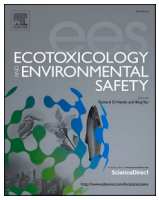
Ecotoxicology and Environmental Safety 267 (2023) 115656

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Adaptation of the metolachlor-degrading fungus *Trichoderma harzianum* to the simultaneous presence of low-density polyethylene (LDPE) microplastics

Przemysław Bernat [a,*], Anna Jasińska [a], Katarzyna Niedziałkowska [a], Mirosława Słaba [a], Sylwia Różalska [a], Katarzyna Paraszkiewicz [a], Lidia Sas-Paszt [b], Hermann J. Heipieper [c]

[a] University of Lodz, Faculty of Biology and Environmental Protection, Department of Industrial Microbiology and Biotechnology, Banacha Street 12/16, 90–237, Lodz, Poland
[b] Department of Microbiology and Rhizosphere, The National Institute of Horticultural Research, Pomologiczna 18, 96-100 Skierniewice, Poland
[c] Department of Environmental Biotechnology, Helmholtz Centre for Environmental Research-UFZ, Permoserstrasse 15, 04318 Leipzig, Germany

ARTICLE INFO

Edited by Muhammad Zia-ur-Rehman

Keywords:
Phospholipids
Extracellular enzyme activities
Lipidomics
Microplastic
Metolachlor

ABSTRACT

Although it is known that microplastics (MPs) in soils cause a threat to this complex environment, the actual effects of MPs on soil microorganisms and their catabolic activities, particularly with the biodegradation of herbicides, remain unclear. Hence, the objective of this study was to investigate the effects of a simultaneous presence of metolachlor and low-density polyethylene (LDPE) microplastics on growth inhibition and adaptive responses of *Trichoderma harzianum* in soil microcosms. Using ergosterol content as an indicator of fungal biomass, it was observed that MPs alone had a marginal inhibitory effect on the growth of the fungus, whereas MET exhibited a dose-dependent inhibitory effect on *T. harzianum*. However, the presence of MPs did not influence the fungal transforming activity toward the herbicide. Conversely, analysis of lipid profiles in the presence of MPs and herbicides revealed a reduction in the overall fluidity of phospholipid fatty acids, primarily attributed to an increase in lysophospholipids. The activities of six extracellular enzymes in the soil, measured using methylumbelliferone-linked substrates, were significantly enhanced in the presence of MET. These findings contribute to a broader understanding of the alterations in fungal activity in soil resulting from the influence of MPs and MET.

1. Introduction

Soil is a fundamental component of terrestrial ecosystems, and relies on the presence of living microorganisms for its vitality and productivity. These microbial communities play a crucial role in determining soil fertility and agricultural yields. However, our understanding of the impact of human-induced pollution on microbial activity remains limited. In recent years, microplastics (MPs), which are plastic particles with a diameter smaller than 5 mm, have emerged as a significant concern in the field of environmental science (Boughattas et al., 2021; Wang et al., 2021). MPs can enter the soil environment, inter alia, through various pathways, such as the use of plastic mulching or the application of sewage sludge and fertilizers (Boughattas et al., 2021). In Europe alone, it is estimated that 63–430 thousand tons of MPs have been introduced into farmlands through the utilization of biosolids (Nizzetto et al., 2016), while the quantities introduced from plastic mulch films are yet to be determined. Given the known adverse effects of plastic pollution on marine organisms, there is a growing concern regarding the potential hazards posed by MPs to soil organisms. However, the exact effects of MPs on soil microorganisms and their catabolic activities, particularly concerning the biodegradation of herbicides, are not yet fully understood.

Studies have shown that both polyethylene (PE) and polyvinyl chloride (PVC) inhibit the activity of bacterial fluorescein diacetate hydrolase in the soil, while simultaneously stimulating the activities of urease and acid phosphatase (ACP) (Fei et al., 2020). Huang et al. (2019) suggested that PE notably enhances the activities of soil catalase and urease and also alters the composition of the soil bacterial community. In addition, also the content of soil microbial carbon and nitrogen decreased significantly with increasing accumulation of plastic residues

* Corresponding author.
E-mail address: przemyslaw.bernat@biol.uni.lodz.pl (P. Bernat).

https://doi.org/10.1016/j.ecoenv.2023.115656
Received 14 August 2023; Received in revised form 24 October 2023; Accepted 30 October 2023
Available online 7 November 2023

(Yang et al., 2018). MPs have been found as the substrates for microorganism colonization, which could alter microbial population and disrupt soil ecosystem. Moreover, it is assumed that MP particles may participate in horizontal gene transfer as "hotspots" (Wang et al., 2020a). The interactions between MPs and microorganisms have also been explored in studies focusing on the degradation, colonization, and ingestion of MPs (Lu et al., 2019). Bandopadhyay et al. (2018) indicated that biodegradable plastic mulches could indirectly affect soil microbial communities by altering the soil microclimate, physical structure, and introducing contaminants that adhere to the film fragments. Moreover, due to their high adsorptive capacity and hydrophobic nature, MPs can accumulate significant amounts of organic pollutants in soil. Wang et al. (2020b) revealed that the co-amendment of ciprofloxacin and MPs reduced the antibiotic degradation efficiency and decreased the microbial diversity in soil.

Research has demonstrated that pesticides such as carbendazim, diflubenzuron, malathion, acetamiprid, and flubendiamide can be adsorbed onto MPs (Śunta et al., 2020; Wang et al., 2020a). Therefore, comprehending the impact of MPs on microbial activity is crucial in mitigating the potential risks associated with the transport of these pollutants within the soil. However, our knowledge about MPs effects on soil microbial microorganisms and their metabolism is still scarce.

Moreover, there is a scarcity of information regarding the impact of MPs on microbial degradation activity towards popular anthropogenic soil pollutants - pesticides. Some studies have mentioned the impact of MPs on extending the effective residual life of cypermethrin and altering specific parameters of soil microbial activity in the presence of glyphosate (Emden and Hadley, 2011; Yang et al., 2018). Nevertheless, there is a dearth of research addressing the potential negative effects of MPs on plant growth-promoting bacteria and fungi, which is an important aspect to consider in future studies.

