Hungarian University of Agriculture and Life Sciences

Doctoral School of Natural Sciences

OPPORTUNISTIC PATHOGEN MICROORGANISMS IN THE ENVIRONMENT – ANTIBIOTIC RESISTANCE, VIRULENCE AND PHYLOGENY

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Table of Contents

1. background and aim 4
2. Materials and Methods 7
   2.1. The effect-based analysis of glyphosate and glyphosate-based herbicides (GBHs) on *Pseudomonas aeruginosa* 7
      2.1.1. Examined microorganisms 7
      2.1.2. Examined pesticides 8
      2.1.3. Preliminary screening of AMR inducing effect of pesticides using pre-exposure and co-exposure 9
      2.1.4. Microplate chequerboard assay 9
      2.1.5. Data analysis and statistics 10
   2.2. Evaluating the *in vivo* virulence of environmental *Pseudomonas aeruginosa* using a newly developed zebrafish (*Danio rerio*) microinjection model 12
      2.2.1. Selection of bacterial strains for virulence testing 12
      2.2.2. Preparation of bacterial suspensions 13
      2.2.3. Maintenance of zebrafish and egg collection 13
      2.2.4. Microinjection of embryos 13
      2.2.5. Experimental endpoints 15
      2.2.6. Statistical analysis 16
3. Results and discussion 17
   3.1. The effect of glyphosate and glyphosate-based herbicides on *P. aeruginosa* 17
      3.1.1. Preliminary screening assays - pre-exposure experiment: increased MIC value of imipenem under the pre-exposure of GBHs 17
      3.1.2. Preliminary screening assays - co-exposure experiment: the increased MIC value of imipenem under the co-exposure with GBHs 19
      3.1.3. Drug interactions in FIC index assay 19
   3.2. Evaluating the zebrafish (*Danio rerio*) microinjection virulence model and its optimization on *P. aeruginosa* 25
      3.2.1. Optimization of the combined zebrafish microinjection virulence model 25
      3.2.2. Sublethal symptoms detected at the end of incubation 31
      3.2.3. Methodological summary of the newly developed, combined virulence model 32
4. Conclusions and recommendations 34
5. New scientific results 37
6. Publications 38

1. BACKGROUND AND AIM

Pseudomonas aeruginosa (*P. aeruginosa*) is a widely known bacterial species in both natural and clinical environments. It is an opportunistic pathogen that might infect plants, animals, and humans if the host immune system is deficient or weak.

P. aeruginosa strains have been known to be highly pathogenic in clinical environments for more than 20 years. At the same time, isolates originating from the natural environment have been often considered as "non-pathogenic" because of lacking virulence-related genes or their non-hemolytic phenotypes. To examine the virulence of environmental *P. aeruginosa*, *in vivo* virulence models still play an important role, therefore numerous infection models have been created and improved in the last decades. Based on the similarity of innate immunity between zebrafish and humans, and the optical transparency of embryos, which enables their easy observation, zebrafish (*Danio rerio*) and its embryos play a very important role among vertebrate animal models to study host-pathogen interactions and infectious diseases. The existing zebrafish embryo virulence assays can be performed through microinjection or bath immersion. Both infection modes have their advantages and disadvantages, but usually they cannot give details about both invasive and cytotoxic features of the examined strains. In order to evaluate the virulence of environmental *P. aeruginosa* and to predict its risk to the natural ecosystem and human health, we developed a novel infection model that combines different zebrafish embryo microinjection methods. The novel model could not only test the effect of environmental *P. aeruginosa* early after infection but in the future, it also enables to determine the infectivity of other fast-growing microorganisms.

Compared to other Gram-negative bacteria, *P. aeruginosa* can live in various, complex environments, even under extremely adverse conditions. Due to its large bacterial genome and metabolic flexibility, *P. aeruginosa* can adapt to various carbon sources and can tolerate or degrade a variety of xenobiotics. One of the greatest consequences of this adaptation capacity with a direct effect on human health is the ability of *P. aeruginosa* to evolve antibiotic resistance.

Based on the scientific literature, the exposure to environmental pollutants, such as pesticides can be a driving force in the development of environmental antimicrobial resistance as it was verified in the case of glyphosate, a widely used herbicide and its formulations: glyphosate salt and glyphosate-based herbicides (GBHs). These chemicals were proved to induce alterations in the antibiotic resistance profiles of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. In the case of *P. aeruginosa*, it is critically important to reveal if there is any correlation between the exposition of strains to these xenobiotics and the developing antibiotic resistance. *P. aeruginosa* can tolerate and degrade glyphosate, but there is a limited knowledge about how the antibiotic resistance of *P. aeruginosa* changes when *P. aeruginosa is* exposed to glyphosate and GBHs at sublethal concentrations.

Based on the above information, the aim of this research work was to fill the knowledge gaps by investigating the *in vivo* virulence of environmental *P. aeruginosa* strains with a newly developed infection model and to reveal the effect of glyphosate and commercially available GBHs' expositions at sublethal concentrations on the phenotype-detectable antibiotic resistance of both environmental and clinical *P. aeruginosa* strains. According to the results from the latest research, additional experiments were set to determine the type of relationship (antagonism, synergism) between the examined GBHs composed by different glyphosate salts and additives) with imipenem, a potent, carbapenem-type, cell wall synthesis inhibitor antibiotic. To fulfil these objectives, the research goals of the thesis work were determined as follows.

Key questions and aims of the research are:

- The development of a novel infection model that combines two different zebrafish embryo infection routes using microinjection method.

- The assessment of the phenotypically-detectable antibiotic resistance alterations when *P. aeruginosa* was exposed to the sublethal concentrations of glyphosate and GBHs.

- The reveal of the relationship (antagonism, synergism) between the examined GBHs

(formed by different glyphosate salts and additives), glyphosate and POE(15) with imipenem.

2. MATERIALS AND METHODS

2.1. The effect-based analysis of glyphosate and glyphosate-based herbicides (GBHs) on *Pseudomonas aeruginosa*

In the first preliminary series, which was performed during my PhD studies, three pesticides (glyphosate, S-metolachlor and terbuthylazine) were screened in a conical flask experiment in one concentration (5 mg/L) to measure their possible effect on the antimicrobial resistance of *P. aeruginosa*. Based on this initial experiment, glyphosate was chosen as the target molecule for further testing, along with several, commercially available GBHs and an additive, POE(15). By performing these experiments, we could get an insight into the potential role of these agrochemicals on antimicrobial resistance (AMR).

In the upcoming sections, I will give an overview of the methodology of these detailed experiments, focusing on glyphosate, GBHs and POE(15).

2.1.1. Examined microorganisms

To analyze the AMR-inducing effect of glyphosate and glyphosate-based herbicides, five selected *P. aeruginosa* strains were used representing different clinical and environmental sources (**Table 1.**). The type strains were acquired from the National Collection of Agricultural and Industrial and Industrial Microorganisms (NCAIM) in Hungary, while the environmental strains were obtained from the strain collection of the Department of Environmental Safety (MATE), Gödöllő, Hungary.

Table 1. Selected *Pseudomonas aeruginosa* strains in the experiments

Strain	Collection	Source
HF234	MATE	Surface water, Hungary
P66	MATE	Hydrocarbon-contaminated groundwater, Hungary
ATCC 27853	NCAIM	Clinical (isolated from blood)
ATCC 10145	NCAIM	Type strain, unknown source

ATCC 15442	NCAIM	Water bottle in the animal room

All the tested strains were representatives of antibiotic-sensitive phenotypes, which was a crucial factor in the subsequent analyses to evaluate the resistance inducing ability of pesticides. To confirm the species level identification, a species-specific PCR-based method (PA-SS PCR) was chosen, specifically targeting the variable regions V2 and V8 of the 16S rDNA.

2.1.2. Examined pesticides

To evaluate the AMR-inducing effect of glyphosate [(N-(phosphonomethyl) glycine) with chemical formula: C3H8NO5P, CAS 1071-83-6], a stock solution was prepared in the MATE's laboratory using Pestanal analytical standard (Merck Ltd., Germany).

To differentiate the effect of glyphosate, the active substance of GBHs, and a commonly used additive, the POE (15), a polymer that contains an average of 15 ethylene oxide groups (chemical formula: R-N(CH2CH2O)m-H (CH2CH2O)n-H; CAS number: 61791-26-2), the additive was also purchased from Greyhound Chromatography and Allied Chemicals and was tested separately.

Moreover, several commercially available GBHs were obtained from the market and examined. A summary of the examined, commercially available GBHs, their declared glyphosate concentrations, and known co-formulants is presented in Table 2.

Table 2. The information of the examined glyphosate-based herbicides used in the experiments, obtained from their Material Safety Data Sheets (MSDS).

