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

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AMPA	Aminomethylphosphonic Acid
AMR	Antimicrobial Resistance
BSE	Bovine Spongiform Encephalopathy
CAS	Chemical Abstract Service
CDC	Centers for Disease Control and Prevention
CF	Cystic Fibrosis
COPD	Chronic Obstructive Pulmonary Disease
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EPSPS	5-Enolpyruvylshikimate-3-Phosphate Synthase
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FICI	Fractional Inhibitory Concentration Index
GBHs	Glyphosate-Based Herbicides
GLY	Glyphosate
HIV	Human Immunodeficiency Virus
ICU	Intensive Care Unit
IUPAC	International Union of Pure and Applied Chemistry
LB	Luria-Bertani
MIC	Minimal Inhibitory Concentration
MLST	Multilocus Sequence Typing
MSDS	Material Data Safety Sheet
MRL	Maximum Residue Limit
NCAIM	National Collection of Agricultural and Industrial Microorganisms
NIOSH	National Institute for Occupational Safety and Health
PCR	Polymerase Chain Reaction
PDR	Pan Drug Resistant
POE(15)	Polyethoxylated tallow amine (with 15 ethylene oxide units)
QS	Quorum Sensing
RSV	Respiratory Syncytial Virus
STEC	Shiga Toxin-Producing *Escherichia Coli*
ST	Sequence Type (In MLST)
XDR	Extensively Drug Resistant

1 INTRODUCTION

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's immune system is deficient or weak.

P. aeruginosa strains have been known to be highly pathogenic in clinical environments for more than 20 years (ALIAGA et al., 2002; THADEN et al., 2017). At the same time, isolates originating from the natural environment have often been considered as "non-pathogenic" because of the lacking virulence-related genes (JOSE et al., 2018) or their non-hemolytic phenotypes (RADHAPRIYA et al., 2015) . To examine the virulence of environmental *P. aeruginosa*, *in vivo* virulence models still play an important role; therefore, various infection models have been created and improved in the last decades, using evolutionary divergent host species (WOOD et al., 2023) . Among them, zebrafish (*Danio rerio*) became an important vertebrate animal model to study host-pathogen interactions and infectious diseases based on the similarity of innate immunity between zebrafish and humans and the optical transparency of the zebrafish embryos, which enables their easy observation (LLAMAS & VAN DER SAR, 2014; MEIJER & SPAINK, 2011). 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, a novel infection model was required that combines different zebrafish embryo microinjection methods. The new model could not only test the effect of environmental *P. aeruginosa* early after infection, but in the future, it can be us to examine 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 (STOVER et al., 2000). 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 to different antibiotics.

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 (QIU et al., 2022), as it was verified in the case of glyphosate (GLY), a widely used herbicide and its formulations: glyphosate salt and glyphosate-based herbicides (GBHs) on *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (KURENBACH et al., 2015; WHITEHEAD et al., 2011). There is evidence that *P. aeruginosa* can tolerate (RAOULT et al., 2021) and degrade (HOODAJI et al., 2012) GLY, but there is 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, this research work aimed 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' exposure 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 of different glyphosate salts/additives and 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.

1.1. Research objectives

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

1.1.2. The reveal of the relationship (antagonism, synergism) between the examined GBHs (formed by different glyphosate salts and additives) with imipenem.

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

2 LITERATURE REVIEW

2.1 Microorganisms in the environment

Microorganisms play a critically important role in nature and in the biogeochemical cycles due to their wide distribution and high diversity (MENG et al., 2022). Certain microorganisms are involved in the environmental cycling and transformation of elements such as carbon, nitrogen, and phosphorus, promoting the flow of these nutrients in the ecosystem (GUPTA et al., 2017). Moreover, microbial species significantly contribute in processes such as waste treatment and biological degradation, detoxification (MAGLIONE et al., 2024). Microorganisms can decompose and transform various organic and inorganic pollutants in an eco-friendly way, helping to maintain the health and balance of the environment (KUPPAN et al., 2024). Furthermore, microorganisms are essential for maintaining the stability of the food web: as producers, consumers, and decomposers, they are involved in the transfer and transformation of energy and matter (GUPTA et al., 2017). Overall, they are an important foundation for the normal operation of the Earth's ecosystem.

2.2 Microorganisms and human health

There are complex interactions between microorganisms and other living organisms, as microbial species can colonize the internal or external surfaces of plants and animals. The type of relationship can be intraspecific or interspecific and can range from short-term, simple to long-term, elaborate interactions (MOËNNE-LOCCOZ et al., 2014).