One group of microorganisms that has gained significant attention in this regard is the fungi belonging to the genus *Trichoderma*. These fungi are frequently utilized to protect plants from pathogens and enhance their resistance to abiotic stress. In a previous study, we revealed that a strain of *Trichoderma harzianum* was capable of partially mitigating the toxic effects of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) on wheat seedlings (Bernat et al., 2018; Mironenka et al., 2020; Mironenka et al., 2021a; Mironenka et al., 2021b). Moreover, this strain exhibited the ability to partially degrade popular chloroacetanilide herbicides, including metolachlor (MET), in liquid cultures (Nykiel-Szymańska et al., 2020).

Literature data reveal that 2.3×10^7 kg of MET were applied in the US in 2015 (Liu et al., 2022). Acetanilide herbicides including metolachlor were used in the midwestern region in the United States at rates between 10 and 30 kg/km^2 (Kalkhoff et al., 2012). Moreover, there have been reports of MET presence in European freshwaters (Liu et al., 2022).

Therefore, the objective of the current study was to assess the effect of MPs on the growth of *T. harzianum* in MET-contaminated soil, as well as its biodegradation ability towards MET, and the synthesis of fungal metabolites: fatty acids, phospholipids and extracellular enzymes.

2. Materials and methods

2.1. Reagents

MET, predominantly S-Metolachlor, Ergosterol, 4-Methylumbelliferone (MUB), 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide, 4-Methylumbelliferyl β-D-glucopyranoside, 4-Methylumbelliferyl β-D-cellobioside, 4-Methylumbelliferyl phosphate, 4-Methylumbelliferyl-β-D-xylopyranoside, and 4-Methylumbelliferyl α-D-glucopyranoside were obtained from Sigma-Aldrich (Merck, Germany). The following lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA): 1,2-Dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol) (sodium salt; 14:0/14:0 PG), 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine (12:0/12:0 PE), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (14:0/14:0 PC), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-myo-inositol) ammonium salt (16:0 PI), 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine sodium salt (14:0/14:0 PS), 1,2-dimyristoyl-sn-glycero-3-phosphate (sodium salt; 14:0/14:0 PA), 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (18:1 LPC), and 1-oleoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (18:1 LPE). All other chemicals were obtained from Avantor Performance Materials (Gliwice, Poland). All chemicals used were of high purity grade. Stock solutions of MET were prepared at a concentration of 5 mg mL^{-1} in 96% ethanol.

2.2. Soil

The soil utilized in this study was collected from the SGGW Experimental Field in Skierniewice, situated in Central Poland (latitude 51.9625 N, longitude 20.1624 E). The soil exhibited the following characteristics: strong clay sand particle size distribution, with a sand content (1–0.1 mm) of 68%, silt content (0.1–0.02 mm) of 15%, and clay content (<0.02 mm) of 17%, including a colloidal clay fraction (<0.002 mm) of 8%. Other soil properties included 8% organic carbon, a pH of 4.5, 4.02/100 g total carbon, and 0.38/100 g total nitrogen (C, N; Dumas"a), Mg 29.6 mg/100 g soil (Schaschabel), K 30.9 mg/100 g, P, and 18.1 mg/100 g (Egner-Riehm). The soil was dried in the open air, sieved through a 1 mm sieve, and subsequently autoclaved before being utilized in the study.

2.3. Strain

The fungal strain *T. harzianum* KKP 534 utilized in this study was obtained from the Collection of Industrial Microorganisms at the Institute of Agricultural and Food Industry (IAFB) (Warsaw, Poland). This collection is a member of the World Data Centre for Microorganisms (WDCM 212). Fungal spores, aged 7 days, were obtained from cultures that had been maintained for 10 days on ZT agar slants. The composition of the ZT agar medium per liter was as follows: glucose 4 g, Difco yeast extract 4 g, agar 25 g, and malt extract 6° Balling (BLG) made up to 1 L (1° BLG corresponds to 1 g of soluble substances extracted from the grain/100 mL of malt extract), with a pH of 7.0. The spores were inoculated into 100 mL Erlenmeyer flasks filled with Sabouraud dextrose broth medium (Difco) at a concentration of 1×10^7 mL^{-1}.

2.4. Microplastic

Three fractions of low-density polyethylene (LDPE) with different particle sizes of 1000, 500, and ≤ 400 μm were purchased as powders from Alfa Aesar (Haverhill, MA, USA). The doses of MPs used in this experiment were as follows: 1000 μm (40% of the total weight of MPs), 500 μm (40% of the total weight of MPs), and ≤ 400 μm (20% of the total weight of MPs). This ratio was selected based on previous studies conducted by other researchers to simulate the heterogeneity of MP sizes found in soils (Meng et al., 2020). Subsequently, the LDPE particles were transferred to Eppendorf tubes at a mass of 50 mg per tube and sterilized using UV light in a laminar airflow chamber for 60 min

2.5. Experimental design

All experiments were conducted in 50 mL Falcon tubes using 5 g of the test soil. The following conditions were maintained, as outlined in

Table 1
Types of cultures used in the present study.

Type of culture	Abbreviation
T. harzianum	Control
T. harzianum + MPs (2%)	MP
T. harzianum + MET (50, 100, 200, or 400 mg kg^{-1})	MET
T. harzianum + MET (50, 100, 200, or 400 mg kg^{-1}) + MPs (2%)	MET MP

Table 1. To achieve a concentration of 2% (w/w) of MPs, 100 mg of MPs was added to each tube and vortexed. This concentration was selected based on the study by de de de Souza Machado et al. (2018), where it was determined as the maximum level of microplastic contamination that did not lead to visible changes in the soil. However, the authors reported that these particles affected soil aggregation and microbial activity. MET was added to the soil at concentrations of 50, 100, 200, or 400 mg kg^{-1} to achieve desired levels. Similar concentrations have been used in previous studies investigating the biodegradation kinetics of herbicides (Zhang et al., 2010). Subsequently, 1 mL of *T. harzianum* spore suspension at a concentration of 1×10^7 mL^{-1} (2×10^6 spores per gram of dry soil) and 0.6 mL of sterilized distilled water were added to the tubes. The contents of the tubes were thoroughly mixed, and the incubations were maintained at a constant temperature of 28 °C and humidity set at 40%. The incubation period lasted for 9 days. The tubes were closed with a cotton wool plug to prevent soil drying while allowing gaseous exchange. The weights of the tubes were recorded, and the moisture content was maintained by rewetting the tubes to the initial moisture level every 3 days with the use of sterilized distilled water.

Noninoculated soil samples with 0, 50, 100, 200, or 400 mg kg^{-1} of metolachlor were prepared as abiotic controls and the incubation period lasted for 9 days. Their moisture content was similar to that of the tested samples (initial gravimetric water content was 32%). After the 9-day incubation period, the soil samples (inoculated and noninoculated) were subjected to further analysis.

