Title: Immobilization of metribuzin degrading bacterial consortium MB3R on biochar enhances bioremediation of potato vegetated soil and restores bacterial community structure

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Abstract

Metribuzin (MB) is a triazinone herbicide used for the eradication of weeds in agriculture. Presence of its residues in agricultural soil can potentially harm the establishment of subsequent crops and structure of soil microbial populations. In this study, remediation potential of an MB degrading bacterial consortium MB3R immobilized on biochar was evaluated in potato vegetated soil. In potato vegetated soil augmented with MB3R alone and MB3R immobilized on biochar, 82 and 96% MB degradation was recorded respectively as compared to only 29.3% in un-augmented soil. Kinetic parameters revealed that MB3R immobilized biochar is highly proficient as indicated by significant increase in the rate of biodegradation and decrease in half-life of MB. Enhanced plant growth was observed when augmented with bacterial consortium either alone or immobilized on biochar. Presence of herbicide negatively affected the soil bacterial community structure. However, MB3R immobilized on biochar proved to be helpful for restoration of soil bacterial community structure affected by MB. This is the very first report that reveals improved remediation of contaminated soil and restoration of soil bacterial populations by use of the MB degrading bacterial consortium immobilized on biochar.

Keywords: metribuzin; bacterial consortium, biochar; biodegradation; rhizosphere microbial communities

1 Introduction

Metribuzin (MB, 4-amino-6-tert-4, 5-dihydro-3-methylthio-1,2,4-triazin-5-one), a triazinone herbicide is applied extensively for control of weeds in crops like potato, sugarcane, maize and tomato [1]. In soil, MB can persist up to 120 days depending on the soil type and climatic conditions [2, 3]. MB residues easily seep to nearby water bodies because of its weak sorption to soil particles (K_{oc}, 53.13 /Kg and K_{ow} 1.70) and high-water solubility (1.05 g/L) causing contamination of surface as well as sub surface water [4, 5]. MB contamination of water bodies is causing concern regarding its long-term/low-dose effects on non-target species. [6] MB is widely reported as endocrine disrupting chemical [7, 8] which implies concerns regarding occurrence of MB residues in fresh water and accompanying public health issues. MB residues in agricultural soil potentially can pose negative effects on the emergence of some rotational crops as well as on soil microbial populations [9, 10]. Therefore, to address concerns regarding MB contamination of soil and water and to develop an approach that can remove metribuzin from agricultural soils efficiently and rapidly at point source i.e. in agricultural fields, that are the primary source of metribuzin pollution, is imperative.

Microbial technologies e.g. bioremediation and rhizoremediation are accepted as better options for the clean-up of polluted soils economically without causing secondary contamination [11]. Usually the ability of native microbes to degrade toxic contaminants is reduced with the increase in contaminants concentration [12]. To overcome this problem, the augmentation with exogenous pollutant degrading microbes is an applied approach [13, 14]. However, environmental conditions of contaminated soil [15], and the survival of exogenously applied bacteria [16, 17] are important factors in successful execution of rhizoremediation. The application of contaminant degrading bacteria by immobilizing onto any appropriate carrier material has been proposed for enhancing their survival and efficiency of bioremediation [18, 19]. Various carrier materials including chitosan, wood chips and wheat straw have been investigated in this regard [20, 21]. However, a novel material having high affinity for bacterial inocula and the contaminant in question is to be identified.

Biochar, prepared by pyrolysis of plant material is widely applied for improving soil quality, agricultural yield and mitigating key international problems related to climate change and environment [22-24]. Unique attributes of biochar like high surface area, internal porosity and capability to adsorb organic compounds and bacteria have increased its feasibility for use as

biocarrier [25]. To date, only few reports regarding its use in bioremediation are available [26, 27].

The present study was based on the hypothesis "Biochar being a porous structure with large surface area would be an important biocarrier of bacterial strains that harbor the catabolic capabilities to detoxify contaminating xenobiotic in soil. This immobilized bacterial-biochar inoculum can be exploited for bioremediation/ biodegradation of the respective contaminant's sites". The study aimed to explore the efficacy of bacterial consortium MB3R alone and after immobilization on biochar for the remediation of metribuzin in potato vegetated soil. Moreover, the effect of metribuzin and biochar/bacterial inoculum MB3R on soil microbial populations of potato rhizosphere was also assessed by 16S rRNA gene amplicons sequencing.

The state of the art in this study is based on the fact that application of biochar immobilized bacterial culture for remediation of herbicide contaminated vegetated soil with concomitant restoration of soil microbial communities is a new approach.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

The HPLC grade chemicals i.e., dichloromethane (DCM), acetonitrile and methanol were purchased from Sigma Aldrich (Germany). Technical grade metribuzin (97.6%) used in this study was provided by Tara Crop Sciences (Lahore, Pakistan). Analytical grade (99.9%) metribuzin was bought from Dr. Ehrenstorfer GmbH (Germany).

2.1.2 Bacterial strains and the consortium MB3R

The metribuzin degrading bacterial consortium MB3R comprising of *Rhodococcus rhodochrous* AQ1, *Bacillus tequilensis* AQ2, *Bacillus aryabhattai* AQ3 and *Bacillus safensis* AQ4 was developed at Biodegradation/Bioremediation lab, National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan earlier as reported by Wahla *et al.* [28]. The bacterial strains were stored in glycerol stocks at -80 °C and revived as and when required. Before the formation of bacterial consortium MB3R, the compatibility of all bacterial strains was checked by cross streak method [29]. All strains were found compatible with each other.

Bacterial strains were cultured in LB medium (pH 7.0) individually at 30°C overnight, centrifuged and resuspended in normal saline (0.85%) to set an OD_{590} at 1.00 with cell densities; AQ1, 7×10^7 CFU ml^{-1}; AQ2, 5 x 10^7 CFU ml^{-1}; AQ3, 5 x 10^7 CFU ml^{-1}, AQ4, 4 x 10^7 CFU ml^{-1}. Finally, these suspensions were mixed to form MB3R inoculum having pH 7.2 and 7 x 10^7 CFU ml^{-1}. The MB3R inoculum (2%) was used for soil inoculation studies to achieve final cell density of 1×10^5 CFU g^{-1} soil.

2.1.3 The consortium MB3R immobilized on biochar

The biochar (BC) prepared by pyrolysis of rice husk at high temperature i.e., 400°C in the absence of oxygen [30] was taken from Soil Fertility Lab, Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Pakistan. For immobilization of bacterial consortium MB3R on biochar, MB3R inoculum (section 2.2) was used. The biochar and MB3R inoculum were mixed in 5:100 (W/V) ratios and placed on a shaker at 150 rpm overnight [31]. To determine immobilization efficiency, the mixture of MB3R and biochar after overnight incubation was centrifuged at 1000 rpm for 10 min. The OD_{590} of supernatant was determined (n=3) and immobilization rate of MB3R on biochar was measured by the formula given below [32].

$$\% \text{ immobilization of MB3R on biochar} = (OD_0\text{-}OD_1)/ OD_0 \times 100 \quad \text{Eq. (1)}$$

OD_0 = initial OD_{590} of bacterial suspension; OD_1= OD_{590} of the supernatant

Immobilization efficiency of MB3R on biochar was calculated as 50%. The MB3R immobilized biochar pellet was stored at 4°C for further use in soil inoculation studies.

2.1.4 Collection and spiking of soil with metribuzin

Soil free of metribuzin residues was collected from a field located at wheat research fields, Ayub Agricultural Research Institute (AARI), Faisalabad (31.4504° N, 73.1350° E), Pakistan. After air drying and sieving, the soil was analysed for various physical and chemical properties (Table 1).

The soil was spiked with metribuzin (technical grade) by following the procedure described earlier [33] with little modifications. Briefly, sand was spiked and stirred thoroughly with MB solution (1% in acetonitrile) in a closed container. After the evaporation of solvent,

sand was added and mixed thoroughly into experimental soil to attain final MB concentration 2.5 mg kg^{-1} soil.

