PCBs increased again (12 months).

The pre-sterilized soil condition was set-up to evaluate how the natural microbial community of this historically contaminated soil had an influence on the plant physiology of this clone. In this condition the poplar cuttings were planted in a soil where the microbial community was not initially present (Figure 4). However, the microbial populations which survived the sterilization treatment (i.e., those able to produce resistant cysts) and derived from the environment developed abundantly, as expected in a non-colonized substratum (Figures 4 and 6) and found in other studies using pre-sterilised soils [55,73,74]. The overall Pre-sterilized results showed that, although more active than in the other conditions, the microbial community colonizing the rhizosphere in the first 6 months (Figure 6) was initially able to stimulate more plant growth than PCB transformation. However, in the subsequent sampling (12 months) the overall increase in PCB markers and the microbial abundance, activity and plant growth and physiology values were comparable to MA, suggesting that the interactions established over time between bacterial populations and the rhizosphere were similar in both treatments. In fact, the roots were able to concentrate PCB markers in the rhizosphere in an amount comparable to those observed in MA at 6 months.

The hypoxic condition was set up for assessing if some PCB congener degradation could be favoured in an anaerobic situation. In this case, each poplar cutting was planted in a soil immersed in water for all the experimental period. The analysis of the six PCB markers at month 6 showed

that their overall decrease was lower than in the MA condition; moreover, at month 12 higher amounts (e.g., of the high-chlorinated 180) were found than in the MA and Pre-sterilized conditions. The hypoxic environmental condition therefore presumably promoted PCB transformations that were more chlorinated (>7 Cl) than the 180 congener and were transformed to some less-chlorinated PCBs, including the 180 one, as also found by other authors [75,76]. In this condition, a marked difference was also found in the PCB markers detected in the roots (less than the other conditions) and in the antioxidants produced by the plant, which were significantly higher than in the other conditions, showing that in this case the plant was more stressed.

The different experimental conditions (natural soil, Pre-sterilized, Hypoxic condition) influenced the development of different microbial populations in the rhizosphere and these in turn affected the plant growth and stress response. For example, in the MA plants as compared to the others a less plant growth occurred. Chekol et al. [8] affirmed that different effects of PCBs on plant biomass can be related to a different sensitivity to PCBs of the root-microorganisms system established in the rhizosphere. For example, there are microorganisms that promote plant growth through the action of 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme can cleave the plant ethylene precursor ACC, and thereby lower the ethylene levels of stressed plants [77]. However, at 12 months, the upper part of plant biomass under the three different treatments was similar, despite the higher PCB accumulation found in roots in the MA and Pre-sterilized soils. This result confirms the adaptation of both rhizosphere microorganisms and this poplar clone to a PCB presence. Other studies considering other plant species have shown no significant differences in plant biomass between plants grown in a control soil and in PCB contaminated soil [8,78].

The photosynthetic performance of the poplar plants was measured in terms of leaf chlorophyll content and chlorophyll fluorescence measurements. Photosynthesis is a pivotal process for plant growth and biomass production, and it has thus often been used as a stress bioindicator [79]. Moreover, it has been shown that, the photosynthetic apparatus is sensitive to different soil conditions, such as hypoxia, soil drought and high soil temperature. For instance, hypoxia can alter the maximum quantum yield of PSII [80], as well as chlorophyll synthesis [81]. Chlorophyll (Chl) content and Chl fluorescence values for the MA treatment turned out to be similar to other values reported for Monviso poplar plants [82], indicating that PCBs did not alter these parameters (and did not cause any severe stress to the poplar plants). However, both the Hypoxic and Pre-sterilized conditions induced a reduction of Chl content in poplar plants 4 months after planting, though leaf chlorosis did not lead to a decrease in maximum quantum yield of PSII photochemistry (Fv/Fm) in hypoxic plants.

The overall results show that, although the soil pre-sterilization and the hypoxic conditions did not promote a general decrease in the soil of the PCBs analysed and instead promoted a general increase in plant oxidants (in particular for the hypoxic one), these conditions did not negatively influence the plant biomass or the plant physiology measurements, highlighting a good adaptability by this clone. The clone tested was therefore able to grow in a low-quality soil, contaminated by PCBs, and to promote the transformation of these contaminants in the presence of the natural soil microbial community. The sterilization and the hypoxic condition acted as selective forces on the soil microbial community and for this reason they were less efficient in PCB transformation.

This paper reports for the first-time data regarding the poplar physiology in a hypoxic condition for its possible application for bioremediation purposes. Watering the soil can be an occasional strategy to improve the anaerobic degradation of PCBs. We cannot exclude a subsequent long-term decrease in PCBs in this historically contaminated soil, as reported in recent review papers [1,9]. However, knowledge of plant-microbe interactions in PCB degradation is far from complete and further studies are necessary to better investigate the chemical dialogue between plant and microbes in the rhizosphere. In any case, thanks to this preliminary greenhouse experiment, a phyto-assisted bioremediation strategy with the Monviso clone has been subsequently performed in the field and is currently in progress.

5. Conclusions

The Monviso clone was able to grow in the low-quality soil studied and to significantly improve microbial activity and promoted an overall PCB transformation (an overall decrease in PCB marker concentration at 6 months and increase at 12 months).

Further research is needed to better understand the metabolic degradation pathways of PCBs in the rhizosphere. In particular, the analysis of a higher number of important congeners, the molecular feedback mechanisms that cause the transformation of PCBs in the rhizosphere and the specific bacterial populations involved, is currently in progress.

This preliminary research was very useful in encouraging us to plant the Monviso poplar cuttings in a field where the soil quality was very low and made it possible to have some information on the physiology of this clone under different conditions. Thanks to this experiment, a phyto-assisted bioremediation strategy using this clone Monviso has been subsequently performed at this experimental site and is currently in progress.

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