2.6. Extraction of metabolites

After the 9-day incubation period, the soil samples (inoculated and noninoculated) were subjected to further analysis. To each sample, 3 mL of methanol, along with a cholesterol standard (25 μg at a concentration of 1 mg mL^{-1}), and glass beads were added. The samples were then homogenized using a ball mill (MM 400, Retsch) for 8 min. Subsequently, the samples were shaken at room temperature for 24 h and then centrifuged (5 min, 2500g). The resulting supernatant was transferred to 2 mL Eppendorf tubes and subjected to a further centrifugation step (10 min, 9000g). The extracted solution obtained through this process was used for the determination of fatty acid, phospholipid, ergosterol, and MET contents.

2.7. Analysis of herbicide

MET was quantified using an Agilent 1200 system (Agilent, USA) coupled with a 3200 Q-TRAP mass spectrometer (Sciex, USA) equipped with an ESI source. The obtained extract was diluted in water:methanol mixture (80:20, v-v). The chromatographic method and MS analysis parameters were as follows: A diluted sample volume of 10 μL was injected onto a Kinetex C18 column (50 mm × 2.1 mm, particle size: 5 μm; Phenomenex, Torrance, CA, USA), which was heated at 35 °C, with a flow rate of 500 μL min^{-1}. The mobile phase consisted of water (A) and methanol (B), both containing 5 mM ammonium formate. The solvent gradient started at 80% A and, after 1 min, decreased to 10% A for 1 min. It was then maintained at 10% A for 2.5 min before returning to the initial solvent composition over 2 min. The ion source of the MS operated in positive mode at a temperature of 500 °C. The multiple reaction monitoring (MRM) pairs for MET were *m/z* 284.09–252.2 and 284.09–176.3. Quantification was based on a five point (n = 3) external standard method for the first MRM ion pair with a linear calibration (R = 0.995) over a concentration range of 25–500 ng mL^{-1}.

The quantitative analysis of MET concentration in the tested samples was performed according to the standard equation and next the metolachlor content was calculated in %. 100% represents the amounts of the herbicide, which were estimated in quality (abiotic) controls.

To explore potential metabolites generated during the degradation of MET, an information-dependent acquisition method called EMS→EPI was utilized. Spectra were acquired in positive mode within a range of *m/z* 100–550 (see Fig S1). The enhanced product ion (EPI) scan rate was set at 1000 amu s^{-1}. By analyzing the product ions, a comprehensive list of MRM transitions for metolachlor-2-hydroxy (*m/z* 266.1–176 and 266.1–234.1) and metolachlor deschloro (*m/z* 250.1–176.1 and 250.1–218.1) was compiled. Subsequently, the quantities of these metabolites were determined and quantified based on the results obtained for MET.

2.8. Determination of ergosterol

For sterol analysis, a QTRAP 3200 mass spectrometer (Sciex) was utilized in combination with a 1200 series HPLC system (Agilent, USA). The separation was carried out on a Kinetex C18 column (50 mm × 2.1 mm, particle size: 5 μm; Phenomenex, Torrance, CA, USA). The solvents consisted of water and methanol, both containing 5 mM ammonium formate. The analytes were separated using the following gradient: starting with 60% solvent A from 0 to 1 min, transitioning to 100% solvent B from 1 to 4 min, returning to 60% solvent A from 4.0 to 4.1 min, and maintaining 60% solvent A from 4.1 to 6 min. The flow rate was set at 0.8 mL min^{-1}.

In positive ion mode, the QTRAP instrument was equipped with an atmospheric pressure chemical ionization (APCI) source operating at a temperature of 550 °C. The monitored MRM pairs were *m/z* 379.3–69.1 and 379.3–81.3. Additionally, cholesterol was used as the internal standard (*m/z* 369.3–147.2).

The method validation was carried out using no-treatment, autoclaved soil samples (5 g) spiked with 25.0 μg ergosterol. Then, the same (point 2.6) sample preparation procedure was applied. Recovery of ergosterol in spiked wet samples was 79%. Ergosterol was not detected in the quality control samples. The LC-MS/MS method was validated for linearity with 0.5–15 μg mL^{-1} ergosterol (R =0.9978).

2.9. Fatty acid analysis

To prepare the methanolic extract sample as described in Section 2.6, 0.75 mL of the sample was transferred to a screw-capped glass test tube. Subsequently, 0.1 mL of toluene and 0.15 mL of an 8.0% HCl solution (Ichihara and Fukubayashim, 2010) were added. An internal standard, nonadecanoic acid (C19:0, at a concentration of 10 μg mL^{-1}), was used. The tube was vortexed and incubated overnight at 45 °C. Once cooled to room temperature, 0.5 mL of hexane and 0.5 mL of deionized water were added to extract the fatty acid methyl esters (FAMEs). The tube was vortexed, and 0.2 mL of the hexane layer was transferred to a chromatographic vial for analysis. A volume of 1.6 μL of the extracted sample was used for analysis.

FAMEs analysis was performed using an Agilent Model 7890 gas chromatograph equipped with a 5975 C mass detector. A capillary column, HP 5 MS methyl polysiloxane (30 m × 0.25 mm i.d. × 0.25 mm ft), was utilized with helium as the carrier gas. The column temperature was initially set at 60 °C for 3 min, then increased to 212 °C at a rate of 6 °C min^{-1}, followed by a further increase to 245 °C at a rate of 2 °C min^{-1}. Finally, the temperature was raised to 280 °C at a rate of 20 °C min^{-1} and held for 10 min. The injection port was set to a split injection at 250 °C. Fungal fatty acids were identified by comparing them with reference standards (Merck).

The calibration curves showed good linearity over a concentration range of 0.2 – 20 μg mL^{-1} with correlation coefficients (R) higher than 0.9950 for all analytes.

