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The potential of enhanced phytoremediation to clean up multi-contaminated soil – insights from metatranscriptomics

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Abstract

This study aimed to (i) investigate the potential for enhanced phytoremediation to remove contaminants from soil historically co-contaminated with petroleum hydrocarbons (PHs) and heavy metals (HMs) and (ii) analyze the expression of crucial bacterial genes and

whole metatranscriptomics profiles for better understanding of soil processes during applied treatment.

Phytoremediation was performed using *Zea mays* and supported by the *Pseudomonas qingdaonensis* ZCR6 strain and a natural biofertilizer: meat and bone meal (MBM). In previous investigations, mechanisms supporting plant growth and PH degradation were described in the ZCR6 strain. Here, ZCR6 survived in the soil throughout the experiment, but the efficacy of PH removal from all soils fertilized with MBM reached 32% regardless of the bacterial inoculation. All experimental groups contained 2% (w/w) MBM. The toxic effect of this amendment on plants was detected 30 days after germination, irrespective of ZCR6 inoculation. Among the 17 genes tested using the qPCR method, only expression of the *acdS* gene, encoding 1-aminocyclopropane-1-carboxylic acid deaminase, and the CYP153 gene, encoding cytochrome P450-type alkane hydroxylase, was detected in soils. Metatranscriptomic analysis of soils indicated increased expression of methane particulated ammonia monooxygenase subunit A (*pmoA-amoA*) by *Nitrosomonadales* bacteria in all soils enriched with MBM compared to the non-fertilized control. We suggest that the addition of 2% (w/w) MBM caused the toxic effect on plants via the rapid release of ammonia, and this led to high *pmoA-amoA* expression. In parallel, due to its wide substrate specificity, enhanced bacterial hydrocarbon removal in MBM-treated soils was observed. The metatranscriptomic results indicate that MBM application should be considered to improve bioremediation of soils polluted with PHs rather than phytoremediation. However, lower concentrations of MBM could be considered for phytoremediation enhancement. From a broader perspective, these results indicated the superior capability of metatranscriptomics to investigate the microbial mechanisms driving various bioremediation techniques.

Keywords: phytoremediation; co-contamination; PGPR; *Pseudomonas qingdaonensis*; meat and bone meal, metatranscriptomics

1. Introduction

The remediation of soil contaminated with a mixture of organic and inorganic pollutants is an unsolved global problem (Chirakkara et al., 2016). Typically, such soils are excavated and sealed *ex situ*, or treated chemically or electrokinetically (Chaukura et al., 2022; Sánchez-Castro et al., 2023). Both techniques obviously destroy soil microbial ecosystems and usually also the soil's physical structure. In turn, phytoremediation is considered as a promising, cost-effective *in situ* method that restores the site while maintaining the physical structure and fertility of the soil (Jia et al., 2023). In the case of co-contaminated areas, it is therefore necessary to use solutions based on phytoremediation or its modification. Among modifications, biostimulation via nutrients and/or oxygen supply or bioaugmentation of plant growth-promoting bacteria (PGPB) with additional beneficial features, e.g., PH degradation and HM tolerance, could be applied.

PGPB are beneficial soil microbes that upgrade plant production, increasing crop yield, being a component of an integrated plant nutrient management system (Vandana et al., 2021). They act via activation of mechanisms involving the production of phytohormones, siderophores, and enzymes. Bacterial PGP mechanisms also involve nitrogen fixation, the solubilization of phosphorus, and the production of antibiotics that protect plants from pathogens (Glick, 1995). They may also reduce abiotic stresses in plants caused by pollutants and can increase the tolerance towards them. Moreover, some auxin-producing plant growth-promoting rhizobacteria (PGPR) can colonize the roots of plants and modify their architecture, which mostly leads to enhanced lateral root branching and the development of root hairs

(Vacheron et al., 2013). This creates more extended niches that can be inhabited by PH-degrading rhizobacteria, thus enhancing the rhizodegradation process. PGPR can also enhance metal bioavailability and increase the metal translocation from roots to shoots of plants (Płociniczak et al., 2019). The amendment of polluted soil with beneficial bacteria and/or specific fertilizers can improve the total plant biomass and the range of the root system and thus enhance the activity of rhizospheric and endophytic microorganisms involved in the phytoremediation process (Marchand et al., 2018; Shtangeeva et al., 2004).

To overcome the difficulties in biological remediation of soils co-contaminated with petroleum hydrocarbons (PHs) and heavy metals (HMs), we aimed to investigate a new, multiway approach which will assess the exact potential of biostimulation and bioaugmentation to increase the efficiency of this phytoremediation. We decided to use phytoremediation because it is the only biological technique that allows for permanent removal of heavy metals from the soil (Ullah et al., 2023). Additionally, the root system of plants creates an appropriate niche for the degradation of organic pollutants by autochthonous microorganisms and/or strains introduced by bioaugmentation (rhizodegradation) (Li et al., 2022). Since the preparation of a bacterial inoculum on a large field scale is a time-consuming and expensive process, we also decided to verify the validity of bioaugmentation by using biostimulation with meat and bone meal (MBM) as a biofertilizer. In our experiments, *Zea mays*, HM-resistant and PH-degrading *Pseudomonas qingdaonensis* ZCR6 strain, a representative of PGPR, and natural biofertilizer (MBM) were applied to soil that had been historically contaminated with PHs and HMs (Zn, Cd, and Cu). Since our main aim was to identify the key mechanisms determining the effectiveness of phytoremediation of co-contaminated areas, we focused our research on determining the impact of the applied treatments on the activity of soil microorganisms, which, despite many studies on phytoremediation, has not been studied in detail so far. The modern molecular techniques we used in our research allow the detailed monitoring of microbial

activity in soil, but they have not been used extensively in phytoremediation studies before. Moreover, there are no reports on the use of metatranscriptomics to explain the impact of applied treatments on the activity of soil microorganisms, which are the main factors degrading PHs and also influencing plant growth, and therefore also the removal of HMs from the soil.

Thus to confirm or exclude the activity of ZCR6 after its introduction into soil, we performed qPCR analysis to monitor the expression of chosen genes detected previously in the genome of the ZCR6 strain, with the potential to enhance the bio- and phyto-remediation processes. What is new in the context of phytoremediation research is the use of global analysis of bacterial gene expression in soil during the bioremediation process.

This method can shed light on the relative importance of mechanisms that could support plant growth or stimulate indigenous microorganisms in experimental groups treated only with MBM, making bioaugmentation unnecessary and phytoremediation more economical. For this reason, applying the metatranscriptomics approach, allowing the global analysis of gene expression in the studied environment and taxonomical analysis, is recommended (Han et al., 2022). Only this approach – rather than cultivation methods and other non-cultivation biochemical methods such as phospholipid fatty acid (PLFA) analysis and community-level physiological profiles (CLPP) – will allow us to determine which processes are crucial for the effective cleaning of contaminated areas and which groups of bacteria are responsible for carrying it out. Designing our experimental groups we hypothesized that the phytoremediation system supported simultaneously with the ZCR6 strain and MBM would show higher efficiency in pollutant removal compared to the control groups separately treated with ZCR6 or MBM. We also assumed that after introduction into the soil, the ZCR6 strain would be able to express the beneficial features and thus support the phytoremediation process. We also hypothesized that the stimulation of autochthonous microorganisms by the addition of MBM may contribute to increasing the effectiveness of phytoremediation to a similar or even greater extent than

bioaugmentation of the ZCR6 strain. Moreover, we expected that the metatranscriptomics approach would reveal crucial pathways activated in soil under the applied biostimulation and bioaugmentation treatments.

2. Materials and methods

2.1 Soil

Soil co-contaminated with hydrocarbons and heavy metals was collected from an industrial area located in the vicinity of the Jadwiga Coke Plant in Zabrze (Upper Silesia, southern Poland, 50.336010, 18.826797). Loamy sand soil samples were collected from a depth of 0.2 m. Before the experiment, the soil was passed through a 2 mm sieve and stored at 4 °C. According to the FAO system, the soil was classified as Albic Luvisol (FAO, 2015). Its detailed chemical and physical parameters are listed in Table S1.

2.2 Bacterial strain

The *Pseudomonas qingdaonensis* ZCR6 strain was isolated from the rhizosphere of *Zea mays* growing in the soil used in this study. In laboratory conditions, the strain showed the activity of several mechanisms potentially involved in plant growth promotion. The ZCR6 was able to produce indole acetic acid (IAA), siderophores, and ammonia, solubilized $Ca_3(PO_4)_2$, and showed activity of cellulase and 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD) at a high level. Apart from PGP activity, it showed surface-active properties, degradation abilities against petroleum hydrocarbons, and resistance to Cd, Zn, and Cu. The detailed genetic and biochemical characteristics of ZCR6 were described by Chlebek et al. (2022).

2.3 Selection of a rifampicin-resistant mutant of the ZCR6 strain and fluorescent labeling

A spontaneous rifampicin-resistant mutant was screened to monitor the fate of the ZCR6 strain after its introduction into the co-contaminated soil. Because resistance to this antibiotic is not common for soil bacteria (no rifampicin-resistant bacteria in the tested soil were detected), it is a good marker of the presence of the inoculated strain in the soil and/or plant tissues. For this purpose, the ZCR6 strain was plated on 0.1 Luria Bertani (LB) medium enriched with 10 µg mL^{-1} rifampicin, 1% (*v/v*) crude oil and 3 mM $ZnSO_4$. The grown colonies were reinoculated on 0.1 LB plates with a progressively higher content of antibiotic (up to 150 µg mL^{-1}). The stability of rifampicin resistance was confirmed by subculturing resistant $ZCR6^{rf}$ on 0.1 LB plates supplemented with crude oil and Zn, without the addition of rifampicin. The $ZCR6^{rf}$ strain had the same biochemical features as the parental ZCR6. The $ZCR6^{rf}$ was then labeled with enhanced green fluorescent protein (EGFP).

To label the rifampicin-resistant ZCR6 strain with EGFP, electrocompetent cells were prepared according to Nigris et al. (2018) and transformed with the pMP4655 plasmid vector with constitutive expression of the *egfp* gene (Bloemberg et al., 2000). Electrotransformation was performed according to the protocol of Prieto and Mercado-Blanco (2008). The labeled cells of the $ZCR6^{rf+egfp}$ harboring plasmids were examined using a Nikon ECLIPSE-Ni-U stereo microscope equipped with epifluorescence detection, 480/40 nm excitation and a 510 nm long pass emission filter (Chlebek et al., 2020).

2.4 Inoculum preparation

Bacteria used for the inoculum preparation were cultured on 0.1 LB broth supplemented with rifampicin (150 µg mL^{-1}), tetracycline (50 µg mL^{-1}), added to maintain the marker resistance and pMP4655 plasmid expression, and crude oil at a concentration of 1% (*v/v*) in an orbital shaker at 120 rpm (28 °C, 48 h). The number of bacteria in the inoculum was established based on the optical density measurement and plating method. The appropriate volume of the bacterial

suspensions was centrifuged (5,000 rcf, 4 °C, 40 min, Sigma 4–16 K), and the bacterial pellet was washed twice with sterile water and resuspended in sterile water. Thermally inactivated bacteria were prepared similarly, and their suspension was then treated at high temperature by autoclaving (121 °C, 1 atm, 30 min). The efficiency of the sterilization process was determined using the plating method, and no bacterial growth was observed.

