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THE EVALUATION OF PESTICIDES ON OPPORTUNISTIC *PSEUDOMONAS AERUGINOSA* AND ITS ANTIBIOTIC RESISTANCE

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Abstract: Background*: Nowadays, researchers have pay attention to the various effects of micro-pollutants in the environment. In the present thesis, I focused on the effect of pesticides on the growth and antibiotic resistance profile of* Pseudomonas aeruginosa*, an emerging opportunistic pathogen microorganism.* ***Method****: 5 ppm concentrations of three pesticides (glyphosate, S-metolachlor, and terbuthylazine) were used for the treatment of 5 clinical and environmental strains of* P. aeruginosa*. Optical density of the treated bacterial cultures was detected after 1, 3, 24, 48, and 120 h to determine growth curves. Antibiotic resistance against 10 chosen agents was tested by MIC test strips (Liofilchem) containing a quantitative gradient of antibacterial drugs. Pesticide degradation rate of the examined strains was determined by gas chromatography.* ***Results****: Glyphosate and S-metolachlor both could be utilized as nutrients by the examined strains; therefore in the examined concentration they did not inhibit the growth of* P. aeruginosa *irrespectively to the origin of the strain (clinical or non-clinical). By contrast, terbuthylazine might have a negative effect on P. aeruginosa and limits its growth, but the differences were not significant.* P. aeruginosa *strains could degrade the S-metolachlor around 34-54% and terbuthylazine around 21-54% in 5 days. In addition, glyphosate caused a significant increase of MIC values on cefepime and ticarcillin and decreased the MIC values on tigecycline. S-metholachlor and terbutylazine have slightly increased the resistance of P. aeruginosa to ticarcillin, too but no other changes were observed.*

Keywords: *Antibiotic resistance, micro-pollutants, pesticides, Pseudomonas aeruginosa*

INTRODUCTION

With the rapid growth of population and the gradual improvement of human living standards all over the world, the requirements for agriculture have been correspondingly increased, thus, a lot of chemical products have been used to increase the amount of agricultural production. One group of the widely used chemical products is pesticides. Pesticides are used to control factors that may have a negative effect on crops or plants. But in recent years, it was increasingly reported that pesticides can

cause serious pollution in the environment. As it was recently verified, pesticides may have various sublethal effects on natural microbiota as well.

Antibiotic resistance, namely the ability of some bacteria to evolve resistance to several classes of antibiotics, is an emerging issue all around the world mainly caused by the improper use the antibiotics. WHO has classified antimicrobial resistance as a widespread and serious threat to humans of any age, in any country (WHO, 2014). One of the most dangerous emerging bacterial species with the ability to cause disease and develop antibiotic resistance is *P. aeruginosa*, an opportunistic microorganism that can be detected in various environments.

Our knowledge about the effects of low concentration agrochemicals on emerging opportunistic pathogens is still limited, therefore our research aimed to determine the effects of some chosen pesticides on the growth rate, antibiotic resistance, and the degradation rate of the chosen bacterial species *P. aeruginosa*. Based on our results, we could analyse the effects of pesticides on the environment from a new aspect. The major steps of our investigation were as follows:

- Based on the scientific literature, we have chosen three pesticides, that are (considering their annual volume and health effects) the most important emerging micro-pollutants in Hungary and Europe: glyphosate, S-metolachlor, and terbuthylazine (Hvězdová et al., 2018).
- We have chosen one of the most important environmentally relevant opportunistic microorganisms, *P. aeruginosa,* and obtained five reference strains of this species for the planned investigations
- We have set a 5-day long experiment and checked the initial and final antibiogram, the growth rate, and the degradation capacity of the examined strains

MATERIALS AND METHODS

The main phases of the experiment are summarized in Figure (1).