The results were presented as a percentage relative to the total amount of fatty acids. Based on the obtained data, the unsaturation index (UI) was calculated (Concha et al., 2018).

$$UI = \frac{(\%C16:1 + \%C18:1) + (\%C18:2 \times 2) + (\%C18:3 \times 3)}{100}$$

2.10. Determination of phospholipids

For phospholipid analysis, methanolic extracts (0.2 mL) were transferred to a chromatographic vial with a glass insert. The measurements were performed using an ExionLC AC UHPLC system (Sciex, USA) coupled with a 4500 QTRAP mass spectrometer (Sciex, USA) equipped with an ESI source. The mobile phase consisted of water (A) and methanol (B), both containing 5 mM ammonium formate, with a flow rate of 500 μL min^{-1}. Fractionation of the samples was achieved using a Kinetex C18 column (50 mm × 2.1 mm, particle size: 5 μm; Phenomenex, USA) heated to 40 °C. The solvent gradient began at 30% A and, after 0.25 min, was increased to 95% B for 1 min. It was then maintained at 95% B for 5 min before returning to the initial solvent composition over 2 min

The following instrumental settings were applied: a spray voltage of − 4500 V, curtain gas at 25, nebulizer gas at 60, auxiliary gas at 50, and an ion source temperature of 600 °C. Data analyses were performed using Analyst™ v1.6.3 software (Sciex, USA) (Jasińska et al., 2022).

For phospholipid analysis, an information-dependent acquisition method known as precursor ion (Prec) → EPI was employed. Spectra were acquired in the range of *m/z* 100–900. A precursor scan for *m/z* 253, 255, 277, 279, 281, and 283 was utilized to detect the subspecies of phospholipids. The mass spectra of PA, PI, LPE, and PE species exhibited ions corresponding to the deprotonated molecules $[M-H]^-$. In contrast, charged LPC and PC species displayed $[M+HCOO]^-$ ions (Fig. S2).

By analyzing the ions from head groups and fatty acyls, a comprehensive list of MRM transitions was created to track the fatty acyl compositions of these lipids. Parent R fatty acyl fragment transitions were used (Figs. S3 and S4). The quantification of lipids within each class was accomplished by comparing them with the internal standard of the respective class. The applied linearity range was 0.05 – 0.75 μg mL^{-1} with R higher than 0.996.

2.11. Enzyme extraction and assays

Extracellular enzyme activities were determined following the methods previously described by Liu et al. (2020) using fluorogenic MUB-based substrates obtained from Sigma-Aldrich. The specific enzymes measured included C-acquiring α-glucosidase (AG), β-glucosidase (BG), β-xylosidase (XYL), cellobiohydrolase (CB), N-acquiring N-acetyl-glucosaminidase (NAG), and P-acquiring acid phosphatase (PHOS).

To perform the analysis, 5 g of soil was suspended in 30 mL of 50 mM acetate buffer (pH 5.0, similar to soil pH). The suspension was then transferred to 50 mL Falcon tubes and homogenized using a ball mill (Ball Mill MM 400, Retsch, Germany) with 1 mm diameter glass beads. Homogenization was conducted for 1 min at a frequency of 30 Hz.

Next, the suspension was moved to 70 mL of acetate buffer and placed on a magnetic stirrer for 60 min. The soil slurry was promptly distributed into 96-well microplates, following a precise order and arrangement on the plate according to ISO-TS-22939 (2010), with the buffer, sample, reference, and substrate in their respective locations. The final concentration of the substrate in each well was 40 μM.

Fluorescence intensity was measured using a FLUOstar Omega computerized microplate fluorimeter (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The measurements were conducted for 30 cycles of 60 s at 30 °C.

Enzyme activities were calculated based on three replicates per soil sample and expressed as μmol h^{-1} g^{-1} using the method described by DeForest (2009).

2.12. Data acquisition

The experimental data presented in this study represent the means of at least three independent experiments. Since the chromatographic determination methods differed from enzyme activity determination methods, a number of samples were prepared taking this fact into account. To compare the multivariate data obtained from phospholipid profiles, principal component analysis (PCA) was employed. Data matrices were constructed, where each column represented a distinct phospholipid molecule and each row represented a sample with a percentage value.

The statistical analyses, including PCA and other tests, were performed using R software version 4.2.1 (R Development Core Team, 2022). Initially, a one-way analysis of variance (ANOVA) was conducted, followed by Dunnett's test for comparisons relative to control samples.

Graphs illustrating the results were created using R and Microsoft Excel for the Microsoft 365 software.

3. Results

3.1. Ergosterol content in inoculated soil

Ergosterol, an essential component of fungal cell membranes, is commonly analyzed to assess the fungal presence and estimate fungal biomass in soil samples (Montgomery et al., 2000). In this study, we utilized APCI-LC-MS/MS to detect ergosterol in all soil samples inoculated with *T. harzianum* spores. However, the quantity of ergosterol varied depending on the type of culturing method used. Therefore, we utilized the ergosterol content as an indicator of *T. harzianum* biomass in the soil microcosms.

Upon examining the impact of different treatments, we observed that MPs alone had only a slight effect on fungal growth. In contrast, significant changes were observed in the presence of MET (Fig. 1). Specifically, MET led to a more pronounced inhibition of ergosterol synthesis and the growth of *T. harzianum*. Interestingly, the addition of MPs also altered the fungal content when combined with the herbicide. Notably, the biomass amount decreased in the presence of 100 and 200 mg MET kg^{-1} soil.

In Sabouraud liquid cultures, we determined that the ergosterol content in *T. harzianum* mycelium (dry mass) reached approximately 4.5 mg g^{-1} (unpublished data). However, it is important to exercise caution when comparing ergosterol amounts between different growth conditions, such as soil and liquid cultures, as suggested by Charcosset and Chauvet (2001) in the literature.

3.2. Degradation of herbicide by the fungus

Previously, we have demonstrated the capacity of *T. harzianum* to degrade MET in a liquid medium in our studies (Nykiel-Szymańska et al., 2020; Jasińska et al., 2022). In the current research, we observed a decrease in the herbicide content in soil samples compared to the abiotic control supplemented with the chemical (Fig. 2A). Furthermore, we found a correlation between the degradation efficiency and the initial concentration of the herbicide, indicating that higher concentrations of MET resulted in lower degradation rates.