2.5 Experimental setup

The phytoremediation experiment was conducted under laboratory conditions using soil co-contaminated with petroleum hydrocarbons and heavy metals, as described in Section 2.1. The experiment included 4 treatments carried out in triplicate: (1) planted soil treated with meat bone meal (MBM) and inoculated with the live ZCR6$^{rf+egfp}$ strain (BL+MBM), (2) planted soil treated with MBM and the thermally inactivated ZCR6$^{rf+egfp}$ strain (BTI+MBM), (3) planted soil treated with MBM and water (W+MBM), and (4) planted soil treated with water (W). In the experiment, 72 separate pots were prepared (4 treatments × 3 replicates × 6 time points). Before the experiment, the soil was air-dried and passed through a 2 mm sieve. Then, the soil moisture was adjusted and maintained at 50% of the maximum water-holding capacity during the entire experimental period. The pots (2 L volume, 20 cm height, 12 cm width, 12 cm length) were filled with 1 kg (dry weight, DW) of polluted soil. In the case of MBM-containing experimental groups, polluted soil was supplemented with this biofertilizer (2%, w/w, provided by Honkajoki Oy, Honkajoki, Finland). Since the ZCR6 strain was isolated from the rhizosphere of maize, *Z. mays* was chosen as the tested plant species to reduce the adverse effects of inoculum incompatibility with the host plant. Additionally, *Zea mays* is a fast-growing, high-biomass-producing species with tolerance to multiple HMs and phytoextraction ability. It develops an extensive fibrous root system and, thus, a large zone of rhizosphere soil, which is highly desirable for the effective rhizodegradation of hydrocarbons by soil

microorganisms (Agnello et al., 2016; Cao et al., 2022). Seeds of *Zea mays* (P9363, Corteva Agriscience) were placed into the soil (2 per pot) and then covered with a 1-cm layer of soil. The experiment was conducted in a growth room under controlled light (14-h photoperiod at 15,000 lux; temperature 23/18 °C; light/dark). After 2 weeks, the following treatments were applied: i) 50 mL of the bacterial solution of the ZCR6 strain (10^8 cfu g^{-1} DW of soil) (BL+MBM), ii) 50 mL of thermally inactivated bacterial solution of the ZCR6 strain (10^8 cfu g^{-1} DW of soil used for preparation) (BTI + MBM), or iii) 50 mL of sterile water was added to the soil (W+MBM and W). After plant treatment the experiment was conducted for 40 days. Soil samples collected on days 3, 10, 20, 30, and 40 were immediately acquired for estimating the ZCR6 strain number. All the soil samples were then stored at -80 °C prior to use. For molecular and biochemical analyses, soil samples taken on days 20 and 40 were used.

2.6 Number of ZCR6 cells in the soil

The number of living cells of the ZCR6 strain in the soil was determined on days 1, 3, 10, 20, 30 and 40 after soil inoculation using the dilution-plate method. For this purpose, LB agar plates supplemented with rifampicin (100 µg mL^{-1}) were used according to Pacwa-Płociniczak et al. (2019).

Additionally, the number of ZCR6 cells was verified using flow cytometry. One gram of soil was mixed with 9 mL of detergent solution (250 mM tetrasodium pyrophosphate [TSP; pH 8.0] containing Tween 80 [0.5% final concentration]). The mixture was shaken for 20 min in a vortex at room temperature. Then, 1 mL of Nycodenz solution (80% [w/vol] prepared in 50 mM TSP buffer) was added to a 15 mL Falcon tube, and after soil sedimentation for 1-2 minutes, 2 mL of soil slurry was added to the Falcon tube with Nycodenz solution. The mixture was centrifuged at 4,650 rcf for 30 min. The supernatant containing bacterial cells was transferred to new Falcon tubes and analyzed by flow cytometry (CyFlow SPACE).

225

226 *2.7 Determination of TPH concentration*

227 The total petroleum hydrocarbon concentration in the soil was determined for each pot before,
228 during, and after the bioremediation study and expressed as the concentration of hydrocarbons
229 with a carbon number between 10 and 40 ($TPH_{C10-C40}$) following ISO 16703:2011 according to
230 Pacwa-Płociniczak et al. (2016a).

231

232 *2.8 Heavy metal concentrations*

233 The Zn, Cd and Cu concentrations were determined in the shoots and roots of the maize. One
234 gram of shoot/root tissues was dried to a constant weight at 105 °C and then digested in a
235 mixture of HNO_3 (65%; 8 mL) and H_2O_2 (30%; 2 mL) using a Milestone Ethos One microwave
236 oven (Milestone, USA). Mineralization was performed according to the manufacturer's
237 recommendation. The total concentrations of Zn, Cd and Cu were estimated using an atomic
238 absorption spectrophotometer (ICP–OES OPTIMA 8300DV, Perkin Elmer, UK). Additionally,
239 the translocation factor (TF), defined as the metal concentration ratio of plant shoots to roots,
240 was calculated.

241

242 *2.9 Fluorescein diacetate hydrolyzing activity*

243 Fluorescein diacetate (FDA) hydrolyzing activity in soil was performed to determine the total
244 activity of soil microorganisms on days 20 and 40 of the experiment according to Sułowicz et
245 al. (2016). The concentration of free fluorescein in the filtered solution was measured at 490
246 nm using a Helios Epsilon (Thermo Fisher Scientific, Waltham, MA USA) spectrophotometer
247 and calculated in reference to the calibration curve.

248

249 *2.10 RNA extraction and RT-qPCR*

RNA was extracted from soils (n = 3 for each treatment and duration) taken on days 20 and 40 of the experiment using an RNeasy PowerSoil Total RNA Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The quantity and quality of the RNA were assessed using an Implen NanoPhotometer spectrophotometer (Implen GmbH, Munich, Germany). For removal of any residual contaminating DNA from the total RNA, RQ1 RNase-free DNase (Promega, Madison, WI, USA; 1 U μg^{-1} RNA for 30 min at 37 °C) was used. The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) was used for synthesis of the cDNA. The remaining RNA samples were stored at −80 °C. The cDNA samples were stored at −20 °C.

Quantification of expression of the 16S rRNA gene and genes encoding the enzymes involved in the degradation of hydrocarbons (alkane monooxygenase (*alkB*), alkane hydroxylase (*alkH*), long-chain alkane monooxygenase (*ladA*), cytochrome P450-type alkane hydroxylase (CYP153), catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O)) was performed in soils from the studied treatments on days 20 and 40 of the experiment using real-time PCR. qPCR was conducted in triplicate using specific primer sets (Table S) (Edwards et al., 1989; Phillips et al., 2008; Powell et al., 2006; Sei et al., 1999; Yousaf et al., 2010) and a LightCycler 96 Real-Time PCR System (Roche Diagnostics, Florham Park, NJ, USA) following the protocol described by Pacwa-Płociniczak et al. (2019).

2.11 cDNA library preparation and RNA sequencing

RNA sequencing was performed for samples extracted from the tested soils taken on day 20 of the experiment. The integrity of RNA was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Ribosomal RNA depletion was performed using the NEBNext rRNA Depletion Kit (Bacteria) (New England BioLabs, Germany); the library was constructed using TruSeq Stranded Total RNA (Illumina, San Diego, CA, USA). The libraries

were sequenced using the Illumina NovaSeq 6000 (2x150 bp, paired-end, Illumina, San Diego, CA, USA). The obtained data were analyzed by an integrated approach of ATLAS (a workflow for assembly, annotation, and genetic binning of metagenome sequence data, https://github.com/metagenome-atlas/atlas) (Kieser et al., 2020). Briefly, quality control of raw sequences, as well as error correction based on k-mer coverage and paired-end read merging, was performed using utilities in the BBTools suite, then metaSPAdes was used for *de novo* assembly. For annotation, the prediction of open reading frames (ORFs) using Prodigal was carried out; the translated gene products were then clustered using linclust to generate non-redundant gene and protein catalogs, which were mapped to the eggNOG catalog. The sequences generated in this study were deposited in the GenBank SRA database under BioProject accession number PRJNA1036546.

2.12 Taxonomic profiling

KneadData (v0.12.0, https://huttenhower.sph.harvard.edu/kneaddata/), a pipeline of both Trimmomatic (Bolger et al., 2014) and Bowtie2 (Langmead and Salzberg, 2012), was used to perform quality control of metatranscriptomic sequencing data. The processed reads were then used to perform the taxonomic analysis using MetaPhlan 4.0 (Blanco-Miguez et al., 2022), a computational tool that aligns metagenome reads to a unique clade-specific marker-gene database to perform taxonomic classification.

2.13 Differential abundance analysis

The differential abundance analysis was performed using the MaAsLin 2 (Microbiome Multivariable Associations with Linear Models) package (Bioinductor) that uses a variance-stabilizing arcsine square root transformation to create continuous abundance profiles that can be analyzed by regular linear models (Mallick et al., 2021) employing the R software. The genes

with a *P* value (*P*-adj) < 0.05 and absolute fold change > 2.0 were considered as differentially expressed. The Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment were conducted using topGO (Alexa and Rahnenführer, 2023) and clusterProfiler (Wu et al., 2021) packages, to identify the biological functions and pathways affected by the differentially expressed genes (DEGs), respectively.

2.11 Statistical analysis

Statistical analysis was performed using STATISTICA 13.3 PL software (StatSoft, Tulsa, USA). An analysis of variance (ANOVA) followed by Tukey's honestly significant difference test (Tukey HSD test) was performed to estimate any significant effect of the individual treatments on the TPH removal, total microbial activity, and the number of 16S rRNA, CYP153, and *acdS* gene transcripts. Differences between treatments with $P < 0.05$ were considered significant. For the experiments, data are presented as the mean ± standard deviation (SD) of three biological replicates.

3. Results

3.1 TPH removal and heavy metal accumulation.

In all experimental groups with the addition of MBM, on the 30th day after germination, the plants began to show symptoms of exposure to toxic substances and finally died shortly after. At the later time points, plant growth was observed only in the experimental group without MBM added, indicating that adding biofertilizer at the tested concentration, but not the presence of pollutants in the soil, is the reason for the toxic effect on plants. Nevertheless, the experiment was continued until the end of the planned period of time.

The initial TPH content in the soil was 2330 ± 331 mg kg^{-1} DW, whereas the initial concentration of Zn, Cd and Cu was 2360 ± 118; 11.7 ± 1 and 41.5 ± 1.2

mg kg^{-1} DW of soil, respectively. PH removal from soil subjected to the different treatments was estimated on days 20 and 40 after bacterial inoculation (Table 1). On day 20, significantly ($P < 0.05$) higher efficiency of hydrocarbon removal was observed in all soils enriched with MBM compared to soil without MBM added, taken from the W group, where maize plants were still alive; however, no significant ($P < 0.05$) differences in the removal efficiency were observed between soils treated with living (BL+MBM, 31.15 ± 2.22%) and thermally inactivated (TI+MBM, 32.70 ± 2.77%) cells of the ZCR6 strain and soil treated with water (W+MBM, 31.96 ± 7.18%). A similar tendency was observed on day 40; however, no further hydrocarbon removal was observed between days 20 and 40.

Table 1. TPH removal efficiency in bioremediation treatments.

Treatment	TPH removal (%)	
	20 days	40 days
BL+MBM	31.15 ± 2.22[a]	32.02 ± 0.30[a]
BTI+MBM	32.70 ± 2.77[a]	33.12 ± 2.77[a]
W+MBM	31.96 ± 7.18[a]	34.67± 1.76[a]
W	18.14 ± 1.50[b]	19.61 ± 1.55[b]

BL – live bacteria; BTI – thermally inactivated bacteria; W – water; MBM – meat and bone meal. Different letters (within each group) indicate significant differences ($P < 0.05$, Tukey test); ± Stand. Dev. of three independent experiments

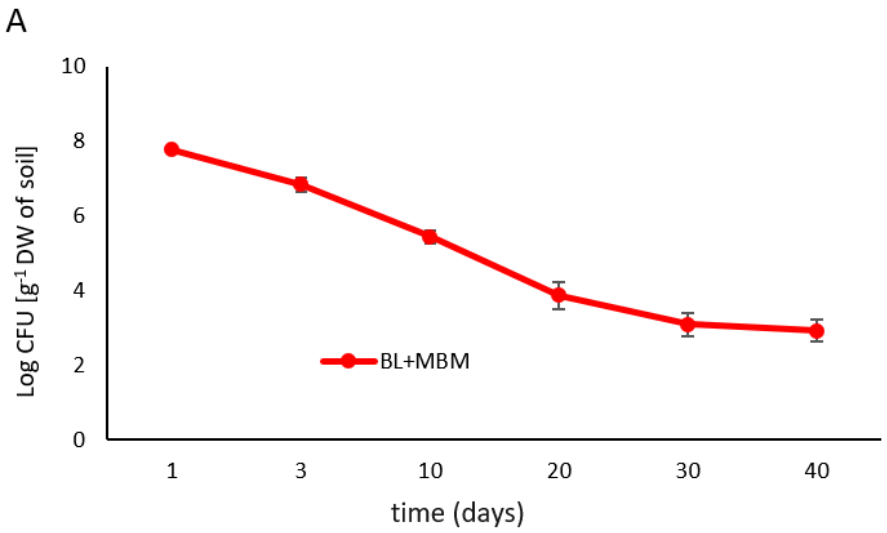
Because plants from the treatments supplemented with MBM did not survive until the end of the experiment, the concentrations of Zn, Cd, and Cu were measured only for the control plants from the W treatment. The accumulation of these metals in non-treated maize was confirmed, but mainly in the roots, where concentrations of Zn, Cd and Cu were 1330 ± 133, 27.4 ± 6.8

345 and 32.4 ± 9.3 mg kg^{-1} DW of plants, respectively. The tested metals' translocation factor (TF)
346 reached values of 0.23 ± 0.02, 0.043 ± 0.00 and 0.34 ± 0.04, respectively (Table 2).
347
348 Table 2. Accumulation of Cd, Zn and Cu in *Z. mays* grown in the soil from treatment W.