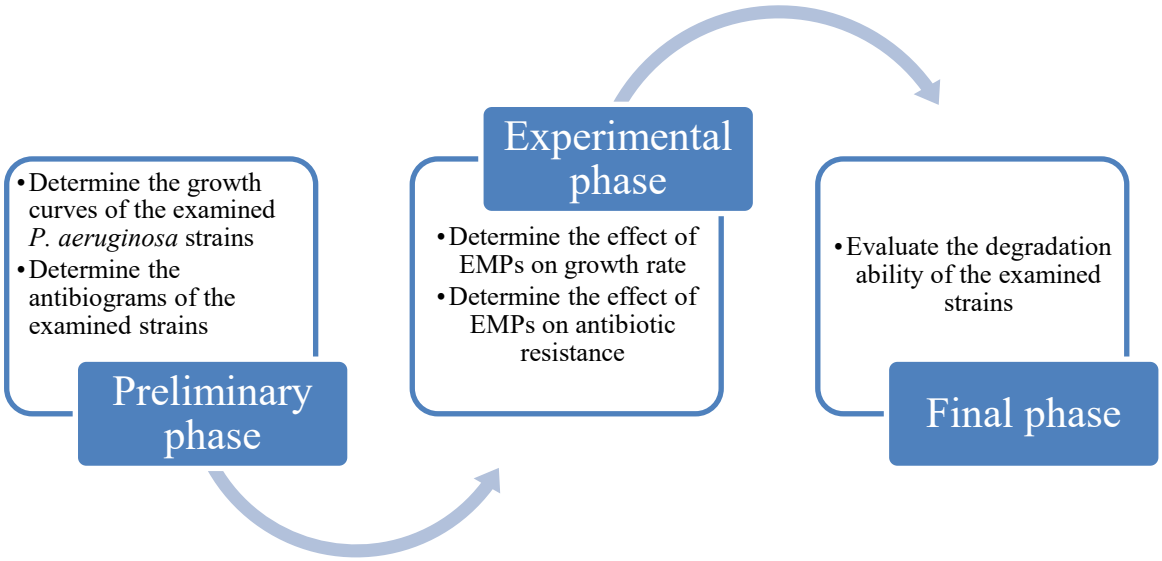


Figure (1): The summary of experimental processes

Bacterial strains and their origin

Five different reference strains of opportunistic pathogen *P. aeruginosa* were obtained from the National Collection of Agricultural and Industrial Microorganisms (NCAIM, MATE, Hungary) and the strain collection of the Department of Environmental Safety (MATE). The origin and the known properties of the examined strains are summarized in Table 1 and Figure 1.

Table (1): The origin and main properties of the examined P. aeruginosa strains

Marks	Culture collection no.	Other collection no.	Origin	Main features
1	ATCC 27853	B.01876, CECT 108, etc.	Clinical (isolated from blood)	Quality control strain for antibiotics susceptibility tests
2	ATCC 15442	B.02060, B.02358, etc.	Animal drinking water	Disinfectant and preservation testing (European standard EN 1040)
3	ATCC 10145	B.02040, DSM 50071	Non-clinical	Quality control strain for API and Sensitise products
4	PAO 11	B.02360, DSM5168	n.d.	Plasmid pMO821
5	P66	-	Zalaegerszeg, Hungary, Hydrocarbon contaminated groundwater	Environmental strain from the Department collection



Figure (2): Liquid cultures of the examined *P. aeruginosa* strains (credit: D. Jiang)