Formulation's name	**Declared glyphosate concentration (g/L)**		**Type of glyphosate salt**	**Declared co-formulant(s) % (w/w)**
	Glyphosate acid (active substance)	**Glyphosate salt**		
Dominator Extra 608 SL	480	608	Dimethylamine (DMA) salt	D-Glucopyranose, oligomers, decyloctyl glycosides (< 5%), disodium cocoamphodipropionate (< 5%) Methyl alcohol (< 1%)

Fozat 480	360	480	Isopropylamine (IPA) salt	Non-declared
Gladiator 480 SL	360	486	Isopropylamine (IPA) salt	Polyethoxylated (15) tallow amine [POE(15)] (13–18%)
Roundup Mega	450	551	Potassium salt	Ethoxylated ether alkylamine (7%)
Total	360	486	Isopropylamine (IPA) salt	Non-declared (inert substance)

2.1.3. Preliminary screening of AMR inducing effect of pesticides using pre-exposure and co-exposure

Preliminary tests were conducted to examine whether five selected commercial GBHs (Table 2.) affect the growth and antibiotic resistance of a representative *P. aeruginosa* isolate (HF234, Table 1.) after pre-exposure. A total of 45 mL Luria–Bertani (LB) medium (10.0 g tryptone, 5.0 g yeast extract, 9.0 g NaCl per 1000 mL distilled water) was used, inoculated with 5.0 mL of overnight bacterial suspension ($OD_{600} = 0.60 \pm 0.02$).To meet the concentration range of the agricultural and household use (0.2–3.5 v/v%), a concentration of 0.5 v/v% GBH was selected, corresponding to 1.8–2.8 g/L glyphosate acid content. Treated bacterial cultures were incubated at 28 °C for 72 h on a horizontal shaker, with regular OD^{600} measurements for growth curve analysis.

To evaluate the alterations in AMR, Liofilchem MIC (Minimal Inhibitory Concentration) test strips were used on Mueller–Hinton agar (without GBH) at 0 h and 72 h of the experiment. MICs for eight antibiotics (represented six classes) were determined following the EUCAST guidelines after 24 h at 35 °C incubation. All experiments were triplicated. Statistical analysis used two-way ANOVA and post-hoc Tukey test ($p < 0.05$) to detect MIC differences between treated and untreated cultures.

For co-exposure tests, three GBHs (Roundup Mega, Dominator Extra 608 SL, Gladiator 480 SL) were chosen that had the most pronounced effect on AMR in pre-exposure assay. For co-cultivation, overnight cultures of five *P. aeruginosa* strains (Table 1.) were directly spread on Mueller–Hinton agar with 0.5 v/v% GBHs and resistance was tested as described above.

2.1.4. Microplate chequerboard assay

According to the results of the preliminary screening assays, a microdilution chequerboard test following a previously described protocol was set with minor modifications. With this method, we could assess the resistance-inducing effect of GBHs, glyphosate, and its formerly used co-formulant POE(15) in a quantitative way with the antibiotic (imipenem) chosen during the preliminary testing phase. The assay was applied in triplicates with freshly prepared solution of glyphosate, POE(15) and GBHs, and overnight bacterial suspensions.

To perform the chequerboard assay, 96-well, clear, U-shaped PS microplates (Greiner Bio-One GmbH, Austria) were used. Considering the water-solubility of the analyzed compounds, the chosen antibiotic (imipenem, CAS 74431-23-5 obtained from Supelco, Sigma-Aldrich Ltd.) was diluted into a two-fold dilution series of a 2 g/L stock solution. Additionally, 10.0 g/L glyphosate, 5 g/L POE(15), and 50v/v% GBH stock solutions were prepared in sterile distilled water to set the experiment. These test solutions were combined in the microplates: imipenem (with a final concentration range of 0–64 mg/L) was added to the x-axis, while glyphosate (0–800 mg/L), POE(15) (0–4 mg/L), and GBH (0–4 v/v%) were added to the y-axis, respectively. Control settings were performed using solo imipenem or glyphosate/POE/GBH solutions without combination. After setting the concentrations of the test materials, each well of the microplate was supplemented with 50 µL of overnight *P. aeruginosa* bacterial suspensions (optical density of $OD_{600} = 0.60 \pm 0.02$) and to make the final volume to reach 250 µL, sterile LB broth medium was added. As the negative control group, a mixture of 200 µL LB supplemented with 50 µL bacterial suspension was used. Microplates were incubated at 28 °C, at a speed of 350 rpm (round per minute) in a microplate shaker thermostat (PST-60HL-4, BioSan, Latvia). To measure the interactions of the different combinations of the antibiotic and the test materials (glyphosate, GBH or POE(15), absorbance measurements were taken by an ELx800 microplate reader at 550 nm at the beginning of the incubation (0 h) and after 24 h of exposure.

2.1.5. Data analysis and statistics

After performing the experiments, raw data were statistically analyzed. The absorbance data were averaged, and then they were visually represented using heat maps. In order to

assess the combined effects of imipenem and the glyphosate-related chemicals (glyphosate, POE(15), GBHs) under investigation, we calculated the Fractional Inhibition Concentration Index (FICI) using the following mathematical expression, where A is compound A, B is compound B, MIC means minimal inhibitory concentration and FIC is the fractional inhibitory concentration.

$$FIC_A = \frac{MIC\,(A\,in\,the\,presence\,of\,B)}{MIC\,(A\,alone)}$$
$$FIC_B = \frac{MIC\,(B\,in\,the\,presence\,of\,A)}{MIC\,(B\,alone)}$$
$$FIC_A + FIC_B = FICI$$

The FICI is used to quantify the interaction between two chemical using the following evaluation categories:

FICI < 0.5	synergism
0.5 < FICI < 4.0	indifference
4.0 ≤ FICI	antagonism

To compare the differences between glyphosate/POE(15)/GBHs and the tested antibiotic (imipenem), we compared the average absorbance values of five examined strains when co-exposed with their respective imipenem control samples (containing bacterial suspension and imipenem). For statistical analysis, we conducted an analysis of variance using a two-way ANOVA followed by Dunnett's multiple comparisons tests, where significance was considered at $p \leq 0.05$.

Based on the above-described protocol, starting with initial screening and finishing with quantitative analysis, a range of antimicrobials could be tested to evaluate the possible AMR inducing effect of glyphosate, POE(15) and GBHs.

2.2. Evaluating the *in vivo* virulence of environmental *Pseudomonas aeruginosa* using a newly developed zebrafish (*Danio rerio*) microinjection model

In the next research, another important scientific question was analyzed, that could clarify the actual virulence (cytotoxic, or invasive feature) of environmental *P. aeruginosa* strains. Based on the scientific literature, the available techniques to determine virulence of environmental *P. aeruginosa* are not always sufficient in providing information about both the invasive and cytotoxic features a given strain. Therefore, to fully assess the complex virulence of *P. aeruginosa* and to predict its ecological risk, novel *in vivo* experimental methods are required. A new virulence model was developed and validated during my PhD research, that can be used to evaluate the hazard of a given *P. aeruginosa* strain on human health and the ecosystem. In the upcoming subsections, the method development process of the new virulence test is summarized in details.

2.2.1. Selection of bacterial strains for virulence testing

In order to develop and validate the new virulence model on environmental pathogens, a diverse set of 15 *P. aeruginosa* was chosen. These isolates varied in origin, phenotypic and genetic traits, multilocus sequence types (MLSTs), antibiotic resistance profiles, virulence factors, biofilm-forming ability, motility, and *in vivo* virulence as previously determined using the *Galleria mellonella* (wax moth) model With molecular typing, we could categorize strains that are closely related into sequence types (STs).

Environmental and clinical *P. aeruginosa* strains were obtained from the National Collection of Agricultural and Industrial Microorganisms (NCAIM), Hungary, and from the collection of the Hungarian University of Agriculture and Life Sciences (MATE), Department of Environmental Safety. All *P. aeruginosa* strains were previously identified at the species level using PA-SS PCR, a species-specific method targeting the variable regions V2 and V8 of 16S rDNA.

In the first step, to optimize the zebrafish embryo microinjection parameters (volume,

concentration, incubation time) a preliminary experiment was set using two environmental strains (P14 and P66) which were represented virulent and avirulent phenotypes based on a previous *G. mellonella* virulence assay.

2.2.2. Preparation of bacterial suspensions

Bacterial strains were grown in Luria-Bertani (LB) broth, a medium that was previously confirmed to be safe (non-toxic) to zebrafish larvae. Overnight cultures of the *P. aeruginosa* strains were diluted with fresh LB until they reached an optical density of $OD_{600} = 0.60 \pm 0.02$. These stock cultures were then further diluted tenfold to reach the required bacterial concentrations.

2.2.3. Maintenance of zebrafish and egg collection

Zebrafish were kept according to the general protocol of the MATE zebrafish lab at the Department of Aquaculture, the Hungarian University of Agriculture and Life Science, Gödöllő, Hungary, in a Tecniplast ZebTEC recirculation system (Tecniplast S.p.A., Italy) at 25.5 °C ± 0.5 °C, pH 7.0 ± 0.2, conductivity 550 ± 50 µS (system water) and light: dark period of 14 h:10 h. Zebrafish were fed twice dry granulate food (Zebrafeed 400–600 µm, Sparos Lda., Portugal), and fed with freshly hatched live *Artemia salina* twice a week. The fish were placed in a breeding tank (Tecniplast S.p.a.) late in the afternoon the day before the experiment and made to spawn the next morning after removing the partition wall. The laying of individual pairs was delayed over time to ensure a continuous supply of single-celled embryos.

2.2.4. Microinjection of embryos

To determine the optimal conditions for virulence testing (bacterial concentration, injection volume, i.e., drop size, incubation time) and for the detection of early-stage symptoms, microinjection was performed immediately after fertilization. This rapid infection protocol allows a large number of zebrafish eggs to be injected in a short time. It is easy to perform and does not require a holder for the injection. In order to evaluate the cytotoxicity of

the examined strains microinjection to the yolk (Y) was performed, while to determine the invasive characteristics of the examined strains, a direct microinjection to the perivitelline (PV) was used.

The bacterial density of the fast-growing *P. aeruginosa* stock solution was set to OD_{600}=0.60 ± 0.02 (equivalent to 4.8× 10^8 ±3.33% CFU/mL as it was verified by colony counting on Luria-Bertani (LB) agar). As zebrafish chorionic pore size (0.77 µm) is smaller than *P. aeruginosa* cells, cross infection was prevented until hatching (72 h).