Interestingly, when performing a posthoc Dunnett's test ($p < 0.05$), we observed differences in MET degradation in soil samples supplemented with MPs. It appeared that the presence of MPs enhanced MET degradation, despite the lower levels of MET metabolites detected (Fig. 2B). The confirmation of MET degradation was obtained through the identification of MET derivatives, including metolachlor deschloro (MDES) and metolachlor-2-hydroxy (M2H), in the methanol extracts of *T. harzianum* samples (Figs. 2B and 3). However, MET oxanilic acid was not detected in the examined samples, although its presence was confirmed in *T. harzianum* liquid cultures (unpublished data). The presence of these MET degradation derivatives was confirmed by comparing the obtained mass spectra with literature data and standard of metolachlor mass spectrum (Fig. S1) (Chang et al., 2020). The fragmentation pattern of the metabolite formed was similar to that of the

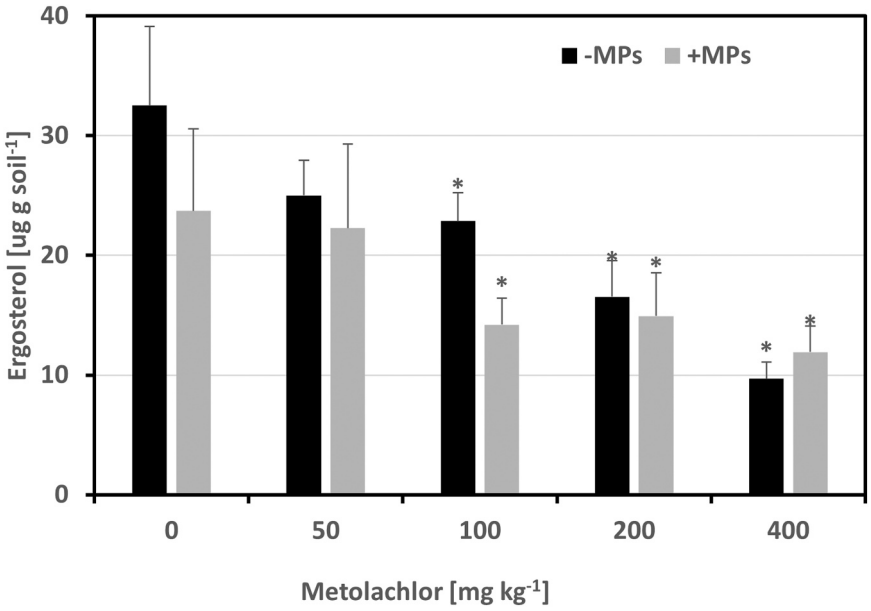


Fig. 1. Ergosterol content determined in soil samples after 9 days of *T. harzianum* culturing. Statistical differences were determined using a one-way ANOVA followed by Dunnett's test relative to the control (sample without MPs and MET) for each data point at $p < 0.05$ (*).

standard, including major fragments with *m/z* of 148 and 176.

3.3. Characterization of the fatty acid profile from soil samples

The increasing concentrations of the herbicide had a significant impact on the fatty acid profile. Specifically, we observed a pronounced reduction in the abundance of 18:3 fatty acid, as well as decreased levels of 18:2 and 18:1 acids. These alterations were reflected in the modification of the UI, which serves as a direct measure of membrane fluidity. As the concentration of MET increased in the culture, the UI decreased accordingly (Fig. 4). Interestingly, the presence of MPs did not inhibit these changes in the fatty acid profile; instead it contributed to a further decrease in the UI of the tested samples.

3.4. Phospholipids

Phospholipids play a crucial role as structural components in fungal cell membranes. In our study, we found that phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the most abundant phospholipids in the control samples. PC accounted for approximately 60% of the total determined phospholipids, followed by PE with a proportion of over 30%. Other phospholipid species, such as phosphatidylserine (PS), phosphatidylinositol (PI), lysophosphatidylethanoloamine (LPE), lysophosphatidylcholine (LPC) and phosphatidic acid (PA), constituted only a small percentage (Fig. 5).

Upon analyzing the specific phospholipid species, we observed that PC 18:2 18:2 and PE 16:0 18:2 were the most prevalent, collectively representing approximately 50% of the total phospholipid pool (Table 2, Fig. S5).

Compared to the control samples, the presence of MPs did not cause significant changes in the phospholipid profile of the tested fungal strain, except for a slight increase in the level of LPC. However, in the presence of MET, drastic alterations in the phospholipid profiles were observed, irrespective of the presence of MPs. As the concentration of MET increased in the cultures, there was a significant decrease in the abundance of PC and PE. Specifically, the main PC species, such as PC 18:2 18:2 and PC 18:2 18:1, showed a three-fold reduction in their levels (Table 2). Similarly, PE 16:0 18:2 exhibited a six-fold decrease. Consequently, there was a rapid increase in the levels of lysophospholipids of the LPC and LPE species (Fig. 5). Among the LPC species, those containing the 18:2 fatty acid were the most dominant, constituting up to 40% of the phospholipid pool (Table 2). Similarly, in the LPE class, phospholipids containing the 18:2 fatty acid were prevalent.

To simplify the data and assess changes in phospholipid abundance, we performed multivariate statistical analysis using PCA. In this analysis, the abundance of 33 phospholipid variables in each culture extract was transformed into 33 principal components. The score plot generated from the PCA analysis confirmed that the lipid profiles of the control samples were significantly different from those treated with the highest concentration of MET (Fig. S6).

3.5. Soil enzyme activity

In the subsequent stage of the research, we examined the activity of six commonly measured extracellular enzymes in soil using MUB-linked substrates (Fig. 6). The results obtained revealed significant impacts of MPs and MET on the activity of the N-acquiring enzyme (NAG) and P-acquiring enzyme (PHOS). Specifically, the activity of NAG increased in samples treated with MPs and MET. Similarly, the activity of PHOS also increased in the presence of MPs and MET. Among the C-acquiring enzymes (BG, AG, CB, and XYL), elevated levels of activity were observed for BG and CB in soil samples supplemented with MPs and/or MET. Overall, the presence of MET led to increased enzyme activity, and the addition of MPs further intensified this effect.

4. Discussion

The present research focused on the potentially harmful outcomes of MPs on the activity of the fungal strain *T. harzianum* and its pesticide degradation abilities.

The results obtained revealed that the growth of *T. harzianum* (with the use of ergosterol as a reliable marker for fungal presence in the soil) was less affected by MPs compared to the herbicide. Interestingly, the concurrent presence of MPs and MET had a distinct impact on the growth of the fungus, enhancing growth inhibition in the presence of MET. This phenomenon may be attributed to the properties of the herbicide and its lipophilicity (Papaefthimiou et al., 2004).

