POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 31(1): 59–74, Y. 2016

HERBICIDE RESISTANCE OF MICROORGANISMS

Małgorzata Baćmaga, Agata Borowik, Monika Tomkiel, Jadwiga Wyszkowska

Department of Microbiology
University of Warmia and Mazury in Olsztyn

Key words: herbicide, microorganisms, sensitivity, resistance, PEC.

Abstract

The aim of study was to evaluate the sensitivity of selected microbial groups cultured on solid media and soil-dwelling microorganisms to metazachlor (Fuego 500 SC), a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium (Alister Grande 190 OD), and a mixture of terbuthylazine + mesotrione + s-metolachlor (Lumax 537.5 SE). The tested microorganisms were: *Azotobacter* spp., *Arthrobacter* spp., *Bradyrhizobium* spp. (lupini), *Rhizobium leguminosarum* bv. *viciae*, *Streptomyces intermedius*, *Streptomyces viridis*, *Streptomyces longisporoflavus*, *Streptomyces odorifer*, *Fusarium* spp., *Aspergillus* spp., *Penicillum* spp., *Rhizopus* spp. The results indicate that fungi were more sensitive to herbicides than bacteria and actinomycetes. The tested microbes were most resistant to increased doses of the mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium. Predicted environmental concentrations (PEC) calculated on day 160 indicate that increased doses of metazachlor posed the greatest threat for soil-dwelling microorganisms. The applied doses of metazachlor resulted in the highest PEC values, which points to a high risk of soil contamination with this weed control agent.

OPORNOŚĆ DROBNOUSTROJÓW NA HERBICYDY

Małgorzata Baćmaga, Agata Borowik, Monika Tomkiel, Jadwiga Wyszkowska

Katedra Mikrobiologii
Uniwersytet Warmińsko-Mazurski w Olsztynie

Słowa kluczowe: herbicydy, drobnoustroje, wrażliwość, oporność, PEC.

Abstrakt

Celem badania była ocena wrażliwości wybranych grup mikroorganizmów hodowanych na podłożach stałych i w środowisku glebowym na metazachlor (Fuego 500 SC), mieszaninę diflufenikanu + mezosulfuroun metylowego + jodosulfuronu metylo-sodowego (Alister Grande 190 OD)

Address: Jadwiga Wyszkowska, Department of Microbiology, University of Warmia and Mazury, pl. Łódzki 3, 10-727 Olsztyn, Poland, phone + 48 (89) 523 39 98,
e-mail: jadwiga.wyszkowska@uwm.edu.pl

i mieszaninę terbutylazyny + mezotrionu + s-metolachloru (Lumax 537.5 SE). Testowanymi drobnoustrojami były: *Azotobacter* spp., *Arthrobacter* spp., *Bradyrhizobium* spp. (lupini), *Rhizobium leguminosarum* bv. *viciae*, *Streptomyces intermedius*, *Streptomyces viridis*, *Streptomyces longisporoflavus*, *Streptomyces odorifer*, *Fusarium* spp., *Aspergillus* spp., *Penicillum* spp., *Rhizopus* spp. Badania te wykazały, że grzyby charakteryzowały się większą wrażliwością na herbicydy niż bakterie i promieniowce. Badane drobnoustroje najbardziej oporne były na zwiększone dawki mieszaniny diflufenikanu + mezosulfuronu metylowego + jodosulfuron metylo-sodowego. Obliczone przewidywane stężenie preparatów w glebie (PEC) w 160 dniu potwierdza, że metazachlor zastosowany w dawkach zanieczyszczających stanowi największe zagrożenie dla bytujących w niej drobnoustrojów. Wartość PEC dla zastosowanych dawek była najwyższa, co dowodzi o możliwości wystąpienia wysokiego ryzyka zanieczyszczenia gleby tym preparatem.

Introduction

The natural environment is increasingly often subjected to anthropogenic contamination, including with herbicides (BAĆMAGA et al. 2014a, KUCHARSKI and WYSZKOWSKA 2008, KUCHARSKI et al. 2009). Due to their widespread use, herbicides are present in various elements of the natural environment, mainly soil and water. Herbicides disrupt the biochemical and physiological responses of weeds, but they can also exert harmful effects on non-target organisms (BAĆMAGA et al. 2012, BAĆMAGA et al. 2014b, WYSZKOWSKA and KUCHARSKI 2004), including microorganisms which quickly respond to environmental changes. Microbes have varied sensitivity to herbicides, and species or strains sensitive to weed control agents are likely to be eliminated from the environment. Resistant organisms are generally characterized by high levels of activity and rapid growth. The responses of microorganisms to herbicides can be indicative of changes taking place in different ecosystems. Variations in microbial activity can be estimated with the use of various tests. Microorganisms play an important role in herbicide degradation, and even the most persistent compounds can be decomposed to forms that are less toxic than the initial substance (DAS and DEY 2013). Microbes can rely on herbicides as sources of nutrients and energy. Microbial consortia decompose herbicides into harmless products more readily than individual species (CASTILLO et al. 2006). Microbes are among the few organisms that can absorb nutrients from various organic and inorganic compounds, which enabled them to colonize all ecosystems and adapt to local conditions. Microorganisms should be used in the process of neutralizing herbicides and other xenobiotics that pose a threat to the environment. Herbicides are generally evaluated for their toxic effects on humans and animals, whereas their impact on microorganisms is rarely investigated. In this study, two laboratory experiments were carried out to evaluate the sensitivity of selected microbial groups cultured on solid media and soil-dwelling microorganisms to metazachlor, a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium, and a mixture of

terbuthylazine + mesotrione + s-metolachlor. The tested substances can exert different effects on microbes cultured under controlled laboratory conditions and soil-dwelling microorganisms. Soil microbes can grow on soil colloids, which can minimize the negative impact of chemical compounds on microbial development. Based on the results of this study, the tested microorganisms could be used in the process of neutralizing pesticides in soil.