In terms of human health, hosts and their microbiota constitute a dynamic system (RACKAITYTE & LYNCH, 2020). The most abundant microorganisms in the human body with significant effects are bacteria, viruses, fungi, and protists (ALBERTS et al., 2002) . Many microorganisms are harmless (e.g., commensals) or even beneficial to humans (e.g., symbionts), but others are potentially harmful, and in some cases are pathogenic to the host (ZHOU et al., 2009). Microbial infection occurs when microorganisms invade a susceptible host, multiply within the body and cause damage (disease) by disrupting the normal physiological functions

(ZACHARY, 2017) . Microbial infection can bring disease and illness. At the beginning of the 21st century, infectious diseases still accounted for about a quarter of global deaths (FENOLLAR & MEDIANNIKOV, 2018) . Microbial infections are often caused by emerging pathogens, where the causative agents of the infectious diseases are showing an increasing number of incidences (DA SILVA & SKOTNES-BROWN, 2023; WOOLHOUSE et al., 2005).

2.3 Pathogen microorganisms

Pathogens, including bacteria, viruses, fungi, protozoa, worms, and infectious proteins called prions are organisms that can cause a disease in a host (ALBERTS et al., 2002). They often have specific mechanisms to interact with their hosts to achieve colonization, survival, reproduction, and spread. For example, bacterial pathogens may carry a set of virulence genes encoding virulence factors that interact directly with the host cells; viruses rely on the mechanisms of the host cell to replicate, while fungi and protozoan parasites have complex life cycles which is hard to combat (ALBERTS et al., 2002). The human body contains a few thousands of microbial species at the same time and a part of them are not members of the healthy microbiome (GILBERT et al., 2018; ROSENBERG, 2024). When the body's immune capacity is reduced, these pathogens can find adequate circumstances to survive and reproduce. In the last decades, at least 50 emerging pathogens have been identified, of which about 10% are bacterial pathogens (ALBERTS et al., 2002).

2.3.1.1 Bacteria and human health

Members of the *Bacteria* domain are the most common types of microorganisms with human health relevance. Bacteria are prokaryotes and simple in structure compared to the eukaryotic cells (ALBERTS et al., 2002). They are ubiquitous in nature (SONI et al., 2024), and many species have great importance in human and environmental health.

Pathogenic bacteria are harmful bacterial species responsible for the majority of waterborne and foodborne diseases (ANELICH, 2014). Depending on the exposition pathway, bacterial pathogens can cause various types of infectious diseases.

In case of inhalation, a possible outcome of the infection is pneumonia which means the largest threat to children worldwide that killed over 808,000 children under the age of 5 in 2017

(HTTP1). A major causative agent of pulmonary infections is the Gram-positive bacterial species *Streptococcus pneumoniae* (MASCINI & WILLEMS, 2010).

Oral exposition usually happens when contaminated food or water is consumed. *Escherichia coli*, a Gram-negative bacterium that is commonly found in the gastrointestinal tract of humans and animals, have a great importance with this exposition route. Most *E. coli* serotypes are harmless, but some strains can be pathogenic, such as Shiga toxin-producing *E. coli* (STEC) responsible for severe diarrheal diseases. The host of this pathogen group appears to be primarily cattle and according to the WHO, one of the most important STEC serotype, *E. coli* O157: H7 is mainly transmitted to humans through the consumption of contaminated food (HTTP2).

Dermal contact through healthy or injured skin is another important exposition pathway that is used by bacterial species like *Clostridium tetani,* a Gram-positive anaerobic bacterium causing the acute infectious disease called tetanus (HTTP3).

2.3.1.2 The transmission of microbial pathogens in the environment

According to the scientific literature, microorganisms have different routes of transmission to reach susceptible hosts in natural settings (ANDERSEN, 2019; HTTP4) . One of the most direct forms of microbial transmission is through direct contact, such as physical contact between people. In the environment, microorganisms can originate from a wide range of sources, including soil, air, and water where some of them do not just survive, but proliferate for a period of time. Therefore, these environmental elements can act as the reservoirs of pathogens and play a significant role in the chain of microbial infections **(Figure 1.**).

Water is one of the most important media in the spread of infectious bacterial species. The degree to which the human body may be affected by waterborne microorganisms varies from mild to fatal, depending on the route of exposure (ingestion, inhalation, contact) and the dose of the infectious agent (LUNN et al., 2019). Most waterborne pathogens, like bacterial species responsible for gastrointestinal infections (e.g., *Vibrio cholerae*), originate from human or animal feces, can be transmitted to humans through contaminated drinking water, or recreational water (HTTP4) and may cause infectious diseases (MARA & HORAN, 2003). The typical diseases caused by waterborne bacterial pathogens are diarrhea, dysentery and typhoid fever (HTTP4).