Two hours post-injection, solidified or unfertilized eggs were removed, and embryos were transferred into sterilized E3 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, 0.16 mM $MgSO_4$). Embryos were incubated at 25 ± 1 °C in a Memmert thermostat. Preliminary tests were performed in triplicates with 10 embryos per group (n = 30).

The experimental settings during the optimization phase are summarized in Table 3.

Table 3. Bacterial concentrations (CFU) drop sizes, final injection volumes and exposition routes used for the optimization of the newly-developed zebrafish microinjection virulence model

Exposition routes	**Drop sizes**	**Levels of bacterial dilutions (CFU) and bacterial doses**			
		10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**
Perivitelline space (PV)	100 µM (0.52 nL)	2.4 × 10^1	2.4× 10^0	2.4·× 10^{-1}	
	150 µM (1.77 nL)	8.4 × 10^1	8.4·× 10^0 *	8.4·× 10^{-1}	
	200 µM (4.17 nL)	2.0 × 10^2	2.0·× 10^1	2.0·× 10^0	
Yolk (Y)	100 µM (0.52 nL)		2.4·× 10^0	2.4·× 10^{-1}	2.4·× 10^{-2}
	150 µM (1.77 nL)		8.4·× 10^0 *	8.4·× 10^{-1}	8.4·× 10^{-2}
	200 µM (4.17 nL)		2.0·× 10^1	2.0·× 10^0	2.0·× 10^{-1}

* Minimum infectious dose (MID) for *P. aeruginosa*

Three bacterial dilutions (Table 3) were used for injection: MID (~10 cells), one lower (10^{-1}), and one higher (10^{-3}). For yolk (Y) injection, one level lower concentration was used than in perivitelline space (PV) injection to observe sublethal effects.

Droplet size is critical for larval survival and influences the infective dose. The maximum safe volume for zebrafish embryos is about 4.2 nL (200 µm diameter), which is about 10% of total yolk volume. Based on prior testing, this and two smaller sizes, 100 µm (0.52 nL) and 150 µm (1.77 nL) were used, with validated volume stability.

In summary, to optimize the infection protocol, environmental strains (P66, P14) and uninoculated control (LB) were tested via two injection routes (PV, Y), three droplet sizes, and

three concentrations (27 settings total). Sublethal symptoms were recorded every 24 h until hatching (72 h). Each embryo received one treatment, resulting in 81 endpoints. Dead embryos were analyzed for pathogen re-isolation to confirm infection.

Figure 1. shows the optimization flowchart.

Based on the preliminary test of microinjection, the optimized microinjection was also conducted as described above, but only with the chosen droplet size, respective volume, bacterial concentrations and incubation time. Treatments in this phase were conducted in five replicates with ten embryos each (n = 50) on 15 representative strains of *P. aeruginosa*.

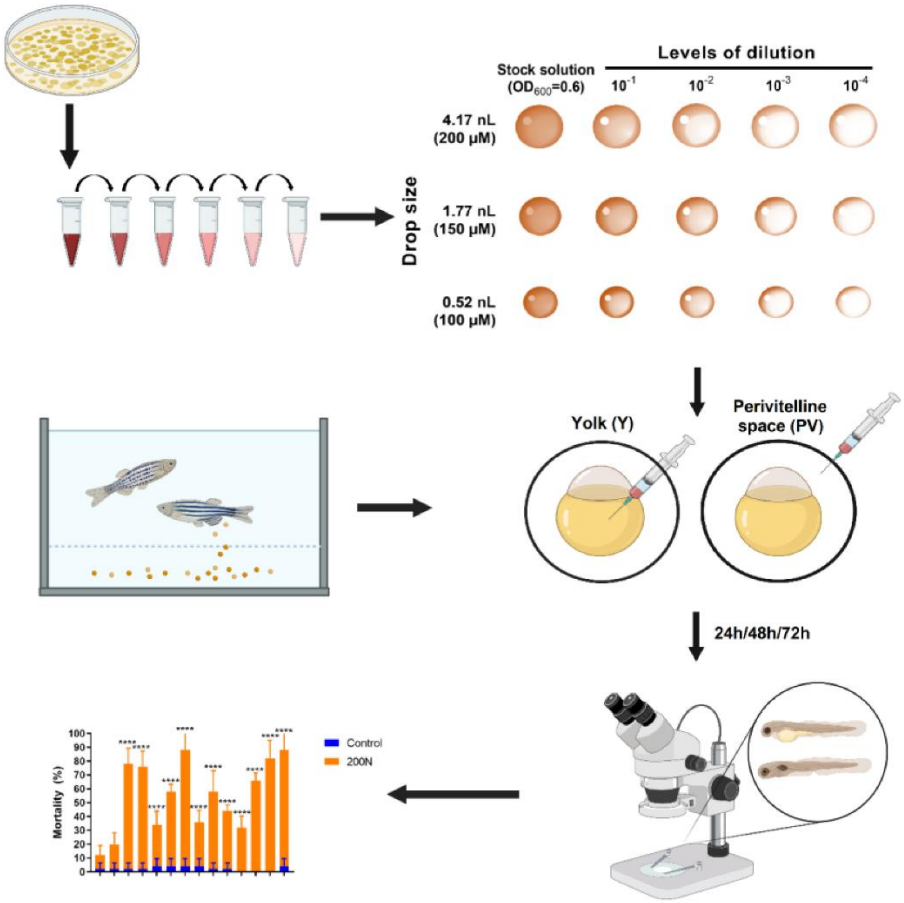

Figure 1. The flowchart of the preliminary testing protocol for the optimization of microinjection (created in Biorender.com by Csenki-Bakos Zs. and Kaszab E.)

2.2.5. Experimental endpoints

Mortality and sublethal symptoms of zebrafish embryos were monitored daily. Embryonic mortality was determined based on egg coagulation, lack of body segment formation, and lack of heart function.

The evaluation of the mortality results followed the existing interpretation criteria used for the *in vivo* virulence testing of *G. mellonella* as follows:

- avirulent (with a survival rate of 75–100%),
- weakly virulent (survival rate: 50–74%),
- moderately virulent (survival rate: 25–49%) and
- virulent (survival rate: 0–24%).

Sublethal endpoints were visually observed using a stereomicroscope (Leica M205 FA, Leica DFC 7000T camera, Leica Application Suite X, Leica Microsystems GmbH, Wetzlar, Germany), digital images of transverse larvae were captured at 30x magnification.

2.2.6. Statistical analysis

Normality of data was analyzed in R software version 4.2.1. with the Shapiro-Wilk normality test. The homogeneity of variance was tested by Bartlett test. Statistical analysis and visualization of exposure pathway, droplet size, bacterial concentration, and incubation time were performed using GraphPad Prism 9 software version 9.5.1. (GraphPad Software Inc., San Diego, California, USA) and R software, version 4.2.1. Ordinary one-way ANOVA was followed by the Dunnet multiple comparison test, with a confidence interval of 95%.

3. RESULTS AND DISCUSSION

3.1. The effect of glyphosate and glyphosate-based herbicides on *P. aeruginosa*

Using the methodology described in section 2.1., the effect of glyphosate, GBHs, and a GBH additive, POE(15) were comprehensively analyzed to determine the effect on growth and phenotypic antibiotic resistance of *P. aeruginosa*, including the effect on AMR using pre-exposure, co-exposure and microplate chequerboard method.

3.1.1. Preliminary screening assays - pre-exposure experiment: increased MIC value of imipenem under the pre-exposure of GBHs

Our results on growth kinetics and AMR obtained after the pre-exposure of a chosen *P. aeruginosa* strain (HF234) with different types of commercially available GBHs ('Roundup Mega', 'Dominator Extra 608 SL', 'Gladiator 480 SL', 'Total', and 'Fozat 480') are summarized in **Figure 2.** As it was a preliminary screening stage, glyphosate and POE(15) were not investigated and only one *P. aeruginosa* strain was involved.

Figure 2. contains A, B, and C subfigures. Figure 2/A shows the growth curve of *P. aeruginosa* strain HF234 over 72 hours when exposed to various glyphosate-based herbicides (GBHs) at a concentration of 0.5 v/v%. The y-axis representing optical density (OD_{600}) of GBH treated and control settings. As shown in Figure 2/A, the HF234 isolate of *P. aeruginosa* could tolerate 0.5 v/v% of 'Roundup Mega', 'Dominator Extra 608 SL', 'Gladiator 480 SL', 'Total', and 'Fozat 480' without a significant inhibitory effect.

Figure 2/B displays the Minimum Inhibitory Concentration (MIC) values for imipenem after 72 hours of pre-exposure to the GBHs. The different MIC values before and after exposure to different GBHs shows the effects on the susceptibility of the examined bacterium under pre-exposure of GBHs. Figure 2/C is illustrating the inhibition zones of imipenem against the HF234 strain pre-exposed to different GBHs. It can be seen that after the

pre-exposure with 0.5 v/v% GBHs, the MIC values of piperacillin and gentamicin increased 1.5-2.0x compared to the non-treated control group, although the difference was not statistically significant (Figure 2/B). Meanwhile, it was observed that the MIC value of imipenem exhibited a notable increase ranging from 2 to 32 times compared to the control group. In contrast, meropenem, doripenem of the same antibiotic class (carbapenems) remained without any significant changes in their MIC values. Based on Tukey's multiple comparison tests, it was evident that pre-exposure to 'Dominator Extra 608 SL', 'Gladiator 480 SL', and 'Roundup Mega' significantly increased the resistance level against imipenem, suggesting a higher degree of resistance induced by these three herbicides compared to the others examined.