In the subsequent phase of the research, we examined the fungus's potential degradation ability toward MET in soil. *T. harzianum* demonstrated the capability to degrade the herbicide in soil, confirming

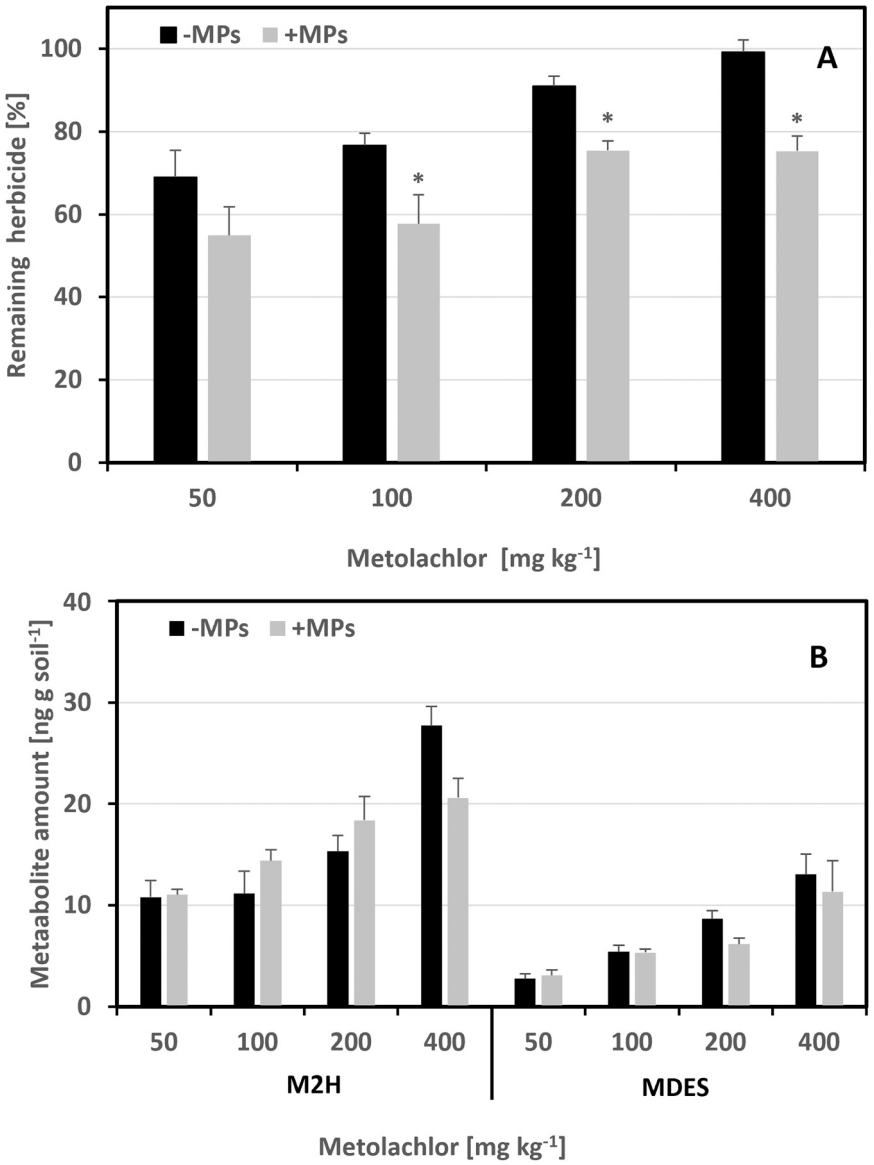


Fig. 2. MET removal by *T. harzianum* cultures incubated in soil for 9 days (A) and metolachlor metabolites identified: metolachlor-2-hydroxy (M2H), metolachlor deschloro (MDES) (B). The (*) marked a significant difference at $p < 0.05$ between the samples without and with MPs for each examined MET concentration (post-hoc Dunnett's Test). "-MPs" – samples without microplastic, "MPs" – samples with added microplastic.

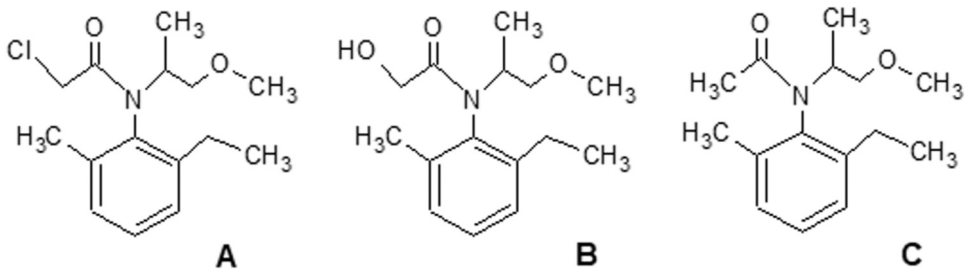


Fig. 3. Structures of MET (A) and its derivatives: metolachlor-2-hydroxy (M2H) (B) and metolachlor deschloro (MDES) (C).

previous findings of MET degradation by this fungal strain in submerged cultures (Nykiel-Szymańska et al., 2020; Jasińska et al., 2022). Interestingly, the presence of MPs increased the elimination of MET by the fungus. A similar trend was observed in the degradation of glyphosate by microbial cultures in Chinese loess soil (Yang et al., 2018).

The confirmation of herbicide elimination was obtained through the detection of MET metabolites. Among these metabolites, MDES and M2H were found in the *Penicillium oxalicum* MET-F-1 culture. It was suggested that MDES was formed through the dechlorination of MET, while M2H was formed through dichlorination and subsequent hydroxylation of the chloroacetyl group (Chang et al., 2020).

The study also examined the fungal adaptation to soil contaminated

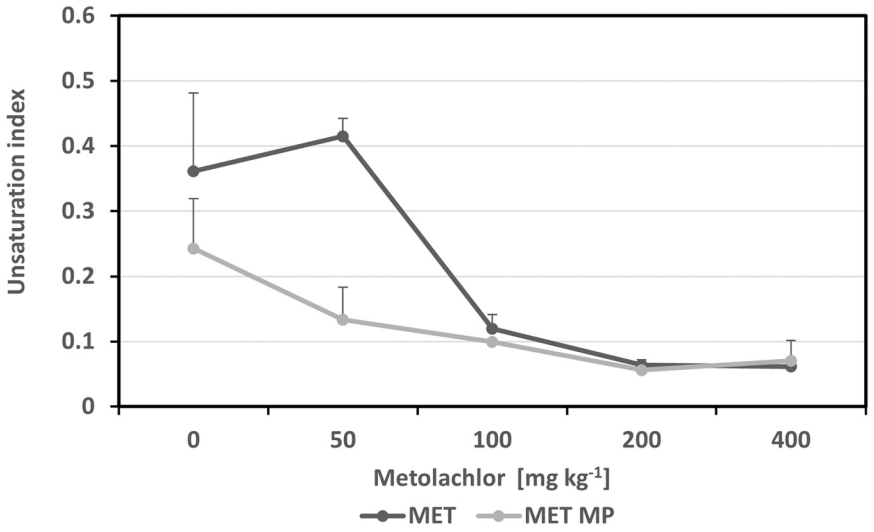


Fig. 4. Effect of MET and MPs on fatty acid unsaturation index of *T. harzianum* after 9 days of incubation. Bars represent the standard error for three independent samples.