Airborne transmission, including droplets and aerosols (HERFST et al., 2017), is responsible

for respiratory infections caused by pathogens such as *Pneumococcus* spp. (LI et al., 2018). Food can also be a vehicle for microbial transmission, as contaminated food can carry a variety of microorganisms such as *E. coli, Salmonella, Listeria, Shigella, Campylobacter* (GORBACH, 1996).

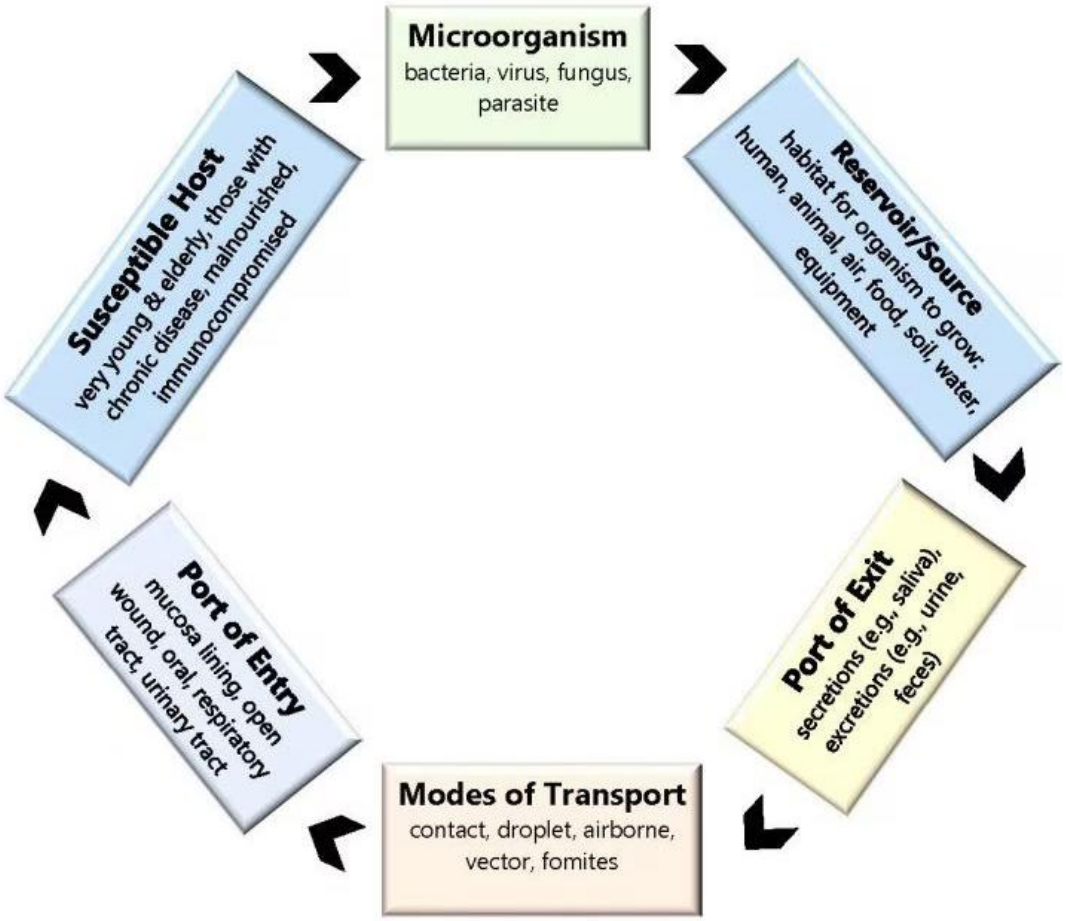

Figure 1. Chain of microbial infections (HTTP5).

Several microorganisms can survive on biotic and abiotic environmental surfaces and equipment and can be indirectly transmitted to humans (KRAMER & ASSADIAN, 2014). For example, surface contamination in hospital environments can lead to the spread of drug-resistant bacteria (e.g., multidrug-resistant Gram-negative rods and methicillin-resistant *Staphylococcus aureus*) (CHEMALY et al., 2014). Other infection routes of microbes are the transmission through wounds, or by vectors, like insects or animals (CAMINADE et al., 2019).