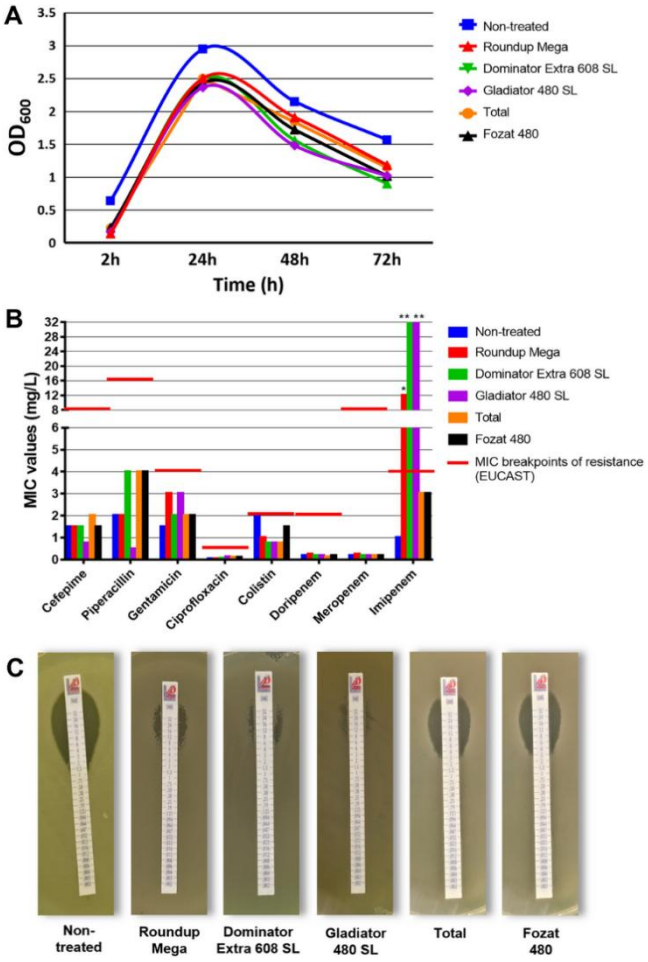

Figure 2. Pre-exposure of *P. aeruginosa* HF234 using 0.5 v/v% GBHs (averaged values obtained from three technical replicates). (A) The optical density (OD_{600}) of broth cultures which was treated with GBHs for 72 h. (B) MIC values of HF234 cultures after 72 h pre-exposure, plated on GBH-free agar plates. (C) Inhibition zones of HF234 cultures after 72 h GBH pre-exposure, plated on GBH-free plates. * and ** mean significant differences compared to the non-treated strain (p=0.0332 – 0.0023). Figure was created by Jiang D. and Kaszab E..

3.1.2. Preliminary screening assays - co-exposure experiment: the increased MIC value of imipenem under the co-exposure with GBHs

Based on the pre-exposure assay of HF234 using 0.5 v/v% GBHs and 8 antibiotics, it was clear that only the phenotypic imipenem resistance of *P. aeruginosa* was significantly influenced by GBHs; therefore, this antibiotic agent was further analyzed during co-exposure studies using the three GBHs ('Dominator Extra 608 SL', 'Gladiator 480 SL', 'Roundup Mega') with the most pronounced effect. Results are summarized in Table 4.

Table 4. Minimal inhibitory concentrations (MIC values) of imipenem on *P. aeruginosa* strains during co-exposure (without pre-exposure) plated on Mueller-Hinton plates supplemented with 0.5 v/v% GBHs

Antibiotic	**GBH (0.5 v/v% content of the plates**	**MIC values of *P. aeruginosa* strains (mg/L)**				
		ATCC 27853	**ATCC 10145**	**ATCC 15442**	**HF234**	**P66**
Imipenem	Non-treated	2	3	0.75	1	1
Imipenem	Gladiator 480 SL	**>32**	**>32**	**>32**	**>32**	**>32**
Imipenem	Roundup Mega	4	4	**>32**	**12**	4
Imipenem	Dominator 608 SL	**>32**	**16**	**16**	**>32**	**>32**

bold - MICs above the EUCAST breakpoints of resistance (4 mg/L)

Based on these results, imipenem under the co-exposure with GBHs shows a significant decrease in effectiveness against the examined *P. aeruginosa* strains.

3.1.3. Drug interactions in FIC index assay

After pre-exposure and co-exposure studies, *P. aeruginosa* was further analyzed with a microplate chequerboard test (as it was described in the Materials and methods, Section 2.1.4.), to verify the presumed drug interactions between GBHs and imipenem. Because GBHs are complex formulations with a variety of active ingredients and additives (Table 2.), not only GBHs, but glyphosate and a previously used additive, POE(15) were analyzed to differentiate between the AMR-inducing effect of these molecules.

Results of the five examined *P. aeruginosa* strains exposed to both imipenem and glyphosate, GBHs, or POE(15) are visualized in **Figure 3.** showing the averaged absorbance values of three replicate measurements as a heatmap in case of each strain. According to this

figure, we can see the interactions between imipenem and various concentrations of glyphosate, POE(15), and GBHs on *P. aeruginosa* in a strain-dependent manner. Relative fold growth is showed by blue bars.

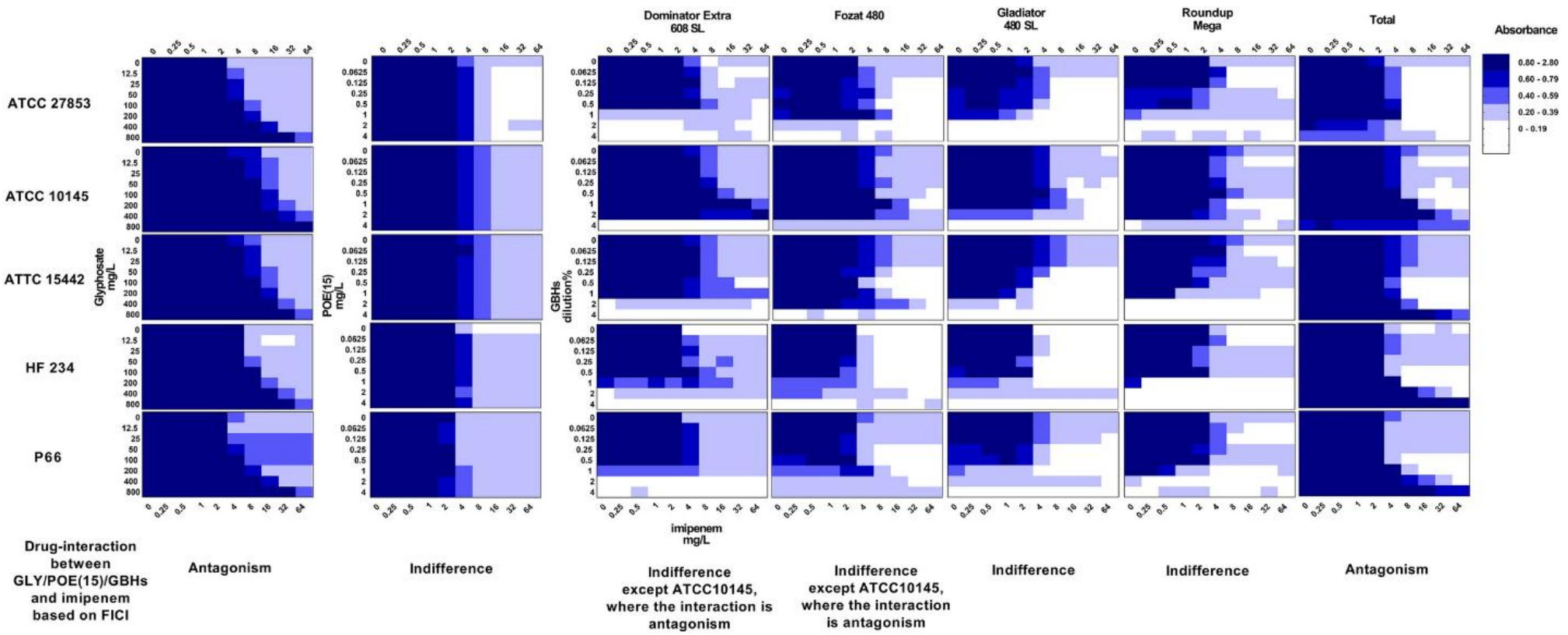

Figure 3. Heatmap of the averaged absorbance values of each tested *P. aeruginosa* strain (ATCC 27853, ATCC 10145, ATCC 15442, HF234, P66) during co-exposure with different concentrations of glyphosate, POE(15), GBHs and imipenem. The X-axis represents imipenem concentration ranging from 0 to 64 mg/L, and the Y-axis indicates the concentration of the test materials (glyphosate: 0–800 mg/L, POE(15): 0–4 mg/L, GBHs: 0–4 v/v%)

The averaged differences (%) of absorbance values of *P. aeruginosa* strains under co-exposition compared with the absorbance values of the relevant solo imipenem exposition are summarized in **Figure 4.** Each bar represents the averaged absorbance values of five *P. aeruginosa* strains co-exposed to imipenem and test materials based on three independent experiments. Bars above the baseline indicate an increase in bacterial growth (reduced effectiveness of imipenem), while bars below indicate decreased growth (enhanced effectiveness of imipenem). With this visualization, we can see the strain-independent effect of co-exposition.

The drug interactions between imipenem and glyphosate/POE(15)/GBHs were assessed using the FIC index to determine the combined effect (synergistic, antagonistic, or additive relations) of multiple chemicals.

