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ORIGINAL ARTICLE

Response of microorganisms and enzymes to soil contamination with a mixture of pethoxamid terbuthylazine

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Abstract This study analysed the effect of a mixture of pethoxamid (P) and terbuthylazine (T) contained in the herbicide Successor T 550 SE on organotrophic bacteria, total oligotrophic bacteria, *Azotobacter* and actinomycetes, oligotrophic sporulating bacteria, fungi and on the activities of dehydrogenases, catalase, urease, alkaline phosphatase, acid phosphatase, arylsulphatase and β-glucosidase. The phytotoxic effect of this pesticide on maize was also determined. The study was undertaken because of a lack of data concerning the effect of a P+T mixture on soil metabolism. The previously undertaken studies concerned only the separate effect of each of these substances. The P+T mixture disturbed soil homeostasis and altered soil stability, resulting in a succession of K-strategy organotrophic bacteria. It had a negative effect on bacteria of the genus *Azotobacter*, oligotrophic sporulating bacteria, actinomycetes and fungi, and a positive effect on oligotrophic bacteria. P+T in doses greater than 0.73 mg kg^{-1} of soil resulted in a strong inhibition of dehydrogenases, catalase, urease, acid phosphatase, alkaline phosphatase, arylsulphatase and β-glucosidase, and significantly inhibited the growth and development of maize.

Keywords Pethoxamid · Terbuthylazine · Herbicide · Soil · Enzymes · Microorganisms

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Introduction

In the last several decades, an increase has been observed in the use of chemical plant protection products in agriculture. Because of such wide use of pesticides, their remains can be found in various elements of the environment, namely in water, soil and the atmosphere (Riah et al. 2014). The time and degree of decomposition of pesticides in the environment depend on many physical and chemical soil factors (Arias-Estévez et al. 2008). Intensive use of pesticides may have serious ecological consequences, which may consequently have an effect on a cultivated plant and on soil microbiome (Kalia and Gosal 2011). However, the impact of plant protection products on microorganisms may have diverse effects. Some pesticides contribute to an increase in the microorganism count, while others may inhibit their development or have no effect (Jastrzębska and Kucharski 2007; Lo 2010). The effect of plant protection products on the soil microbiome largely depends on the dose and type of product used and on the type of the microorganisms (Lone et al. 2014). Not only pesticides themselves pose a threat to the environment, but also their metabolites.

Soil enzymes are a very good indicator of soil fertility and quality. They are very sensitive to various stress factors, including contaminants (Jyot et al. 2015). Enzymes such as: dehydrogenases, acid and alkaline phosphatase, β-glucosidase, urease and arylsulphatase participate in the turnover of the basic elements (phosphorus, carbon, nitrogen and sulphur) in the environment. For this reason, they play such an important role in the proper functioning of ecosystems (Riah et al. 2014). According to Floch et al. (2011), the activities of enzymes are the most credible and reliable indicator for assessing the effect of pesticides on a soil environment.

Pethoxamid [2-chloro-N-(2-ethoxyethyl)-N-(2-phenyl-prop-1-enyl-2-methyl) acetamide] is a compound

belonging to the chloroacetamides. This substance is often used to combat weeds in maize and soybean (Kato et al. 2001). Its half-life is from 6.1 to 14.2 days (Jursík et al. 2013). Terbuthylazine [2-tert-butylamine-4-chloro-6-ethylamine-1,3,5-triazine] is a substance introduced in place of atrazine (Caracciolo et al. 2005). It belongs to the group of s-triazine herbicides. In the EU countries, it has been successfully used for over 10 years (Jurina et al. 2014). According to the literature data (Dousset et al. 1997; James et al. 1998; Sahid and Teoh 1994), the half-life of terbuthylazine is from 5 to 116 days. The half-life is closely connected with the type of soil and the temperature.

In the literature (Caracciolo et al. 2005; Dousset et al. 1997; James et al. 1998; Jursík et al. 2013; Sahid and Teoh 1994; Skrzypczak et al. 2007), reports can be found concerning the herbicidal effectiveness or degradation in soil of both studied substances, but only of them used separately. However, there is no information concerning the synergic or additive effect of either of these substances on soil microorganisms, enzymatic activity or the growth and development of plants. It was precisely this lack of information that prompted the need for a study to unequivocally define the interaction of pethoxamid and terbuthylazine on soil microorganisms, soil enzymatic activity and the yielding of maize.

Materials and methods

Characteristics of soil

The research material was soil collected in the Research and Education Centre in Tomaszkowo (the NE part of Poland, 53.71610 N, 20.41670 E), from a depth of 0 to 20 cm, from the arable–humus horizon. Tomaszkowo is located in the area of the Olsztyn Lakeland which constitutes the western part of the Masurian Lakeland. The object is located within the Pomeranian phase of the Main Stadial of the Vistulian Glaciation. In this landscape, ground moraine prevails, where the dominant type of soils is brown soils with crude soils and leached brown soils. In this part of the region, areas used for agriculture prevail. The soil used for the study, according to the WRB classification (World Reference Base for Soil Resources 2014), was classified as Eutric Cambisols. Ratio C:N-8:1. The physicochemical properties of the soil are shown in Table 1.