2.2 Experimental layout

Microcosm experiment was performed in potato vegetated soil during Nov-2016 to Feb-2017 to evaluate metribuzin remediation by the application of bacterial consortium MB3R alone and immobilized onto biochar separately. The experiment consisted of five treatments at initial metribuzin concentration of 2.5 mg kg^{-1} soil. The experiment was conducted in plastic pots (8″ x 6″) containing 2.5 Kg soil using complete randomized design (CRD). The experimental lay out was as follows:

1. Potato vegetated native soil (C)
2. Potato vegetated soil spiked with MB (P-UI)
3. Potato vegetated MB spiked soil treated with biochar (P-UI_BC)
4. Potato vegetated MB spiked soil treated with MB3R (P-I)
5. Potato vegetated MB spiked soil with MB3R immobilized on biochar (P-I_BC)

The potato seeds were obtained from Vegetable Research Institute, Ayub Agricultural Research Institute (AARI), Faisalabad, treated with 1% H_2O_2 and sown in pots (3 seeds/pot). The pots were augmented with 2% MB3R inoculum (section 2.1.2) where required. To assure equivalent inoculum in treatments 4 and 5, amount of biochar immobilized with MB3R required for augmentation was calculated (section 2.1.3). Based on this, 2 g kg^{-1} soil biochar immobilized with the consortium and same concentration of biochar alone were used in respective treatments.

Data related to root length (RL), shoot length (SL), root fresh mass (RFM), shoot fresh mass (SFM), root dry mass (RDM) and shoot dry mass (SDM) was recorded by harvesting plants after 30, 60 and 90 days of sowing. For the determination of residual MB, plant tissue (roots and shoots) samples and rhizospheric soil samples were also taken at same intervals after sowing.

To assess the effect of MB and bacterial consortium MB3R immobilized onto biochar on the soil bacteria, soil samples (n=3) were collected at the end of the experiment using a spatula within a 1-mm vicinity of the primary and lateral roots and stored at -80 °C until further processing.

2.3 Methods

2.3.1 Extraction of MB from plant tissues and soil

Dichloromethane (DCM) was used to extract of MB from soil and plant tissues [34]. Briefly, soil samples (20 g) were extracted with equal volume of DCM twice. For the extraction of MB from roots and shoots of potato plants, the plant tissues were washed with autoclaved water and crushed into paste with the help of pestle and mortar. This paste was extracted twice with equal volume of DCM. The extracts thus obtained were evaporated under nitrogen, dissolved in acetonitrile (1 ml) and filtered through 0.45 µm filter before analysis by HPLC.

2.3.2 HPLC analysis of residual MB

The DCM extracts were subjected to quantitative analysis of MB using Perkin Elmer HPLC coupled with diode array detector (DAD) at 280 nm. Acetonitrile: water (80:20) acidified with acetic acid was used as mobile phase on reverse-phase ODS2 C18 column with an isocratic flow rate of 1 mL min^{-1}. The retention time of the MB peaks was 3.12 min at 280 nm wavelength.

A standard curve was drawn by plotting peak area versus concentrations of MB analytical standards dissolved in acetonitrile at 1.25, 2.5, 5.0, 7.5, 10, 20, 40 and 50 mg L^{-1}. The concentration of MB in unknown samples was calculated with the help of quadratic equation mentioned below.

$$y = 28091x - 2844.5 \qquad \text{Eq. (3)}$$

Where y = MB concentration in unknown samples and x= area of the peak

2.3.3 Biochar analysis by scanning electron microscope (SEM)

The biochar was analyzed by SEM (Model XC-30 ETAX, Philips, USA). The samples were dried at 35 °C and coated with 15 nm gold layer followed by scanning electron microscopy analysis (SEM, S-3000N, Hitachi Ltd., Tokyo, Japan) [31] and presented in Supplementary figure 1.

2.3.4 Extraction of total soil DNA and PCR amplification

To determine entire bacterial community in potato rhizospheric soil, total DNA of the samples was extracted using Power Soil DNA Isolation Kit (MP BIO Laboratories) according to

manufacturer instructions. The quality of DNA was assured by agarose gel (2%) and each DNA preparation was quantified with the Qubit fluorimeter (Invitrogen).

2.3.5 Library construction and sequencing

For the characterization of potato rhizospheric bacterial community under various treatments, V1-V2 hypervariable region of the 16S rRNA gene was amplified by PCR using the 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 338R (ATGCTGCCTCCCGTAGGAGT) primers [35]. The V1-V2 hypervariable region of the 16S rRNA gene was considered for amplification based on earlier studies in which better resolution of 16S amplicons and bacterial diversity was seen for the plant rhizosphere [36].The amplicons were pooled and used to generate Illumina pair end libraries by targeting the hypervariable region V1-V2 of the 16S rRNA and sequenced on an Illumina MiSeq platform (2 × 250 bp, Illumina, California, USA).

2.3.6 Bioinformatic analysis and data processing

The partial 16S rRNA gene sequences representing V1-V2 hypervariable region were subjected to bioinformatics analysis to generate operational taxonomic unit (OTU) tables. Briefly, Ribosomal Database Project (RDP) assembler was applied to merge raw reads [37] while the sequences were aligned by MOTHUR pipeline [38] which uses SILVA reference database (Gotoh algorithm) [39]. The sequences were pre-clustered to yield so-called phylotypes, which were filtered for a sequence length of ≥250 bp and the average abundance of ≥0.02% before analysis. For assigning taxonomy to Phylotypes, the naïve Bayesian RDP classifier with a pseudo-bootstrap threshold of 80% was used [40]. Only those genus names were assigned phylotype whose 16S rRNA gene fragments have only up to two mismatches with the previous submitted 16S rRNA gene fragments of the same genus isolates [41]. The relative abundance of phylotypes along with alpha and beta diversity analysis were carried out considering whole OTUs composition by using package shiny-ampvis2 [42] (https://kasperskytte.shinyapps.io/shinyampvis/). For alpha diversity analysis, three widely used indices in microbial ecology namely Chao1, Shannon and Simpson were calculated to compare the richness, diversity and evenness in different treatments. On the other hand, beta diversity was tested via principal component analysis (PCA) which is the first multivariate

approach for community comparison studies. The bar plots were made in Microsoft Excel 2016.

2.3.7 MB degradation kinetics and statistical analysis

Kinetics parameters for MB biodegradation in soil were determined by plotting ln [Ct/C0] over time (days). Equation 2 and 3 were used to determine degradation rate constant (k, h^{-1}) and half-life ($T_{1/2}$, h) correspondingly.

$$Ct = C0 \times e^{-kt} \qquad \text{Eq. (2)}$$

$$T_{1/2} = \ln (2)/k \qquad \text{Eq. (3)}$$

Where Ct represents concentration of MB (mg kg^{-1}) at time "t" and C0 represents concentration of MB (mg kg^{-1}) at time "zero".

Minitab 17 software was used for statistical analysis of the data related to plant biomass, MB contents in rhizospheric soil as well as in plants tissues and rhizospheric bacterial populations. Further, Tukey's test was employed to check the significance of results at $P < 0.05$.

3 Results

3.1 Effect of metribuzin and MB3R immobilzed biochar on growth of potato plants

A microcosm experiment was conducted to study the effect of various treatments (section 2.3.3) on the growth of potato plants. Data regarding the root length (RL), shoot length (SL), root fresh mass (RFM), shoot fresh mass (SFM), root dry mass (RDM) and shoot dry mass (SDM) of potato plants at 30, 60, 90 days after sowing (DAS) is presented in Table 2. At 30 DAS, RL and SL of potato plants growing in MB spiked soil (P-UI) were reduced by 38 and 43% respectively as compared to native soil (C). Inoculation of soil with the consortium MB3R (P-I) significantly ($p>0.05$) enhanced RL and SL as compared to unincoulated soil (P-UI). A further 10 and 9% increase in RL and SL was observed respectively when MB3R immobilized biochar was used (treatment P-I_BC). Similar trend was observed at all sampling times. The plants grown in MB contaminated soil (P-UI) remained stunted as compared to those of control treatment.

Both RFM and SFM of plants growing in soil spiked with MB reduced significantly ($p>0.05$) as compared to control. RFM and SFM was significantly higher in inoculated treatment (P-I) than unincoulated treatment (P-UI). A further increase in these two parameters was observed in treatment P-I_BC i.e. where consortium immobilized on biochar was applied. Similar trend was observed for the RDM and SDM at all samplings. Generally, bioaugmentation

with bacterial consortium MB3R (P-I), addition of biochar alone (P-UI_BC) and the consortium immobilized onto biochar (P-I_BC) showed positive effects on plants biomass whereby this effect was more pronounced when the bacterial consortium immobilized onto biochar was applied. Further, the augmentation of PB contaminated soil with bacterial consortium immobilized onto biochar (P-I_BC) significantly ($P > 0.05$) enhanced the number as well as mass of potato tubers as compared to the uninoculated soil i.e., treatment P-UI (Supplementary Fig. 2).