2.4 Virulence of environmental microorganisms

Virulence, namely the ability of a microorganism to cause disease in a host can vary significantly both within a single bacterial species and between different bacterial species due to the genetic diversity, environmental factors, and host interactions (CASADEVALL & PIROFSKI, 1999). Virulence is determined by various factors, including the microorganism's capacity to invade host tissues, adhere to biotic surfaces, evade the immune response, or produce toxins (FINLAY & FALKOW, 1997). The study of virulence is critical for understanding infectious diseases and developing strategies for their prevention and control. Virulence is usually associated with clinical pathogens, but it is also a significant trait among environmental microorganisms. Understanding the virulence of environmental microorganisms is crucial for public health, particularly with the increasing occurrence of opportunistic pathogen infections. Opportunistic pathogens are microorganisms that usually do not infect healthy hosts, but only immunocompromised patients and as commensals and they often originate from the environment (MARTÍNEZ, 2014). The best studied, environmentally transmitted opportunistic pathogens are species *Pseudomonas aeruginosa* and *Burkholderia cepacia* (BROWN et al., 2012).

2.4.1.1 The role of *in vivo* models in virulence investigations of bacteria

Several molecular and phenotypic tests are used to determine the presence or absence of virulence traits (e.g., virulence genes) of bacterial strains (HUANG et al., 2019), but due to the complexity of virulence mechanisms, the results of these individual assays are not necessarily correlated with the ability of the organism to cause disease (KASZAB et al., 2021). To clarify the actual virulence of environmental strains, *in vivo* virulence models can be used. Among these model systems, mammalian test organisms, such as rodents, were the first choice, but due to ethical constrains, non-mammalian models came to the fore. Nematodes, fruit flies (*Drosophila*) and wax moth larvae (*Galleria mellonella*) were the first widely used invertebrate model organisms. Non-mammalian vertebrates like fish and amphibians, with adaptive immunity and complement systems, are more recent models in the research of infectious diseases caused by bacteria (O'CALLAGHAN & VERGUNST, 2010). These models offer cost and ethical benefits however, they have their limitations: due to their evolutionary distance from human pathology,

they cannot address all specific research questions effectively. In the upcoming chapter, zebrafish, a commonly used model organism will be introduced in details including the advantages and disadvantages compared to other test organisms.

2.4.1.2 Zebrafish as a model organism

Zebrafish (*Danio rerio*) (**Figure 2.**) is a freshwater fish that is widely used in scientific research. It is one of the most important vertebrate animal models to study host-pathogen interactions and infectious diseases (LLAMAS & VAN DER SAR, 2014) . In the natural environment, zebrafish is native to the Indian subcontinent of South Asia, including India, Nepal, and Bangladesh (TEAME et al., 2019) . There are many advantages of using zebrafish as an experimental model: it is relatively small (3-4 cm), reaches sexual maturity within 2-3 months, and has high efficiency in reproduction (FINLEY & ZON, 2004). Zebrafish share a high genetic similarity with humans and have further advantages as a model organism: the embryos are almost transparent and 82% of genes associated with human disease have at least one zebrafish orthologue (HOWE et al., 2013) . Meanwhile, zebrafish are easy to genetically modify, and compared to other animal models, their maintenance is relatively cheap (DRIEVER et al., 1996; MACRAE & PETERSON, 2015).

Figure 2. Adult male and female zebrafish (*Danio rerio*) (TEAME et al., 2019).

Due to its unique biological characteristics, zebrafish have become an important model organism in genetic research, developmental biology, disease modelling, toxicology, and drug screening studies (DOOLEY, 2000; HOWE et al., 2013; MACRAE & PETERSON, 2015; SANTORIELLO & ZON, 2012; TEAME et al., 2019).

Examining the bacterial virulence in zebrafish larvae is a promising option because, based on European legislation, zebrafish larvae models are not regulated for experimental use before the stage of independent feeding (HTTP6).

Microorganisms can be tested to infect zebrafish embryos using different exposure pathways, such as immersion or microinjection as it is summarized in **Figure 3**.