Plant part	Heavy metal		
	Cd	Zn	Cu
roots	27.4 ± 6.8	1330.3 ± 433.1	32.4 ± 9.3
shoots	1.18 ± 0.1	300.3 ± 30.2	10.9 ± 1.1
TF	0.04 ± 0.00	0.23 ± 0.02	0.34 ± 0.04

349 TF – translocation factor; ± Stand. Dev. of three independent experiments
350
351
352 *3.2 Fate of the ZCR6 strain in the soil*
353
354 The rifampicin-resistant mutant of the ZCR6 strain harboring the pMP4655 plasmid vector with
355 constitutive expression of the *egfp* gene was monitored for its ability to survive in the soil and
356 to colonize roots and shoots of the maize after its bioaugmentation. The cells of the inoculated
357 strain were detected in the soil until the end of the experiment using the plating method and
358 fluorescence microscopy. The results from the dilution plate method on LB plates supplemented
359 with rifampicin showed that three days after soil inoculation, the number of ZCR6 cells
360 decreased one order of magnitude from the initial 7.8 to 6.8 $\log_{10}$ cfu g^{-1} DW of soil in treatment
361 BL+MBM (Fig. 1a). At days 10, 20, 30 and 40 of the experiment, the number of cells decreased
362 gradually, reaching values of 5.6, 3.4, 3.9 and 3.7 $\log_{10}$ cfu g^{-1} DW of soil, respectively. The
363 results from flow cytometry analysis confirmed a decrease in the number of ZCR6 cells on day
364 3 of the experiment to 6.9 $\log_{10}$ cell number g^{-1} DW of soil (Fig. 1b); however, at the following

365 sampling points, until the end of the experiment, the number of ZCR6 cells remained at a stable
366 level of approximately 6.7 $\log_{10}$ cell number g^{-1} DW of soil.
367



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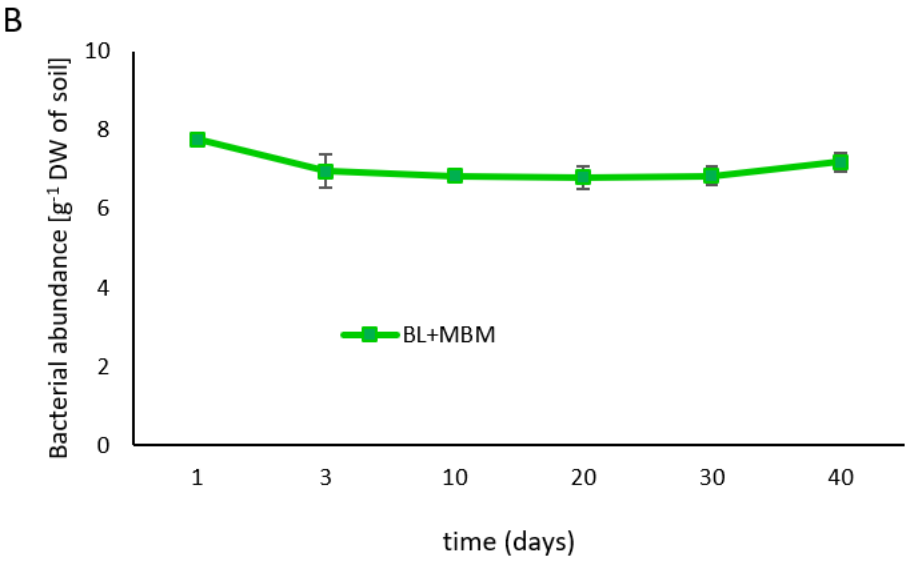


369

370 Fig. 1 Number of ZCR6 strain cells detected by plating method (a) and flow cytometry (b) in
371 the soil; BL – live bacteria; MBM – meat and bone meal. The data presented are the means and
372 standard deviations (SD) of three replicates.

373

374 *3.3 Total activity of soil microorganisms*

375 The total microbial activity on days 20 and 40 of the experiment was determined by FDA
376 hydrolysis. For both sampling days, significantly ($P < 0.05$) higher microbial activity was
377 reported for all soils enriched with MBM, compared to soil not supplemented with MBM. On
378 day 20, the total activity calculated for soil BL+MBM was 9 times higher, whereas for soils
379 BTI+MBM and W+MBM it was 11 times higher, compared to soil not treated with meat and
380 bone meal. On the other hand, among treatments with MBM, soil BL+MBM was characterized
381 by significantly lower microbial activity compared with soil W+MBM. The analysis performed
382 for the samples from day 40 showed no significant differences in the activity of microorganisms
383 in all the experimental groups treated with MBM; however, the microbial activity calculated
384 for the MBM-treated soils was still about 12 higher than for soil W (Table 3).

385

386 Table 3. Total microbial activity for the soil from different treatments.

Treatment	FDA hydrolysis [µg fluorescein g^{-1} d. w.soil]	
	20 days	40 days
BL+MBM	25.99 ± 1.48^{b}	26.42 ± 0.39^{a}
BTI+MBM	31.00 ± 3.16^{ab}	24.77 ± 2.79^{a}
W+MBM	32.53 ± 3.36^{a}	26.10 ± 0.40^{a}
W	2.87 ± 0.83^{c}	2.18 ± 0.40^{b}

387 BL – live bacteria; BTI – thermally inactivated bacteria; W – water; MBM – meat and bone meal.
388 Different letters (within each group) indicate significant differences ($P < 0.05$, Tukey test); ± Stand.
389 Dev. of three independent experiments
390

391

392

393

394 *3.4 Quantification of gene expression levels*

395 On day 20, the expression of the 16S rRNA gene in all tested samples was in the range of 12.06-
396 12.21 $\log_{10}$ copies g^{-1} DW of soil, and no significant differences were found between treatments.
397 In turn, on day 40, the expression level of the 16S rRNA gene was significantly ($P < 0.05$)
398 higher in soils BL+MBM and BTI+MBM than in soils W and W+MBM (Fig. 2).

399 Among the tested genes involved in PH degradation, only the CYP153 gene was detected in
400 the analyzed soil. It was demonstrated that on day 20 the number of transcripts of this gene was
401 significantly ($P < 0.05$) lower in all soils supplemented with MBM than in the soil not enriched
402 with this fertilizer (20, 18, and 35 times lower in the BL+MBM, BTI+MBM and W+MBM soils
403 than in the W soil, respectively). On day 40, a significantly ($P < 0.05$) lower expression level
404 of the CYP153 gene in soils enriched with MBM compared to nonsupplemented soil was
405 detected only in the W+MBM treatment. The number of transcripts of this gene estimated in
406 the W+MBM soil was 5 times lower than that in the W soil (Fig. 2).

407 Among the tested genes involved in plant growth promotion, only the expression of the *acdS*
408 gene was observed in the tested soils. A lower number of *acdS* transcripts was also noted in
409 soils with MBM than in soil without the addition of fertilizer (W). On day 20, these differences
410 were significant ($P < 0.05$) only between soil W+MBM (5.38 $\log_{10}$ copies g^{-1} DW of the soil)
411 and treatment W (6.02 $\log_{10}$ copies g^{-1} DW of the soil), while on day 40, they were observed
412 between soil W (6.25 $\log_{10}$ copies g^{-1} DW of the soil) and treatments BL+MBM (5.45 $\log_{10}$
413 copies g^{-1} DW of the soil) and W+MBM (5.43 $\log_{10}$ copies g^{-1} DW of the soil) (Fig. 2).

414

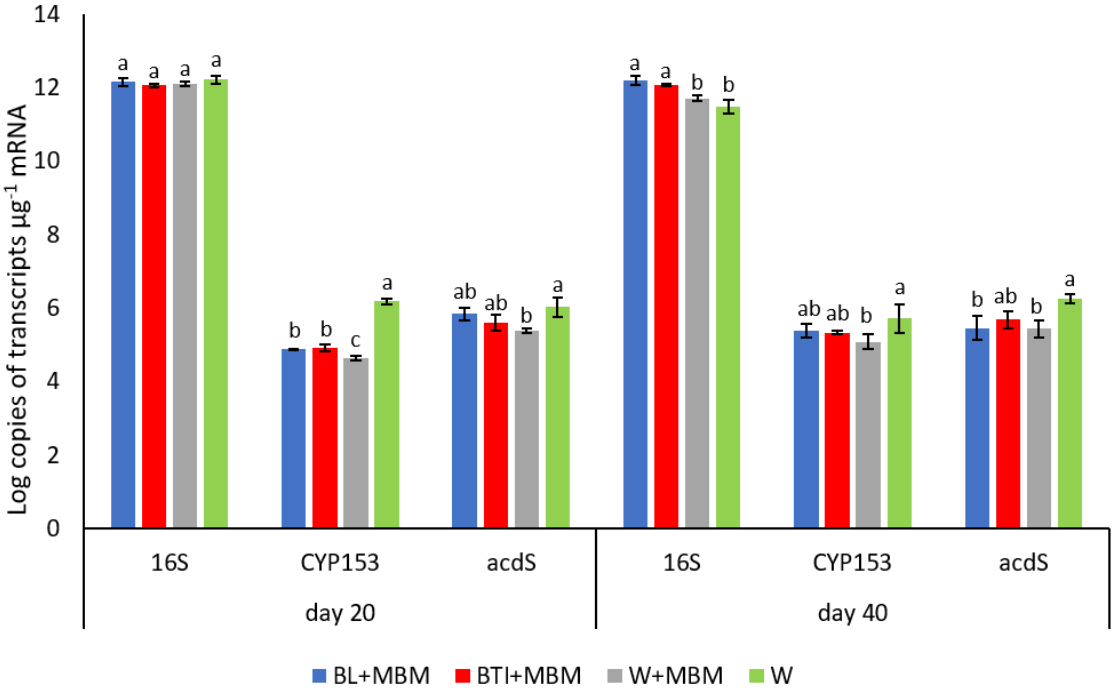


Fig. 2 Number of 16S rRNA, CYP153 and *acdS* transcript copies in soils during the experiment; BL – live bacteria; BTI – thermally inactivated bacteria; W – water; MBM – meat and bone meal. The data presented are the means and standard deviations (SD) of three replicates. Different letters (within each group) indicate significant differences ($P < 0.05$, Tukey HSD-test).

3.5 Bacterial community structure

The taxonomic classification performed for the cDNA sequences from day 20 of the experiment revealed that fertilization of soils with MBM changed the composition of the bacterial community, causing the appearance of *Nitrospira and Albibacterium* bacteria, as well as an increase in the abundance of *Rhodanobacter* and *Lysobacter* genera compared to soil W (Fig. 3). Nevertheless, the abundance of the sequences characteristic for all these genera differed between MBM-treated soils. The highest abundance of *Nitrospira* was estimated for soil BL+MBM (51.48%), whereas in soils BTI+MBM and W+MBM it was 23.60% and 34.22%, respectively. For *Albibacterium* the abundance ranged between 1.94% (BTI+MBM) and about

4.2% (BL+MBM and W+MBM). In the case of *Rhodanobacter* the highest abundance of sequences characteristic for this genus was observed in soil W+MBM (58.67%), whereas in soils BL+MBM and BTI+MBM it reached values of 17.35% and 22.95%, respectively. Abundance of the genus *Lysobacter* reached the values of 9.44% in the soil BTI+MBM and about 2.3% in soils BL+MBM and W+MBM. In soils treated with MBM and both variants of bacteria, the presence of the genera *Rhodococcus* (6.75% for BL+MBM and 31.14% for BTI+MBM) and *Eoetvoesia* (17.51% for BL+MBM and 7.52% for BTI+MBM) was observed. Bacteria from the *Rhodococcus* genus dominated in the soil not treated with MBM, where they accounted for 64.71% of all detected bacteria, while they were not observed in the MBM+W soil. In turn, sequences characteristic for *Eoetvoesia* were not detected either in the MBM+W or in the W soil.