3.2 Remediation of MB contaminated potato vegetated soil by MB3R immobilized biochar

Efficiency of bacterial consortium MB3R immobilized on biochar, for the remediation **of** potato vegetated soil contaminated with MB (2.5 mg kg^{-1}), was studied in a pot experiment as explained in section 2.3.3.

At 30 DAS, the highest MB removal (60.7%) was observed in treatment P-I_BC followed by treatment P-I where 43.5% of the applied MB was degraded (Fig. 1) whereas MB removal was lowest (15.0%) in treatment P-UI. At 90 DAS, in the soil augmented with bacterial consortium MB3R immobilized on biochar (P-I_BC), MB degradation was 96.1% whereas in the soil augmented with MB3R alone (P-I), the MB degradation was 81.8%. In contrast, only 29.3% MB removal was observed in uninoculated soil (P-UI; control), which can be attributed to the presence of indigenous microflora and other abiotic factors.

The results demonstrated that immobilization of bacterial consortium MB3R on biochar enhanced MB removal from soil. It may be concluded that combining biochar and bacterial consortium could efficiently remediate MB contaminated soil.

3.3 Influence of MB3R immobilized biochar on the metribuzin degradation kinetics

Dynamics model used to fit the changes of residual MB concentration in soil versus time indicated that degradation followed the first-order kinetics (Fig. 2). The kinetic parameters i.e., MB removal rate constant (K d^{-1}), half-life ($T_{1/2,}$ days) and regression coefficient in various treatments were calculated using first-order model equation and presented in Table 3. In treatment P-I_BC, an increase in the rate constant up to 0.036 d^{-1} and reduction in the half-life of MB up to 19 days was observed as compared to the control treatment (P-UI) in which the rate

constant and half-life of MB was 0.004 d^{-1} and 179 days respectively. The half-life of MB in treatments P-UI_BC and P-I reduced to 110 and 36 days respectively.

3.4 Effect of MB3R immobilized biochar on MB concentrations in potato plant tissues

The residual metribuzin concentration in the roots and shoots of potato plants at 30, 60 and 90 DAS as affected by different treatments was presented in Fig. 3. Significantly higher ($p>0.05$) MB residues both in roots and shoots of potato plants were detected in the treatment P-UI as compared to other treatments at each sampling stage. The Lowest MB concentrations were observed in plants growing under treatment P-I_BC, the one bioaugmented with MB3R immobilized on biochar. The concentration of residual MB in roots was higher as compared to shoots in all treatments. Moreover, a gradual decrease in MB residues (in roots and shoots) was observed in plants harvested at 30, 60 and 90 DAS.

3.5 Effect of MB and MB3R immobilized biochar on soil microbial populations

In the current study, the effect of various treatments on rhizospheric bacterial community of potato vegetated soil was assessed by 16S rRNA gene amplicons sequencing. The Operational Taxonomic Units (OTUs) richness and Chao1 described the number of species within a sample, while Shannon's and Simpson's indices described the evenness of microbial communities. From alpha diversity indices, it was inferred that in all treatments, the diversity of bacterial communities did not vary significantly ($P< 0.05$) as compared to control (Table 4). This showed that application of metribuzin alone or in combination with biochar/bacterial consortium have no significant effect on the diversity of soil bacterial populations. On the other hand, results of beta diversity (PCA) showed that control (C), P-UI_BC and P-I_BC treatments were similar because they were clustered together whereas treatments P-UI and P-I were different. This clustering was based on ~67.6% of the data variance by first two principal components (factors), i.e., 46.4% and 21.2% respectively. It was suggested from the results that biochar had apparent effect on the bacterial communities.

The composition of bacterial communities in various treatments was also analysed at phylum, class and family levels (Fig. 5a, 5b, 5c). Firstly, the prokaryotic community was described by considering the most abundant phyla (higher taxonomic level) having relative abundance >1%. Bacterial communities were quite similar in soil samples collected from various treatments with respect to dominant phyla but different in terms of abundance of these phyla.

Overall, the rhizosphere of potato was mainly occupied by seven bacterial phyla including Proteobacteria, Bacteriodetes, Actinobacteria, Acidobacteria, Chloroflexi, Gemmatimonadetes and Firmicutes (Fig. 5a). Among these, Proteobacteria was the most abundant followed by Bacteriodetes and Actinobacteria with an overall average abundance of 35%, 12.8% and 11.2% respectively.

Population of Proteobacteria and Actinobacteria reduced by 7.6% and 16.3% in soil contaminated with metribuzin (P-UI) whereas 26.2% increase in Bacteriodetes was observed. In treatment P-I_BC, a respective increase of 5.6 and 10.8% in populations of Proteobacteria and Actinobacteria as compared to treatment P-UI was recorded. Relative abundance of Bacteriodetes reduced up to 10.6% in soil amended with bacterial consortium MB3R immobilized onto biochar (P-I_BC) in contrast to treatment P-UI.

Among phylum Proteobacteria, these classes Alphaproteobacteria, Gammaproteobacteria and Betaproteobacteria were found highly abundant (Fig. 5b). The number of Alpha proteobacteria, Gamma proteobacteria and Beta proteobacteria classes in unspiked soil samples (C) was 26.3%, 15.3% and 3.4% respectively. The contamination of soil with MB (P-UI) resulted in the lowering of Alpha proteobacteria and Gamma proteobacteria whereas the beta proteobacteria increased in response to MB. The Gamma proteobacteria was highly influenced by the MB i.e., reduced 9.1% in treatment P-UI as compared to control (C). While at family level, the families *Sphingomonadaceae* and *Xanthomonadaceae* showed higher abundance among Proteobacteria followed by *Sinobacteraceae*, *Erythrobacteracea*e and *Phyllobacteriaceae* (Fig. 5c). In MB contaminated soil unincoulated soil (P-UI), the abundance of S*phingomonadaceae* decreased from 7.7% to 6.0% while restored to 8.45% in treatment P-I_BC. Similarly, the population of family *Xanthomonadaceae* lowered from 5.4% to 4.2% in treatment P-UI as compared to control whereas its abundance in the treatment P-I_BC was 5.28% (Fig.5c).

In contrast, sequences related to phylum Bacteriodetes increased from 11.3% to 14.3% in metribuzin-spiked samples (Fig. 5a). Majority of the sequences of this phylum belonged to class Flavobacteria, Cytophagia and Sphingobacteia (Fig. 5b). An increase of 20.8 and 15.4% were seen in the abundance of class Cytophagia and Sphingobacteria respectively in soil polluted with MB (P-UI). Families like *Chitinophagaceae* and *Saprospiraceae* were found to be most

abundant within phylum Bacteriodetes whose abundance increased in treatment P-UI as compared to control (Fig. 5c).

4 Discussion

The experiment was conducted to assess the influence of MB degrading bacterial consortium MB3R, biochar and the consortium immobilized biochar, on plant biomass and remediation of potato vegetated soil. Changes in soil bacterial community structure in response to the treatments were also investigated. Plant biomass enhanced significantly over the control when biochar immobilized with bacterial consortium was applied. As observed by retrieval of plant growth, the detrimental effect of metribuzin observed at 30 DAS was restored at 60 DAS in the treatments where MB3R either alone or immobilized on biochar was augmented. Potentially, it was due to alleviation of metribuzin-induced stress owing to its degradation by virtue of the inoculated bacteria. Moreover, bacterial strains comprising the consortium MB3R also possess ACC deaminase activity, that might have helped the plant to overcome contaminant induced stress.