Materials and Methods

The responses of microorganisms cultured on solid media to herbicides

A laboratory experiment was carried out to analyze the effect of metazachlor (active ingredient in the Fuego 500 SC herbicide), a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium (active ingredients in the Alister Grande 190 OD herbicide) and a mixture of terbuthylazine + mesotrione + s-metolachlor (active ingredients in the Lumax 537.5 SE herbicide) on the growth of the following microorganisms: *Azotobacter* spp., *Arthrobacter* spp., *Bradyrhizobium* spp. (lupini), *Rhizobium leguminosarum* bv. *vicie*, *Streptomyces intermedius, Streptomyces longisporoflavus, Streptomyces odorifer, Streptomyces viridis*, *Rhizopus* spp., *Aspergillus* spp., *Penicillum* spp. and *Fusarium* spp. The tested herbicides are characterized in Table 1. The herbicides were applied in four different doses (Table 2).

Pure microbial cultures were cultivated on agar slants in a thermostat at 28°C (fungi and *Azotobacter* spp. – for 48 h, the remaining bacteria – for 72 h, actinomycetes – for 168 h). The resulting cultures were transferred to agar slants with different media and incubated under identical conditions. The cultures were rinsed off agar slants with 5 cm^3 of aqueous solution of 0.85% NaCl, and they were placed in flasks containing different media in the amount of 1 cm^3 of microorganisms per 100 cm^3 of the medium. Culture media with the microorganisms were poured onto Petri plates in the amount of 15 cm^3. After media solidification, three filter paper discs saturated with different herbicide doses were placed on the plates. Each disc, 6 mm in diameter, was saturated with 5 cm^3 of aqueous herbicide solution. Petri plates were incubated at 28°C (fungi – for 24 h, bacteria – for 48 h, actinomycetes – for 72 h). After incubation, the zones of inhibition created by the tested herbicides for each microbial group were measured in mm. The experiment was performed *in vitro* by the disc diffusion method described by BOROS et al. (2007), in three replications, on six strains of each tested microorganism from the collection of the Department of Microbiology. This qualitative method relies on herbicide

Table 1

General characteristics of the tested herbicides

Name of herbicide	Active ingredient	Chemical group	Action	Dose recommended by manufacturer [dm^3 ha^{-1}]	Manufacturer
Fuego 500 SC	metazachlor	chloroacetanilides	Inhibits mitosis and cell division	2.00	Feinchemia Schwebda GmbH
Alister Grande 190 OD	diflufenican	phenoxy nicotinic acid-amides	Inhibits carotenoid biosynthesis	0.90	BayerCrop Science
	mesosulfuron--methyl	sulfonylureas	Inhibits acetolactate synthase		
	iodosulfuron--methyl-sodium	sulfonylureas	Inhibits acetolactate synthase		
Lumax 537.5 SE	terbuthylazine	triazines	Inhibits photosynthesis in photosystem II A	3.75	Syngenta
	mesotrione	triketones	Inhibits carotenoid and chlorophyll biosynthesis		
	s-metolachlor	chloroacetamides	Inhibits chlorophyll, protein and lipid synthesis		

Table 2

Active ingredient doses applied to filter paper discs on solid media, mg $disc^{-1}$

Name of herbicide	Active ingredient	Active ingredient dose			
		1	2	3	4
Fuego 500 SC	metazachlor	2.500	1.2500	0.6250	0.4160
Alister Grande 190 OD	diflufenican	0.9000	0.4500	0.3000	0.2250
	mesosulfuron-methyl	0.0300	0.0150	0.0100	0.0075
	iodosulfuron-methyl-sodium	0.0225	0.0112	0.0075	0.0056
Lumax 537.5 SE	terbuthylazine	0.9375	0.4687	0.3125	0.2344
	mesotrione	0.1875	0.0937	0.0625	0.0469
	s-metolachlor	1.5625	0.7812	0.5208	0.3906

diffusion from a saturated filter paper disc to a solid culture medium. Herbicides are diffused in a radial pattern and create zones with a concentration gradient. The larger the zone of inhibition, the more sensitive the analyzed microorganism.

Microorganisms were grown and proliferated on the following solid artificial media: *Azotobacter* spp. – on Fenglerowa's medium (1965), *Arthrobacter* spp. – on the medium developed by MULDER and ANTHEUMISSE (1963), *Bradyrhizobium* spp. (lupini) and *Rhizobium leguminosarum* bv. *viciae* – on the YEMB – Vincent medium (1970), *Streptomyces intermedius*, *Streptomyces viridis*, *Streptomyces longisporoflavus* and *Streptomyces odorifer* - on the medium developed by Küster and Williams (PARKINSON et al. 1971), and *Fusarium* spp., *Aspergillus* spp., *Penicillum* spp. and *Rhizopus* spp. on Martin's medium (1950).

Herbicide resistance of soil-dwelling microorganisms

The experiment was performed on sandy loam (Table 3) classified as Eutric Cambisol by the World Reference Base of Soil Resources (2014). Soil samples were collected from the humus horizon at a depth of 0-20 cm, in Tomaszkowo near Olsztyn in north-eastern Poland. Air-dried soil samples of 100 g were passed through a sieve with 2 mm mesh size, placed in 150 cm^3 beakers and combined with different doses of the tested herbicides (Table 1). The applied doses are described in Table 4, and the predicted environmental concentrations (PEC) of active ingredients on day 160 are given in Table 5. Soil was combined with herbicides and brought to 50% capillary capacity with the use of distilled water. Beakers were covered with perforated film and incubated at 25°C for 160 days. After incubation, the counts of organotrophic bacteria were determined on the Bunt and Rovira medium with the addition of soil extract (ALEXANDER 1973), the counts of actinomycetes were determined on the Küster and Williams medium with the addition of antibiotics nystatin and actidione (PARKINSON et al. 1971), and fungal counts were determined on Martin's glucose-peptone agar (1950) with the addition of rose bengal and aureomycin. Petri plates were incubated at 28°C for 7 days (organotrophic bacteria and actinomycetes) and 5 days (fungi). After incubation, the number of colony forming units (CFU) was determined in nine replications. The results were used to calculate the index of microbial resistance (RS) to soil contamination with herbicides according to the formula developed by ORWIN and WARDLE (2004):

$$RS = 1 - \frac{2|D_0|}{C_0 + |D_0|}$$

where:
C_0 – is the soil resistance under natural conditions over time t_0;
P_0 – is the resistance of soil subjected to pressure over time t_0;
$D_0 = C_0 - P_0$.