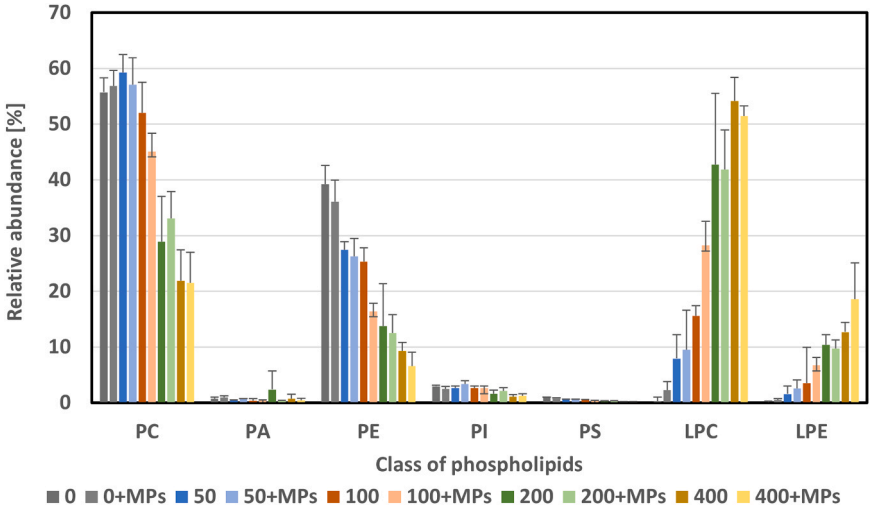


Fig. 5. Comparison of the main phospholipid classes of *T. harzianum* exposed to 0, 50, 100, 200, and 400 mg L^{-1} MET and MET with MPs after 9 days of incubation. Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), lysophosphatydylcholine (LPC), lysophosphatydylethanoloamine (LPE), and phosphatidic acid (PA).

Table 2
Comparison of phospholipid composition [percentage of the total phospholipids from species with a content of more than 5%] of *T. harzianum* from soil samples after 9 days of incubation. Statistical differences were determined using a one-way ANOVA followed by Dunnett's test relative to the control (sample without MPs and MET) for each phospholipid species at $p < 0.05$ (numbers in bold).

	Phospholipid species [%]							
	PC 18;2 18;2	PC 18;2 18;1	PE 16;0 18;2	PE 18;2 18;2	LPC 18;2	LPC 18:1	LPE 18;2	LPE 18:1
-MPs								
0	30.34 ± 1.04	9.9 ± 0.52	19.15 ± 1.94	7.5 ± 1.07	0.15 ± 0.03	0.08 ± 0.02	0.02 ± 0.01	0.01 ± 0.01
50	33.81 ± 3.55	**14.71 ± 1.18**	**12.07 ± 1.4**	6.08 ± 0.42	**5.53 ± 1.62**	2.13 ± 0.95	0.86 ± 0.34	0.6 ± 0.28
100	**31.9 ± 2.27**	**9.12 ± 1.21**	**10.83 ± 1.0**	6.78 ± 1.66	**14.07 ± 0.7**	**3.91 ± 0.69**	**2.84 ± 0.07**	**1.2 ± 0.05**
200	**18.62 ± 2.25**	**4.89 ± 0.43**	**5.38 ± 1.21**	**3.65 ± 1.22**	**32.46 ± 3.74**	**8.73 ± 1.54**	**6.82 ± 1.25**	**2.98 ± 0.37**
400	**12.71 ± 3.93**	**3.78 ± 1.02**	**3.42 ± 0.9**	**1.83 ± 0.52**	**40.43 ± 3.54**	**11.67 ± 2.74**	**8.11 ± 1.15**	**3.66 ± 0.67**
+MPs								
0	34.6 ± 2.32	10.48 ± 0.46	19.68 ± 1.72	7.15 ± 0.68	1.59 ± 0.57	0.5 ± 0.14	0.34 ± 0.15	0.15 ± 0.06
50	**37.38 ± 1.21**	11.29 ± 1.77	**12.21 ± 2.43**	6.89 ± 1.54	**6.9 ± 1.08**	2.08 ± 0.46	1.63 ± 0.03	0.76 ± 0.12
100	**24.31 ± 2.77**	**8.57 ± 0.62**	**5.76 ± 0.62**	**2.97 ± 0.64**	**25.74 ± 3.15**	**8.89 ± 0.88**	**4.91 ± 0.72**	**2.6 ± 0.7**
200	**18.68 ± 2.37**	**7.73 ± 1.2**	**5.33 ± 1.25**	**3.05 ± 0.97**	**29.54 ± 4.81**	**10.67 ± 1.96**	**6.09 ± 1.06**	**3.21 ± 0.54**
400	**13.95 ± 1.17**	**3.91 ± 1.6**	**2.84 ± 1.08**	**1.57 ± 0.42**	**38.02 ± 3.44**	**11.79 ± 1.13**	**12.05 ± 4.22**	**5.85 ± 1.08**