Description of experiment

The next stage of the study was to conduct a pot culture experiment in the vegetation hall of the University of Warmia and Mazury in Olsztyn. In each of 3.5 dm^3 polyethylene pots, 3 kg of air dry mass of soil were placed. The experiment was conducted in five repetitions. Before being placed in the pot, the soil was thoroughly mixed with macro- and microelements. For all objects, the same fertilisation level was used, in mg kg^{-1} of soil: N—200 (100 mg—before sowing and 100 mg at the BBCH 19 phase of maize), P—44, K—100, Mg—20, Cu—5, Zn—5, Mo—5, Mo—2.5 and B—0.33. Nitrogen was used in the form of $CO(NH_2)_2$, phosphorus—KH_2PO_4, potassium—$KH_2PO_4 + KCl$, magnesium—$MgSO_4 \cdot 7H_2O$, copper—$CuSO_4 \cdot 5H_2O$, zinc—$ZnSO_4$, manganese—$MnCl_2 \cdot 4H_2O$, molybdenum—$NaMoO_4 \cdot 5H_2O$ and boron—H_3BO_3. To the prepared soil, a mixture of pethoxamid and terbuthylazine was added, in amounts of: 0; 0.73; 14.63; 29.26; 58.52; 117.04; 234.08; 468.16 mg kg^{-1} of DM of soil. The control object was soil without the herbicide added. The soil was then brought to moisture equal to 50 % capillary water capacity, and maize of the variety LG 32.58 was sown. After the emergence phase, thinning of plants was carried out to leave only five plants in each pot. The plants were vegetated for 60 days. The maize was harvested at the BBCH 53 phase (the top of the ear can be seen). Microbiological and biochemical analyses were performed twice during the plant vegetation (on days 30 and 60). The date of performance of microbiological and biochemical analyses was determined based on the PEC results Table 2. Therefore, microbiological and biochemical tests on the soil were performed twice during the vegetation of crop plants, in two different maize development phases, i.e. with nine leaves unfolded (30th day of the experiment—BBCH 19 phase) and the tip of inflorescence emerged (60th day of the experiment—BBCH 53 phase).

Characteristics of the herbicide

In the pot culture experiment, the herbicide Successor T 550 SE was used which contained two active substances:

Table 1 Selected physicochemical properties of soil used in experiment

Granulometric composition (%)			C_{org}	N_{og}	pH_{KCl}	HAC	EBC	CEC	BS (%)
Sand	Silt	Clay	(g kg^{-1} soil DM)	(g kg^{-1} soil DM)		($mMol_{(+)}$ kg^{-1} soil DM)	($mMol_{(+)}$ kg^{-1} soil DM)	($mMol_{(+)}$ kg^{-1} soil DM)	
72	21	7	7.05	0.86	7.00	8.00	111.00	119.00	93.28

C_{org} organic carbon, *N_{og}* total nitrogen, *HAC* hydrolytic acidity, *EBC* sum of exchangeable cations, *CEC* cation exchange capacity, *BS* saturation with base cations, *pH_{KCl}* soil pH

Table 2 Predicted environmental concentration (PEC) of active ingredients of the herbicide Successor T 550 SE in soil

Active ingredient dose (mg kg^{-1} DM soil)	Days	
	30 PEC	60
Pethoxamide (mg kg^{-1})		
0.40	0.092	0.021
7.98	1.845	0.427
15.96	3.690	0.853
31.92	7.380	1.706
63.84	14.761	3.413
127.68	29.522	6.826
255.36	59.044	13.652
Terbuthylazine (mg kg^{-1})		
0.33	0.130	0.052
6.65	2.628	1.039
13.30	5.256	2.077
26.60	10.513	4.155
53.20	21.026	8.310
106.40	42.051	16.619
212.80	84.102	33.238

pethoxamid (300 g dm^{-3}) and terbuthylazine (250 g dm^{-3}). The predicted environmental concentrations (PEC) in the soil of these substances are presented in Table 2. Predicted environmental concentrations (PEC) were determined with the use of the below formula: $PEC = PEC_{initial} \times (1-e^{-kt})/kt$, $PEC_{initial}$—predicted concentration of preparation in soil after single application (mg kg^{-1} of soil); $k-\ln 2/DT_{50}$ (DT_{50}—time for disappearance of half the chemical (days); t—time between applications (days) proposed by the European Comission—EC Document UE.7617/VI/96 (FOCUS 1997).

Terbuthylazine (T) is a compound from the group of triazines. It is taken up by roots of weeds. Pethoxamid (P) is a compound from the group of chloroacetamides. It is taken up by seedlings. Successor T 550 SE is produced by the company Stähler International GmbH & Co. KG (Germany). The recommended dose of this herbicide is 4 dm^3 ha^{-1}, or 1.33 mm^3 kg^{-1}. The dose of the herbicide per 1 kg was calculated based on the soil bulk density, which was 1.5 g cm^{-3}. Successor T 550 SE is a concentrate in the form of suspo-emulsion to be diluted with water. It is intended for combating weeds in maize crops.

Determination of the soil microorganism counts

On the days 30 and 60 of the experiment, the counts of organotrophic bacteria, total oligotrophic bacteria and oligotrophic sporulating bacteria, *Azotobacter,* actinomycetes and fungi were determined. In five repetitions, the respective dilutions of soil were sown onto Petri dishes. On the media characterised by Baćmaga et al. (2015).

Diversity of soil microorganisms

On days 30 and 60, the structure and biodiversity of organotrophic bacteria, actinomycetes and fungi were determined. The biodiversity of microorganisms was determined based on a 10-day observation and on daily counting of the grown microorganism colonies for each of the experimental days. Then, based on the formula proposed by Sarathachandra et al. (1997), the colony development index (CD) for the microorganisms was determined $CD = [N_1/1 + N_2/2 + N_3/3 \ldots N_{10}/10] \times 100$, where N_1, N_2, N_3,…N_{10} were proportional counts of colonies that emerged on day 1, 2, 3,…,10. According to the formula proposed by De Leij et al. (1993) the ecophysiological diversity index (EP) for the microorganisms was determined $EP = -\sum(pi \times \log pi)$ where pi—counts of colonies that emerged on a given day divided by total counts of colonies.