Table 3

General characteristics of experimental soil

Parameter	Value
sand [2000–50 μm] %	72.00
silt [50–2 μm] %	21.00
clay [<2 μm] %	7.00
pH_{KCl}	7.00
HAC [mmol(+) kg^{-1}]	8.00
TEB [mmol(+) kg^{-1}]	111.00
C_{org} kg^{-1}]	7.05
N_{total} kg^{-1}]	0.86

Explanation: HAC – hydrolytic acidity, TEB – total exchangeable bases, C_{org} – organic carbon content, N_{total} – total nitrogen content

Table 4

Active ingredient doses applied to soil, mg kg^{-1}

Name of herbicide	Active ingredient	Active ingredient dose				
		1	20x	40x	80x	160x
Fuego 500 SC	metazachlor	0.4150	8.3000	16.600	33.200	66.400
Alister Grande 190 OD	diflufenican	0.0540	1.0800	2.1600	4.3200	8.6400
	mesosulfuron-methyl	0.0018	0.0360	0.0720	0.1440	0.2880
	iodosulfuron-methyl-sodium	0.0013	0.0260	0.0520	0.1040	0.2080
Lumax 537.5 SE	terbuthylazine	0.2344	4.6887	9.3750	18.750	37.500
	mesotrione	0.0471	0.9425	1.8850	3.7700	7.5400
	s-metolachlor	0.3906	7.8125	15.625	31.250	62.500

Explanation: 1 – dose recommended by the manufacturer, doses 20-, 40-, 80- and 160-higher than recommended by the manufacturer

Table 5

Predicted environmental concentrations (PEC) in soil on day 160, mg kg^{-1}

Name of herbicide	Active ingredient	Active ingredient dose				
		1	20x	40x	80x	160x
Fuego 500 SC	metazachlor	0.0258	0.5154	1.0309	2.0618	4.1335
Alister Grande 190 OD	diflufenican	0.0144	0.2877	0.5755	1.1509	2.3019
	mesosulfuron-methyl	0.0002	0.0035	0.0070	0.0140	0.0280
	iodosulfuron-methyl--sodium	6.6068E-07	1.3213-05	2.6427E-05	5.2854E-05	0.0001
Lumax 537.5 SE	terbuthylazine	0.0114	0.2283	0.4568	0.9135	1.8270
	mesotrione	0.0013	0.0263	0.0525	0.1051	0.2102
	s-metolachlor	0.0090	0.1822	0.3643	0.7287	1.4573

Explanation: 1 – dose recommended by the manufacturer, doses 20-, 40-, 80- and 160-higher than recommended by the manufacturer

Statistical analyses

The results were processed in the Statistica 10.0 application (StatSoft, Inc. 2011). Homogeneous groups were identified by Tukey's test at a significance level of $p = 0.01$. Microbial responses to the tested herbicides were described by hierarchical cluster analysis (CA) with the use of Ward's method and Euclidean distance. Microbial resistance to herbicides was compared by principal component analysis (PCA). CA and PCA were conducted by exploring multidimensional data sets. Pearson's coefficients of correlation between herbicide dose and microbial resistance were calculated.

Results and Discussion

The responses of microorganisms cultured on solid media to herbicides

Widespread pesticide use in agriculture poses a serious environmental problem. New research is needed to determine the impact of those chemical substances on various organisms, including soil-dwelling microbes, which are reliable indicators of changes in soil environments exposed to stressors (WYSZKOWSKA 2002, ZHANG et al. 2012). Pesticides are degraded in the soil environment, mainly by soil-dwelling microorganisms. Selected bacterial strains, such as *Azotobacter*, *Arthrobacter*, *Pseudomonas* and *Rhodococcus*, are capable of decomposing pesticides, and they are frequently used in bioremediation of pesticide-contaminated soil (PAL et al. 2006). In this study, microbial responses to higher herbicide concentrations were determined by the dose and type of the applied product (Figure 1). In the group of the analyzed bacteria, *Azotobacter* was most resistant to herbicides, and its growth was inhibited only by a mixture of terbuthylazine + mesotrione + s-metolachlor. CHENNAPPA et al. (2014) demonstrated that selected *Azotobacter* species can survive and proliferate in the presence of pesticides. The cited authors tested five *Azotobacter* species (*Azotobacter vinelandii*, *Azotobacter salinestris*, *Azotobacter* sp., *Azotobacter nigricans* subsp. *nigricans*, *Azotobacter tropicalis*) to determine their ability to proliferate on culture media containing pendimethalin, glyphosate, chlorpyrifos and phorate. Thirteen of the 14 evaluated strains proliferated on pesticide-containing media. In our study, bacteria of the genus *Arthrobacter* were most sensitive to the tested herbicides. When the highest herbicide doses were applied, the zone of inhibition for *Arthrobacter* spp. was determined at 22.056 mm (metazachlor), 24.500 mm (diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium) and 24.389 mm (terbuthylazine + mesotrione +