Furthermore, effect of augmentation on plant growth was greater when biochar was used to immobilize the bacterial consortium MB3R. Biochar is known to exhibit many characteristics, which are beneficial for agriculture. Biochar is reported to improve soil physio-chemical properties like soil pH, electrical conductivity, organic carbon, total nitrogen, available phosphorous, cation exchange capacity etc. [43]. Due to improved soil characteristics, the availability and uptake of nutrients by plants is increased resulting in better plant biomass [44]. Also, biochar acts as safe habitat for bacteria due to its porous nature and high surface area that save microbes within biochar pores [45]. Survival and activity of biochar immobilized bacteria further increases due to enhanced nutrient availability and inactivation of microbial growth inhibiting substances [46]. Moreover, biochar can also improve plant-microbe interactions that leads to enhanced plant growth [47]. Recent field studies done on switchgrass *(Panicum virgatum* cv. Cave-in-Rock) and french beans (*Phaseolus vulgaris*) reported a significant increase in plant biomass/yield when a combination of biochar and bacterial consortium was applied [48, 49].

The results showed that MB removal was 96% as compared to control (29.9% removal) when bacterial consortium MB3R immobilized on biochar was applied. The improved microbial degradation of pollutants in the presence of biochar is considered to be because of activation of persistent free radicals and hence facilitating the electron transport between microorganisms and

pollutants [50]. In addition, the reduction of hazardous compounds poses positive effects on soil microbes that could also be a possible reason for enhanced microbial degradation of pollutants in the presence of biochar [51]. The enhanced degradation of organic contaminants like petroleum hydrocarbon and cypermethrin by bacteria immobilized onto biochar have been reported earlier [31, 52].

The half-life of MB in untreated planted soil was 179 days depicting its persistence in soil. The MB half-life reduced to 36 days when soil was inoculated with bacterial consortium MB3R alone. The application of bacterial consortium MB3R immobilized on biochar further reduced the half-life up to 19 days. These results demonstrated that MB contaminated soils can be remediated more efficiently by the combined use of biochar and bacteria. The results were consistent with a previous study where Liu *et al.,* reported a significant decrease in the half-life of cypermethrin by using bacteria immobilized onto biochar [52]. The Reduction of MB concentration in the roots and shoots of potato plants at 60 and 90 DAS as compared to 30 DAS can be attributed to its degradation due to enzymatic activities of plants. Metabolism of various xenobiotics including pesticides by plants have been well documented [53]. Moreover, the consortium MB3R used for MB degradation in vegetated soil comprised of four bacterial strains three of which i.e., *Bacillus tequilensis* AQ2, *Bacillus aryabhattai* AQ3 and *Bacillus safensis* AQ4 were endophytes. Presence of these bacteria in roots and shoots of plant parts was also observed at 90 DAS (data not shown) which might have metabolized the herbicide taken up by the plant roots and shoots.

The analysis of prokaryotic community in the potato rhizosphere under various treatments depicted the dominance of phylum Proteobacteria, followed by Bacteriodetes and Acidobacteria. The taxa of these phyla generally have been found in rhizospheric soil and have direct negative or positive effects to plant health [54, 55]. For example, members of phylum Proteobacteria like *Pseudomonas* have been reported extensively for their plant growth promoting properties and use as biological control agent for many plant diseases [56].

The composition of various bacterial phyla was significantly ($p<0.05$) affected in potato vegetated MB contaminated soil as compared to native soil. For example, a significant reduction in the abundance of Gamma proteobacteria was observed in the treatment P-UI as compared to control suggesting that members of this phylum may be sensitive to MB. Proteobacteria have

earlier been reported to decrease under the stress of sulphonamides and sulfamethazine in two different studies [57, 58]. In contrast to Proteobacteria, the relative abundance of Bacteriodetes (families *Chitinophagaceae* and *Saprospiraceae*) increased under MB stress suggesting that Bacteriodetes adapted to and/ or were enriched MB. An increase in abundance of Bacteriodetes under the influence of oligomeric herbicidal ionic liquids with MCPA and Dicamba anions [59] have already been reported. Moreover, strong association of bacteria belonging this phylum with atrazine biodegradation was also reported [60]. The variation in relative abundance of prokaryotic communities at phylum level indicated that MB could change their composition. Usually, these types of changes may result in the suppression of plant beneficial bacteria and promotion of others that compete for soil resources.

The abundance of the prokaryotic bacterial community in treatments P-UI_BC and P-I_BC seems to restore similar proportion as observed for control treatments. Interestingly, the effects of BC on soil microbial communities have shown considerable variability in several studies. The results of few studies demonstrated the increase in abundance of Proteobacteria, Bacteriodetes, Actinobacteria, Gemmatimonadetes, and Planctomycetes in response to biochar amendment [61-63]. Whereas the negative effect of biochar on the abundance of Proteobacteria, Acidobacteria, Firmicutes and Bacteroidetes was also reported in other studies [64-66]. In the present study, the abundance of Proteobacteria, Actinobacteria and Firmicutes increased while the population of Bacteriodetes decreased in treatment P-I_BC than P-UI. This suggests that biochar/bacterial consortium can play a potential role in mitigating the toxic effects of MB on the composition of soil bacterial communities.

5 Conclusions

Bacterial consortium MB3R comprising of four MB degrading bacterial strains showed potential for the remediation of metribuzin in potato vegetated soil. Moreover, MB remediation potential of the consortium MB3R was improved when it was immobilized on biochar. The consortium MB3R immobilized on biochar also restored the detrimental effects of MB on soil microbial populations and plant growth.

It is concluded that immobilization of MB3R on biochar is a novel approach for the decontamination of MB at point source i.e., in agricultural soil. Its potential can be exploited for the rehabilitation of soil microbial populations and mitigating toxic effects of MB residues on the

subsequent crops and reducing its risk of leaching into nearby waterbodies. Microplot studies and field demonstration at multiple locations are under way, to further assess if the process could be up-scaled for field application.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table

Table 1: Physio-chemical properties of soil used in MB biodegradation microcosm studies

Sr. No.	Treatments	Remarks
1	pH	7.6 ± 0.06
2	Electrical conductivity (EC, dS m^{-1})	6.4 ± 0.12
3	Total nitrogen (%)	0.056 ± 0.003
4	Available phosphorus (mg kg^{-1})	0.08 ± 0.005
5	Extractable potassium (mg kg^{-1})	1.44 ± 0.07
6	Organic matter (%)	0.077 ± 0.005
7	Sand (%)	68
8	Silt (%)	16
9	Clay (%)	15
10	Texture class	Sandy clay loam
11	Moisture content (%)	5.85 ± 0.23
12	Maximum water holding capacity (MWHC, %)	38 ± 0.87

Table 2: Effect of metribuzin and immobilized bacterial consortium MB3R with biochar as carrier on the agronomic parameters of potato plants at 30, 60 and 90 days

Days	Treatments	RL (cm)	SL (cm)	RFM (g)	SFM (g)	RDM (g)	SDM (g)
30	C	27.5^{b} (0.41)	38.8^{a} (0.96)	4.9^{ab} (0.27)	30.6^{b} (0.61)	2.0^{b} (0.01)	5.2^{b} (0.38)
	P-UI	17.0^{d} (0.81)	22.1^{d} (0.65)	2.6^{c} (0.26)	16.7^{d} (0.61)	1.2^{c} (0.05)	2.6^{d} (0.11)
	P-UI_BC	21.6^{c} (0.56)	27.4^{c} (0.56)	3.6^{bc} (0.43)	21.0^{c} (0.83)	1.3^{c} (0.18)	3.3^{cd} (0.07)
	P-I	28.3^{ab} (1.20)	33.7^{ab} (1.76)	4.7^{ab} (0.56)	31.5^{b} (0.76)	1.9^{b} (0.08)	4.5^{bc} (0.12)
	P-I_BC	31.2^{a} (0.83)	36.7^{a} (0.56)	5.7^{a} (0.47)	35.5^{a} (0.60)	2.8^{a} (0.27)	8.4^{a} (0.46)
60	C	34.3^{b} (0.52)	42.9^{b} (1.21)	6.4^{b} (0.26)	35.7^{b} (1.06)	2.3^{b} (0.19)	6.3^{b} (0.23)
	P-UI	20.4^{d} (0.57)	25.5^{d} (0.35)	3.1^{d} (0.21)	19.2^{d} (0.27)	1.4^{c}(0.13)	3.1^{c} (0.11)
	P-UI_BC	24.6^{c} (0.75)	30.5^{c} (0.93)	4.4^{c} (0.23)	23.4^{c} (0.16)	1.5^{c} (0.21)	3.9^{c} (0.12)
	P-I	35.5^{ab} (0.64)	43.4^{ab} (1.13)	6.5^{b} (0.32)	36.0^{b} (0.85)	2.6^{ab} (0.31)	6.9^{b} (0.52)
	P-I_BC	38.9^{a} (1.09)	46.4^{a} (1.00)	7.7^{a} (0.48)	40.2^{a} (0.68)	3.3^{a} (0.27)	8.8^{a} (0.26)
90	C	35.4^{b} (0.36)	44.3^{b} (0.46)	7.2^{b} (0.36)	38.2^{b} (0.63)	3.0^{b} (0.28)	7.1^{b} (0.48)
	P-UI	20.7^{d} (0.50)	26.5^{d} (0.61)	3.5^{d} (0.28)	19.8^{d} (0.44)	1.4^{c} (0.15)	3.9^{c} (0.16)
	P-UI_BC	25.3^{c} (0.49)	31.7^{c} (0.69)	5.0^{c} (0.29)	24.5^{c} (0.28)	1.6^{c} (0.10)	4.7^{c} (0.41)
	P-I	36.6^{b} (0.59)	43.8^{b} (0.96)	7.5^{b} (0.47)	38.7^{b} (0.54)	3.1^{b} (0.43)	7.5^{b} (0.31)
	P-I_BC	40.7^{a} (0.94)	48.5^{a} (0.69)	9.1^{a} (0.28)	42.3^{a} (0.56)	4.2^{a} (0.42)	9.4^{a} (0.32)