Experimental settings

Each experiment was set in Luria-Bertani (LB) medium (10.0 g tryptone, 5.0 g yeast extract, and 9.0 g NaCl in 1000 ml distilled water). The experiment was performed in triplicates with all five bacterial strains therefore, 15 flasks with 45 ml LB were used for each herbicide experiment with an abiotic control of herbicides. LB medium in conical flasks was supplemented with 5 ml of overnight (<24h) bacterial suspension with an optical density of OD_{600}: 0.6 checked on GenesysTM 10S UV-Vis Spectrophotometer (Thermo Scientific). See Figure (3).
Glyphosate (Pestanal®, CAS 1071-83-6), S-metolachlor (Pestanal®, CAS 87392-12-9), and terbuthylazine (Pestanal®, CAS 5915-41-3) were obtained from Sigma Aldrich Ltd. Stock solutions were prepared in dimethyl sulfoxide (DMSO, CAS 67-68-5, purity ≥99.99%, Fisher Scientific) with a final concentration of 2500ppm (Tóth et al., 2019). Herbicide concentrations in conical flasks were set to 5 ppm. Flasks were inoculated in Certomat BS-1 rotary shakers (Sartorius) at 28°C, with 170 rpm (round per minute) for 120h.

Determination of growth curves

This experiment was set to determine the growth curves of the selected strains of *P. aeruginosa* which were treated by glyphosate, S-metolachlor, and terbuthylazine separately. The growth rate was determined using the optical density of bacterial cultures determined by GENESYS™ 10S UV-Vis Spectrophotometer at 600 nm wavelength (Figure 3 and Figure 4).

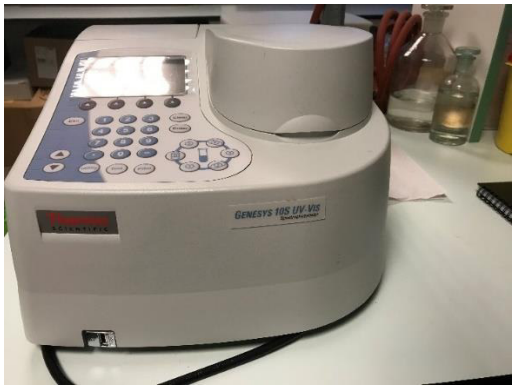


Figure (3): The Genesys 10S UV-Vis spectrophotometer which was used during the lab work (credit: D. Jiang)

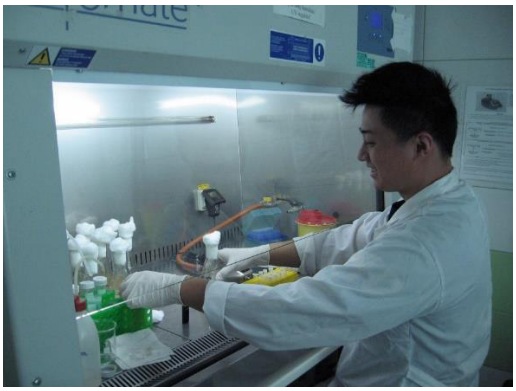


Figure (4): The operational process of measuring the optical density (credit: E. Kaszab)

During the 120 hour long experimental period, OD values were determined in the 1st, 2nd, 4th, 8th, 16th hours and then once in every 24 hours. Data on the detected OD values were visualized and analysed by GraphPad Prism 7. Software.

Antibiotic resistance

The antibiotic resistance assay was performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006) using MIC Test Strips (MTS) (Liofilchem) to determine Minimal Inhibitory Concentrations. The principle of the antibiotic resistance investigation is to prepare a bacterial suspension of the examined strain in physiological saline with 0.5 McFarland density and spread onto the surface of Mueller Hinton agar (Sigma Aldrich). MTS is positioned by sterile forceps on the surface of the medium (see Figure 5.) and plates are incubated at 35°C for 24h. According to the visible breakpoints, we can determine and compare the minimal inhibitory concentrations (MIC values) to the available database of EUCAST to characterize the bacterial strain as resistant (R), intermediate (I), or sensitive (S).
The list applied antibiotics and their evaluation based on their MIC values are summarized in Table (2). (source: http://www.eucast.org/clinical_breakpoints/)
As a baseline, we have determined the antibiotic resistance profiles (antibiograms) of each strain without pesticide treatment. Treated bacterial strains were re-isolated after 120h incubation. Triplicate samples were mixed, and their mixture was used for antibiotic resistance assay.