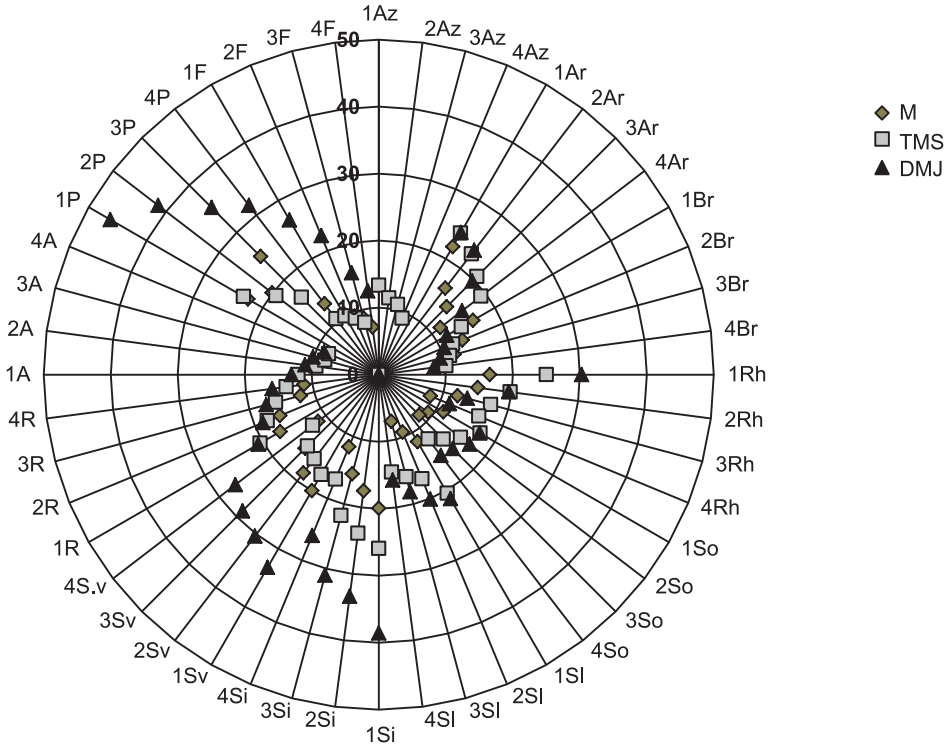

Fig. 1. The effect of herbicides on the growth of microorganisms cultured on solid media
Explanation: 1–4 – herbicide dose, M – metazachlor, DMJ – diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium, TMS – terbuthylazine + mesotrione + s-metolachlor, 0–50 – zone of growth inhibition [mm], *Az – Azotobacter* spp., *Ar – Arthrobacter* spp., *Br – Bradyrhizobium* spp. *(lupine), Rh – Rhizobium leguminosarum* bv. *vicie, Sl – Streptomyces longisporoflavus, Si – Streptomyces intermedius, Sv – Streptomyces viridis, So – Streptomyces odorifer, R – Rhizopus* spp., *A – Aspergillus* spp., *P – Penicillium* spp., *F – Fusarium* spp.

s-metolachlor). *Rhizobium leguminosarum* bv. v*icie* and *Bradyrhizobium* spp. (*lupini*) responded similarly to all evaluated herbicides. The most notable changes in *Rhizobium leguminosarum* bv. v*icie* were observed after the addition of the diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium mixture to the culture medium, and in *Bradyrhizobium* spp. (*lupini*) – after the addition of metazachlor. The varied influence of the analyzed herbicides can be attributed to differences in dose as well as microbial species or strain. SHARMA and KHANNA (2011) compared the impact of two herbicides, fluchloralin and pendimethalin, on the growth of bacteria of the genus *Rhizobium*. They concluded that fluchloralin ($20.25 \cdot 10^4$ mg kg^{-1}) and the lowest dose of pendimethalin ($9.00 \cdot 10^4$ mg kg^{-1}) did not exert a negative influence on the analyzed bacteria, but higher doses of pendimethalin ($15.9 \cdot 10^4$ mg kg^{-1}) inhibited the

growth of *Rhizobium* bacteria. An in vitro experiment conducted by ALLIEVI and GIGLIOTTI (2001) demonstrated that cinosulfuron applied in the amount of 100 mg dm^{-3} had an adverse effect on the growth of *Rhizobium leguminosarum* and *Bradyrhizobium japonicum*.

Actinomycetes were also sensitive to herbicides that diffused from filter paper discs into culture media, in particular at the highest doses. *Streptomyces odorifer* and *Streptomyces longisporoflavus* were most resistant, whereas *Streptomyces intermedius* and *Streptomyces viridis* were most sensitive to the tested substances. The mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium had the most inhibitory effect on the growth of actinomycetes, and it produced inhibition zones with an average diameter of: 16.32 mm for *Streptomyces odorifer*, 18.88 mm for *Streptomyces longisporoflavus*, 29.85 mm for *Streptomyces viridis,* and 32.24 mm for *Streptomyces intermedius*. Metazachlor had the least inhibitory effect on the tested species of actinomycetes. According to SETTE et al. (2004), actinomycetes are characterized by considerable physiological and metabolic diversity, which is why they play an important role in the degradation of chemical compounds that are released into the environment. The cited authors demonstrated that *Streptomyces* spp. strains are resistant to increased doses of alachlor. An alachlor dose of 144 mg dm^{-3} was degraded in 60–75% in 14 days.

In the present study, the tested herbicides also induced changes in the growth pattern of molds. Metazachlor did not exert a negative effect on *Aspergillus* spp., and it had only a minor influence on *Fusarium* spp. (the average zone of inhibition in response to the highest metazachlor dose was 10.56 mm). The mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium had the least toxic effect on *Aspergillus* spp., whereas the mixture of terbuthylazine + mesotrione + s-metolachlor was least toxic for *Fusarium* spp. *Penicillium* spp. was most sensitive to all of the analyzed herbicides, and the diameter of its inhibition zone was determined at 22.556 mm for metazachlor, 46.278 mm for the diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium mixture, and 23.333 mm for the terbuthylazine + mesotrione + s-metolachlor mixture. Fungi, in particular *Penicillium* spp., were highly sensitive to the tested herbicides. Somewhat different results were reported by KODAMA et al. (2001), in whose study, only the DS6F *Penicillium steckii* strain was capable of degrading simazine, and the rate of decomposition increased after glucose was introduced to the substrate as a source of carbon. Simazine, which was added to the substrate in the amount of 25 mg dm^{-3} and 50 mg dm^{-3}, was degraded in 53% after 5 days.