Determination of the soil enzyme activities

On the days 30 and 60 of the experiment, the activities of the following soil enzymes were determined: dehydrogenases (EC 1.1), catalase (EC1.11.1.6), urease (EC 3.5.1.5), arylsulphatase (EC 3.1.6.1), β-glucosidase (EC 3.2.1.21), acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1). The exact assay procedure of enzyme activity is described in the publication Kucharski et al. (2016).

Statistical analysis of the results

All of the obtained results were statistically analysed using the Statistica 12.5 (StatSoft 2016) software package. The analysis of variance ANOVA was used using Duncan's multiple range test, at a significance level of $p = 0.05$. Based on the obtained results, the percentage of the observed variation η^2 was determined using the two-way ANOVA method. The responses of microorganisms to the tested herbicide were compared using the cluster method—the Ward dendrogram. The activities of the soil enzymes were shown using principal component analysis (PCA) performed using multi-dimensional exploratory techniques.

Results and discussion

The counts of soil microorganisms

The use of herbicides may result in changes, both quantitative and qualitative, of microorganisms living in the soil

environment (Vlad et al. 2012). The performed studies demonstrated that the count of soil microorganisms was determined by the dose of a mixture of pethoxamid (P) and terbuthylazine (T) within the range from 15.98 % (organotrophic bacteria) to 64.71 % (*Azotobacter*), and the duration of herbicide persistence in soil from 6.77 to 58.65 % (Table 3). The response of the microorganisms to P+T are displayed on a cluster analysis diagram, with the Ward method (Fig. 1). This method allows one to generalise and properly interpret the obtained results. It is aimed at the determination of a similar response of microorganisms to herbicide contamination. Three separate clusters were created consisting of several sub-clusters with homogeneous variances. The first cluster was formed by oligotrophic bacteria (the days 30 and 60), organotrophic bacteria (the day 60) and fungi (the day 30). The second cluster was formed with oligotrophic sporulating bacteria (days 30 and 60), organotrophic bacteria, bacteria of the genus *Azotobacter*, actinomycetes (the day 30), the third cluster: bacteria of the genus Azotobacter, actinomycetes and fungi (the day 60). Contamination of the soil with the tested herbicide had a significant effect on the count of microorganisms on both tested days. On both the 30th and 60th day of the experiment, the counts of oligotrophic spore-forming bacteria, *Azotobacter*, actinomycetes and fungi were negatively correlated with the P+T dose, while the count of oligotrophic bacteria was positively correlated. Apart from the duration of the experiment, it was found that soil contamination with P+T contributed to a decrease in the count of oligotrophic sporulating bacteria, *Azotobacter*, actinomycetes and fungi, and to an increase in total oligotrophic bacteria (Fig. 2). The response of oligotrophic bacteria different from other groups of microorganisms may result from different abilities of microorganisms to degrade plant protection products and from oligotrophic bacteria being microorganisms with low nutritional requirements. In addition, they have a membrane which constitutes a barrier against harmful environmental factors (HuiXia et al. 2007). An increase in the count of oligotrophic bacteria in soil supplemented with pethoxamid and terbuthylazine (the active substances of the herbicide Successor T 550 SE) was also observed in the previous studies by our team, conducted under strictly controlled conditions (Tomkiel et al. 2014). The response of microorganisms in the vegetation experiment which tested the effects of a mixture of pethoxamid and terbuthylazine was different from that in the laboratory model experiment, which is due to the fact that these are experiments of a different type. The experiment described in the presented

Table 3 Percentage of the observed variability η^2 of microorganisms in soil contaminated with a mixture of Pethoxamid (P) + Terbuthylazine (T)

Variable factors	Microorganisms					
	Olig	$Olig_p$	Org	Act	Fun	*Az*
Dose P+T	60.80	55.39	15.98	57.47	48.18	64.71
Time	12.74	34.68	58.65	10.89	26.54	6.77
Dose P+T × Time	24.26	8.56	19.94	30.59	20.41	26.10
Error	2.20	1.37	5.43	1.05	4.87	2.42

olig oligotrophic bacteria, $Olig_p$ spore-forming bacteria, *Org* organotrophic bacteria, *Act* actinomycetes, *Fun* fungi, *Az Azotobacter*

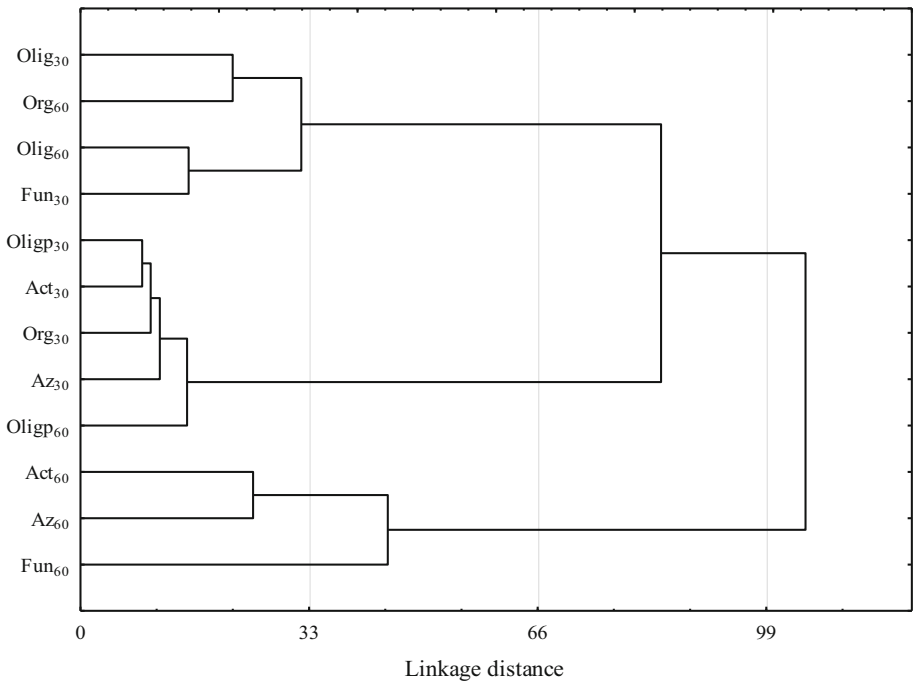

Fig. 1 Similarity of microbial reaction to contamination of soil with Pethoxamid (P) and Terbuthylazine (T). *Act* Actinomycetes, *Az Azotobacter* sp., *Olig* oligotrophic bacteria, *Olig p* spore-forming oligotrophic bacteria, *Org* organotrophic bacteria, *Fun* fungi, soil incubation time, days: 30 and 60