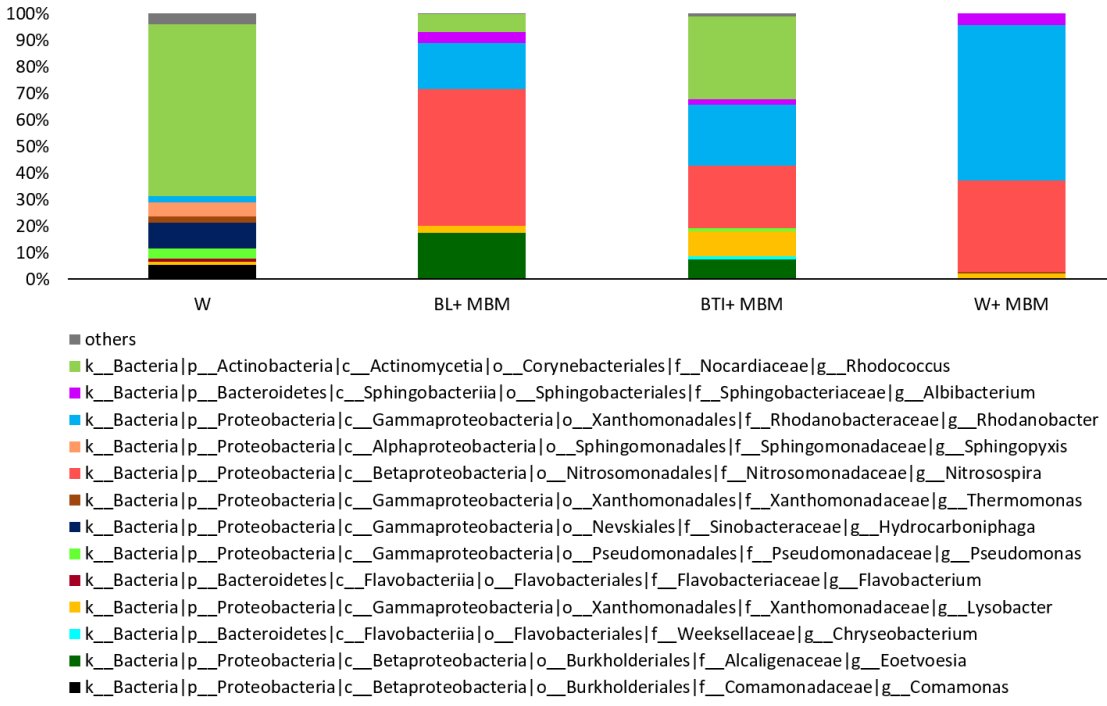


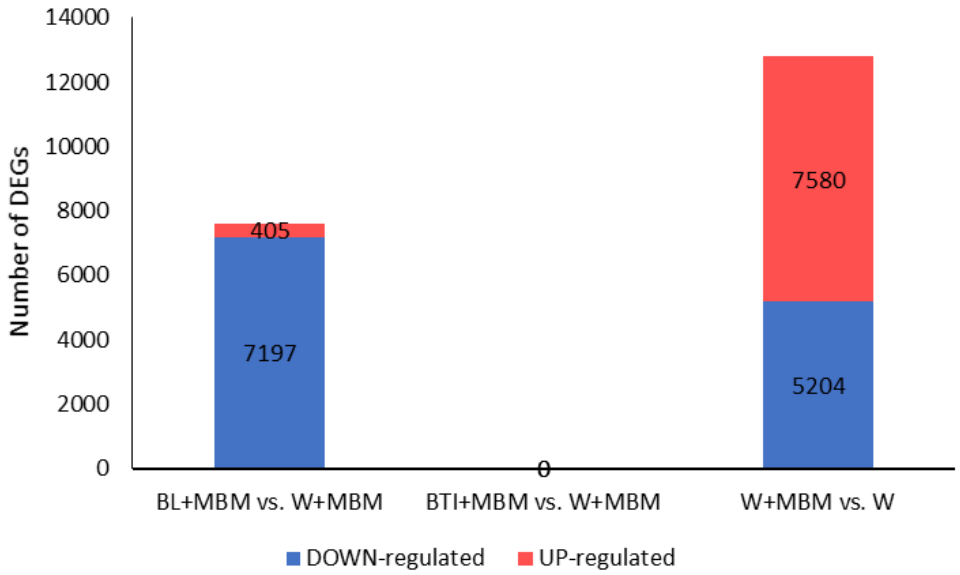
Fig. 3 Relative abundance of the bacterial genera in soils on day 20 of the experiment; W – water; BL – live bacteria; BTI – thermally inactivated bacteria; MBM – meat and bone meal.

446

447 *3.6 Differentially expressed genes detected in metatranscriptomic analysis*

448 To compare the gene expression changes between treatments on day 20 of the experiment, three
449 contrast groups were established: 1) BL+MBM vs. W+MBM, 2) BTI+MBM vs. W+MBM, 3)
450 W+MBM vs. W. A comparison of BL+MBM soil with W soil revealed 7,602 (405 up- and
451 7,197 down-regulated) genes that were differentially expressed (DEGs); no DEGs were
452 detected when comparing BT+MBM versus W soil, whereas 11,095 genes (7,580 up-, and
453 5,204 down-regulated) were differentially expressed between soils W+MBM and W (Fig. 4).

454



455

456 Fig. 4 Comparative analysis of differentially expressed genes (DEGs) identified within three
457 contrast groups; BL – live bacteria; BTI – thermally inactivated bacteria; W – water; MBM –
458 meat and bone meal.

459

460 *3.7. GO enrichment analysis*

461 GO enrichment analysis (enrichment *p.adjust* < 0.05) of DEGs was carried out to find
462 significant GO terms (in each of the GO categories: Biological Process (BP), Cellular

463 Component (CC) and Molecular Function (MF)) specific for applied treatment on day 20 of the
464 experiment.
465 Among the significant BPs associated with up-regulated genes in the BL+MBM soil were those
466 associated with growth (GO:0040007). Among MFs the most represented were transcripts
467 related to ATP-dependent activity (GO:0140657), ATPase-coupled ion transmembrane
468 transporter activity (GO:0042625), and zymogen binding (GO:0035375). In the case of CC, the
469 most enriched functional categories were plasma membrane (GO: 0005886), cell wall
470 (GO:0005618), intracellular non-membrane-bound organelle (GO: 0043232), and cytosolic
471 large ribosomal subunit (GO:0022625) (Fig. S1).
472 The most enriched significant BPs associated with down-regulated genes in BL+MBM
473 soil were nicotinamide nucleotide metabolic process (GO:0046496), cell division
474 (GO:0051301), and protein maturation (GO:0051604). Among MFs the most enriched
475 functional categories were unfolded protein binding (GO:0051082), catalytic activity, acting on
476 a protein (GO:0140096), as well as carbamoyl-phosphate synthase (ammonia, GO:0004087),
477 and carbamoyl-phosphate synthase (glutamine-hydrolyzing, GO:0004088) activity, whereas
478 with CC the most represented were transcripts related to cytoplasm (GO:0005737), protein-
479 containing complex (GO:0032991), and membrane (GO:0016020) (Fig. S2).
480 GO enrichment analysis performed for up-regulated genes in the W+MBM soil revealed
481 the predominance of the following functional categories among BPs: '*de novo*' protein folding
482 (GO:0006458) and chaperone-mediated protein folding (GO:0061077). Among MFs the most
483 enriched categories were protein folding chaperone (GO:0044183), unfolded protein binding
484 (GO:0051082), and ATP hydrolysis activity (GO:0016887), whereas among CC the most
485 represented were intracellular anatomical structure (GO:0005622), cytoplasm (GO:0005737),
486 protein-containing complex (GO:0032991), and intracellular organelle (GO:0043229) (Fig.
487 S3). On the other hand, the most significant BP associated with down-regulated genes enriched

in W+MBM soil was that associated with growth (GO:0040007). In the case of MFs, the most enriched functional category was structural constituent of ribosome (GO:0003735), and among CCs the most significant were cytosol (GO:0005829), plasma membrane (GO:0005886), cell wall (GO:0005618), cytosolic small ribosomal subunit (GO:0022627), and extracellular region (GO:0022627) (Fig. S4).

3.8 KEGG enrichment analysis

KEGG enrichment analysis (enrichment *p.adjust* < 0.05) of DEGs was performed to investigate the effect of applied treatment on the pathway network in tested soil taken on day 20 of the experiment. Among the significant pathways associated with up-regulated transcripts in the BL+MBM soil only three pathways were indicated: caprolactam degradation, fatty acid degradation and glycine, serine and threonine metabolism. In the caprolactam degradation pathway, partial DEGs were involved in metabolizing cyclohexane (*alkB*, *chnB*), whereas among DEGs associated with fatty acid degradation genes encoding alkane 1-monooxygenase (*alkB*) and butyryl-CoA dehydrogenase (*bcd*) were detected (Fig. 5a).

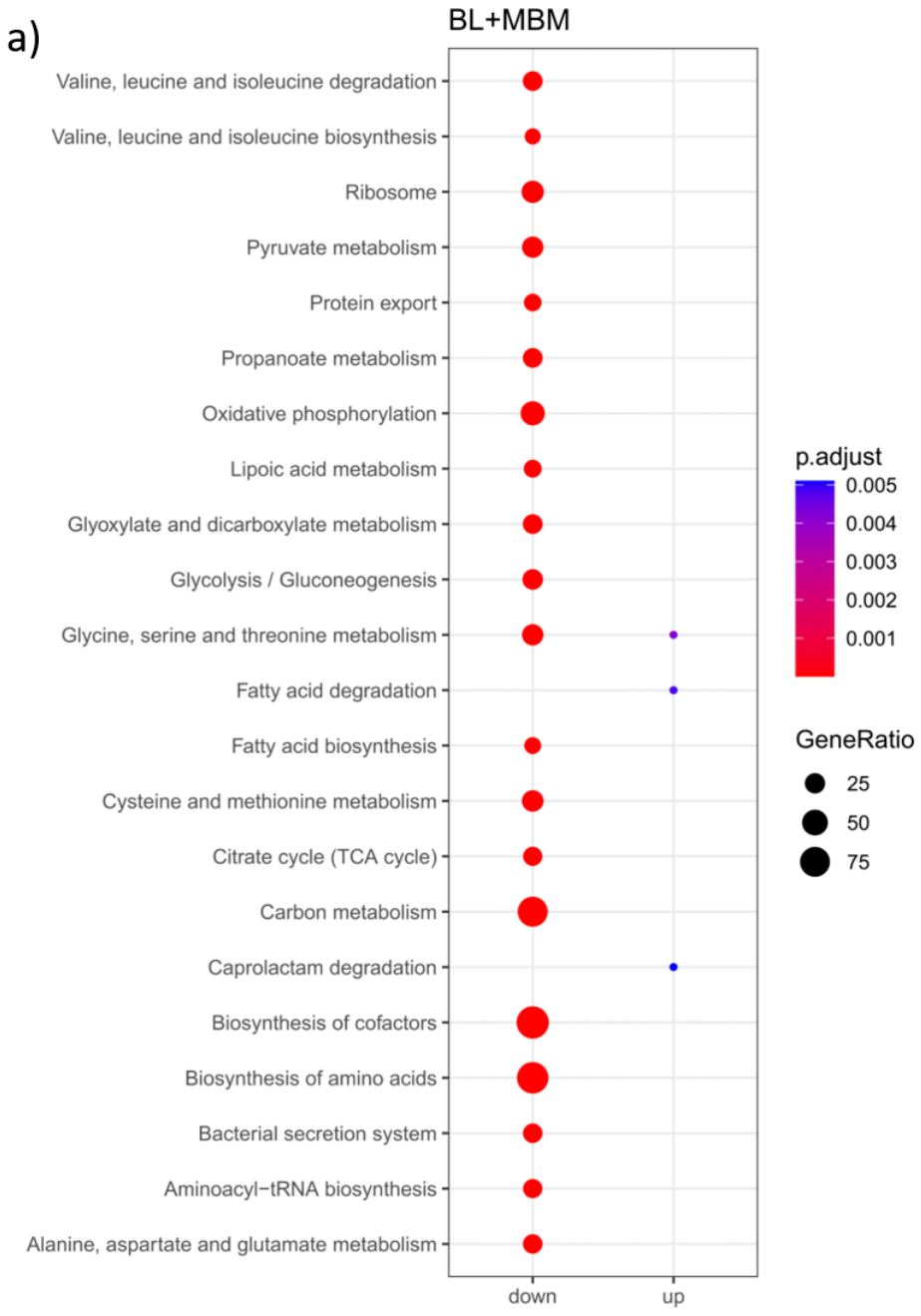
In the case of significant pathways associated with down-regulated genes in BL+MBM soil, the most represented were: biosynthesis of cofactors, biosynthesis of amino acids, and carbon metabolism. In the mentioned pathways numerous DEGs were associated; nevertheless, it can be summarized that down-regulated genes were involved in glycolysis [encoding fructose-bisphosphate aldolase (ALDO), glyceraldehyde 3-phosphate dehydrogenase (*gapA*), phosphoglycerate kinase (*pgk*), 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase (*gpmA*), enolase (*eno*), pyruvate kinase (*pyk*), glucose-6-phosphate isomerase (*pgi*) and 6-phosphofructokinase 1 (*pfkA*)], the Entner-Doudoroff pathway [encoding glucose-6-phosphate 1-dehydrogenase (*zwf*), 6-phosphogluconolactonase (*pgl*), phosphogluconate dehydratase (*edd*)], pyruvate oxidation [encoding pyruvate dehydrogenase E1 component (*aceE*)], citrate