RL = Root length, SL = Shoot length, RFM = Root fresh mass, SFM = Shoot fresh mass, RDM = Root dry mass, SDM = Shoot dry mass
C = Potato vegetated soil
P-UI = Potato vegetated metribuzin contaminated soil
P-UI_BC = Potato vegetated metribuzin contaminated soil supplemented with biochar
P-I = Potato vegetated metribuzin contaminated soil inoculated with bacterial consortium MB3R
P-I_BC = Potato vegetated metribuzin contaminated soil inoculated by immobilized bacterial consortium MB3R with biochar as carrier
Each value is mean of three replicates; standard error of the replicates is presented in parenthesis (±). Means followed by different letters are significantly different ($p<0.05$).

Table 3: First order kinetic parameters for Metribuzin (MB) degradation in various treatments

Treatments	Regression equation	Rate constant ($K\ d^{-1}$)	Half-life ($T_{1/2}$, days)	Regression coefficient
P-UI	$C_t = 2.5e^{-0.004}$	0.004	179.7	0.974
P-UI_BC	$C_t = 2.5e^{-0.006}$	0.006	110.3	0.981
P-I	$C_t = 2.5e^{-0.019}$	0.019	36.7	0.996
P-I_BC	$C_t = 2.5e^{-0.036}$	0.036	19.2	0.998

under planted (potato) soil

P-UI = Potato vegetated metribuzin contaminated soil
P-UI_BC = Potato vegetated metribuzin contaminated soil supplemented with biochar
P-I = Potato vegetated metribuzin contaminated soil inoculated with bacterial consortium MB3R
P-I_BC = Potato vegetated metribuzin contaminated soil inoculated by immobilized bacterial consortium MB3R biochar as carrier

Table 4: Alpha diversity metrics of the bacterial communities of potato rhizosphere in the different treatments

Treatments	Observed	Chao1	Shannon	Simpson
C	1671a (094)	1751a (085)	6.76a (0.38)	0.9976a (0.008)
P-UI	1700a (004)	1770a (095)	6.83a (0.41)	0.9980a (0.005)
P-I	1699a (092)	1794a (114)	6.62a (0.54)	0.9968a (0.009)
P-UI_BC	1740a (080)	1797a (103)	6.87a (0.67)	0.9980a (0.007)
P-I_BC	1730a (110)	1804a (088)	6.73a (0.61)	0.9974a (0.008)

C = Potato vegetated soil
P-UI = Potato vegetated metribuzin contaminated soil
P-UI_BC = Potato vegetated metribuzin contaminated soil supplemented with biochar
P-I = Potato vegetated metribuzin contaminated soil inoculated with bacterial consortium MB3R
P-I_BC = Potato vegetated metribuzin contaminated soil inoculated by immobilized bacterial consortium MB3R with biochar as carrier
Each value is mean of three replicates; standard error of the replicates is presented in parenthesis (±). Means followed by different letters are significantly different ($p<0.05$).

Figure
Click here to download Figure: figures_3.pptx

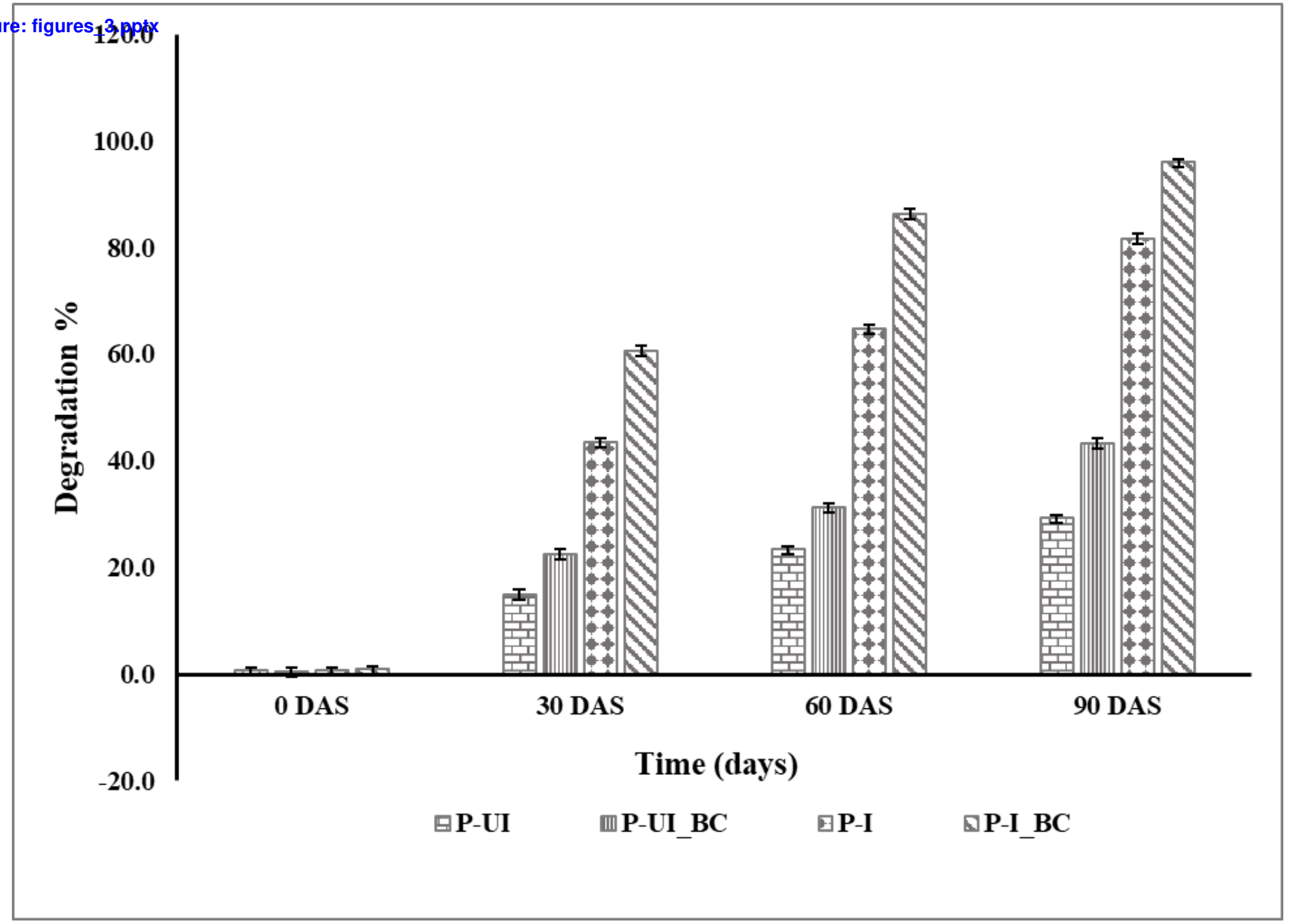
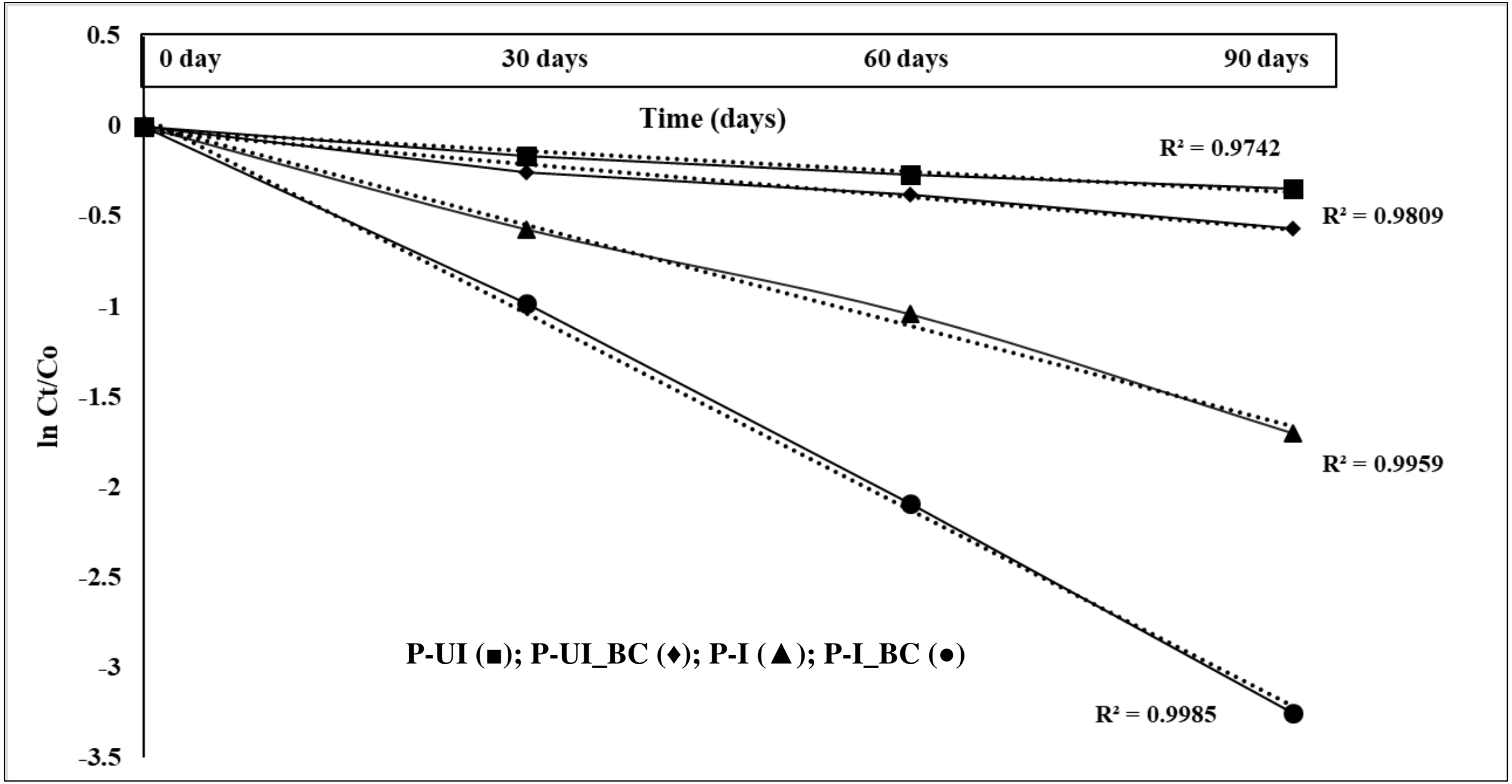