The dispersal of objects in a system of two principal components is presented in Figure 2. The horizontal axis explains 61.15% of total variance, the vertical axis – 21.24% of total variance, and the two explain 82.39% of variance in

primary variables. An analysis of the first principal component revealed two homogeneous groups. The first group is represented by *Rhizobium leguminosarum* bv. v*icie*, *Aspergillus* spp., *Rhizopus* spp., *Arthrobacter* spp., *Streptomyces odorifer* and *Streptomyces longisporoflavus,* and the second group – by *Fusarium* spp., *Penicillium* spp., *Streptomyces intermedius* and *Streptomyces viridis*. A homogeneous group comprising *Bradyrhizobium* spp. (*lupini*) and *Azotobacter* spp. was formed around the second principal component. The location of vectors along the axes of the coordinate system indicates that the

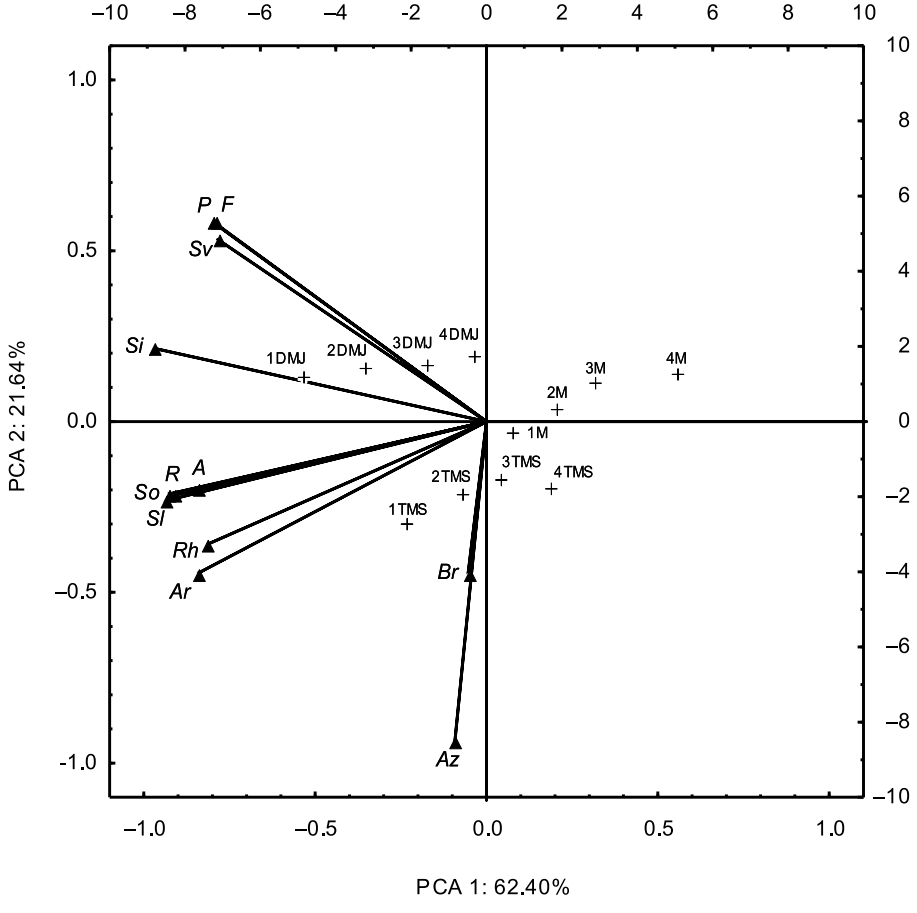

Fig. 2. A comparison of the sensitivity of microorganisms cultured on solid media to metazachlor, a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium, and a mixture of terbuthylazine + mesotrione + s-metolachlor, determined by PCA

Explanation: 1–4 – herbicide dose, M – metazachlor, DMJ – diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium, TMS – terbuthylazine + mesotrione + s-metolachlor, *Az – Azotobacter* spp., *Ar – Arthrobacter* spp., *Br – Bradyrhizobium* spp. *(lupine), Rh – Rhizobium leguminosarum* bv. *vicie, Sl – Streptomyces longisporoflavus, Si – Streptomyces intermedius, Sv – Streptomyces viridis, So – Streptomyces odorifer, R – Rhizopus spp., A – Aspergillus* spp., *P – Penicillium* spp., *F – Fusarium* spp.

tested herbicides influenced microbial growth. The distribution of cases in the four quarters also suggests that the analyzed substances had a varied effect on the growth and development of microorganisms.

The responses of the tested microbes to herbicides were confirmed by cluster analysis involving Ward's method. The results are presented in a dendrogram in Figure 3. The analysis led to the identification of five clusters. A high degree of similarity was observed between *Azotobacter* spp. and *Aspergillus* spp., between *Arthrobacter* spp., *Rhizobium leguminosarum* bv. *viceae* and *Rhizopus* spp., between *Bradyrhizobium* spp. (lupini) and *Fusarium* spp., between *Streptomyces odorifer* and *Streptomyces longisporoflavus,* and between *Streptomyces intermedium* and *Streptomyces viridis*. *Penicillium* spp. fungi were most sensitive to the analyzed herbicides, and they differed most significantly from the remaining microorganisms.