cycle [encoding aconitate hydratase (*acnA*), isocitrate dehydrogenase (*icd*), 2-oxoglutarate dehydrogenase E1 component (*sucA*), succinyl-CoA synthetase alpha subunit (*sucD*), succinate dehydrogenase flavoprotein subunit (*sdhA*), fumarate hydratase (*fumA*), malate dehydrogenase (MDH1) and citrate synthase (*gltA*)], as well as the pentose phosphate pathway (encoding glucose-6-phosphate 1-dehydrogenase (*zwf*), 6-phosphogluconolactonase (*pgl*), 6-phosphogluconate dehydrogenase (*gnd*), glucose-6-phosphate isomerase (*pgi*), transaldolase (*talB*), fructose-bisphosphate aldolase (ALDO), transketolase (*tktA*), ribose 5-phosphate isomerase A (*rpiA*), and ribulose-phosphate 3-epimerase (*rpe*)]. The other significant down-regulated pathways in BL+MBM soil were related to oxidative phosphorylation, ribosome, metabolism of amino acids (cysteine, methionine, glycine, serine, threonine, alanine, aspartate, glutamate), pyruvate, propanoate, glyoxylate, dicarboxylate, and lipoic acid, as well as bacterial secretion system, biosynthesis of aminoacyl-tRNA, fatty acid, and amino acids (valine, leucine and isoleucine), and protein export. Down-regulated DEGs involved in the bacterial secretion system included genes associated with secretion system Type I [encoding outer membrane protein (*tolC*)], Type II [encoding general secretion pathway protein D (*gspD*), general secretion pathway protein G (*gspG*) and general secretion pathway protein H (*gspH*)], Type VI [encoding type VI secretion system secreted protein Hcp (*hcp*), ImpL (*impL*), and VasG (*vasG*)], secretion system Sec-SRP [encoding preprotein translocase subunit SecD (*secD*), SecG (*secG*), SecY (*secY*), and YajC (*yajC*), YidC/Oxa1 family membrane protein insertase (*yidC*), fused signal recognition particle receptor (*ftsY*), preprotein translocase subunit SecA (*secA*), and SecB (*secB*), and signal recognition particle subunit SRP54 (*ffh*)], as well as Twin arginine targeting (Tat) secretion system [encoding sec-independent protein translocase protein TatA (*tatA*), TatB (*tatB*), TatC (*tatC*) and TatE (*tatE*)] (Fig. 5a)

KEGG enrichment analysis of DEGs in the W+MBM soil revealed that among the most significant pathways associated with up-regulated genes were biosynthesis of cofactors,

biosynthesis of amino acids, and carbon metabolism. Among DEGs involved in carbon metabolism up-regulated genes were associated with methane oxidation [encoding methane/ammonia monooxygenase subunit A (*pmoA-amoA*)], glycolysis [*pgi*, *pfkA*, ALDO, *gpmA*], pyruvate oxidation [*aceE*], and the pentose phosphate pathway [*pgi*, *talB*, *rpiA*]. The other significant up-regulated pathways in W+MBM soil were associated with biosynthesis of nucleotide sugars, ubiquinone and other terpenoid-quinones, amino acids, O-antigen nucleotide sugars, the peptidoglycan and terpenoid backbone, metabolism of amino acids, amino sugars, nucleotide sugars, nucleotides, lipoic acid, and nitrogen, as well as degradation of amino acids, base excision repair and carbon fixation in photosynthetic organisms. Among up-regulated genes related to nitrogen metabolism were those associated with nitrification [*pmoA-amoA*, and *hao* (encoding hydroxylamine dehydrogenase)], and partially with denitrification (encoding nitric oxide reductase subunit B (*norB*)) (Fig. 5b). Among significantly down-regulated pathways the most represented were related to the two-component system, degradation of aromatic compounds and the carbon system. Additionally, in the W+MBM soils down-regulated genes were also associated with degradation of different compounds (i.e. benzoate, xylene, dioxin, naphthalene, fatty acids, amino acids, ethylbenzene, atrazine, and caprolactam), metabolism of pyruvate, nitrogen, glyoxylate, dicarboxylate, purine, butanoate, and phenylalanine, as well as biosynthesis of amino acids. Among down-regulated DEGs involved in nitrogen metabolism were genes related to dissimilatory nitrate reduction [encoding nitrate reductase/nitrite oxidoreductase, alpha subunit (*narG*), nitrate reductase (*napA*), nitrite reductase (NADH) large subunit (*nirB*)], denitrification [*narG*, *napA* and *nirS* (encoding nitrite reductase (NO-forming)/hydroxylamine reductase)], and related to the last step of nitrification (*narG*) and the first step of anammox (*nirS*). Moreover, genes encoding nitronate monooxygenase (NMO), the NNP family, nitrate/nitrite transporter (*nark*), nitrate/nitrite transport system substrate-binding protein (*nrtA*) and hydroxylamine reductase (*hcp*) were

down-regulated. It was observed that some of the pathways were associated with both up- and down-regulated DEGs; nevertheless, within a given pathway, some processes were up- while others were down-regulated (Fig. 5b).



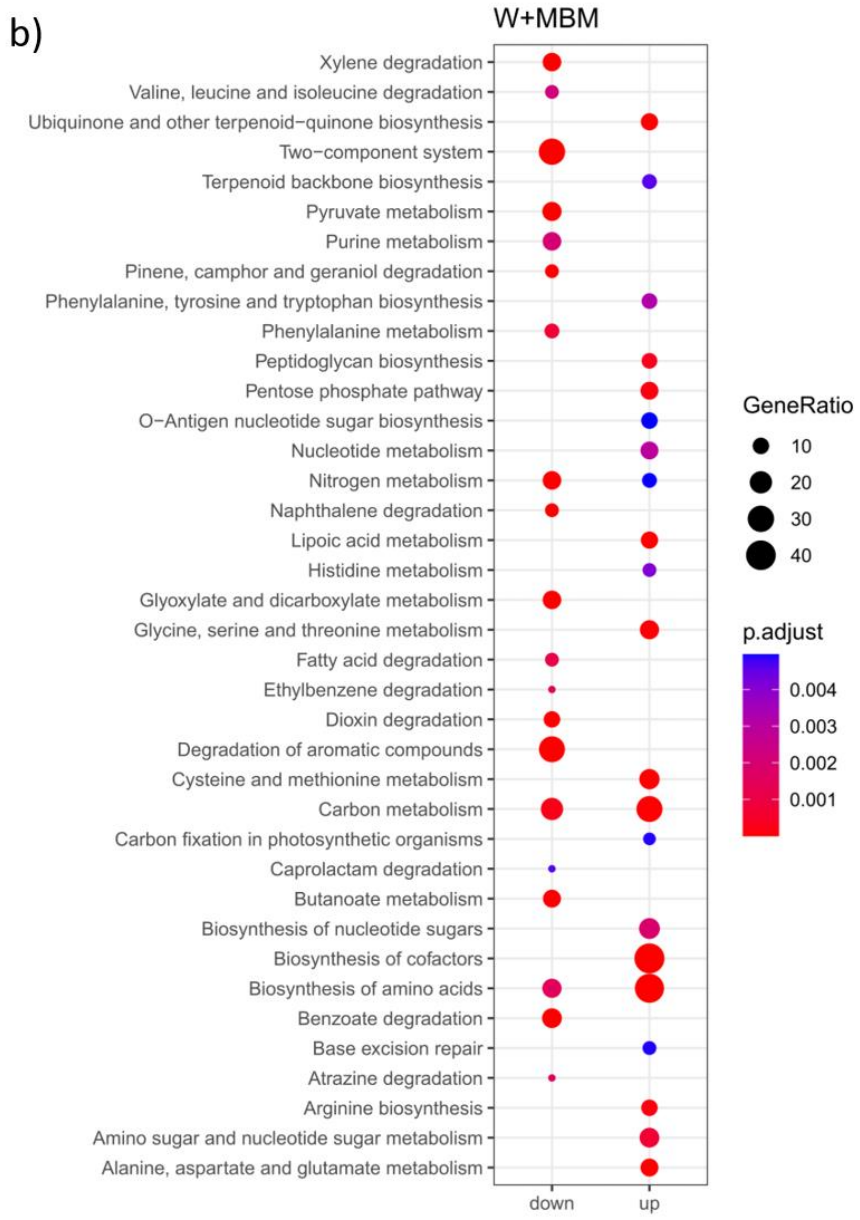


Fig. 5 KEGG enrichment analysis of Differentially Expressed Genes (DEGs) in response to BL+MBM (a) and W+MBM (b) treatments; BL – live bacteria; W – water; MBM – meat and bone meal.

3.9 Transcription of selected genes crucial for phytoremediation efficiency

The abundance of hydrocarbon degradation-related gene transcripts, as well as transcripts of ACC deaminase gene, was studied (Fig.6). Within the group of genes associated with PH

degradation, expression of *pmoA-amoA*, encoding methane particulated ammonia monooxygenase subunit A, CYP153, encoding cytochrome P450 alkane hydroxylase 1, and *alkB*, encoding alkane 1-monooxygenase, was reported in all tested soils. Expression of the mentioned genes differed between treatments, and a significantly higher number of *pmoA-amoA* transcripts (417-954 reads per million, rpm) was observed in soils amended with MBM, compared to soil W (85 rpm). In turn, in soil non-treated with MBM, the significantly higher abundance of CYP153 (365 rpm) and *alkB* (290 rpm) transcripts was revealed, compared to MBM soils (26-68 rpm for CYP153, and 0.4-2 rpm for *alkB*). In the case of *acdS* gene, a low number of transcripts (max. 6 rpm) was estimated in all tested soils.