It can be seen from Figure 3., that solo glyphosate treatment was not toxic to *P. aeruginosa* strains within the tested concentration range of 12.5-800 mg/L because it did not inhibit the growth of *P. aeruginosa* in this concentration range. Based on our results for imipenem alone, at higher concentrations (above 1-3 mg/L), the absorbance values were generally lower, so the solo antibiotic treatment inhibited the growth of the test organism.

When combined with glyphosate, even at low concentrations of imipenem, the absorbance values increased, suggesting that glyphosate reduced the inhibitory effect of imipenem: MIC values of imipenem increased from 1-3 mg/L to 32-64 mg/L. According to the results of Figure 4., among all the strains tested, the strongest antagonistic effect between glyphosate and imipenem was determined in the case of *P. aeruginosa* ATTC10145. The negative correlation was more pronounced at higher glyphosate concentrations (400-800 mg/L). The high FICI value (18.6) of glyphosate versus imipenem also indicates the antagonistic effect, which means that there is a significant decrease in the effectiveness of imipenem when combined with glyphosate, irrespectively to the bacterial strains involved (Figure 3.).

In case of POE(15), a slight increase in absorbance was detected with the co-exposition of 4 mg/L imipenem, but there was no concentration-dependent effect on the bacterial growth.

For GBHs, there was a noticeable increase in absorbance values at higher concentrations (2-4 v/v%), which indicates GBHs ability to decrease the effectiveness of imipenem, but there were notable differences between difference types of GBHs.

‘Total’, that was non-toxic to the examined *P. aeruginosa* strains in the examined

concentration range, clearly induced resistance to imipenem with a significant increase in absorbance (up to 192 %) and depending on the tested bacterial strain, a simultaneous increase was detected in imipenem MIC values (4-64 mg/L). The calculation of FIC index proved the antagonistic effect with a FICI value of 12.8.

'Dominator Extra 608 SL' and 'Fozat 480' also decreased the antibiotic susceptibility of *P. aeruginosa* but only in the case of strain ATCC10145. Specifically, the FICI for 'Dominator Extra 608 SL' on strain ATCC10145 was determined at 32.5 while the FICI for 'Fozat 480' was recorded at 4.03 indicating an antagonistic effect. With others strains, the interaction was "indifferent"; there were no significant interactions between imipenem and glyphosate-related chemicals.

'Gladiator 480 SL' and 'Roundup Mega' were cytotoxic to the examined strains, therefore, their co-exposition with imipenem concentrations led to a significant decrease in absorbance at higher (2-4 v/v%) concentrations, which is equivalent to 7.2 -19.2 g/L glyphosate acid. This cytotoxic effect disabled the determination of the effect on AMR in that concentration range. Interestingly, at lower concentrations of GBHs (up to 1.0 v/v%), it was observed that these GBHs slightly increased the resistance to imipenem compared to when the antibiotic was used alone. However, this effect was only significant in the case of 'Roundup Mega' and 'Fozat 480' when co-exposed with imipenem at concentrations of 4-8 mg/L.

Based on our results, 'Dominator Extra 608 SL' and 'Total' showed a significant antagonistic effect on imipenem, which can be attributed to their lower cytotoxicity towards *P. aeruginosa.* Even at a higher concentration (2.0–4.0 v/v% equivalent to 7.2–14.4 g/L glyphosate acid), the cytotoxicity of these substances was not found to be significant. As a result, these tested substances could potentially stimulate a more pronounced resistance to imipenem in *P. aeruginosa*, with resistance levels reaching up to 64 mg/L.

Altogether we can see that all the tested GBHs reduced the efficiency of imipenem, but the trend in high imipenem concentrations was not as pronounced as in low imipenem concentrations. Based on our results, it can be concluded that the major interaction between imipenem and GBHs were "antagonism" or "indifference".

Our results were published in the journal *Scientific Reports* (D1, impact factor: 4.493) in 2022.

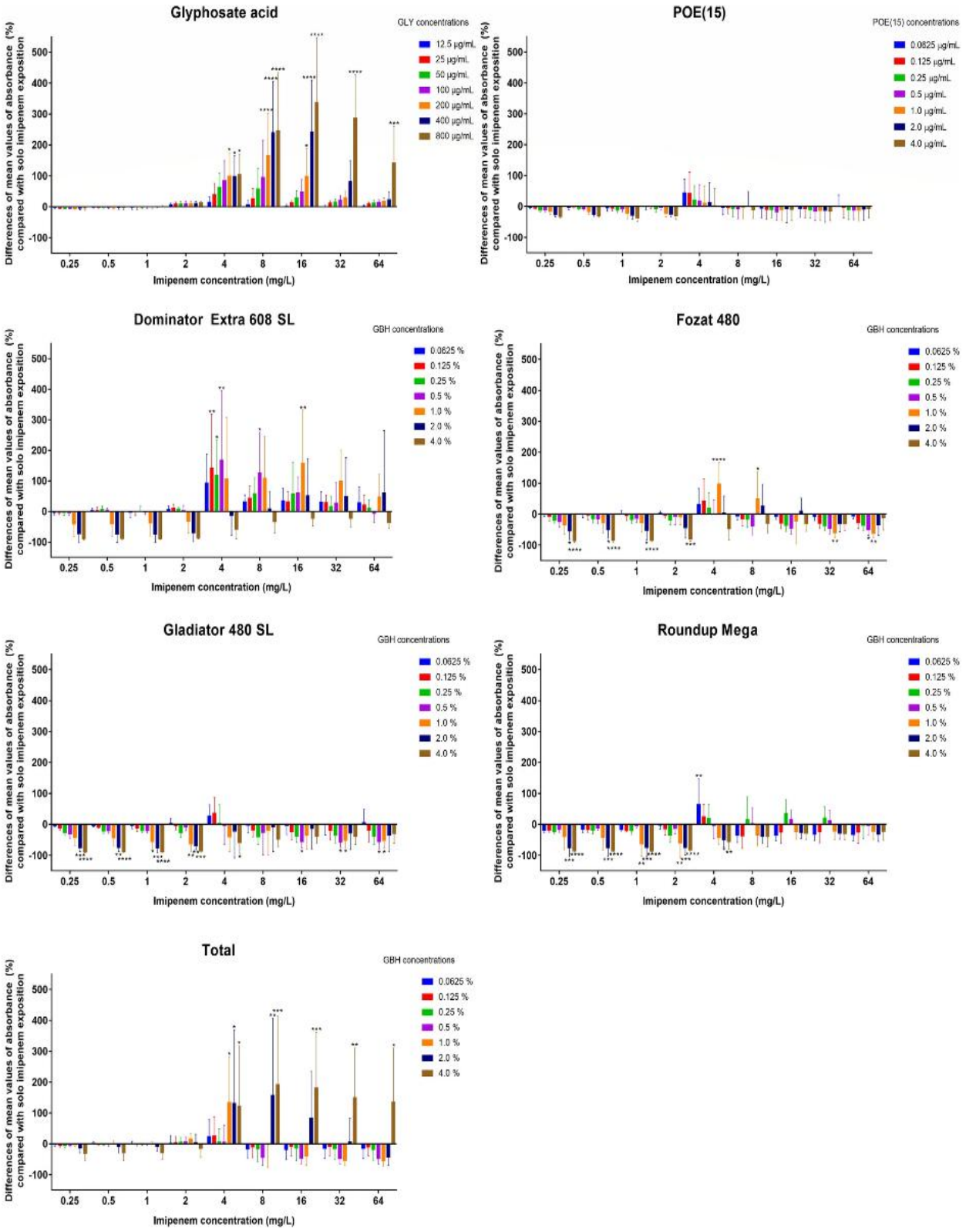

Figure 4. Differences in averaged absorbance values of the five *P. aeruginosa* strains (ATCC 27853, ATCC 10145, ATCC 15442, HF234, P66) during co-exposure with the test materials (glyphosate, POE(15), GBHs) and imipenem compared to solo imipenem exposure with the same concentrations (expressed in %).

3.2. Evaluating the zebrafish (*Danio rerio*) microinjection virulence model and its optimization on *P. aeruginosa*

In the upcoming section, the results of the new microinjection-based *P. aeruginosa* virulence assay's development are summarized, which includes the optimization and validation stages and their relevant results.

3.2.1. Optimization of the combined zebrafish microinjection virulence model

The new virulence model was first developed using two environmental *P. aeruginosa* strains to optimize the minimum observation period of the embryos and the bacterial cell count for injection, based on bacterial density and droplet size, as described in the Materials and Methods chapter. (Section 2.2.4). The optimization was required to make the test's application easy, while enabling the determination of statistically significant differences compared to untreated controls. With this optimization, the virulence model was aimed to reach high infection success with a relatively low mortality rate in a short period of time. To reach this goal, the mean mortality (%) of zebrafish embryo groups injected with P14 and P66 *P. aeruginosa* to the perivitelline (PV) or to the yolk (Y) was statistically assessed to determine the optimal incubation time, dilution level, and drop size. The tested *P. aeruginosa* strains (P66 and P14) both contained determinants of virulence, but based on their *in vivo* toxic effects in *G. mellonella*, P66 was identified as virulent and P14 as avirulent. The previous results showed that strain P66 had an antibiotic sensitive phenotype, while strain P14 had a resistant phenotype.