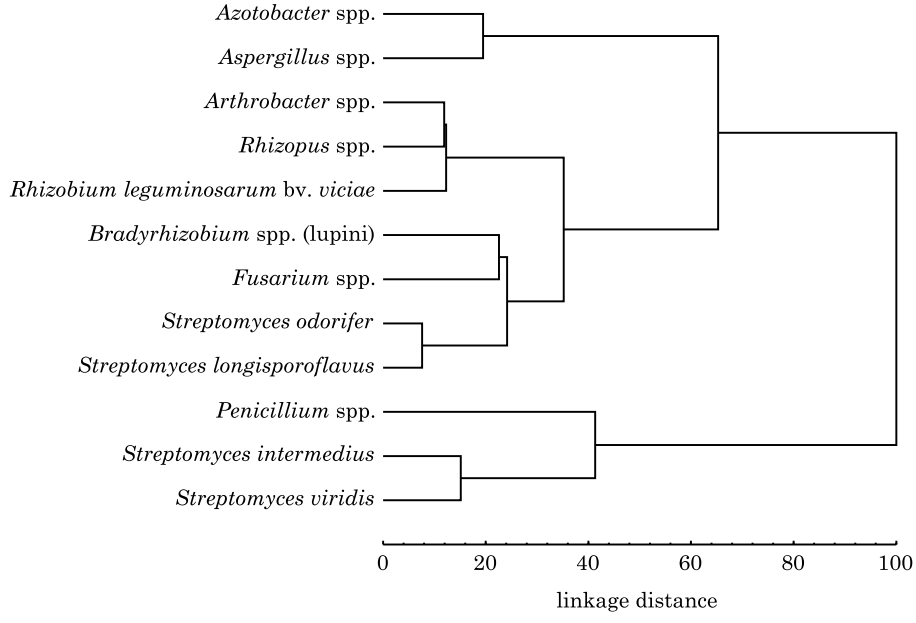

Fig. 3. Similarities in the responses of microorganisms cultured on solid media to the tested herbicides

Herbicide resistance of soil-dwelling microorganisms

Organic compounds, including herbicides, can pose a serious threat to the soil environment by causing a long-term disruption of the soil's biological balance (BAĆMAGA et al. 2014a, BAĆMAGA et al. 2014b, CHOWDHURY et al. 2008, CYCOŃ and PIOTROWSKA-SEGET 2007, JASTRZĘBSKA and KUCHARSKI 2007). The

herbicides tested in this study had a significant impact on soil-dwelling microorganisms (Table 6). The herbicide resistance of microorganisms was determined by the type and dose of the applied weed control agent. In metazachlor treatments, the lowest values of the RS index for actinomycetes (0.224) and fungi (0.281) were observed after the application of a dose that was 160-higher than the dose recommended by the manufacturer. Organotrophic bacteria were most resistant to the same dose of metazachlor, and their RS index reached 0.708. The mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium contributed to an increase in the value of the RS index. The only exception were organotrophic bacteria whose RS index decreased after the application of doses that were 20- and 40-fold higher than the recommended dose. The tested microbes were characterized by varied resistance to soil contamination with a mixture of terbuthylazine + mesotrione + s-metolachlor. When introduced to the soil at high doses, the mixture increased the RS index of actinomycetes and fungi, and lowered the RS index of organotrophic bacteria. The highest value of the RS index for organotrophic bacteria was noted in treatments with the optimal herbicide dose (RS = 0.915), for actinomycetes – in treatments were the herbicide dose was 80-times higher than the recommended dose (RS = 0.558), and for fungi – in treatments where the herbicide dose was 20- and 40-times higher than the recommended dose (RS = 0.696 and RS = 0.697, respectively). Regardless of herbicide dose, metazachlor had the most inhibitory influence on microbial development. The RS index assumed the lowest average values in metazachlor treatments: 0.537 for organotrophic bacteria, 0.288 for actinomycetes and 0.469 for fungi. The mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium had the least significant impact on the values of the RS index. The average values of the RS index indicate that organotrophic bacteria and fungi were most resistant to the above mixture. Microbial resistance to the examined herbicides, determined by PCA, is presented in Figure 4. The first two principal components explained 82.44% of total variance. The length of primary variable vectors determines their influence on the distribution of principal components. The first principal component was negatively correlated with actinomycetes and fungi, and the second principal component – with organotrophic bacteria. The distribution of cases in the chart indicates that herbicide doses exerted a varied effect on soil-dwelling microorganisms. The influence of weed control products on soil microbes could be attributed to microbial tolerance of active ingredients in the analyzed preparations. Those compounds could be an excellent source of nutrients for selected microorganisms, but they could be toxic and lethal for other microbial groups (CROUZET et al. 2010, ZABALOY et al. 2010). According to GRIFFITHS and PHILIPPOT (2013), and ORWIN and WARDLE (2004), soil resistance and resilience values can be used to determine the analyzed ecosystem's sensitivity to various

stressors. In this study, the values of the RS index indicate that metazachlor had the most inhibitory effect on the proliferation of soil microbes. Soil resistance and resilience indicators provide information about the status of soil environments contaminated with organic compounds, including pesticides (BAĆMAGA et al. 2015, ORWIN and WARDLE 2004). There is a general scarcity of published data about the influence of herbicides on soil resistance, and the results of this study can expand our knowledge about the resistance of soil contaminated with metazachlor, a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium and a mixture of terbuthylazine + mesotrione + s-metolachlor.

Table 6

Microbial resistance (RS) to soil contamination with herbicides

Dose of herbicide	Microorganisms		
	B_{org}	Act	Fun
Metazachlor (M)			
1	0.452^{c}	0.335^{def}	0.578^{abc}
20	0.398^{c}	0.324^{def}	0.227^{c}
40	0.543^{abc}	0.296^{efg}	0.850^{ab}
80	0.586^{abc}	0.263^{fg}	0.406^{abc}
160	0.708^{abc}	0.224^{g}	0.281^{bc}
Average	0.537	0.288	0.468
r	0.938*	-0.982*	-0.384
Diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium (DMJ)			
1	0.709^{abc}	0.360^{de}	0.762^{abc}
20	0.599^{abc}	0.365^{de}	0.948^{a}
40	0.620^{abc}	0.366^{de}	0.877^{a}
80	0.862^{ab}	0.483^{ab}	0.863^{ab}
160	0.904^{a}	0.386^{cde}	0.882^{a}
Average	0.739	0.392	0.866
r	0.821	0.357	0.250
Terbuthylazine + mesotrione + s-metolachlor (TMS)			
1	0.915^{a}	0.360^{de}	0.550^{abc}
20	0.574^{abc}	0.374^{cde}	0.696^{abc}
40	0.549^{bc}	0.406^{bcd}	0.697^{abc}
80	0.546^{bc}	0.558^{a}	0.673^{abc}
160	0.542^{bc}	0.463^{bc}	0.514^{abc}
Average	0.625	0.432	0.626
r	-0.572	0.625	-0.456