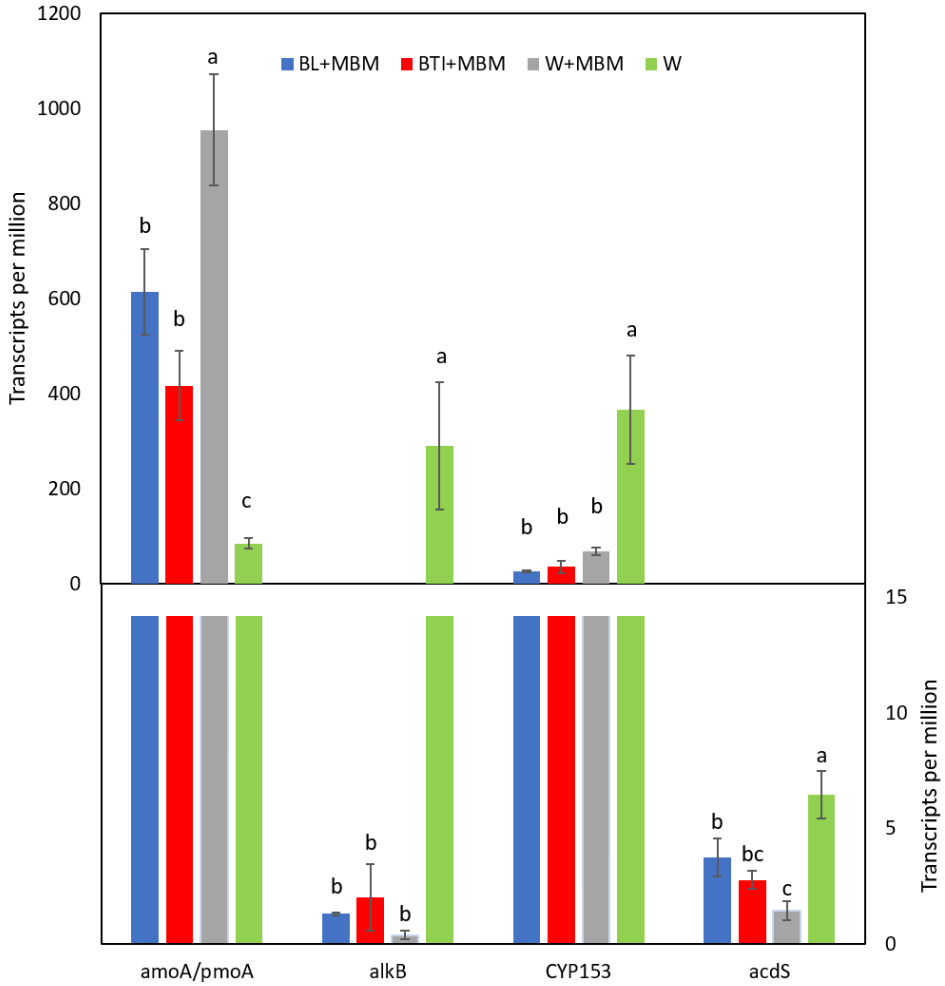


Fig. 6 Abundance of transcripts of *pmoA-amoA*, *alkB*, CYP153 and *acdS* gene. BL – live bacteria; BTI – thermally inactivated bacteria; W – water; MBM – meat and bone meal.

Discussion

To the best of our knowledge, the current study is the first attempt to combine the remediation capacity of a fast-growing plant (maize), a multi-way supporting PGPR strain (*Pseudomonas qingdaonensis* ZCR6), and the addition of a slow-release fertilizer (MBM) to clean up soil historically co-contaminated with PHs and HMs. A further novelty of our study is the use of whole metatranscriptomics analysis for a better understanding of ongoing processes during applied treatments. Even though our goal was to investigate the simultaneous use of bioaugmentation and biostimulation to support the phytoremediation of co-polluted areas, due to a more accessible interpretation of the obtained results, we decided to divide the discussion into two subsections, each focusing separately on the treatment used.

Consequences of the bioaugmentation for the remediation process

In the laboratory, the ZCR6 strain expressed the activity of several mechanisms crucial to promote plant growth, and thus potentially supporting the phytoremediation of co-contaminated soil. Despite its promising characteristics, we did not observe a positive effect of the living cells of the ZCR6 strain on the phytoremediation efficiency of soil contaminated with PH and HM treated with MBM. Such a positive effect of the ZCR6 strain was observed earlier in our preliminary tests using maize and single pollutant-containing soil (data not shown). Similar observations reported previously by Liu et al. (2021) were explained as a result of the low survival of the endophytic bacterial strain after its inoculation into the soil. It is well known that the most important factors influencing the efficiency of bioaugmentation during bacterial-assisted phytoremediation include the survival of the introduced bacteria after soil inoculation, their ability to multiply in the soil, and their metabolic activity in such environment (Płociniczak et al., 2017). In survival studies using plating and flow cytometry techniques, we recorded a one-order-of-magnitude reduction in ZCR6 strain abundance directly after introduction into the

soil; which means that the number of CFU of the ZCR6 strain was reduced by 90%. This is a frequent observed tendency in bioaugmentation experiments in which the fate of inoculants is monitored. Most of the bacteria did not survive the soil inoculation, but still they could support the plants in two ways, mainly because the bacterial necromass enhanced the pool of nutrients in the soil and through the metabolic activity of the remaining 10% of inoculants. This was a similar effect to that caused by the thermally inactivated strain. However, it is an open question whether the remaining 10% of bacteria is a sufficient number of cells to cause a visible effect detected as increased efficiency of this process. Another issue is to determine whether the survived inoculants and/or indigenous bacteria showed the activity of beneficial mechanisms supporting phyto- and bio-remediation of co-contaminated areas. This was analyzed using RT-qPCR and metatranscriptomics techniques.

In the current study, we quantified the expression level of several PGP genes, but only the expression of the *acdS* gene, encoding ACC deaminase, was detected in soils. However, metatranscriptomic analysis revealed its expression at a minimal level, which indicates a marginal impact on conducted processes. The higher abundance of *acdS* gene transcripts in W soil may be related to the fact that the plants survived in this experimental group until the end of the experiment and thereby could shape the composition of rhizosphere microorganisms.

Based on the results regarding the survival of the strain and the expression of selected PGP mechanisms in the soil, we suspect that the introduced strain, due to adverse environmental conditions, gradually shifted into the VBNC (viable but non-culturable) state (Su et al., 2019). In this state, bacteria partially reduce their metabolism, which may reduce the activity of selected metabolic pathways and cause difficulties in their cultivation on microbiological media, especially supplemented with selective factors. Since rifampicin is not present in the soil at concentrations that require the activation of bacterial resistance mechanisms to this antibiotic, the growth of inoculants on media with high (100 µg mL^{-1} of medium) Rif content

may be additionally constricted. Because the method of determining the number of inoculants using flow cytometry does not require their cultivation on a selective medium, the number of the EGFP-marked ZCR6 cells was significantly higher and remained constant until the end of the experiment. The higher decrease in the number of rifampicin-resistant cells of the ZCR6 strain detected in the later phase of the experiment compared to EGFP-marked cells supports such a statement.

Another factor that may have an impact on the efficiency of phytoremediation is additional soil fertilization by dead bacteria. Bacterial necromass, being an additional source of energy and carbon, is thought to serve as a biofertilizer that leads to increased activity of autochthonous hydrocarbon-degrading microorganisms for a short time (Liang et al., 2019). Nevertheless, such an effect was not observed in our experiment. It has been assumed that soil fertilization with MBM played a crucial role in supporting the microbial degradation of PHs. This was also confirmed by the significantly higher loss of PHs in MBM treatments compared to the untreated control.

An interesting finding from the RT-qPCR analysis is that among tested genes involved in hydrocarbon degradation, only the CYP153 gene was expressed in soil. Surprisingly, it was at a significantly higher level in soil not enriched with MBM than in soils supplemented with this fertilizer. Moreover, PH removal was higher in MBM soils. Since there was no difference in PH removal between living bacteria and necromass treatments in MBM soils, introduced living cells of *P. qingdaonensis* ZCR6 obviously did not contribute to the removal of hydrocarbons from soil. This is not surprising, as microbial degraders often underperform *in situ* compared with laboratory conditions (Bodor et al., 2021). Notably, since the *alkB*, *alkH*, C120, and C230 genes were not expressed in the studied soils, we assume that the degradation of hydrocarbons was the effect of the activity of enzymes characteristic for autochthonous soil microorganisms, which have not been tested in this study. Therefore, to better understand the mechanisms of

hydrocarbon decomposition in the analyzed soils, a metatranscriptomic analysis was performed. The varied expression of CYP153, *alkB*, and *pmoA-amoA* was revealed in all tested soils. Metatranscriptomics showed the presence of *alkB* gene transcripts in the soil, which was contrary to the findings from the RT-qPCR; however, observed differences may be because the used primer pair captured only part of the analyzed gene, which was also reported by Frostegård et al. (2022). In the W soil, hydrocarbon degradation was mainly driven by cytochrome P450 alkane hydroxylase 1, and alkane 1-monooxygenase. In turn, in MBM-supplemented soils, higher expression of methane particulated ammonia monooxygenase subunit A (*pmoA-amoA*) by bacteria from the order *Nitrosomonadales* was reported. These integral membrane proteins have a relatively wide substrate specificity and can oxidize a broad range of substrates. It can catalyze oxidation of both ammonia, methane, alkyl, aryl and halogenated hydrocarbons and even aromatic compounds (Arp et al., 2002). This observation suggests the important role of these enzymes in the oxidation process in MBM soils. The higher expression level of *pmoA-amoA* in soils supplemented with MBM was correlated with the changes in the bacterial community composition, observed as increased abundance of bacteria from the order *Nitrosomonadales* in these soils. Additionally, in MBM soils, expression of cytochrome P450 alkane hydroxylase 1 and alkane 1-monooxygenase was detected at a low level, which suggests that these enzymes did not significantly contribute to the hydrocarbon removal process. In turn, a high abundance of *pmoA-amoA* gene transcripts in MBM soils indicated its crucial role in PH removal.

Effect of MBM biofertilizer on remediation efficiency

Unpredictable interactions among different types of pollutants bring more challenges to plant growth or even survival; therefore, the appropriate intensification of phytoremediation helps to overcome these limitations. In addition to the introduction of PGPB, the amendment of soil

688 with fertilizers can significantly increase plant biomass production and soil microbial activity
689 (Cao et al., 2022; Shtangeeva et al., 2004); therefore, in our experiment, we studied the impact
690 of MBM addition on phytoremediation efficiency. The enrichment of soil co-contaminated with
691 PHs and HMs with MBM had a negative effect on the growth of maize plants, which, however,
692 at this stage of research does not completely exclude its usefulness in supporting this process,
693 and lower concentrations of MBM should also be tested. A possible explanation for the plant
694 dieback is the increased toxicity caused by the rapid release of a large amount of ammonia from
695 the MBM, and its further transformation into highly toxic nitrites. This assumption is supported
696 by the metatranscriptomic results showing increased expression of methane/ammonia
697 monooxygenase subunit A (AMO) and hydroxylamine dehydrogenase (HAO) observed in all
698 soils fertilized with MBM. These two enzymes are involved in two-step oxidation of NH_3;
699 AMO catalyzes the oxidation of NH_3 to NH_2OH, and HAO catalyzes further oxidation to NO_2.
700 Freichs et al. (2020) observed that a short time after germination seedlings of basil were
701 suffering from high NH_3 and NH_4^+ exposure caused by rapid ammonification after addition of
702 plant available N as amino acid fertilizer manufactured from enzymatically hydrolyzed animal
703 proteins. In their study strong inhibition of development of the radicle, hypocotyl, and
704 cotyledons of basil was observed. Similarly, adverse effects of ammonical N forms on
705 germination and seedling development have also been reported for cucumber, wheat, rye,
706 barley, maize and rice (Bremner and Krogmeier, 1989; Ells et al., 1991; Qi et al., 2012).
707 Despite the toxic effect of MBM on plant growth in co-contaminated soil, its positive influence
708 on the PH degradation rate *via* indigenous microorganisms in the soil was confirmed in our
709 experiment. The assumption is that MBM releases nutrients that enhance the degradation of
710 organic contaminants in mild climatic conditions (Liu et al., 2019; Sun et al., 2018). Similarly
711 to Liu et al. (2019), we found that the MBM stimulation effect was particularly evident in the
712 early remediation phase. It thus seems that MBM addition as a source of nutrients to PH

713 contaminated soil is a better strategy than the use of maize. Nevertheless, since phytoextraction
714 is the only biological method used to permanently remove HMs from soil, it is not possible to
715 abandon the use of plants in the remediation of co-contaminated areas. However, based on our
716 results, phytoremediation of co-contaminated soils should not be started simultaneously with
717 MBM application. Instead, the plant (e.g., maize) cultivation a few months after soil
718 biofertilization with MBM could lead to optimal remediation of co-contaminated soils. In such
719 an approach the MBM would stimulate microbial activity and enhance PH degradation, and
720 then plants would take up HMs and support PH rhizodegradation from pre-cleaned soil without
721 suffering from the excess of N-rich components and potential toxic products of PH degradation.
722 Despite the difficulties in using MBM in our research, the usefulness of this biofertilizer has
723 been confirmed previously. In the study of Mondini et al. (2008), the amendment of arable soils
724 with MBM caused an overall increase in the activity of soil microorganisms (determined based
725 on the hydrolysis of FDA), indicating an enhanced capacity of the soil for element cycling and
726 an improvement of its quality. An increase in FDA hydrolysis also directly indicates an
727 enhanced ability of the soil to degrade and transform organic substrates and pollutants
728 (Sánchez-Monedero et al., 2008), which is convergent with our results. Nevertheless, we also
729 observed significant differences in the microbial activity between MBM-treated soils. Lower
730 FDA hydrolysis was reported for BL+MBM soil compared to W+MBM soil. Effective
731 bioaugmentation, except for successful soil colonization, also depends on the interaction
732 between inoculants and autochthonous microorganisms. Because most of the soil bacteria are
733 unculturable, estimating such dependencies in laboratory conditions is impossible. In our
734 experiment, the ZCR6 inoculation resulted in lower soil microbial activity compared to non-
735 inoculated soil. Similarly, in the experimental group without MBM, the soil bioaugmentation
736 with live ZCR6 strain caused an even higher reduction of resulted in reduced microbial activity,
737 estimated on the level of 0.87 µg fluorescein g^{-1} DW of soil and was 30 times lower compared