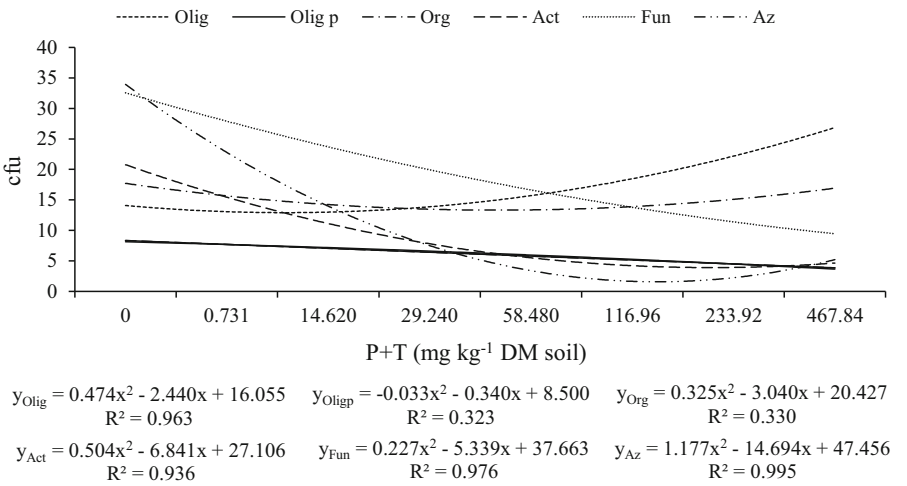

Fig. 2 Average microbial counts in soil contaminated with the herbicide Successor T 550 SE. cfu 10^n kg^{-1} DM of soil *Olig* oligotrophic bacteria (10^9), *Oligp* spore-forming bacteria (10^8), *Org* organotrophic bacteria (10^9), *Act* actinomycetes (10^9), *Fun* fungi (10^7), *Az Azotobacter* (10^3)

manuscript is a vegetation experiment in which, as compared to the studies conducted previously under laboratory conditions, another element was introduced which affected the soil microbiota. This element was the cultivated crop plant. If the plant did not affect the soil microbiota, the commonly known rhizosphere effect (R:S ratio) would not be experienced. Thus, both our own studies and the literature (Baćmaga et al. 2015; Kucharski et al. 2016; Sebiomo et al. 2011) demonstrate that herbicides are not always toxic to soil microbiome. Some species or strains may display increased tolerance to herbicides. The differentiated response of microorganisms to the excessive content of herbicides in soil may result from both their species characteristics and morphological characteristics. The effect of herbicides on microbiological properties is dictated by the type of active substance, its dose and physicochemical properties of soil (Kucharski et al. 2016). For example: carfentrazone-ethyl (the active substance of the preparation Aurora 40 WG) had an effect on an increase in the counts of total oligotrophic bacteria and organotrophic bacteria, while it decreased the counts of *Azotobacter*, fungi, oligotrophic sporulating bacteria and actinomycetes (Tomkiel et al. 2015). Metazachlor had a negative effect on the development of oligotrophic bacteria and their spore forms, *Azotobacter*, organotrophic bacteria and actinomycetes (Baćmaga et al. 2014a). Atrazine, primextra, paraquat and glyphosate also had a negative effect on the development of bacteria, actinomycetes and fungi, according to Sebiomo et al. (2011). In contrast, Baćmaga et al. (2015) following the use of a mixture of active substances named diflufenican, mesosulphuron-methyl and iodosulphuron-methyl-sodium observed an increase in the counts of total oligotrophic bacteria and their spore forms, organotrophic bacteria and actinomycetes, and a decrease in the counts of fungi and *Azotobacter*. Martinez et al. (2008) studying sulfentrazone found that this product stimulates the growth of actinomycetes, but it does not have an effect on the count of fungi. Araújo et al. (2003) noted a significant increase in the count of actinomycetes following the application of glyphosate. Crouzet et al. (2010) studying mesotrione at increased doses (tenfold, 100-fold higher than the recommended) found an increase in the count of fungi. Milošević et al. (2004) and Elbashier et al. (2016) also noted the sensitivity of bacteria of the genus *Azotobacter* to pesticides. Thus, it can be clearly stated that the responses of microorganisms to contamination of a soil environment with herbicides are very diverse. It should also be noted that root exudates such as: carbohydrates, organic acids, amino acids, enzymes and flavonoids, accumulating in the maize rhizosphere had a significant effect on the responses of particular groups of microorganisms to a mixture of pethoxamid and terbuthylazine. Root exudates may increase the ability of microorganisms to degrade plant protection products and increase their bioavailability.