Fig. 1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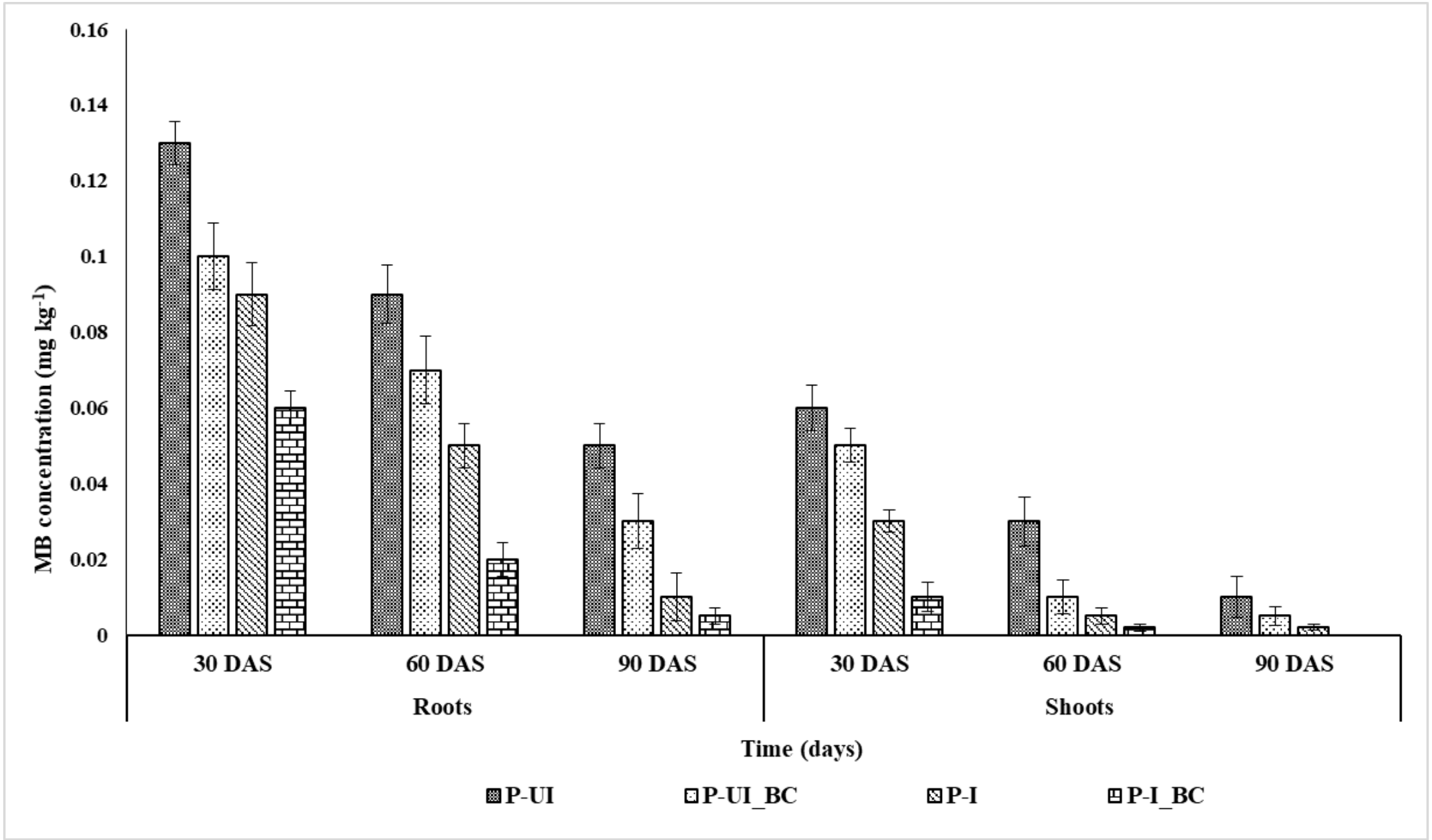
ln Ct/Co
R² = 0.9742
R² = 0.9809
R² = 0.9959
R² = 0.9985
P-UI (■); P-UI_BC (♦); P-I (▲); P-I_BC (●)

Fig. 2


0.16
0.14
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MB concentration (mg kg-1)
30 DAS
60 DAS
90 DAS
30 DAS
60 DAS
90 DAS
Roots
Shoots
Time (days)
P-UI
P-UI_BC
P-I
P-I_BC