Table (2). The examined antibiotics and their evaluation based on their MIC values (EUCAST)

Name of antibiotic	MIC breakpoints	
	S ≤	R >
CEFTAZIDIME	0.001	8
CEFEPIME	0.001	8
IMIPENEM	0.001	4
MEROPENEM	2	8
TICARCILLIN	0.001	16
GENTAMICIN	4	4
TOBRAMYCIN	4	4
CIPROFLOXACIN	0.001	0.5
COLISTIN	2	2
TIGECYCLINE	1	2



Figure (5): The results of the experiment of antibiotic resistance

Examination of degradation rate

After determining the growth curves and the antibiotic resistance profile of the examined strains, samples were centrifugated for 30 min at 3220g, supernatant was filtered (using a syringe filter with 0.45 µm pore size) and sent to the Wessling Hungary Ltd. analytical laboratory to determine the residual amounts of pesticides. Analysis was performed according to the MSZ EN 15662:2018 standard for the determination of pesticide-residues using GC- and LC-based analysis.

RESULTS AND DISCUSSION

The effect on micro-pollutants on the growth rate of *P. aeruginosa*

Results of growth rate experiments of Control (untreated) group, Glyphosate group, S-metolachlor group, and Terbuthylazine group are summarized in Figure (6).

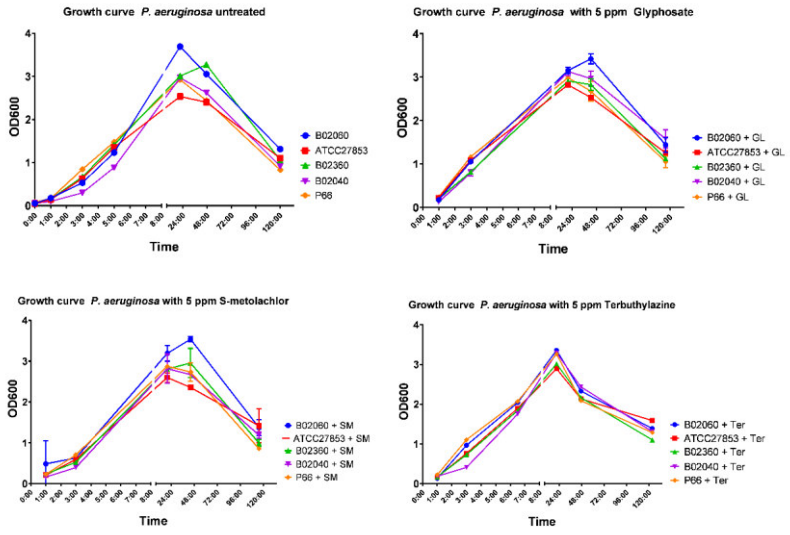


Figure (6): Growth curves of the examined strains of *P. aeruginosa* with different treatments

There were many similarities of these four sets of growth curves, for example, all tested strains were intensively developing in the first 24 hours, and then the inflection point occurred from 24 hours to 48 hours.

To give an overall evaluation of the 4 treatment, strain B02060 was detected as the most intensively growing strain in all groups with different treatments. Strain ATCC27853 had the lowest peak point comparing to other *P. aeruginosa* strains in all groups. Based on scientific sources, glyphosate could be utilized as a nutrient by various microbial species (Martinez et al., 2018) and S-metolachlor could also promote the growth of some plants as nutrition (Clewis et al., 2006). Thus, it is possible that the glyphosate and S-metolachlor might be used by *P. aeruginosa* as nutrients. At the same time, terbuthylazine may have slightly negative effect on *P. aeruginosa* strains. According to Tasca et al. (2018), the reason might relate to that the terbuthylazine is a chemical of emerging concern due to its persistence, and toxicity to aquatic organisms. Furthermore, the terbuthylazine has endocrine disruption capacity to wildlife and humans (Tasca et al., 2018).