Diversity of soil microorganisms

The common use of pesticides in plant protection has an effect not only on a change in the count of autochthonous microorganisms in soil, but it can cause physiological and biochemical changes in soil microorganisms and change their diversity (Kucharski et al. 2016). In our own studies, in order to determine the diversity of soil microorganisms, the colony development index (CD) and the ecophysiological diversity index (EP) of microorganisms were computed. The CD and EP indices provide information concerning changes in the proportions between the slow-growing (K-strategists) and the fast-growing (r-strategists) microorganisms. The CD index ranges from 0 to 100, and the EP index from 0 to 1. If the CD values are close to 100, it indicates the dominance of the fast-growing microorganisms and if the EP values are close to 1, then the growth of microorganisms in a given environment is more even.

Table 4 Ecophysiological biodiversity index (EP) in soil contaminated with the Pethoxamid (P) and Terbuthylazine (T)

Dose of P+T (mg kg^{-1} DM soil)	Microorganisms					
	Organotrophic bacteria		Actinomycetes		Fungi	
	Soil incubation time, days					
	30	60	30	60	30	60
0.00	0.857	0.742	0.855	0.788	0.540	0.568
0.73	0.875	0.809	0.798	0.842	0.623	0.588
14.63	0.875	0.893	0.889	0.851	0.566	0.572
29.26	0.898	0.933	0.840	0.879	0.588	0.558
58.52	0.856	0.911	0.885	0.917	0.563	0.535
117.04	0.860	0.778	0.837	0.865	0.553	0.567
234.08	0.858	0.829	0.880	0.887	0.594	0.553
468.16	0.857	0.748	0.867	0.792	0.537	0.474
Average	0.867	0.830	0.856	0.853	0.571	0.552

In the conducted studies, pethoxamid and terbuthylazine to a slight degree changed the ecophysiological diversity index of microorganisms (Table 4). Of all the studied microorganisms, the lowest EP values were found in fungi. Low values of this index may indicate that sensitive microorganisms were replaced with microorganisms more resistant to a stress factor (in this case, the tested herbicide). For fungi, both on the day 30 and 60, the (EP) index value was highest for soil to which a mixture P+T was applied, in an amount of 0.73 mg kg^{-1}. The EP index was 0.623 and 0.588, respectively. For organotrophic bacteria, the EP values were highest for objects contaminated with a dose of P+T in the amount of 29.26 mg kg^{-1}. This was observed for the whole duration of the study (60 days). The ecophysiological diversity index of bacteria on day 30 of herbicide persistence in soil was 0.898, and on day 60 it was 0.933. The EP index of actinomycetes on day 30 had the highest values following the application of the preparation in a dose of 14.63 mg P+T kg^{-1} (EP = 0.889) and on day 60 following the use of the product in the amount of 58.52 mg P+T kg^{-1} (EP = 0.917).

The tested herbicide contributed to changes in the value of the colony development index (CD) of all the three studied groups of microorganisms (Table 5). Regardless of the dose of herbicide or the time of analysis, the highest values of the colony development index CD were noted in fungi (CD = 40.116), organotrophic bacteria (CD = 34.395) and the lowest values were in actinomycetes (CD = 24.566). The colony development index CD of fungi varied from 32.155 to 48.759. In fungi, the lowest values of this index were noted following the application of the two highest doses of P+T (234.08 and 468.16 mg kg^{-1}). The CD values for actinomycetes were within the range from 20.201 (a dose of 58.52 mg kg^{-1} on day 30) to 32.853 (the control object on day 60). In organotrophic bacteria on day 30, the highest values of this index were noted following the application of a dose of 234.08 mg kg^{-1} (CD = 37.063) and on day 60 in the control object (CD = 43.219). The greatest changes in the CD index value occurred in organotrophic bacteria, after relatively slight fluctuations between objects on day 30. On day 60, a drastic change occurred in the growth rate of the colonies. The rate decreased with an increasing dose of P+T. The CD index for organotrophic bacteria on the 30th day of the experiment increased following the application of doses from 58.52 to 234.08 mg kg^{-1} DM soil as compared to the control sample, while on the 60th day, the addition of the tested herbicide resulted in a decrease in this index. The CD index for actinomycetes on both days decreased following the application of the herbicide in an amount from 14.63 to 468.16 mg kg^{-1} DM soil. In turn, the CD index for fungi on the 30th day decreased following the application of the tested preparation as compared to the control object, while on the 60th day, the trend was reversed. To conclude, it can be stated that the values of the CD and EP indices prove that the tested herbicide Successor T 550 SE had an effect on the structure of microorganisms. The changes in diversity of microorganisms, observed in our studies, in soil supplemented with herbicide, are concordant with the literature data (Kucharski et al. 2016; Lone et al. 2014; Ratcliff et al. 2006). Organotrophic bacteria, as opposed to fungi, were characterised by the greatest diversity in soil with an addition of the herbicide Boreal 58 WG (flufenacet + isoxaflutole) (Kucharski et al. 2016) and a mixture of diflufenican + mesosulphuron-methyl + iodosulphuron-methyl-sodium (Baćmaga et al. 2015). In contrast, in studies using herbicides Alister Grande 190 OD (diflufenican + mesosulphuron-methyl + iodosulphuron-methyl-sodium), Fuego 500 SC (matazachlor) and Lumax 537,5 SC (terbuthilazine + mesatrione + s-metolachlor) it was observed that the values of the ecophysiological

Tabele 5 Colony development index (CD) in soil contaminated with the Pethoxamid (P) and Terbuthylazine (T)

Dose of P+T (mg kg^{-1} DM soil)	Microorganisms					
	Organotrophic bacteria		Actinomycetes		Fungi	
	Soil incubation time, days					
	30	60	30	60	30	60
0.00	34.250	43.219	24.807	32.853	38.636	44.964
0.73	33.074	39.427	25.431	29.328	34.286	44.869
14.63	33.317	37.128	23.509	28.580	35.624	46.224
29.26	33.706	35.865	23.659	27.133	35.527	48.059
58.52	36.314	35.186	20.201	24.463	36.243	48.759
117.04	36.582	27.661	23.617	21.982	33.660	48.573
234.08	37.063	27.442	22.146	22.422	33.413	37.489
468.16	34.120	25.975	24.189	18.743	32.155	43.379
Average	34.803	33.988	23.445	25.688	34.943	45.290