3.2.1.1. Determination of the optimal incubation time

To determine the optimal incubation time for testing, multiple comparisons of 24-hour, 48-hour, and 72-hour mean mortality values were made between the PV/Y injected groups and the control group. Results are shown as histograms in **Figure 5.** that illustrates the mortality rates of zebrafish embryos injected with *P. aeruginosa* strains at three different time points. According to the results, mortality rates were relatively low for both PV and Y injections at 24 hours applied to both strains tested (P66 and P14), and there were no significant differences in

mortality compared to the control group. Using a longer incubation time, the mortality rates increased, but the difference compared to the control group was still not statistically significant for both infection routes at 48 hours. Significant increases in mortality for both strains (P66 and P14) and both injection routes (PV and Y) were only observed at 72 hours (P-value：0.0332-0.0021).

Therefore, a 72-hour incubation period (equivalent to the hatching period) was selected as optimal for further experiments. In further statistical analysis, mortality results from shorter incubations (24 and 48 hours) were excluded to reduce the standard deviation in the data.

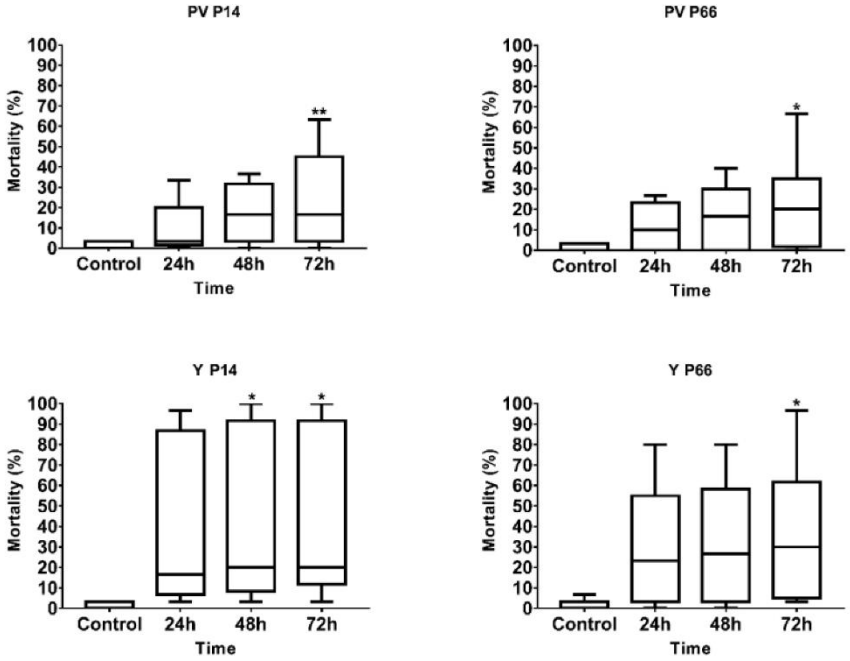

Figure 5. Mortality results of the examined *P. aeruginosa* strains during optimization of zebrafish microinjection model with different times of incubations (24, 48 and 72 h). Mean values were analyzed with One-way ANOVA followed by Dunne's multiple-comparison test at 95% confidence interval.

P-value: 0.1234 (ns), 0.0332 (*), 0.0021 (**)

PV—perivitelline injection; Y—yolk injection.

3.2.1.2. Determination of the optimal level of dilution of *P. aeruginosa*

In the next step, tenfold dilutions of the bacterial stock solutions (10^{-1} to 10^{-4}) were tested to determine the optimal bacterial concentration for microinjection. **Figure 6.** illustrates the mortality rates of zebrafish embryos injected with *P. aeruginosa* strains at various levels of dilution (10^{-1}, 10^{-2}, 10^{-3}, and $10^{-4,}$ respectively). As it can be seen in Figure 6., mortality rates at the 10^{-1} dilution level to both strains (P14 and P66), with the PV microinjection route, were

significantly higher compared to the control group, indicating strong virulence at this dilution level. Mortality rates were lower at the 10^{-2}, 10^{-3}, and 10^{-4} dilution levels and were not significantly different from the control group. Regarding the Y microinjection, the mortality rates were significantly higher compared to the control groups at the 10^{-2} dilution level for both strains (P14 and P66). Similar to the PV injections, the lower dilution levels (10^{-3} and 10^{-4}) did not result in significant mortality. Thus, the optimal bacterial dilutions were determined to be 10^{-1} for PV injection and 10^{-2} for Y injection.

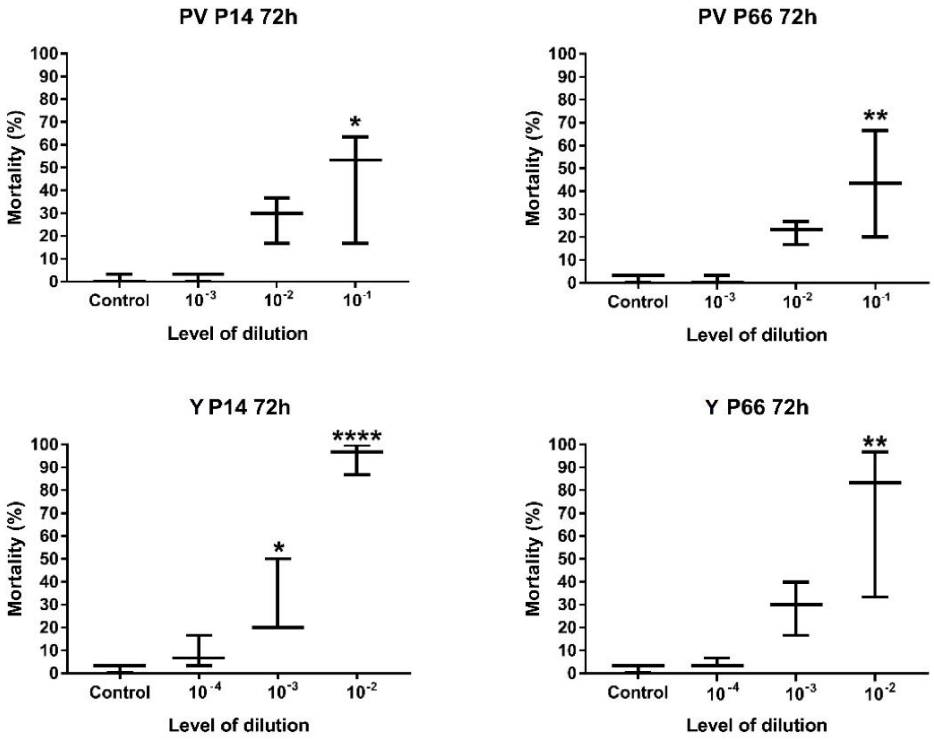

Figure 6. Mortality results of the examined *P. aeruginosa* strains during optimization of zebrafish microinjection model with different levels dilutions of bacterial strains (10^{-1}–10^{-4}). Mean values were analyzed with One-way ANOVA followed by Dunnett's multiple-comparison test at 95% confidence interval.
P-values: 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0001 (****)
PV—perivitelline injection; Y—yolk injection.

3.2.1.3. Determination of the optimal drop size

Based on the previous steps of optimization process, the optimal drop size of the infectious material used for microinjections was determined, calculated at the dilution level of 10^{-1} (PV) and 10^{-2} (Y) within 72 hours of incubation. According to **Figure 7.**, mortality rates increased as the drop size increased from 100 µM (0.52 nL) to 200 µM (4.17 nL). Only the 100 µM drop size didn't show a statistically significant difference in mortality compared to the

control on P14 by PV injection. The 150 µM (1.77 nL) and 200 µM sizes, however, showed significant increases (P-value: 0.0002–0.0001) in the embryo mortality of zebrafish at both microinjection routes and strains. Considering the need to reduce errors and increase the speed of the process, we decided to use only one drop size for microinjection. After the consideration of effectiveness and safety, the 150 µM drop size (1.77 nL volume) was chosen, because it was shown to be effective by statistical evaluation, and compared to the 200 µM droplet, it has a lower chance of causing a lesion in the embryo during microinjection.

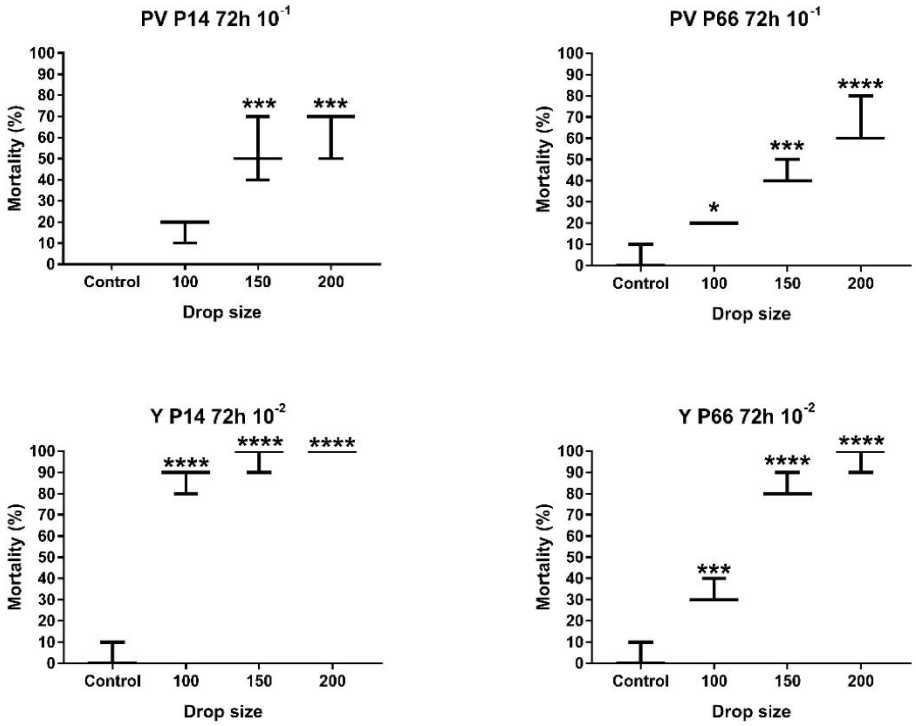

Figure 7. Mortality results of the examined *P. aeruginosa* strains during optimization of zebrafish microinjection model with different drop sizes (100, 150, 200 µL). Mean values were analyzed with one-way ANOVA followed by Dunnett's multiple-comparison test at 95% confidence interval.
P-value: 0.1234 (ns), 0.0332 (*), 0.0002 (***), 0.0001 (****).
PV—perivitelline injection; Y—yolk injection.