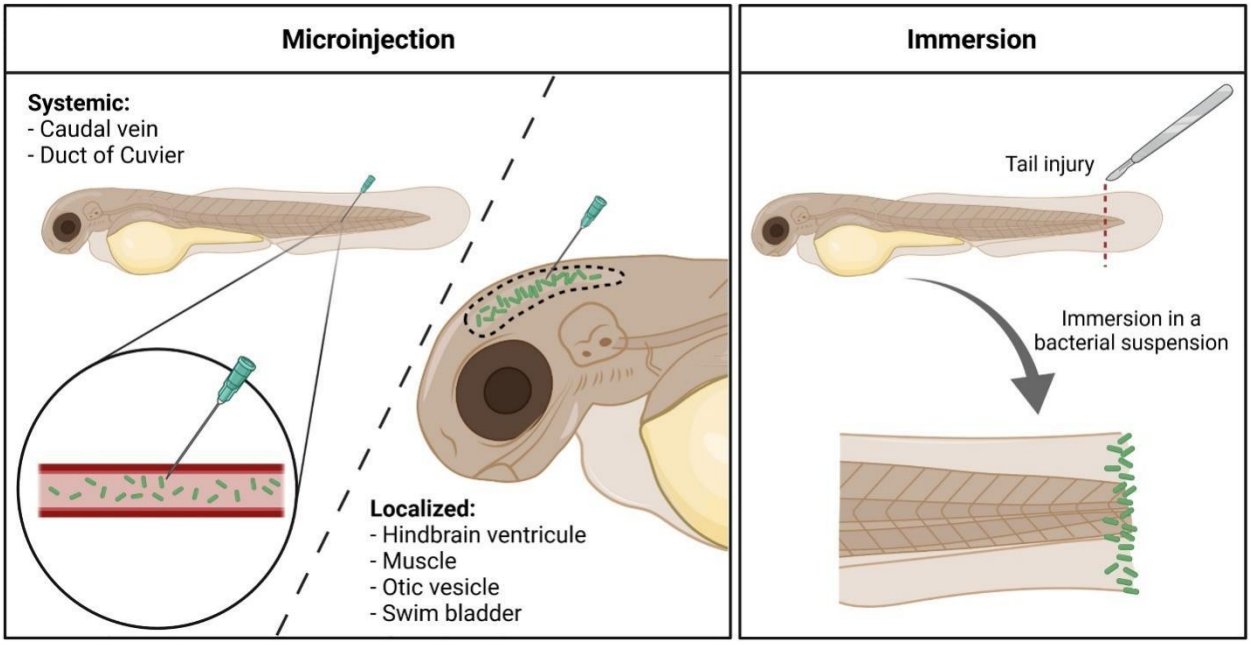

Figure 3. Possible infection routes in zebrafish (*Danio rerio*) embryo (PONT & BLANC-POTARD, 2021).

By the microinjection method, which is a common experimental protocol used in *in vivo* model studies, pathogen microorganisms can be artificially introduced to the test organism to simulate the infection process (pathogenesis) in the host body. In most cases, the tested microorganism can be injected into the caudal vein, the Duct of Cuvier, the hindbrain ventricle, the tail muscle, the notochord, or the otic vesicle. These procedures are usually performed during the first 3 days of embryonic development (BENARD et al., 2012). In other cases, if the growth rate of the bacteria is very slow, the microbe can be injected into the yolk of the embryo at the stage of 1-1024 cells (CARVALHO et al., 2011). Zebrafish microinjection can be used not only

to observe lethal outcomes but also to detect sub-lethal symptoms (CSENKI et al., 2019), providing a more comprehensive insight into complex host-pathogen interactions. However, except for injections in the early embryonic stage, these techniques require a lot of practice to perform and are relatively slow (NOGARET et al., 2021). Another disadvantage of zebrafish embryo microinjection is the direct introduction of microorganisms into the embryo. This direct inoculation disables to determine whether the infection becomes systematic and induces death or remains local (PONT & BLANC-POTARD, 2021). As an alternative, bath immersion (DÍAZ-PASCUAL et al., 2017; PONT & BLANC-POTARD, 2021; ROWE et al., 2014) was developed, which mimics the natural infection path, easier to perform, and has been further developed into a wound infection model using embryos with tail injuries (NOGARET et al., 2021; POPLIMONT et al., 2020). Although many different routes of infection have been used for virulence testing, these techniques still do not provide information about both the invasive and cytotoxic characteristics of a given bacterial strain. Therefore, to fully assess the complex virulence of our target organism, species *P. aeruginosa* and predict its potential risk to environmental health, more refined *in vivo* experimental methods need to be developed.

However, virulence is just one factor affecting the hazard of bacterial infections originating from the environment. Another significant problem is the spread of antimicrobial resistance mechanisms in clinical and non-clinical compartments, including the natural and built environment which means one of the gravest threats to human health leading to the spread of uncurable infections. In the upcoming sections antimicrobial resistance and its relevance to the thesis research is presented.