Table 6 Percentage of the observed variability η^2 of enzymes activity in soil contaminated with a mixture of Pethoxamid (P) + Terbuthylazine (T)

Variable factors	Enzymes						
	Deh	Cat	Ure	Pal	Pac	Aryl	Glu
Dose of P+T	85.68	43.20	90.56	65.33	53.59	14.94	42.80
Time	0.11	0.26	3.19	0.54	25.99	74.51	39.41
Dose P+T × Time	14.04	55.37	5.81	33.11	9.68	1.16	16.79
Error	0.17	1.17	0.44	1.02	10.74	9.39	1.00

Deh dehydrogenases, *Cat* catalase, *Ure* urease, *Pal* alkaline phosphatase, *Pac* acid phosphatase, *Aryl* arylsulfatase, *Glu* β-glucosidase

diversity index EP of fungi decreased, but the colony development index CD increased (Baćmaga et al. 2014b). Following the use of preparations soproturon, metribuzin, clodinafop propargyl, atlantis and sulfosulfuron, Lone et al. (2014) observed their differentiated effect on the biodiversity of microorganisms.

The activities of soil enzymes

Due to their protein nature, enzymes secreted to the environment by microorganisms, plants and soil fauna respond quickly to various environmental factors, both natural and anthropogenic. For this reason, enzymes are among the most important indicators determining soil quality (Kucharski et al. 2016). The enzymes actively participating in the turnover of elements, the transformations of organic matter and transformation of xenobiotics entering soil include, among others, dehydrogenases, catalase, urease, acid phosphatase, alkaline phosphatase, arylsulphatase and β-glucosidase. Because of new plant protection products coming onto the market all the time, their effect on the activities of soil enzymes has not yet been sufficiently explained. In our own studies, it was demonstrated that the activities of enzymes (Table 6) were shaped by the dose of the P+T mixture within a range from 14.95 % (arylsulphatase) to 90.56 % (urease), and by the duration of herbicide persistence in soil from 0.11 % (dehydrogenases) to 74.51 % (arylsulphatase). The data presented in Table 7 clearly show that a dose of 0.73 mg P+T kg^{-1} already destabilised the activities of most enzymes. When the substances were used in higher doses (from 14.63 to 468.16 mg P+T kg^{-1} of soil), strong inhibition of all enzymes was found. On average, regardless of the time of study, in most contaminated soil (a dose of 468.16 mg P+T mg kg^{-1} of soil) the mixture caused inhibition of enzymatic activity within a range from 21 % (catalase) to 90 % (dehydrogenases). In terms of sensitivity to a P+T mixture, enzymes can be ordered as follows (from the most to the least sensitive): dehydrogenases > acid phosphatase > urease > alkaline phosphatase > β-glucosidase > arylsulphatase > catalase. Principal component analysis (PCA) indicates not only a negative correlation between the activities of particular enzymes and the degree of soil contamination with pethoxamid and terbuthylazine, but also a significant positive correlation of enzymes between each other (Fig. 3). The first two components together constituted 83.03 % of the total variation. The vectors representing the primary variables of the activities

Table 7 Enzyme activity in soil contaminated with the Pethoxamid (P) and Terbuthylazine (T), kg^{-1} DM soil h^{-1}

Dose of P+T (mg kg^{-1} DM soil)	Enzymes*													
	Deh μmol TFF		Cat mol O_2		Ure mmol N-NH_4		Pal mmol PNP		Pac mmol PNP		Aryl mmol PNP		Glu mmol PNP	
	Soil incubation time, days													
	30	60	30	60	30	60	30	60	30	60	30	60	30	60
0.00	14.154	30.176	0.212	0.309	0.607	0.781	2.980	2.682	1.654	3.121	0.141	0.264	0.285	0.467
0.73	17.243	25.445	0.197	0.360	0.694	0.832	2.224	1.866	0.878	1.650	0.111	0.215	0.278	0.488
14.63	12.238	6.452	0.194	0.210	0.600	0.354	2.253	1.751	0.869	1.632	0.100	0.198	0.278	0.364
29.26	10.379	6.350	0.211	0.202	0.471	0.331	1.904	1.748	0.865	1.488	0.098	0.189	0.276	0.334
58.52	7.725	2.856	0.212	0.132	0.286	0.170	1.803	1.746	0.865	1.279	0.099	0.187	0.276	0.295
117.04	6.018	1.623	0.213	0.154	0.278	0.122	1.715	1.752	0.830	1.244	0.098	0.177	0.264	0.287
234.08	5.154	1.664	0.222	0.156	0.293	0.321	1.647	1.709	0.829	1.102	0.083	0.184	0.106	0.281
468.16	3.365	1.092	0.215	0.195	0.386	0.350	1.604	1.571	0.797	1.027	0.080	0.180	0.100	0.326
Average	9.535	9.457	0.210	0.215	0.452	0.408	2.016	1.853	0.948	1.568	0.101	0.199	0.233	0.355
r	−0.796	−0.543	0.551	−0.388	−0.504	−0.327	−0.623	−0.480	−0.371	−0.549	−0.690	−0.494	−0.914	−0.469
$LSD^{*}_{0.05}$	0.500^{a}; 0.250^{b}; 0.708^{ab}		0.008^{a}; 0.004^{b}; 0.012^{ab}		0.022^{a}; 0.011^{b}; 0.032^{ab}		0.495^{a}; 0.248^{b}; 0.700^{ab}		0.495^{a}; 0.248^{b}; 0.700^{ab}		0.025^{a}; 0.012^{b}; n.s.^{ab}		0.014^{a}; 0.007^{b}; 0.020^{ab}	