738 to soil BL+MBM (data not shown). The confirmation for this also comes from the
739 metatranscriptomic results showing significantly lower expression of genes involved in
740 glycolysis, the Entner-Doudoroff pathway, pyruvate oxidation, and the pentose phosphate
741 pathway observed in BL+MBM treatment, as compared with the number of their transcripts in
742 W+MBM soil. Moreover, decreased expression of genes encoding proteins constituting part of
743 secretion systems (Type II, Type VI, Sec-SRP), a multi-protein complex used by bacteria to
744 move substances across their cell membrane, was observed in soil BL+MBM, compared to soil
745 W+MBM. Since these systems are associated with the secretion of many diverse substrates
746 involved in bacterial adhesion, biofilm formation, and *quorum sensing* (Naskar et al., 2021),
747 they have an important role in bacterial communication (Pena et al., 2019). Thus, decreased
748 expression of genes encoding secretion systems could consequently lead to impaired
749 interactions between bacteria acting as whole communities.
750 In our experiment, we noted significantly higher hydrocarbon degradation in all soils enriched
751 with MBM, independently of the soil treatment (BL, BTI, and W), even though the plants were
752 only able to survive in this soil for 10 days and did not support this process in the most
753 experimental period. This result also indicated that hydrocarbon removal from soil co-
754 contaminated with PHs and HMs supplemented with MBM resulted mainly from the enhanced
755 activity of autochthonous microorganisms, biostimulted by the MBM addition. Such
756 observations are consistent with results from our previous studies on the bioaugmentation of
757 soil contaminated only with hydrocarbons, in which further PH loss was also noted after the
758 death of the inoculants (Pacwa-Płociniczak et al., 2019). We also reported the lack of
759 correlation between PH removal efficiency and microbial activity measured by FDA
760 hydrolysis. The FDA decomposition is a consequence of the metabolic activity of all soil
761 microorganisms (inoculants, indigenous bacteria, fungi, etc.), and only part of them is involved
762 in hydrocarbon degradation. Moreover, the changes in microbial community composition may

763 unpredictably influence the microbial activity and PH removal, which was observed in the
764 ZCR6 bioaugmented soil, non-treated with MBM, in which hydrocarbon loss reached a value
765 of 7.95% (data not shown).
766 The analysis of plants from non-treated W soil showed a limited potential of maize to remove
767 Cd, Zn, and Cu from the soil in the presence of PHs, with the highest accumulation capacity for
768 Zn. Nevertheless, the translocation factor calculated for all tested HMs was far from the value
769 of 1 desired for plants used in phytoextraction. However, the large biomass of maize and its
770 extensive root system, compared to the most effective hyperaccumulators, can compensate for
771 the lower ability to accumulate metals compared to hyperaccumulating plants. Also, the low
772 translocation factor for Cd, Zn, and Cu does not disqualify these plants, because most of the
773 roots can be removed together with aboveground parts using typical agricultural equipment,
774 and similar results confirming these features of maize were also observed in other studies on
775 phytoextraction of HMs from co-contaminated soils (Hechmi et al., 2013; Lin et al., 2008;
776 Nwosu et al., 2018; Zhang et al., 2009).
777
778
779 *5. Conclusions*
780 Although our research did not confirm the usefulness of MBM at the tested concentration to
781 support phytoremediation of co-contaminated soil, we demonstrated the potential of its use to
782 support the removal of petroleum hydrocarbons by microbial degradation. This was confirmed
783 by metatranscriptomics, which showed increased expression of genes encoding enzymes
784 potentially involved in the removal of hydrocarbons in MBM-treated soils. Our results provide
785 a rationale to perform further phytoremediation tests using a lower amount of MBM and/or soil
786 pre-treated with MBM. This approach will avoid toxic effects of high ammonia concentration
787 on plants and will enable effective removal of HMs by phytoextraction and PHs by

rhizodegradation due to autochthonous microorganisms. Our findings indicate that optimal biofertilization of co-contaminated soil can effectively replace expensive and time-consuming soil bioaugmentation with bacterial strains, such as ZCR6. Since the number of remediation methods to clean up co-contaminated areas is limited, further research on the optimization of phytoremediation is strongly recommended.

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CRediT author statement

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Supplementary materials

The potential of enhanced phytoremediation to clean up multi-contaminated soil – insights from metatranscriptomics

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1031 Table S1. Selected physicochemical properties of the soil used in the experiment.

Parameter	Soil	Soil+MBM
Sand [%]	80.37 ± 0.06	79.88 ± 0.17
Silt [%]	17.83 ± 0.06	17.95 ± 0.03
Clay [%]	1.8 ± 0.00	2.17 ± 0.15
Textural classification	Loamy sand	Loamy sand
Organic matter [%]	4.03 ± 0.15	17.03 ± 0.47
pH_{H2O}	6.97 ± 0.15	6.33 ± 0.21
N total [% N]	0.63 ± 0.10	2.47 ± 0.40
Fe [mg kg^{-1}]	11402 ± 509	9701 ± 214
P total [%]	0.039 ± 0.003	1.01 ± 0.08
C organic [%]	2.62 ± 0.19	6.88 ± 0.42

1032 ± Stand. Dev. of three independent experiments; ; MBM – meat and bone meal.

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1051 Table S2**.** Primers used for PCR amplification.

Primer	Sequence (5'-3')	Gene	Annealing (°C)	Reference
pvdF-F	TACATCCTCGATGCCGACCT	*pvd*	57	MBG8561616.1*
pvdF-R	CAGGTCGATCATGGTAGCCC			
dcyD-F	CTCGAACGCTTCAAACGCC	*dcyD*	57	MBG8561384.1*
dcyD-R	GCTTGATGTAGATGTCGCGC			
bfr-F	TGCACAAGGACTACGTCAGC	*bfr*	57	MBG8560256.1*
bfr-R	TTGATCAGACCCTGCTGCTT			
tonB-F	CTACGAGTTCGGCTACAGCG	*tonB*	57	MBG8561215.1*
tonB-R	CACGGTGACCTTCTGGATGG			
cysA-F	TCAAGAACACCCAGGGCAAG	*cysA*	57	MBG8561413.1*
cysA-R	TCAAGAACACCCAGGGCAAG			
pstA-F	CGCGAAGTCGAGATCAACCT	*pstA*	57	MBG8558409.1*
pstA-R	CCCACACCTTGGCGAAGTAG			
gcd-F	AGATCACCCCGAACAACGTG	*gcd*	57	MBG8562075.1*
gcd-R	TTCTCGTTGGTCAGCTCGAC			
pqqA-F	CGTCTTCTACTGTCGCTCCG	*pqqA*	57	MBG8558041.1*
pqqA-R	CAGGCCATCACGGTACCAG			
acdS-F	GATTTGCCCTCGTAGACCGG	*acdS*	57	MBG8559878.1*
acdS-R	ATCCGGAATATGGCTTGCCC			
entD-F	CGCTGACCTTCTCCCTCAAG	*entD*	57	MBG8562760.1*
entD-R	GCGGACCATTCCAGCAGTTC			
pE	AAACTCAAAGGAATTGACGG	16S rRNA	57	(Edwards et al.,
pF•	ACGAGCTGACGACAGCCATG			1989)
alkB-F	AACTACMTCGARCAYTACGG	*alkB*	57	(Powell et al.,
alkB-R	TGAMGATGTGGTYRCTGTTCC			2006)
alk-H1-F	CIGIICACGAIITIGGICACAAGAAGG	*alkH*	55	(Phillips et al.,
alk-H3-R	IGCITGITGATCIIIGTGICGCTGIAG			2008)

ladA-F	GCAGTTCGTGAAGCTGTTCA	*ladA*	57	MBG8560652.1*
ladA-R	GAGAAGGTGACAGCGAATGC			
P450fw1	GTSGGCGGCAACGACACSAC	CYP153	58	(Yousaf et al.,
P450rv3	GCASCGGTGGATGCCGAAGCCRA			2010)
C120F	GCCAACGTCGACGTCTGGCAGCA	C120	59	(Sei et al., 1999)
C120R	CGCCTTCAAAGTTGATCTGCGTGGTTGGT			
C23OF	AAGAGGCATGGGGGCGCACCGGTTCGA	C230	57	(Sei et al., 1999)
C23OR	TCACCAGCAAACACCTCGTTGCGGTTGCC			

* accession numbers from GenBank

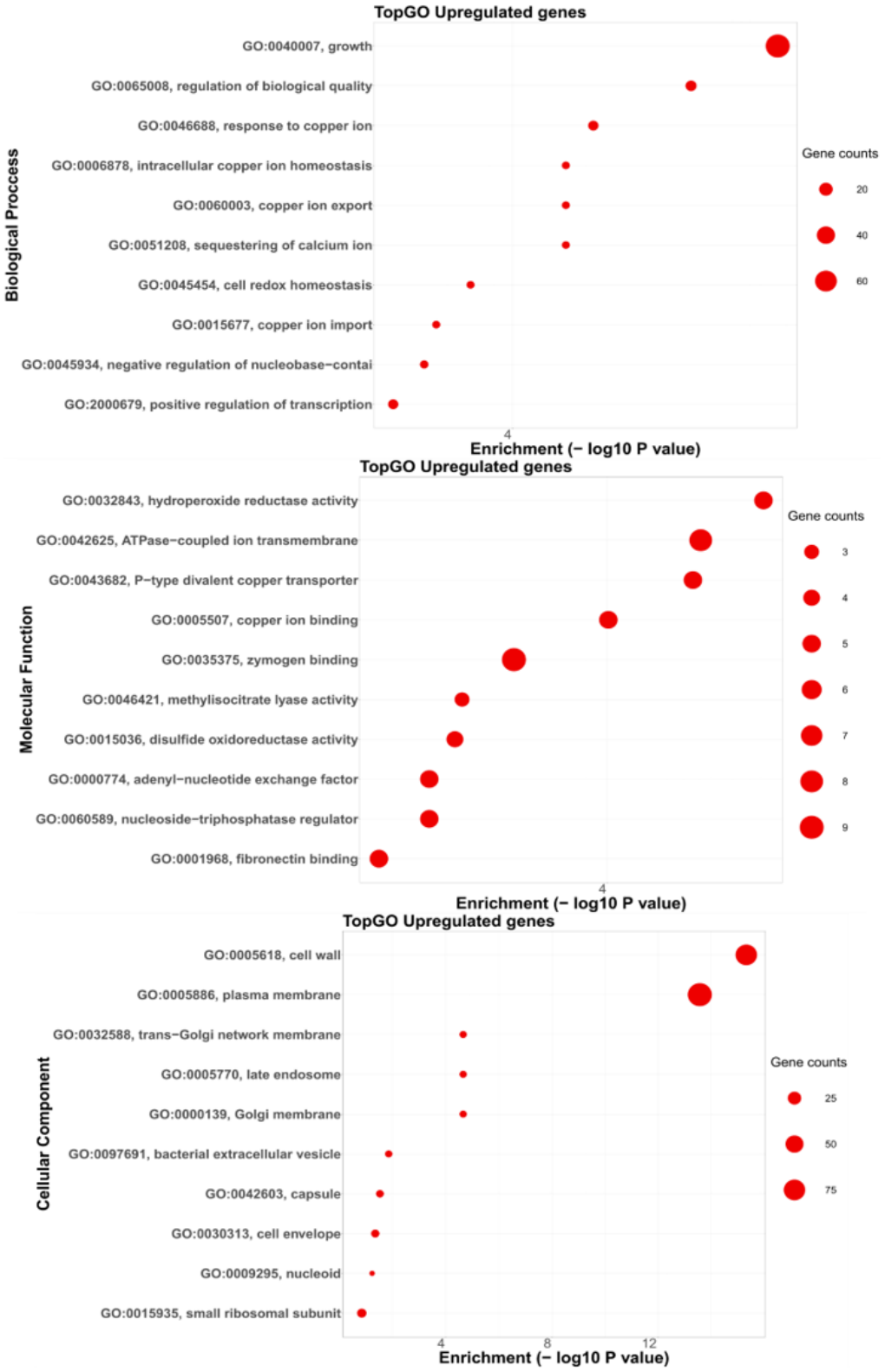

Fig. S1 GO enrichment analysis of up-regulated genes in response to BL+MBM treatment; BL – live bacteria; MBM – meat and bone meal.