Fig. 3

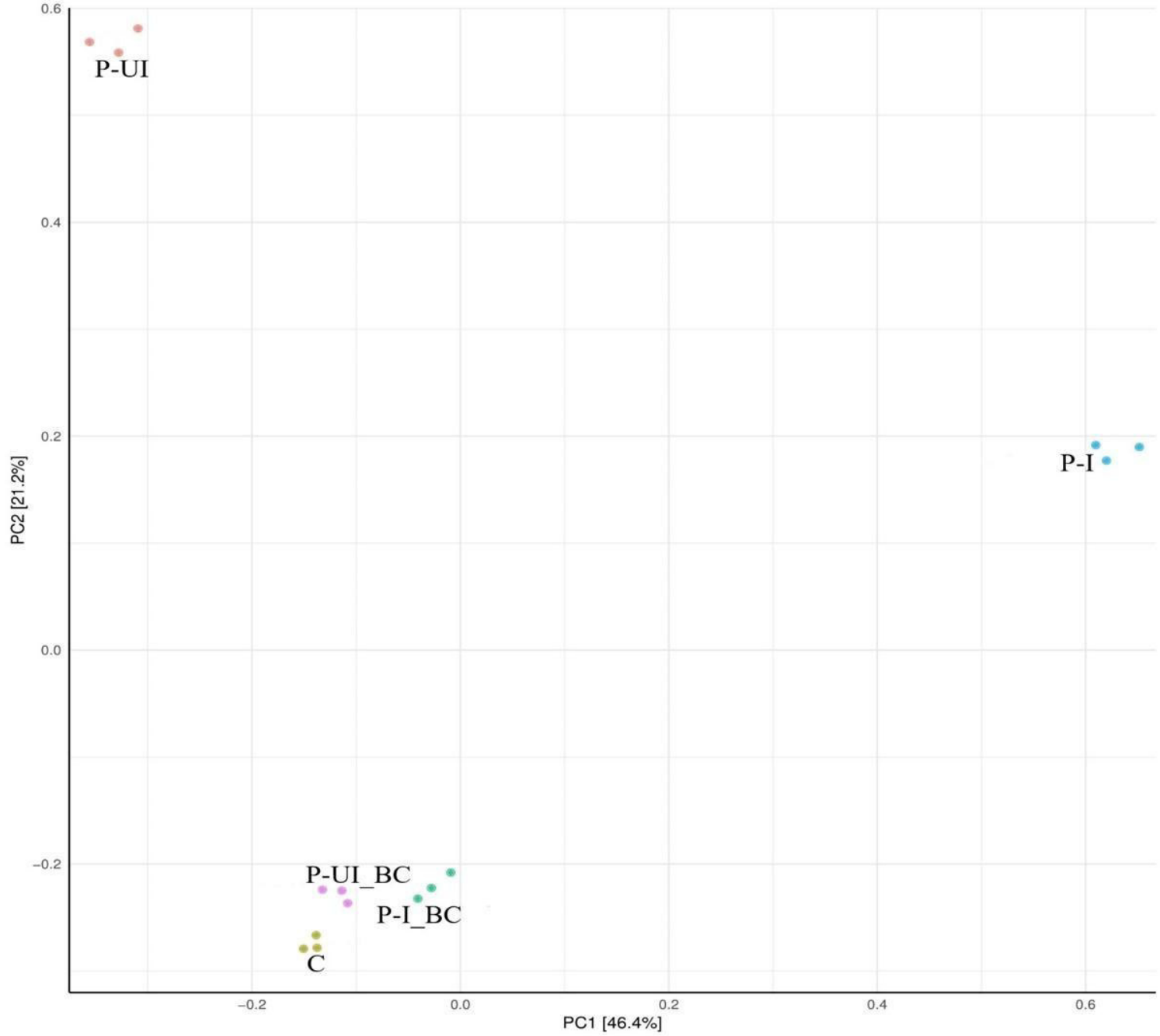

Fig. 4

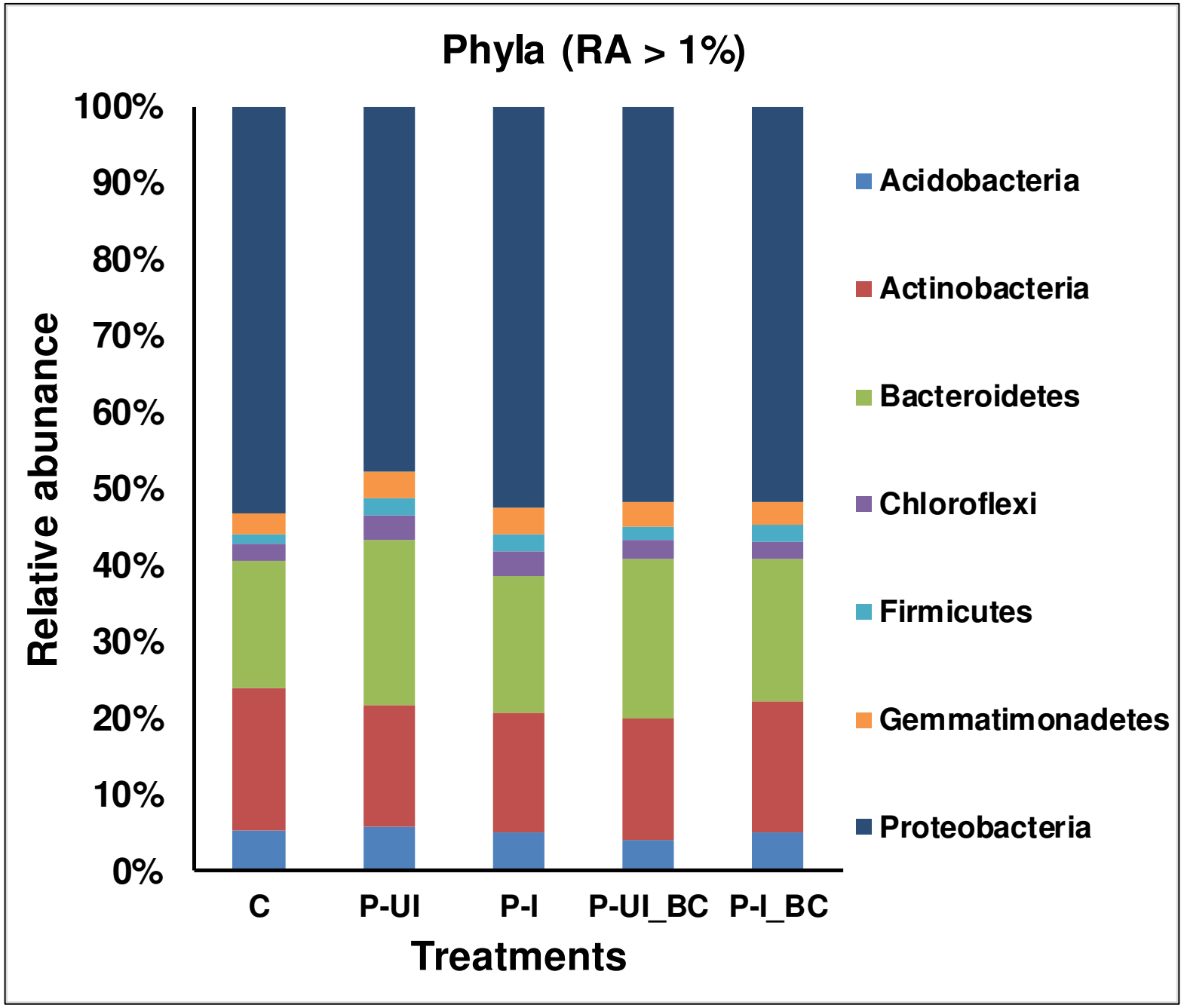
Phyla (RA > 1%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abunance
C
P-UI
P-I
P-UI_BC
P-I_BC
Treatments
Acidobacteria
Actinobacteria
Bacteroidetes
Chloroflexi
Firmicutes
Gemmatimonadetes
Proteobacteria