* *Deh* dehydrogenases, *Ure* urease, *Cat* catalase, *Pal* alkaline phosphatase, *Pac* acid phosphatase, *Aryl* arylosulfatase, *Glu* β-glucosidase

$LSD_{0.05}$ for: [a] dose of P+T; [b] incubation time; [ab] interaction factors; *r* coefficient of correlation

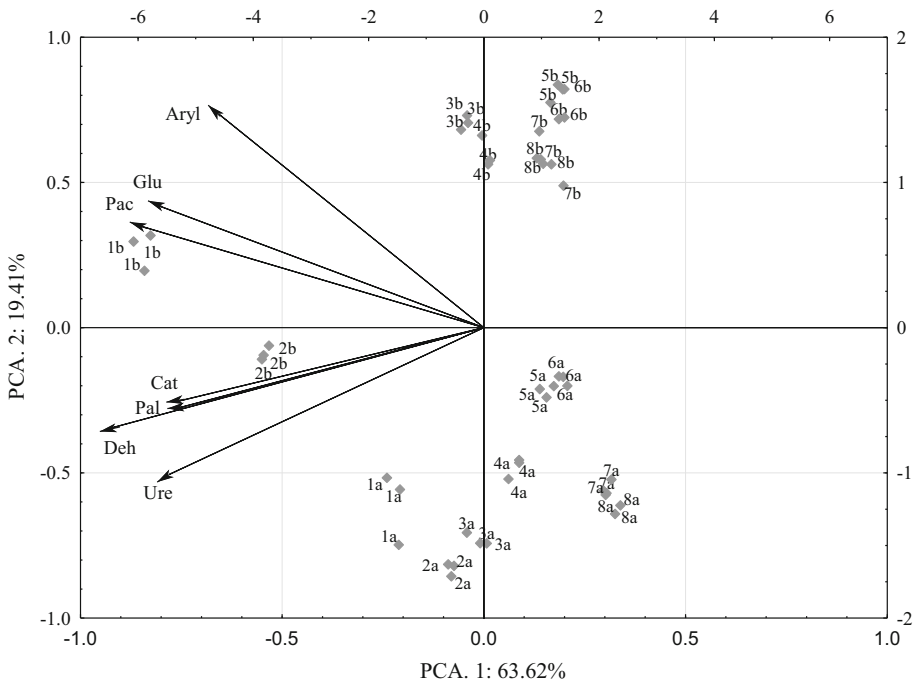

Fig. 3 Enzyme activity in soil with the Pethoxamid (P) and Terbuthylazine (T)—PCA method. *Deh* dehydrogenases, *Ure* urease, *Cat* catalase, *Pal* alkaline phosphatase, *Pac* acid phosphatase, *Aryl* arylosulfatase, *Glu* β-glucosidase, dose of P+T (mg kg^{-1} DM soil): 1–0 mg; 2–0.732 mg; 3–14.630 mg; 4–29.260 mg; 5–58.520 mg; 6–117.04 mg; 7–234.08 mg; 8–468.16; soil incubation time, days: 30 and 60

of catalase, alkaline phosphatase, dehydrogenases and urease were negatively correlated with both the first and the second principal component, and the vectors representing the activities of arylsulphatase, β-glucosidase and acid phosphatase were negatively correlated with the first principal component, but positively with the second principal component. It is not an isolated fact that contamination of soil on which plants are cultivated with a P+T mixture, results in disturbances in soil metabolism. The unfavourable effect of these substances, though tested under strictly controlled laboratory conditions, was observed by Tomkiel et al. (2015). Kucharski et al. (2016) and Wyszkowska (2002) also noted the differentiated effect of herbicides on enzyme activities. Kucharski et al. (2016) demonstrated that contamination with the herbicide Boreal 58 WG (40 mg kg^{-1} of soil) contributed to a decrease in the activities of dehydrogenases, catalase, urease, arylsulphatase and β-glucosidase, but the preparation did not have a negative effect on the acid phosphatase activity.

The preparation Reglone 200 SL (diquat) resulted in an increase in the activities of dehydrogenases, acid phosphatase and alkaline phosphatase, and Elastiq 550 EC (synthetic latex + alkoxylated alcohol)—in a decrease in their activities (Jezierska-Tys and Rutkowska 2013). The sensitivity of dehydrogenases to atrazine, primextra, paraquat and glyphosate was also demonstrated by Sebiomo et al. (2011). Similar results were obtained by Lone et al. (2014), testing six different herbicides (soproturon, metribuzin, clodinafop propargyl, atlantis and sulfosulfuron). They proved that dehydrogenases were the most sensitive to the used preparations. Acid phosphatase responded more negatively to these products than alkaline phosphatase. A similar result was noted in our studies with P+T. A negative effect on phosphatases was also demonstrated by Yao et al. (2006) when testing acetamiprid and by Wyszkowska and Kucharski (2004) testing Triflurotox 250 EC (trifluranine).

Baćmaga et al. (2014a) demonstrated a negative effect of metazachlor on the activities of dehydrogenases, catalase, urease, acid and alkaline phosphatase, arylsulphatse and β-glucosidase, and Muñoz-Leoz et al. (2012) found a negative effect of tebuconazole on the β-glucosidase activity. In contrast, Saha et al. (2012), testing chloroacetanilide herbicides (alachlor, butachlor and pretilachlor), observed a stimulating effect of the preparations on the β-glucosidase activity. In our own studies, the β-glucosidase activity was not disturbed before a dose of P+T higher than 0.73 mg kg^{-1} of soil was used.