Antibiotic resistance

Antibiotic resistance results of the examined strains (ATCC27853, B02040, B02060, B02360, and P66) in all settings (Control, Glyphosate, S-metolachlor, Terbuthylazine) for the nine examined antibiotics are shown in Figure 7. Average values with standard deviations are summarized in Figure (8). As supporting information, threshold values of antibiotic sensitivity (green line) and antibiotic resistance (red line) according to EUCAST are displayed.

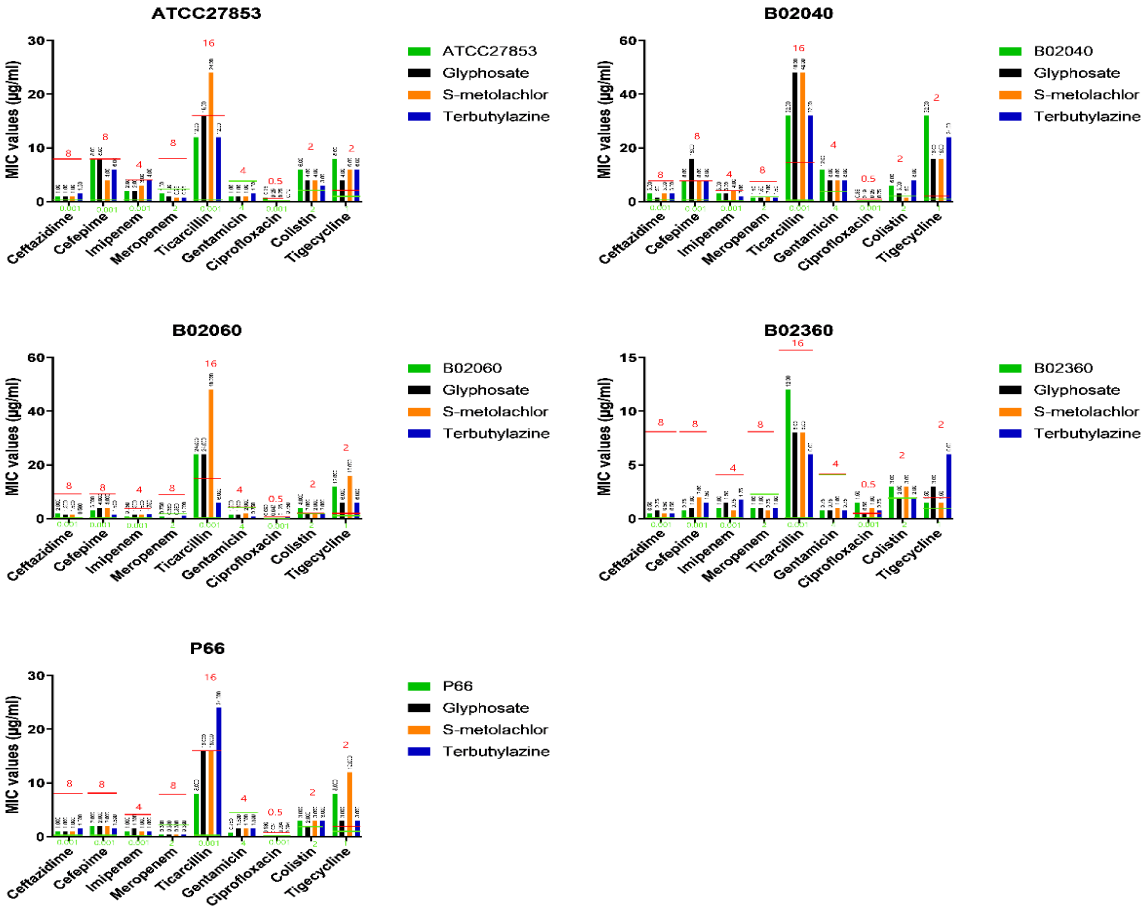


Figure (7): Antibiotic resistance of the examined *P. aeruginosa* strains ATCC27853, B02040, B02060, B02360, and P66 to antibiotic Ceftazidime, Cefepime, Imipenem, Meropenem, Ticarcillin, Gentamicin, Ciprofloxacin, Colistin, and Tigecycline respectively, under the condition of untreated, Glyphosate-treated, S-metolachlor-treated, and Terbuthylazine-treated.
Resistant threshold - red line; sensitive threshold - green line.