2.5 Antibiotics and antimicrobial resistance

2.5.1.1 The history of antibiotics

In 1941, Selman Waxman first used the term “antibiotic” as a noun to describe any small molecule produced by a microbe that inhibits the growth of other microbes (CLARDY et al., 2009). In our days, antimicrobials, a wider term is commonly used to describe medicines used for the treatment of various infections in humans, animals, and plants. Antimicrobials include several subsets, like antibiotics that are used to treat infections caused by bacteria, antivirals that

could treat viral infections, antifungals that can be used in the case of infections induced by fungi, and antiparasitics to treat parasite infections HTTP7).

The first antibiotic, penicillin, was accidentally discovered in 1928 by a Scottish scientist, Alexander Fleming, who observed that *Penicillium notatum* produces a substance capable of inhibiting the growth of *Staphylococcus* bacteria on a culture plate. After a series of examinations, the first antibiotic was described and named as penicillin (FLEMING, 1980) and later it was introduced to treat bacterial infections (CHAIN et al., 1940). The mechanism of action in the case of penicillin is mainly to play a bactericidal role by interfering with the synthesis of bacterial cell walls (LOBANOVSKA & PILLA, 2017).

Although the newly developed antibiotics, such as penicillin, were very effective in treating bacterial infections, in the past decades, due to overly intensive and inappropriate use, some bacteria have gradually developed non-susceptibility, leading to the occurrence of a worldwide phenomenon, antimicrobial resistance (AMR) (HUTCHINGS et al., 2019).

When the same antimicrobials start to be less efficient or do not work anymore with the same concentration on the same targets as before, it means the target developed antimicrobial resistance. Antimicrobial resistance is a natural process, but it is an emerging issue, and can be considered as an "invisible pollution" worldwide. Moreover, while the number of microorganisms developing antibiotic resistance continues to rise, the discovery of new antimicrobials has declined, making antimicrobial resistance one of the most serious threats to human health (MAY, 2014; O'NEILL, 2016).

2.5.1.2 The history of antimicrobial resistance

Antibiotic-resistance genes have been known to exist in nature long before antibiotics were developed (AMINOV & MACKIE, 2007) . The first documented case of AMR systematically studied was penicillin resistance, in 1940, only a few years after penicillin became widely available. Ernest Chain, Howard Florey, and their co-workers discovered that some *Staphylococcus aureus* strains were resistant to penicillin and discovered an enzyme (beta-lactamase or penicillinase) capable of destroying the antibiotic's molecule (ABRAHAM & CHAIN, 1940). Penicillin was mass-produced and sold in the market in 1945 (AMINOV, 2010). In the same year, Fleming himself warned in his Nobel lecture about the need to be vigilant

about the use of penicillin to prevent bacteria from developing resistance. He mentioned that a patient was given a low dose of penicillin, but it failed to kill the infection, so instead of curing the disease, the bacteria developed resistance (HTTP8).

The earliest antibiotic used in hospitals was Pyocyanase, extracted by Emmerich and Löw in 1899 from *P. aeruginosa* (formerly known as *Bacillus pyocyaneus*) (AMINOV, 2010) . Emmerich and Löw found that the bacteria and their extracts were effective against many pathogenic bacteria and tried to use them to treat various infections. However, this approach was eventually dropped due to inconsistent treatment results and the toxicity of the extracts to humans. Later, further studies confirmed that *P. aeruginosa* produced antibiotic substances (AMINOV, 2010; HAYS et al., 1945).

More examples can be seen in **Figure 4.** below, summarizing the identification timeline of antibiotic resistance compared with the introduction time of corresponding antibiotics. The left side lists the year when resistance was first identified and the corresponding bacterial species, while the right side lists the year when antibiotics were introduced. The “gold era” in the development of antibiotics was from the 1940s to the 1980s. The significant increase and spread in antibiotic resistance began within a few years after the introduction of each novel type of antibiotic (VENTOLA, 2015).

The emergence and spread of resistance have several implications for public health and clinical treatment. Drug-resistant bacterial infections lead to treatment failure, prolonged disease duration, increased medical costs, and increased mortality. About 25,000 patients in the EU die from an infection caused by a multidrug-resistant bacteria each year (ECDC, 2009). Globally, in 2019, the estimated number of AMR-related casualties reached 1.2 million, while by 2050, the predicted annual death attributed to AMR is approximately 10 million (AHMED et al., 2024). If we analyze the global distribution of AMR, it can be seen that in the same region, the frequency of antimicrobial resistance is inversely correlated with the income levels: regions with lower income tend to experience higher rates of AMR and its associated impacts which further increase the consequence of AMR on global health (HTTP7).