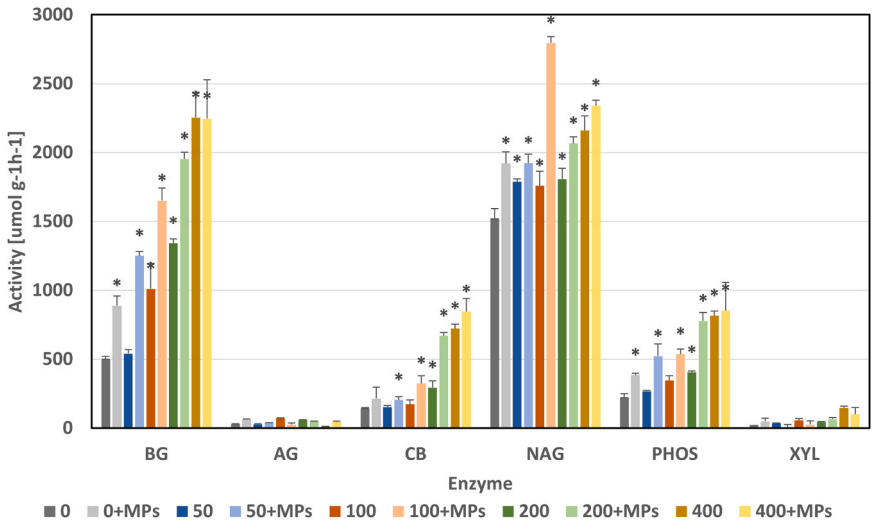


Fig. 6. Enzyme activity of soil samples after 9 days of incubation of *T. harzianum* exposed to 0, 50, 100, 200, and 400 mg L^{-1} MET and MET with MPs. α-glucosidase (AG), β-glucosidase (BG), β-xylosidase (XYL), cellobiohydrolase (CB), N-acetyl-glucosaminidase (NAG), and acid phosphatase (PHOS). Statistical differences were determined using a one-way ANOVA followed by Dunnett's test relative to the control (sample without MPs and MET) for each data point at $p < 0.05$ (*).

with MPs and the herbicide by analyzing its fatty acid profile. In response to increasing concentrations of MET, the fungus reduced the level of unsaturation in its membrane lipid fatty acids. This adjustment results in a rigidified membrane, counteracting the fluidizing effect of the hydrophobic MET. This adaptive response at the level of fatty acid composition had been previously observed in bacteria, fungi, algae, and even zebrafish embryos (Frostegard et al., 1993; Sikkema et al., 1994, 1995; Frostegard and Baath, 1996; Heipieper et al., 2000; Kabelitz et al., 2003; Piotrowska et al., 2018; Viegas et al., 2005).

Phospholipids, as the predominant membrane lipids, reflect the modifications occurring in the membranes of the tested microorganism in response to changing environmental conditions. It was observed that MPs did not statistically significantly modify the phospholipid profiles. However, the introduction of MET to the culture resulted in noteworthy changes in the profiles.

When discussing the changes in the profile, particular attention should be given to the main classes of phospholipids in cell membranes, namely PC and PE. Higher concentrations of MET resulted in an increased PC-to-PE ratio, indicating that the membrane became more fluid and less rigid. PC has a cylindrical molecular shape and is known for its role in membrane stabilization, while PE possesses a more conical molecular shape and is involved in forming nonbilayer hexagonal phases (Bernat et al., 2018; Yu et al., 2021). A higher PC ratio was also observed in the liquid cultures of the strain of *T. harzianum* when cultured with MET (Nykiel-Szymańska et al., 2019; Jasińska et al., 2022).

A surprising observation was the increasing levels of *lyso* forms of phospholipids with the gradual increase in the MET concentrations in the soil. Unlike PC and PE, the functions of lysophospholipids in membranes are not extensively described. They possess an inverted conical molecular shape and exhibit different properties compared to PC and PE (Yu et al., 2021). Excessive amounts of lysophospholipids in cells are believed to contribute to the breakdown of cell membranes (Tan et al., 2020). Normally, their levels are kept low due to the intense activity of lysoacyltransferases, which attach a second fatty acid and convert lysophospholipids into phospholipids.

A significant increase in LPC and LPE levels was observed in *T. harzianum* cells, accompanied by a decrease in PC and PE. LPC and LPE are formed through hydrolysis, which involves the removal of one acyl group with the involvement of phospholipase A2 (PLA2). However, it is unclear what inhibits the activity of lysoacyltransferase, which normally converts lysophospholipids back into phospholipids (Yu et al., 2021).

Lysophospholipids are typically found in membranes in trace amounts, as observed in the control group of this study. However, their concentration can increase during stressful environmental conditions. High levels of LPE were found in *Yersinia pseudotuberculosis* and *Escherichia coli* cells in response to heat shock (Kern et al., 2001; Davydova et al., 2016). Lysophospholipid levels were also elevated in *Arabidopsis* plant membranes in response to freezing (Welti et al., 2002). On the other hand, higher levels of LPC and LPE were synthesized in psychrophilic yeasts *Cryptococcus vishniacii* and *Dioszegia cryoxerica* (Dalluge and Connell, 2013). It cannot be excluded that the presence of MET is also a stress factor for *T. harzianum*.

Based on the PCA results of the phospholipid profiles, it appears that MPs and MET have different effects on fungal phospholipids. MET was found to induce the most detrimental changes, potentially due to its low solubility in water and high partition coefficient in octanol/water, leading to incorporation of the herbicide into microbial membranes (Pereira et al., 2009). It seems that MPs enhance the toxic effect of the herbicide, similar to what has been described for combined exposure to MPs and atrazine, which increased oxidative stress in earthworms (Cheng et al., 2020).

Soil enzyme activities are commonly used as indicators of soil microbial status (Gu et al., 2019). Microorganisms synthesize extracellular enzymes that break down organic macromolecules in order to use them for their growth. Surprisingly, however, MPs did not significantly inhibit the growth of *T. harzianum*, its addition to the soil increased the activity of C, P and N-acquiring enzymes synthetized by the fungus. Moreover, although MET inhibited the growth of the fungus, the presence of MET increased the activity of β-glucosidase, cellobiohydrolase, N-acetyl--glucosaminidase, and acid phosphatase.

5. Conclusion

The present study showed that MPs did not have a disruptive effect on T. *harzianum* growth. However, they did influence the activity of β-glucosidase, acid phosphatase and N-acetyl-glucosaminidase extracellular enzymes. Furthermore, the study revealed that MET decreased fungal growth but, increased enzyme activity. The fungi adapted to its presence by decreasing the unsaturation index of their membrane fatty acids and by an increase in the content of *lyso* forms of phospholipids. Interestingly, the presence of MPs in herbicide-polluted soil did not alleviate the negative effects of MET; instead, it increased the elimination of the herbicide. The authors anticipate that the presented data will

make a noteworthy contribution to describe certain aspects of fungal activity under the influence of MPs and herbicides in the soil environment.

CRediT authorship contribution statement

PB contributed to the design of experiments, collected and analyzed data, secured funding, and wrote the manuscript. AJ designed the experiment and collected and analyzed data. KN designed the experiment and analyzed the data. MS, SR, and KP designed the experiment and analyzed the data. LS-P secured soil and edited the manuscript. HJH designed the experiments, analyzed data, and edited the manuscript.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Acknowledgments

This research was funded by the National Science Centre, Poland, grant number 2020/39/B/NZ9/00471.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115656.

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