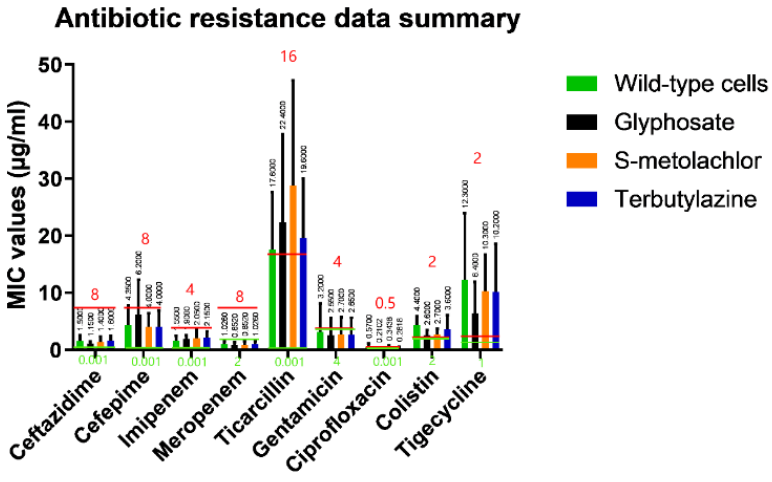


Figure (8): The average values of antibiotic resistance data of *P. aeruginosa* strains for different treatments

Based on the results displayed on Figure (7) and Figure (8), we can conclude that the vast majority of the examined *P. aeruginosa* were resistant to colistin irrespectively to their origin or treatment. For colistin resistance, antibiotic resistance is known and naturally occurs in some genera such as *Proteus, Serratia,* and *Burkholderia*, but not *P. aeruginosa,* which is well known to develop acquired resistance (Olaitan et al., 2014). In future experiments, it is crucial to evaluate the mechanism behind the detected colistin resistance (Gharaibeh and Shatnawi, 2019).

Tigecycline resistance was also detectable among untreated strains. Based on scientific literature, it can be a result of efflux-mediated mechanisms (Dean et al., 2003). The control cases were intermediate to ceftazidime, cefepime, imipenem, and were sensitive to meropenem in all the experiments. In control cases, nearly all strains were sensitive to gentamicin, with the exception of B02040 isolated from non-clinical sources. Overall, the control cases were resistant to colistin and tigecycline in all cases and were occasionally resistant to ticarcillin and ciprofloxacin.

Based on Figure 8, and according to the analysis of Figure 8, we could see some minor effects of the three tested pesticides on antibiotic resistance.

- Glyphosate caused a significant increase of MIC values on cefepime and ticarcillin and decreased the MIC values on tigecycline, however, but these slight changes had no effect on the interpretation categories of these antibiotics. There was no obvious change in the susceptibility to other antibiotics.
- S-metolachlor treatment caused an increased level of antibiotic resistance in one case only, (ticarcillin) and the effect was more pronounced than in the glyphosate and terbutylazine-treated groups. Other changes in susceptibility were not significant.
- In the terbuthylazine-treated group, the examined strains increased their resistance to ticarcillin just like in the S-metolachlor-treated group. However, the alteration in antibiotic resistance was insignificant.

Examination of the pesticide degradation

Comparing the residual amounts of pesticides with the initial concentrations (5 ppm) corrected with the abiotic loss measured in the non-inoculated control samples; we could calculate the rates of pesticide-biodegradation for S-metolachlor and terbuthylazine (Table 3). Due to technical problems, glyphosate degradation could not have been determined.