Improving this situation is a huge challenge, and it requires not only government policy support, academics, and experts, but also public response and collective efforts to tackle the problem of antibiotic resistance (AMINOV, 2010).

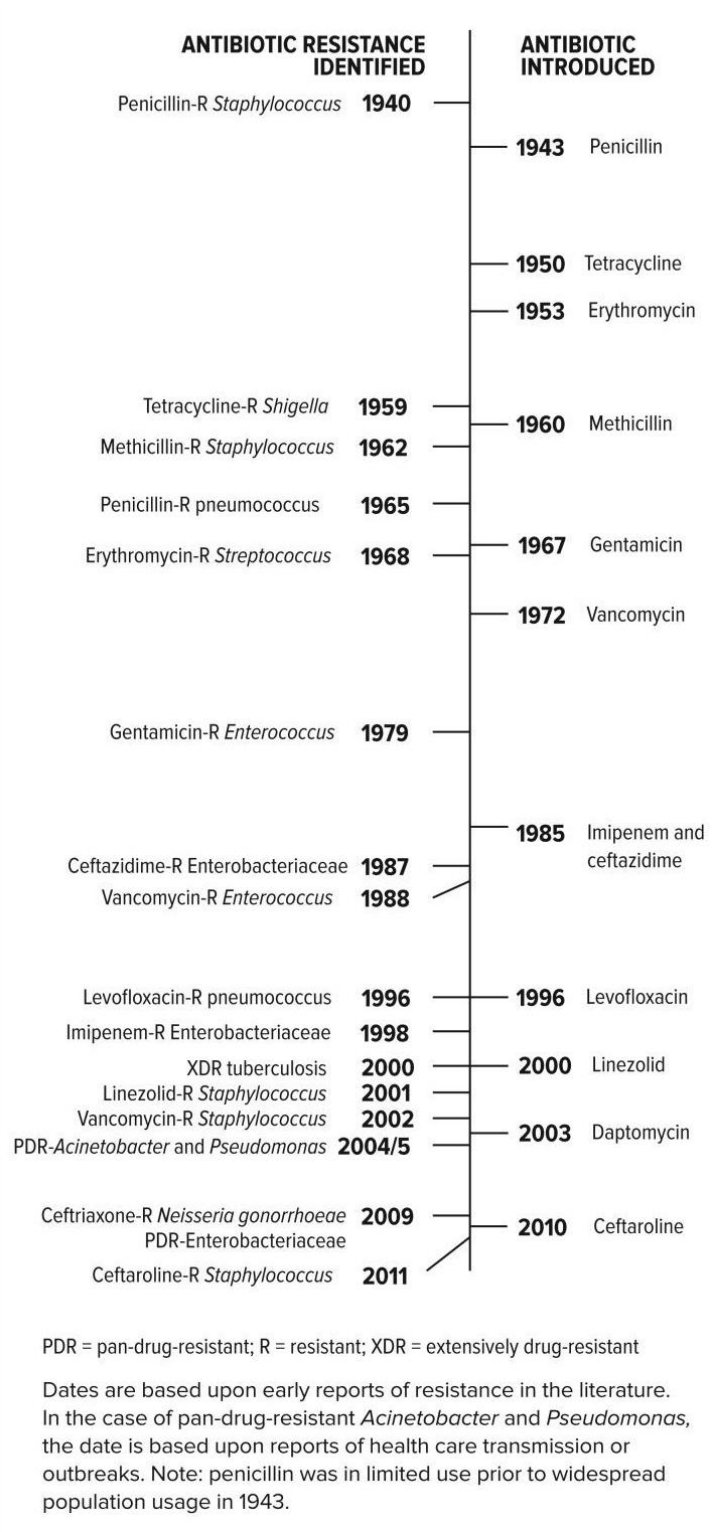

Figure 4. The development of antibiotics and antimicrobial resistance (VENTOLA, 2015) .

2.5.1.3 Drivers of antimicrobial resistance

In clinical settings, antimicrobial resistance is mainly triggered by the overly intensive use of antibiotics (MUTEEB et al, 2023). According to KLEIN et al. (2018), in the period between

2000 and 2015, the total amount of antibiotics used for human therapy increased to 10.3 billion defined daily doses in high income countries showing the increasing trend of antimicrobial consumption. But following the "One Health" approach, the clinical environment cannot be discussed without the careful analysis of animal and environmental health (MACKENZIE & JEGGO, 2019).