3.2.1.4. Development of the recommended zebrafish embryo infection model

After conducting the optimization process and statistical analysis, a combined microinjection protocol was applied for zebrafish larvae exposed to environmental *P. aeruginosa* in the following specific conditions. The protocol involves parallel perivitelline (PV) and yolk (Y) exposition with two different dilutions of the overnight OD_{600} = 0.6 ± 0.02 bacterial stock solution (10^{-1} for PV and 10^{-2} for Y) in 150 µL drop size, followed by 72 hours of incubation.

The optimized infection protocol was confirmed by testing with 15 different environmental and clinical strains of *P. aeruginosa* representing a range of phenotypic, genetic, and phylogenetic characteristics. **Figure 8.** illustrates the examined *P. aeruginosa* strains across their MLST phylogenetic tree and their virulence obtained by using the newly developed, combined microinjection method in zebrafish embryos. Both environmental and clinical strains of *P. aeruginosa* showed a wide range of virulence.

In our evaluation system we used the same categorization for the newly developed virulence model as it can be seen on the color distribution on Figure 8. Based on our results, yolk injection (Y) generally resulted in lower survival rates compared to perivitelline injection (PV). This suggests that injecting directly into the yolk (Y) is more lethal than injecting into the perivitelline (PV) as it was presumed. Differences between the the examined strains were detected across the phylogenetic tree, supporting the theory that several virulence characteristics can be correlated with the population structure of *P. aeruginosa*. This finding suggests that virulence characteristics of *P. aeruginosa* can be highly strain specific. Meanwhile, no connection was found between the range of phenotypic antibiotic resistance and the *in vivo* virulence.

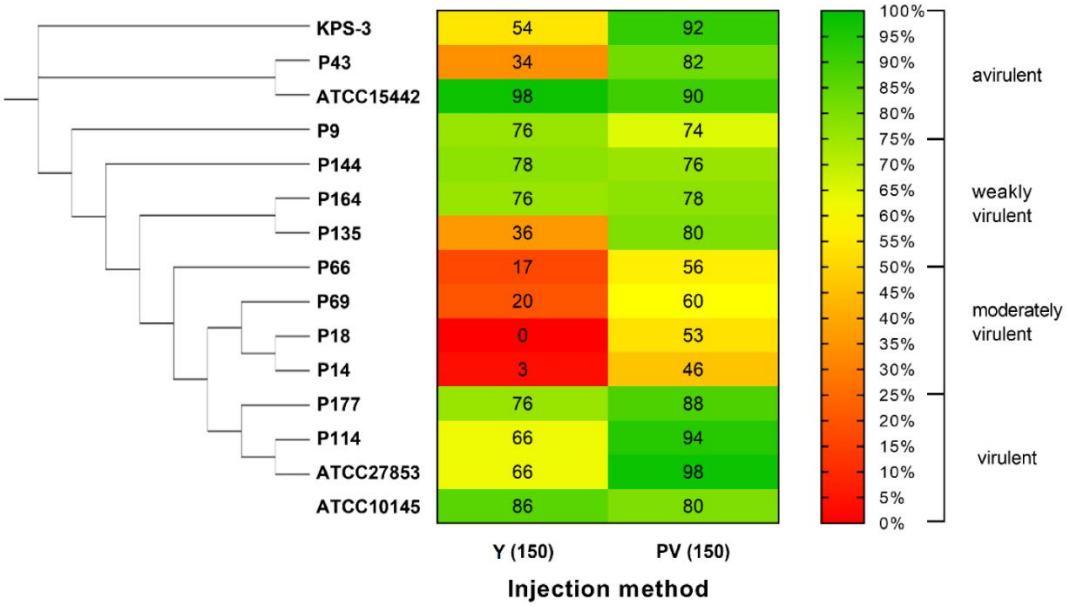

Figure 8. Virulence of the examined environmental and clinical *P. aeruginosa* strains using the combined microinjection virulence model and the spread of the *in vivo* virulence (mortality) across the phylogenetic tree obtained based on the multilocus sequence types (STs) of the examined strains (ATCC10145 was not classified). Y—yolk injection; PV—perivitelline injection. Categories of virulence based on the survival rates (%) of the embryos.

3.2.2. Sublethal symptoms detected at the end of incubation

In addition to the embryo mortality results, we also observed the sublethal symptoms of the surviving embryos at the end of incubation. Although all embryos were treated with the same bacterial species and strains, symptoms were not uniform within the treatment group, and there were individuals in each case that did not show any phenotypic differences compared to the control group. Therefore, sublethal symptoms were not applicable to be a part in the newly developed virulence protocol.

Symptoms mentioned in the literature for bacterial infections of zebrafish larvae were natal sepsis, tachycardia, edema, and vascular leakage. In our study, yolk and pericardial edema were the most significant malformations. In addition, small and not well-defined, common abnormalities such as stunted growth in the head and tail regions were observed, while some larvae also had curved bodies and abnormal blood vessels in the tail. Other embryos could not hatch properly into larvae.

. **Figure 9.** visualizes the main types of malformations detected at the end of the experiment.

Figure 9. Representative phenotypic malformations caused by *P. aeruginosa* after 72 h on zebrafish embryos. (A) Control, (B) pericardial and yolk edema (injected strain: P14), (C) head and tail malformations (injected strain: P132), (D) vascular disorders in the tail region (injected strain: P43), (E) edemas and curved tail (injected strain: P26), (F) hatching disorders (injected strain: P66).
PE: pericardial edema; YE: yolk edema; HM: head malformation; TM: tail malformation; VD: vascular disorder; CT: curved tail. Scale bar: 500 µM. (Photo: Csenki-Bakos Zs.)

3.2.3. Methodological summary of the newly developed, combined virulence model

After completing the optimization experiments and validation studies, the new infection protocol was developed. The methodology can be summarized as follows. Treatments are performed in five replicates in groups of 10 (n=50). *P. aeruginosa* is grown overnight and diluted to the desired optical density (OD_{600}= 0.60 ± 0.02). The *P. aeruginosa* stock suspensions are diluted tenfold to reach the required dilution levels (10^{-1} dilution for PV injections, and 10^{-2} dilution for Y injections). After collecting the eggs of zebrafish, 1-cell stage zebrafish embryos are maintained under controlled laboratory conditions. The microinjections are performed at two specific pathways into the zebrafish embryos using 150 µM (1.77 nL) drop size to the yolk (Y) and the perivitelline space (PV). After the microinjection, the embryos are incubated for 72 hours to allow the infection to develop. Then, mortality is recorded to assess the virulence of the strains.

According to our optimization and validation studies, the flowchart of the comprehensive analysis and evaluation of the combined zebrafish embryo microinjection model of *P. aeruginosa* is shown in **Figure 10.**