Explanation: Homogeneous microbial groups are marked with the same letters in columns: B_{org} – organotrophic bacteria, Act – actinomycetes, Fun – fungi; 1 – dose recommended by the manufacturer, doses 20-, 40-, 80- and 160-higher than recommended by the manufacturer; *r* – coefficient of correlation significant at $*p=0.01$

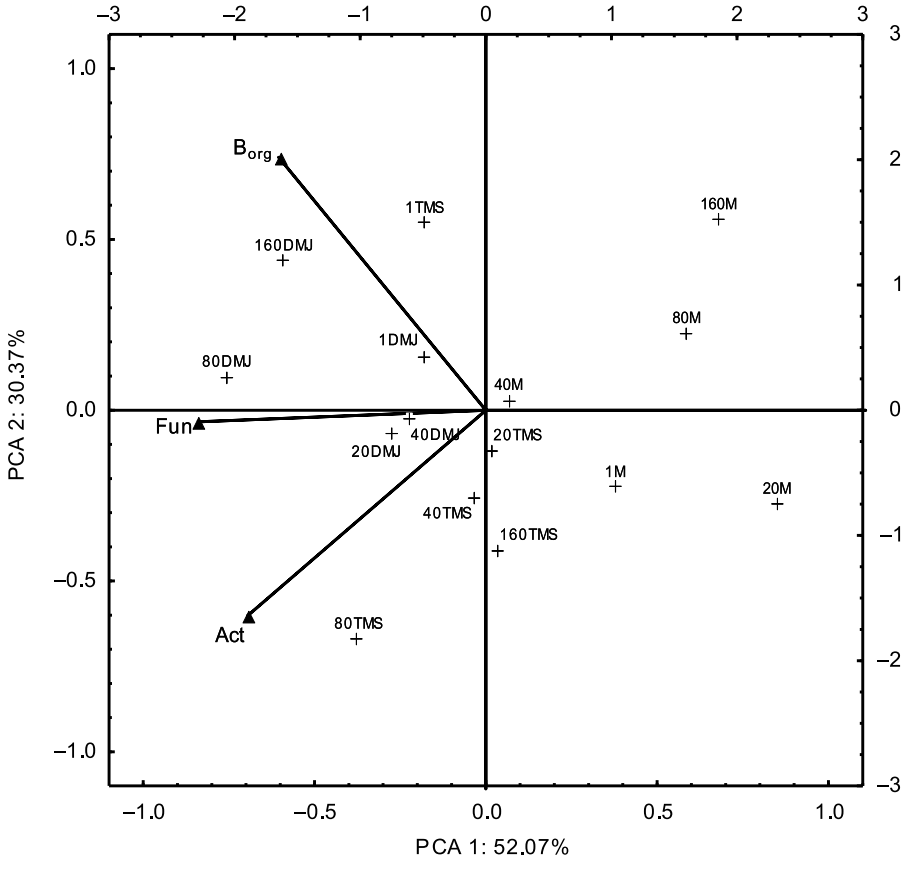

Fig. 4. Microbial resistance to soil contamination with metazachlor, a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium, and a mixture of terbuthylazine + mesotrione + s-metolachlor, determined by PCA

Explanation: B_{org} – organotrophic bacteria, Act – actinomycetes, Fun – fungi; 1 – dose recommended by the manufacturer, doses 20-, 40-, 80- and 160-higher than recommended by the manufacturer, M – metazachlor, DMJ – diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium, TMS – terbuthylazine + mesotrione + s-metolachlor

Conclusions

In this experiment, microorganisms responded differently to the tested herbicides, subject to the type and dose of the applied product. *Penicillium* spp. fungi cultured on soil media *in vitro* were most sensitive to herbicides, in particular to the mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium. The mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium had the most inhibitory effect on actinomycetes

and fungi, whereas the mixture of terbuthylazine + mesotrione + s-metolachlor was most toxic for *Azotobacter* spp. and *Arthrobacter* spp. Metazachlor diffusing from filter paper discs into culture media had the least inhibitory effect on microorganisms, excluding *Bradyrhizobium* spp. (*lupini*) bacteria whose growth was most severely impaired by the above compound. The application of metazachlor to soil exerted the most negative influence on organotrophic bacteria, actinomycetes and fungi. The tested microbes were most resistant to increased doses of the mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium. Predicted environmental concentrations (PEC) calculated on day 160 indicate that increased doses of metazachlor posed the greatest threat for soil-dwelling microorganisms. The applied doses of metazachlor resulted in the highest PEC values, which points to a high risk of soil contamination with this weed control agent.

Acknowledgements

This study was supported by research grant No. N N305 386138 from the Polish National Science Center (NCN).

Translated by ALEKSANDRA POPRAWSKA

Accepted for print 22.10.2015

References

ALEXANDER M. 1973. *Microorganisms and chemical pollution*. Biosci., 23: 509–515.

ALLIEVI L., GIGLIOTTI C. 2001. *Response of the bacteria and fungi of two soils to the sulfonylurea herbicide cinosulfuron*. J. Environ. Sci. Health B., 36: 161–175.

BAĆMAGA M., BOROS E., KUCHARSKI J., WYSZKOWSKA J. 2012. *Enzymatic activity in soil contaminated with the Aurora 40 WG herbicide*. Environ. Protec. Eng., 38(1): 91–102.

BAĆMAGA M., BOROWIK A., KUCHARSKI J., TOMKIEL M., WYSZKOWSKA J. 2015. *Microbial and enzymatic activity of soil contaminated with a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium*. Environ. Sci. Pollut. Res., 22: 643–656.