Fig. 5a

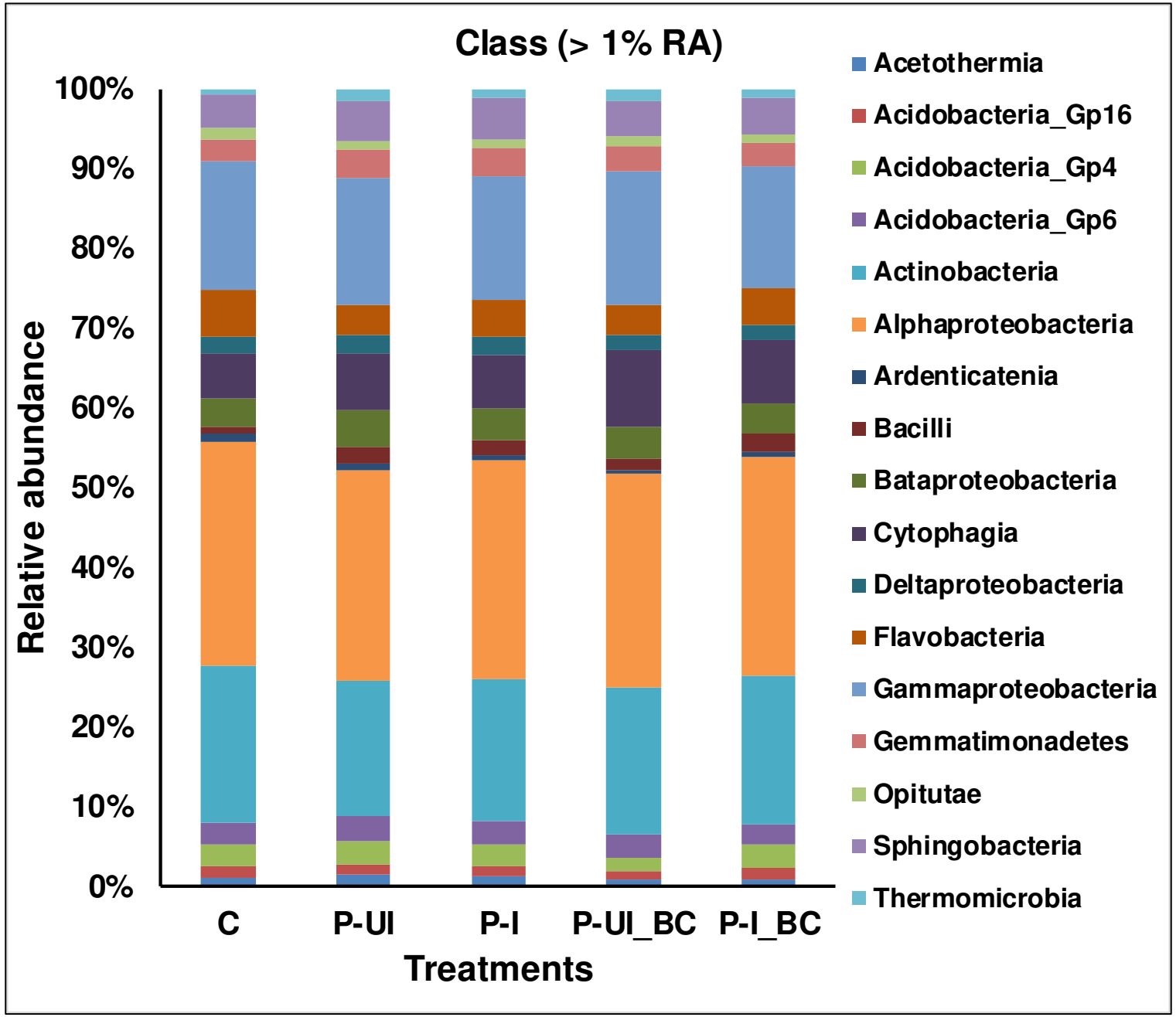
Class (> 1% RA)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance
C
P-UI
P-I
P-UI_BC
P-I_BC
Treatments
Acetothermia
Acidobacteria_Gp16
Acidobacteria_Gp4
Acidobacteria_Gp6
Actinobacteria
Alphaproteobacteria
Ardenticatenia
Bacilli
Bataproteobacteria
Cytophagia
Deltaproteobacteria
Flavobacteria
Gammaproteobacteria
Gemmatimonadetes
Opitutae
Sphingobacteria
Thermomicrobia

Fig. 5b

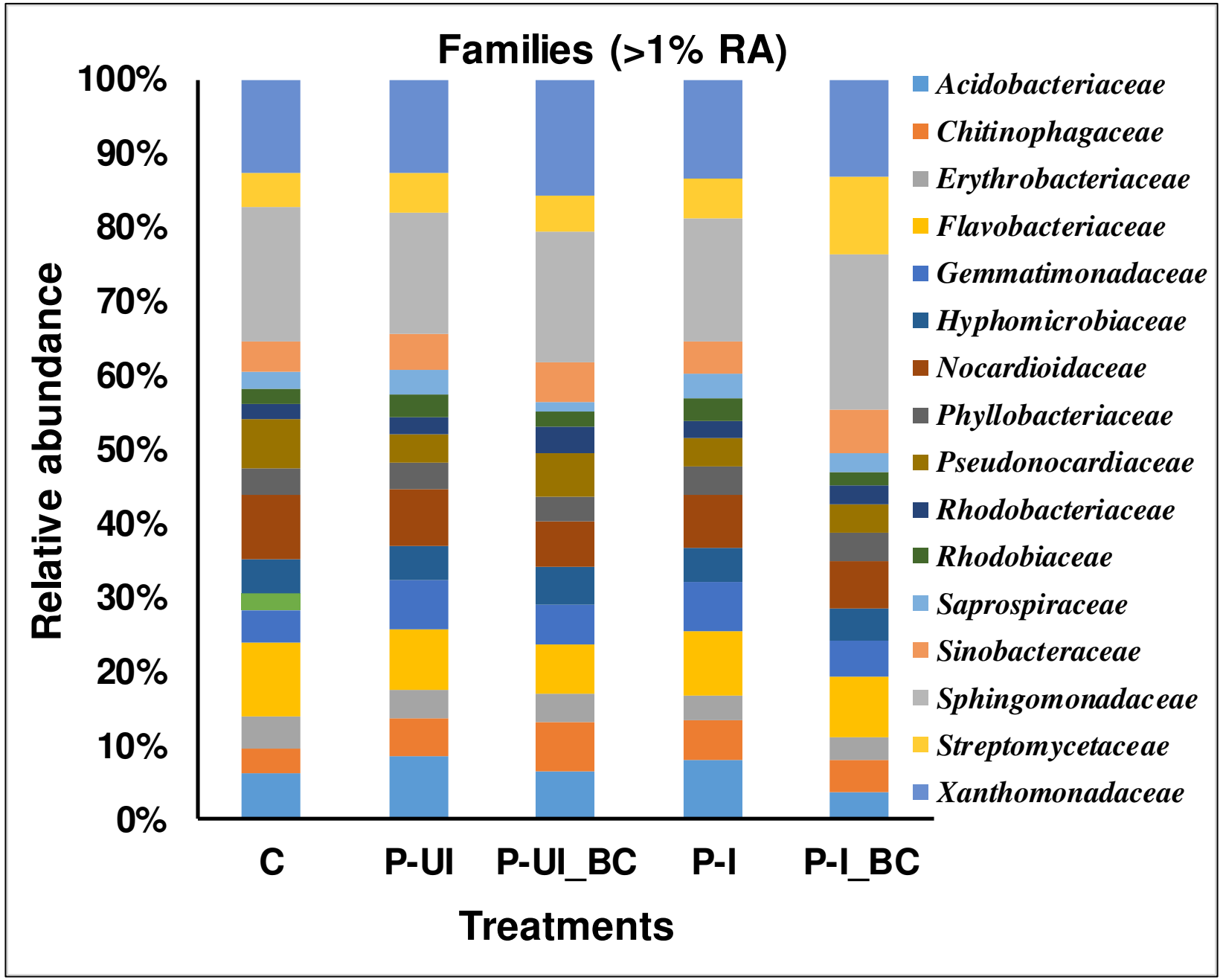
Families (>1% RA)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance
C
P-UI
P-UI_BC
P-I
P-I_BC
Treatments
Acidobacteriaceae
Chitinophagaceae
Erythrobacteriaceae
Flavobacteriaceae
Gemmatimonadaceae
Hyphomicrobiaceae
Nocardioidaceae
Phyllobacteriaceae
Pseudonocardiaceae
Rhodobacteriaceae
Rhodobiaceae
Saprospiraceae
Sinobacteraceae
Sphingomonadaceae
Streptomycetaceae
Xanthomonadaceae

Fig. 5c

Supplementary Material

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Supplementary Material

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