Table (3): The S-metolachlor and terbuthylazine degradation rates of the examined P. aeruginosa strains

P. aeruginosa strains	S-metolachlor average degradation rate (%)	Terbuthylazine average degradation rate (%)
1. B02060	34.0%	35.2%
2. ATCC27853	44.6%	54.2%
3. B02360	45.8%	21.3%
4. B02040	54.6%	39.0%
5. P66	35.7%	24.0%

According to our calculation, strain B02040 could degrade the highest rate of S-metolachlor during the experiment with an average degradation rate of 54.6%. Comparing to other strains, B02060 and P66 were weak degraders of S-metolachlor, the average of degradation rates reached 34% and 36%, respectively. Regarding terbuthylazine, degradation rates varied between 54.2% and 21.3%.
Overall, *P. aeruginosa* strains had a higher potential to degrade S-metolachlor than terbuthylazine, but there was no clear correlation between the origin and the degradation capability of the examined strains.
Considering the relatively low residual concentrations in the abiotic control samples, degradation assay should be repeated to get a more reliable view on the degradation capacity of *P. aeruginosa* strains.

CONCLUSIONS AND RECOMMENDATIONS

Nowadays, in the healthcare system infections caused by antibiotic-resistant bacteria mean a severe problem, threaten personal health, and have serious economic effects including the cost of therapy and all additional expendables. Treatment of resistant infections is associated with higher costs for second-line drugs, additional investigations, and longer hospitalization (Coast et al., 1996). Other indirect costs associated with AMR include productivity losses due to excess morbidity and premature mortality (Shrestha et al., 2018). Therefore, it is important to gain knowledge about emerging opportunistic bacteria, such as species *P. aeruginosa*, and the factors affecting its survival and antibiotic resistance profile. Antibiotic resistance could be developed by enhanced evolvability (Spiers et al., 2000), or acquiring from genotypic diversity (mutation or horizontal gene transfer) (Carmeli et al., 1999), normally the resistance will be increased continuously (Hughes and Andersson, 2017).
Based on our experiences there are cases that pesticides can enhance antibiotic resistance of *P. aeruginosa* which is in accordance with the explanation that environmental factors around bacteria increasing the selection of antibiotic resistant strains (Kurenbach et al., 2018). Strains that were used in the series of experiments were the same species, *P. aeruginosa*, but the origin of strains was different. It was reported that the different origins of isolation may cause the different phenotypical traits of *P. aeruginosa*, even if genomes are almost identical (Grosso-Becerra et al., 2014) that can be the reason of the slight differences in growth and antibiotic resistance.
In conclusion, based on the results and analyses in my thesis work, the micro-pollutants did affect species *P. aeruginosa.* According to our experiments, glyphosate and S-metolachlor did not inhibit the growth of the examined *P. aeruginosa* strains in the applied concentration (5 ppm). It is presumed that P. aeruginosa can utilize the examined pesticides as a carbon source, since *P. aeruginosa* strains could degrade S-metolachlor around 34-54% in 5 days.
By contrast, terbuthylazine may have a slightly negative effect on *P. aeruginosa* to limit its growth, but still *P. aeruginosa* strains could degrade terbuthylazine around 21-54% in 5 days. Altogether, we can conclude that the examined pesticide concentrations did not inhibit the growth of *P. aeruginosa*.

Based on the antibiotic resistance assay, there were only a few cases when the antibiotic resistance was increased by the examined pesticides.
The detected changes in MIC values were not significant, which is not supporting the findings of Plotkin et al. (2003). But still, we have found alterations and the reason for these differences should be clarified in future experiments with the application of a larger set of *P. aeruginosa* strains from various origins. In the future, another set of antibiotics should be also tested to get a better picture of this topic.
Our investigations prove that our knowledge is still limited on the sublethal effects of micro-pollutants. In the future it is important to reach deeper and extended data to identify the potential consequences arising from the application of pesticides.

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