The inhibitory effect of the P+T mixture used in excessive doses on the activities of the tested soil enzymes may result from not only a direct inhibitory effect of these substances, but also from the effect of intermediary products formed during the decomposition of pethoxamid and terbuthylazine. The negative effect of this herbicide may also depend on the duration of its persistence in a soil environment (Kucharski et al. 2016). The authors demonstrated that, over time, in soil treated with the herbicide Boreal 58 WG, the activities of urease and catalase decreased, and the activities of acid phosphatase and β-glucosidase increased. Also, the P+T mixture (the object of this study) resulted in changes in the activities of soil

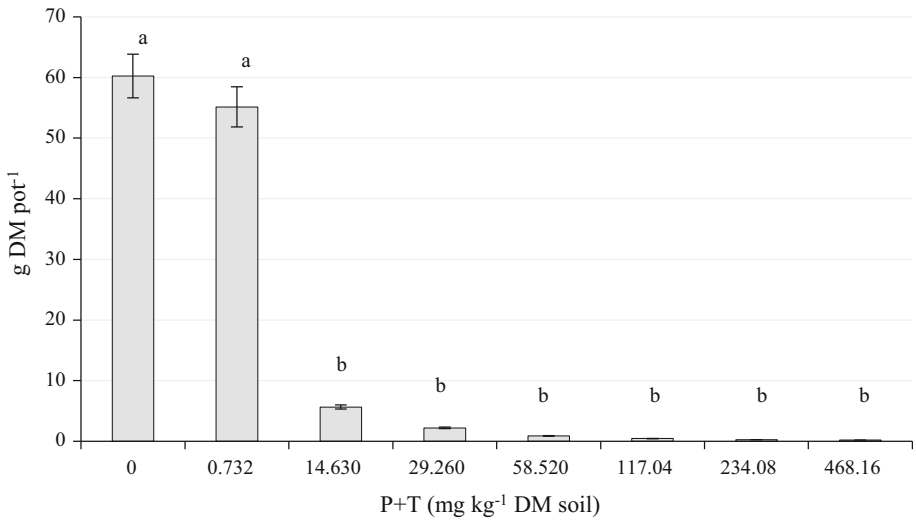

Fig. 4 Yield of maize grown on soil contaminated with the Pethoxamid (P) and Terbuthylazine (T), g DM per pot

enzymes during the experiment. With the increased duration of persistence in soil of pethoxamid and terbuthylazine, the activities of dehydrogenases, catalase, urease and alkaline phosphatase decreased and the activities of acid phosphatase, arylsulphatase and β-glucosidase increased. Based on the presented predicted environmental concentrations (PEC) (Table 2) of the active substances, it can be stated that terbuthylazine had a greater effect on the activities of soil enzymes than pethoxamid. It was demonstrated that on day 60 of the experiment, following the application of the highest dose of the tested preparation, the predicted concentration of pethoxamid in soil was 13.652 mg kg^{-1} and terbuthylazine was 33.238 mg kg^{-1}. In conclusion, it can be stated that both microorganisms and enzymes are very good potential indicators of soil quality. This results mainly from them being more sensitive to stress and responding more quickly to contamination of the environment with biocides than other parameters.

Growth and development of maize

Soil contamination with a mixture of pethoxamid and terbuthylazine changed not only the soil microbiome, but also had a significant effect on the growth and development of maize (Fig. 4). The key factor determining the growth and development of maize was the dose of the applied herbicide. Only the dose of herbicide recommended by the producer did not significantly disturb plant growth. Skrzypczak et al. (2007), trying to determine the herbicidal effectiveness of a mixture of two herbicides, Callisto 100 SC and Successor T 550 SE, applied in different doses (150 + 1650; 100 + 1650; 100 + 1375 g ha^{-1}) also did not find a phytotoxic effect on maize.

In the presented studies, a feedback was observed between the degree of soil contamination with the tested product and the yield of plants. Consequently, in the most contaminated objects, maize plants died out at the seedling stage. In the literature, confirmation can be found of a negative effect of excessive amounts of herbicides on the yield of plants. Baćmaga et al. (2014a) studying the effect of metazachlor on the yield of spring rape demonstrated an unfavourable effect of the preparation on plants. Wyszkowska and Kucharski (2004) also reported a decrease in the fresh mass of spring rape and white mustard under the effect of excessive amounts of the herbicide Triflurotox 250 EC. Similar results were obtained by Baćmaga et al. (2014b) in an experiment using a mixture of diflufenican + mesosulphuron-methyl + iodosulphuron-methyl-sodium. They observed a decrease in the yield of spring wheat, and the application of the two highest doses (18.24 and 36.48 mg kg^{-1} of DM of soil) resulted in complete dying out of plants. The pesticide Sevin (carbaryl) applied in a dose of 5 dm^3 ha^{-1} also had a negative effect on carrot growth (Elbashier et al. 2016).

Summary

Herbicides may be toxic not only to the target organisms, but also to non-targeted organisms. When entering soil ecosystems, herbicides become a threat to all organisms. These studies describe the effect of a mixture of two active substances (pethoxamid and terbuthylazine) contained in the herbicide Successor T 550 SE on soil microorganisms, soil enzymes and plants. Excessive amounts of this herbicide disturb the biological equilibrium of soil, by disturbing the soil microbiological and biochemical properties. They shift a succession of organotrophic bacteria from the r-strategy to the K-strategy. They also have a negative effect on bacteria of the genus *Azotobacter*, oligotrophic

sporulating bacteria, actinomycetes and fungi and a positive effect on oligotrophic bacteria. A mixture of pethoxamid and terbuthylazine in doses higher than 0.73 mg kg^{-1} of soil resulted in a strong inhibition of dehydrogenases, catalases, ureases, acid phosphatase, alkaline phosphatase, arylsulphatase and β-glucosidase and significantly inhibited the growth and development of maize.

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