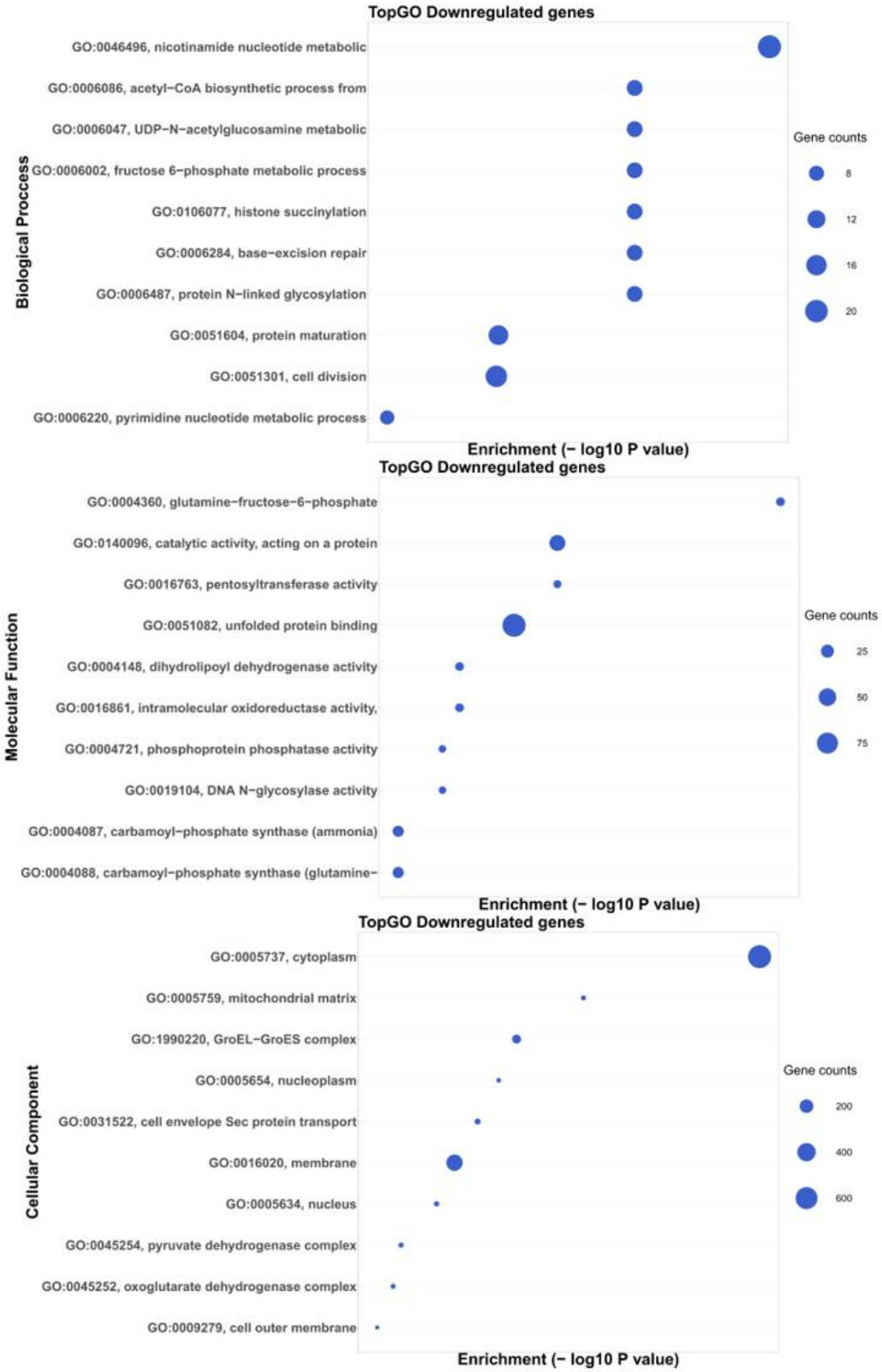

Fig. S2 GO enrichment analysis of down-regulated genes in response to BL+MBM treatment; BL – live bacteria; MBM – meat and bone meal.

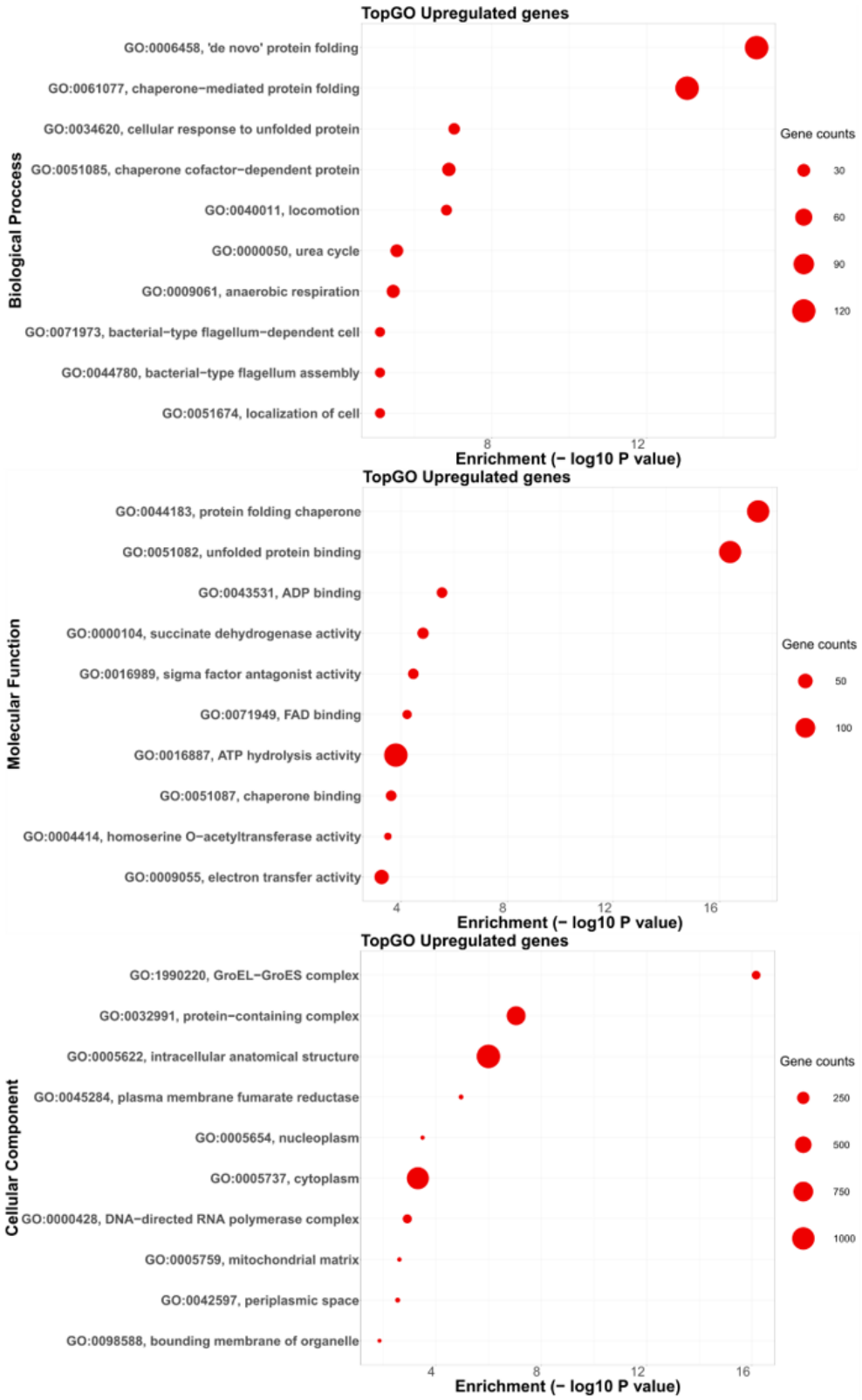

Fig. S3 GO enrichment analysis of up-regulated genes in response to W+MBM treatment; W – water; MBM – meat and bone meal.

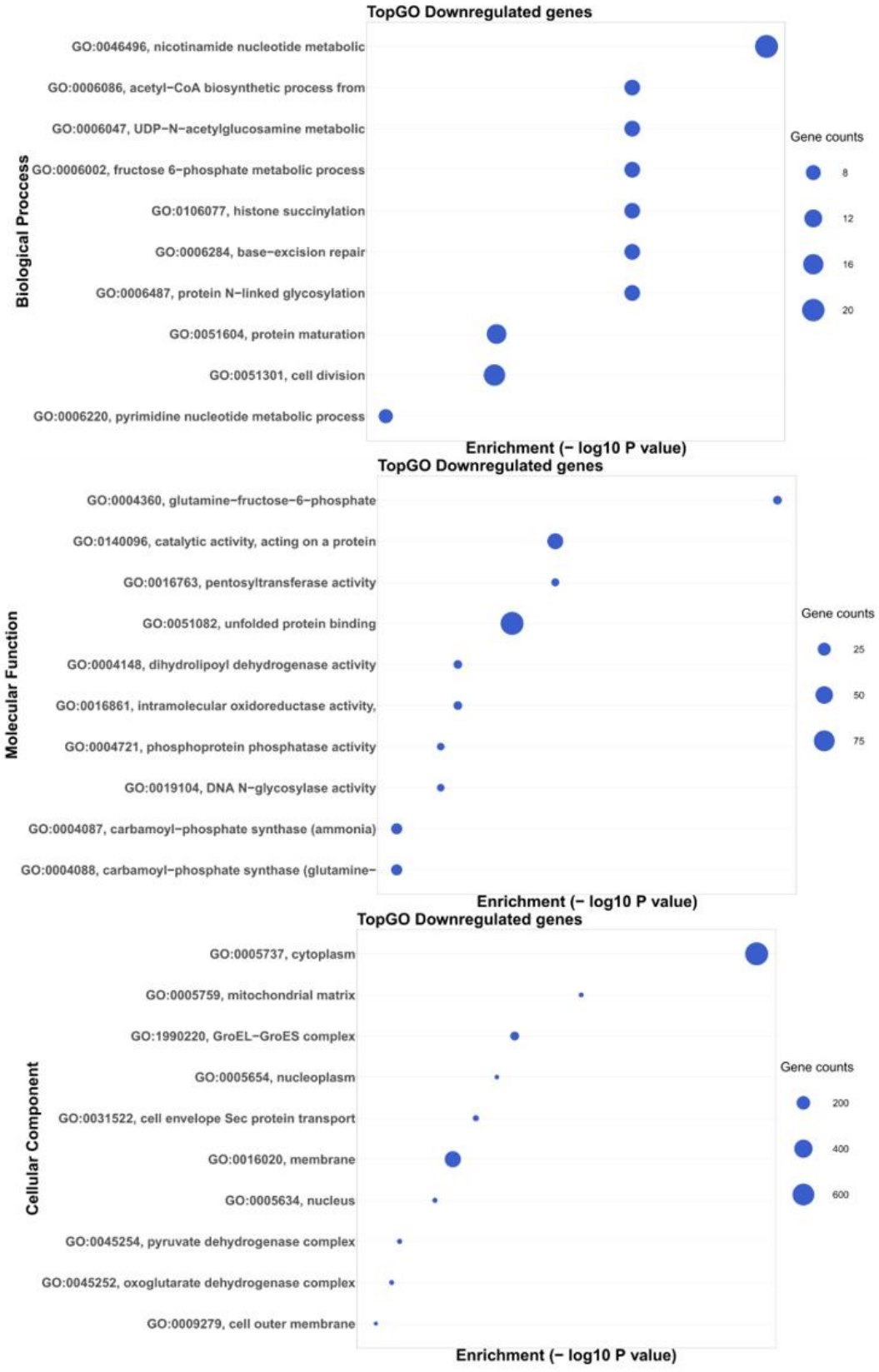

Fig. S4 GO enrichment analysis of down-regulated genes in response to W+MBM treatment; W – water; MBM – meat and bone meal.