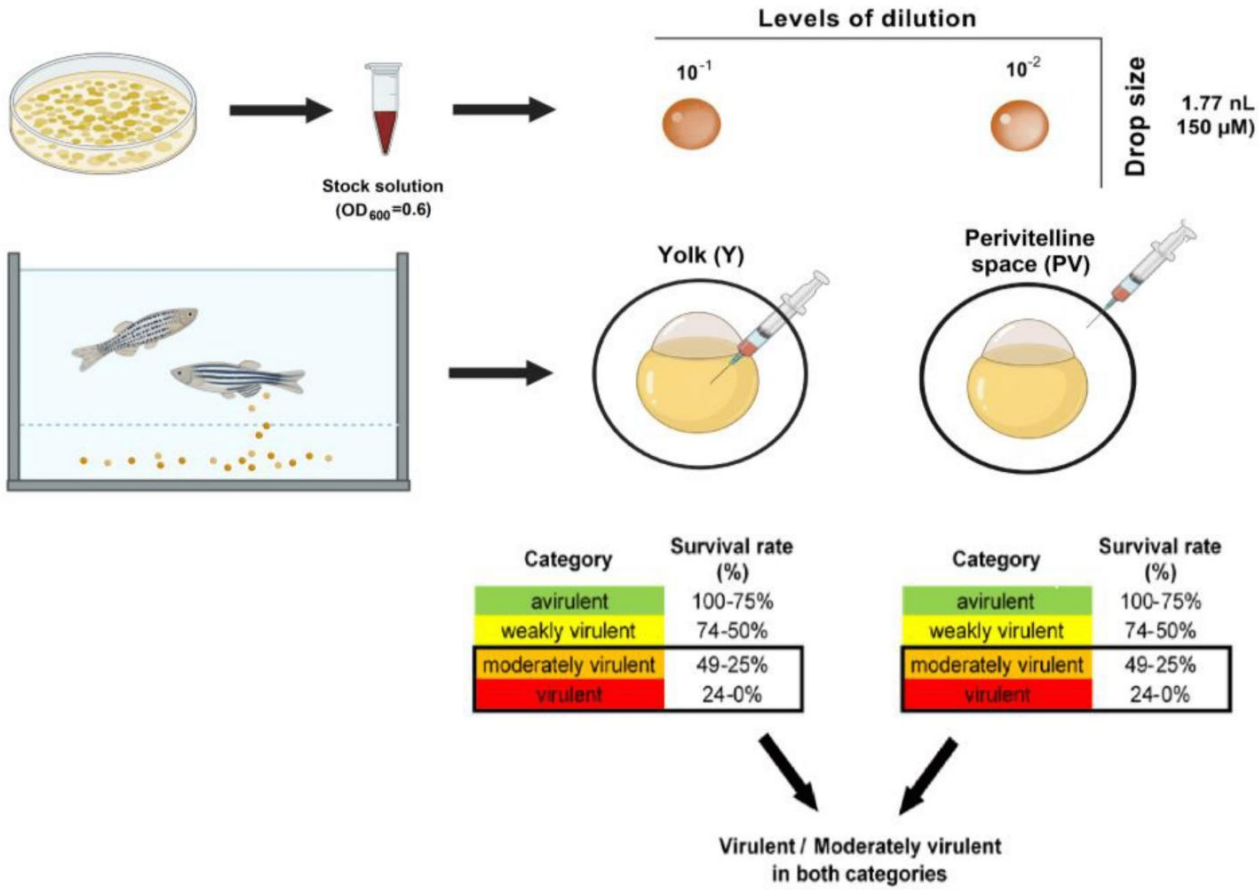

Figure 10. The recommended flowchart of the newly developed virulence infection model using zebrafish larvae to evaluate the virulence of *P. aeruginosa*.

In this newly developed virulence model, conclusions can be drawn regarding the virulence of the tested bacterial strains with categorizing them as avirulent (75–100%), weakly virulent (50-74%), moderately virulent (25-49%), or virulent (0-24%). A *P. aeruginosa* isolate can be evaluated as having high environmental and ecological risks if it demonstrates at least moderate toxicity (with a survival rate of 50% or less) in both PV and Y microinjection routes.

Our results were published in *Antibiotics* (Q1, impact factor: 4.3), 2023.

4. CONCLUSIONS AND RECOMMENDATIONS

In our days, environmental safety in harmed by several factors such as the increasing rate of pesticide use worldwide and the spread of antibiotic resistant, virulent microorganisms such as species *Pseudomonas aeruginosa*. My thesis work aimed to analyze the interactions between pesticide use and antimicrobial resistance and to develop a new virulence model to get a more detailed picture of the environmental hazard of this opportunistic pathogenic species.

Many other studies show the adverse biological effects of glyphosate and GBHs or co-formulants used in GBHs in recent years. We reported for the first time that glyphosate acid and commercially available GBHs (containing a mixture of additives) induce significant, phenotypically detectable, discrepant imipenem resistance in clinical and environmental *P. aeruginosa* strains, while POE(15), a formerly used and banned co-formulant does not affect imipenem sensitivity. Specifically, glyphosate, the active ingredient and the formulated GBHs significantly reduced the susceptibility of *P. aeruginosa* to imipenem. The changes in resistance were measurable as an increase in the MIC values. The resistance to imipenem was found to be concentration-dependent: the higher the concentration of glyphosate or GBHs induced a higher level of resistance of *P. aeruginosa* to imipenem. Moreover, we presume that the development of this resistance may be related to the regulation of oprD porin, or it may be related to the efflux mechanism in this bacterial species. Different GBHs formulations showed variances in the effects on imipenem sensitivity of *P. aeruginosa* that was mainly connected to their cytotoxic features.

Our study provides new insights into how glyphosate and GBHs affect *P. aeruginosa* resistance to imipenem and gives valuable data for understanding the influences of pesticides on the antibiotic resistance of bacteria. It may help prevent the problem of antibiotic resistance caused by environmental pollution. Although this study provides initial insights into how glyphosate affects *P. aeruginosa* resistance, the sample size was limited and focused on specific clinical and environmental strains, and may not be fully representative of strains in other environments. Considering the global use of glyphosate and GBH, as well as the

simultaneous emergence of antibiotic-resistant bacteria in environmental matrices, the detected interactions between these chemicals may affect microbial communities, leading to increasing levels of environmental and human health risks. Exploring the potential mechanisms of this phenomenon is essential to further improve risk management strategies.

Similar to antimicrobial resistance, virulence investigations are also coming into the fore to evaluate the environmental hazard of a given bacterial strain. Methods using zebrafish embryos by microinjection are important for evaluating the virulence of different microorganisms, particularly in assessing nosocomial and environmental isolates. Zebrafish embryos share certain similarities with higher vertebrates. Models take advantage of these immunological similarities and can help study the mechanisms of infection. Although the zebrafish embryo microinjection model offers many advantages, this approach can be difficult in practice.

In our study, the virulence of *P. aeruginosa* was investigated by using a newly developed zebrafish embryo microinjection model using two different infection routes (yolk and perivitelline) to identify the infective and cytotoxic features of the examined microbial strains. Additionally, sublethal effects, after early infection, were determined. From our results, we can see that significant differences can be detected between different types of exposure, even in the case of the same strain. The virulence was generally moderate to high under Y injection, while PV injection resulted in lower virulence compared to Y injections. The parallel application of these infection routes provides a better understanding of the virulence of *P. aeruginosa*.

The main advantages of *in vivo* methods such as microinjection are low budget, no special equipment required and that they are easy to learn. By optimizing the experiment step by step, adjusting the volume and concentration of the microbial suspension, identifying specific biochemical markers, and modifying the duration of observation after injection, we gradually developed an optimal experimental method. The newly developed experimental method can be quickly and easily adapted to other microbial species. Meanwhile, the method enables the virulence characterization of a specific microbial strain within 72 hours which is a short period compared to other vertebrate models. Using only mortality as the endpoint for assessing results is a simple way to evaluate the virulence of a strain. This newly developed method is

particularly suitable for the characterization of microbial strains isolated from the environment.

Altogether, my PhD thesis work is providing novel data on a critically important opportunistic pathogen species in accordance with the One Health approach and with analyzing antimicrobial resistance and virulence, may help to combat *P. aeruginosa* infections in the future.

5. NEW SCIENTIFIC RESULTS

1) It was first detected that both clinical and environmental *Pseudomonas aeruginosa* strains can tolerate the environmentally relevant concentrations of glyphosate and glyphosate-based herbicides; glyphosate treatment was not toxic to *P. aeruginosa* strains within the tested concentration range of 12.5-800 mg/L, and 0.5 v/v% concentration of GBHs did not inhibit the growth of the test organisms. In higher concentrations, GHBs have various effects: 'Total' was proved to be non-toxic to *P. aeruginosa*, while 'Gladiator 480 SL' and 'Roundup Mega' were cytotoxic to the examined strains.
2) During pre-exposition and co-exposition, glyphosate and GBHs have an antagonistic effect on imipenem, and can significantly reduce the phenotypically detectable imipenem susceptibility of *P. aeruginosa* in a concentration dependent manner.
3) A novel zebrafish embryo microinjection protocol was developed and validated that can be used effectively to evaluate the cytotoxic and invasive characteristics of clinical and environmental *P. aeruginosa* strains using parallel infection routes.
4) Yolk microinjections into zebrafish embryos using the same bacterial density and drop size of *Pseudomonas aeruginosa* consistently caused a higher mortality rate compared to perivitelline injections.
5) Perivitellinar and yolk injection of environmental and clinical strains of *P. aeruginosa* into zebrafish larvae can cause various sublethal symptoms and abnormalities, which were described here for the first time, such as yolk, and pericardial edema, stunted growth in the head and tail regions, curved body, abnormal blood vessels in the tail and hatching anomalies.

6. PUBLICATIONS

1) **Jiang, Dongze**; Yi, Yang; Cserháti, Mátyás; Kriszt, Balázs; Kaszab, Edit (2025): OxiTop microcosm model as a possible tool to study the effect of antibiotic exposure on the microbial community. *HUNGARIAN JOURNAL OF HYDROLOGY (HIDROLÓGIAI KÖZLÖNY)*, full-length research paper accepted for publication.

2) **Jiang, Dongze**; Kaszab, Edit; Szoboszlay, Sándor (2021). The evaluation of pesticides on opportunistic *Pseudomonas aeruginosa* and its antibiotic resistance. In: Hosam, E.A.F. Bayoumi Hamuda (ed.) Proceedings Book "Environmental Quality and Public Health": The Vth International Symposium-2021. Budapest, 304-313.

3) Kaszab, Edit†; **Jiang, Dongze**†; Szabó, István; Kriszt, Balázs✉; Urbányi, Béla; Szoboszlay, Sándor; Sebők, Rózsa; Bock, Illés; Csenki-Bakos, Zsolt (2023): Evaluating the *in vivo* virulence of environmental *Pseudomonas aeruginosa* using microinjection model of zebrafish (*Danio rerio*). *ANTIBIOTICS*, 12: 1740.
Citations: 2, Independent citations: 2
Quartile: Q1, IF: 4.3

4) Háhn, Judit†; Kriszt, Balázs†; Tóth, Gergő; **Jiang, Dongze;** Fekete, Márton; Szabó, István; Göbölös, Balázs; Urbányi, Béla; Szoboszlay, Sándor†; Kaszab, Edit†,✉ (2022): Glyphosate and glyphosate-based herbicides (GBHs) induce phenotypic imipenem resistance in *Pseudomonas aeruginosa*. *SCIENTIFIC REPORTS*, 12(1): 18258.
Citations: 7, Independent citations: 7
Quartile: Q1/D1, IF: 4.493

† these authors contributed equally to this work

✉corresponding author