BAĆMAGA M., WYSZKOWSKA J., BOROWIK A., TOMKIEL M., KUCHARSKI J. 2014a. *Response of fungi, β-glucosidase and arylsulfatase to soil contamination by Alister Grande 190 OD, Fuego 500 SC and Lumax 357.5 SE herbicides*. Pol. J. Environ. Stud., 23(1): 19–25.

BAĆMAGA M., KUCHARSKI J., WYSZKOWSKA J., BOROWIK A., TOMKIEL M. 2014b. *Responses of microorganisms and enzymes to soil contamination with metazachlor*. Environ. Earth Sci. 72: 2251–2262.

BOROS E., WYSZKOWSKA J., KUCHARSKI J. 2007. *Influence of nickel on the growth of microorganisms in solid media*. J. Elem., 12(3): 167–180.

CASTILLO M.A., FELIS N., ARAGON P., CUESTA G., SABATER C. 2006. *Biodegradation of the herbicide diuron by streptomycetes isolated from soil*. Int. Biodeterior. Biodegrad., 58: 196–202.

CHENNAPPA G., ADKAR-PURUSHOTHAMA C.R., SURAJ U., TAMILVENDAN K., SREENIVASA M.Y. 2014. *Pesticide tolerant Azotobacter isolated from paddy growing areas of northern Karnataka, India*. World J. Microbiol. Biotechnol., 30(1): 1–7.

CHOWDHURY A., PRADHAN S., SAHA M., SANYAL N. 2008. *Impact of pesticides on soil microbiological parameters and possible bioremediation strategies*. Indian J. Microbiol., 48: 114–127.

Crouzet O., Batisson I., Besse-Hoggan P., Bonnemoy F., Bardot C., Poly F., Bohatier J., Mallet C. 2010. *Response of soil microbial communities to the herbicide mesotrione: A dose-effect microcosm approach*. Soil Biol. Biochem., 42: 193–202.

Cycoń M., Piotrowska-Seget Z. 2007. *Effect of selected pesticides on soil microflora involved in organic matter and nitrogen transformations: pot experiment*. Pol. J. Ecol., 55(2): 207–220.

Das A.C., Dey S. 2013. *Effect of systemic herbicides on microbial biomass in relation to availability of some plant nutrients in an alluvial soil of West Bengal*. Bull. Environ. Contam. Toxicol., 90: 666–672.

Fenglerowa W. 1965. *Simple method for counting Azotobacter in soil samples*. Acta Microbiol. Polon., 14: 203–206.

Griffiths B.S., Philippot L. 2013. *Insights into the resistance and resilience of the soil microbial community*. FEMS Microbiol. Rev., 37: 112–129.

Jastrzębska E., Kucharski J. 2007. *Dehydrogenases, urease and phosphatases activities of soil contaminated with fungicides*. Plant Soil Environ., 53(2): 51–57.

Kodama T., Ding L., Yoshida M., Yajima M. 2001. *Biodegradation of an s-triazine herbicide, simazine*. J. Mol. Catal. B: Enzymatic, 11: 1073–1078.

Kucharski J., Baćmaga M., Wyszkowska J. 2009. *Effect of hericydes of the course of ammonification in soil*. J. Elem., 14(3): 477–487.

Kucharski J., Wyszkowska J. 2008. *Biological properties of soil contaminated with the herbicide Apyros 75 WG*. J. Elem., 13(3): 357–371.

Martin J. 1950. *Use of acid rose bengal and streptomycin in the plate method for estimating soil fungi*. Soil Sci., 69: 215–233.

Mulder E.G., Antheumisse J. 1963. *Morphologie, physiologie et ecologie des Arthrobacter*. Ann. de Institut Pasteur, 105: 46–74.

Orwin K.H., Wardle D.A. 2004. *New indices for quantifying the resistance and resilience of soil biota to exogenous disturbance*. Soil Biol. Biochem., 36: 1907–1912.

Pal R., Chakrabarti K., Chakraborty A., Chowdhury A. 2006. *Degradation and effects of pesticides on soil mocrobiological parameters – a review*. Int. J. Agric. Res., 1: 240–258.

Parkinson D., Gray F.R.G., Williams S.T. 1971. *Methods for studying the ecology of soil micro-organism*. Blackweel Scientific Publication Oxford and Edinburgh, IBP Handbook 19.

Sette L.D., Mendonça Alves Da Costa L.A., Marsaioli A.J., Manfio G.P. 2004. *Biodegradation of alachlor by soil streptomycetes*. Appl. Microbiol. Biotechnol., 64: 712–717.

Statsoft, Inc, Statistica. 2011. *Data Analysis Software System, version 10.0.,* <http://www.statsoft.com.>.

Sharma J.P., Khanna V. 2011. *In vitro of Rhizobium and phosphate solubilising bacteria of herbicides*. Indian J. Microbiol., 51(2): 230–233.

Vincent J.M. 1970. *A manual for the practical study of root-nodule bacteria*. IBP Handbook, 15 Blackweel, Oxford.

World Reference Base of Soil Resources. 2014. *A framework for international classification, correlation and communication*. World Soils Resources Raport. 103, FAO, Rome.

Wyszkowska J. 2002. *Effect of soil contamination with Treflan 480 EC on biochemical properties of soil*. Pol. J. Environ. Stud., 11(1): 71–77.

Wyszkowska J., Kucharski J. 2004. *Biochemical and physicochemical properties of soil contaminated with herbicide Triflurotox 250 EC*. Pol. J. Environ. Stud., 13(2): 223–231.

Zabaloy M.C., Garland J.L., Gomez M.A. 2010. *Assessment of the impact of 2,4-dichlorophenoxyacetic acid (2,4-D) on indigenous herbicide-degrading bacteria and microbial community function in an agricultural soil*. Appl. Soil Ecol., 46: 240–246.

Zhang Y., Mend D., Wang Z., Guo H., Wang Y., Wang X., Dong X. 2012. *Oxidative stress response atrazine-degrading bacteria exposed to atrazine*. J. Hazard. Mater., 229–230: 434–438.