In livestock, antibiotic use is also a significant factor: in 2013, the estimated volume of antimicrobials used for food animals was approximately 131,000 tons (VAN BOECKEL et al., 2017). In the environment, several factors have been described as potential drivers of antimicrobial resistance, such as biocide application, heavy metal contamination or the spread of antibiotic resistance genes. The environmental drivers and hot-spots, based on SINGER et al. (2016), are visualized in **Figure 5**.

Similar to clinical compartments, the key environmental drivers of AMR include the discharge of antibiotics, resistant bacteria, and resistance genes, but they can be introduced into natural ecosystems not only from humans, but also from agricultural and pharmaceutical sources. Wastewater treatment plants, agricultural runoff, and effluents from pharmaceutical manufacturing are also major contributors.

Moreover, various environmental pollutants can create selective pressure that promotes the survival and proliferation of resistant microorganisms.

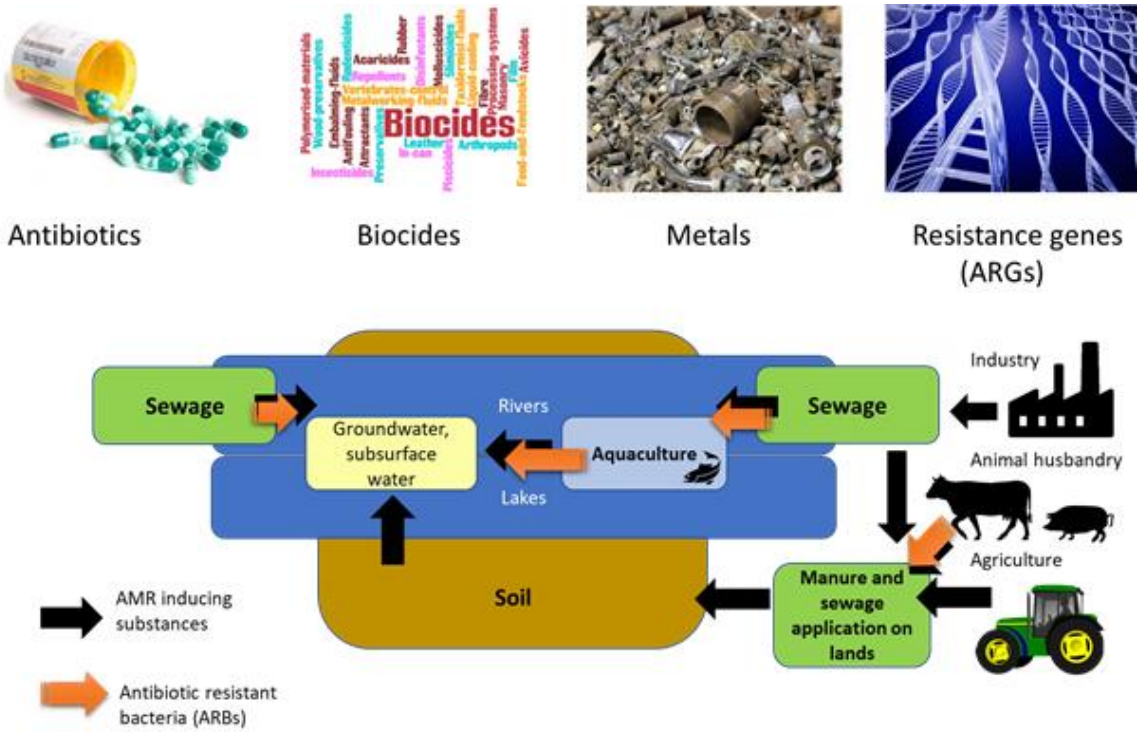

Figure 5. Environmental drivers and hot-spots of antimicrobial resistance (based on SINGER et al., 2016)

SAMREEN et al. (2021) noted that besides the previously revealed factors, herbicides, various pharmaceuticals and pesticides can also lead to the selection of resistant microorganisms, which means a serious threat to natural environments. Therefore, testing the effect of the widely used pesticides, such as the globally distributed glyphosate, on AMR is becoming increasingly important in terms of antimicrobial resistance. In the upcoming section, the significance and the potential role of glyphosate, a commonly used pesticide, on environmental safety, were summarized.

2.6 Glyphosate and glyphosate-based herbicides (GBHs)

2.6.1.1 The brief history and introduction

Glyphosate (CAS number: 1071-83-6; IUPAC name: N-(phosphonomethyl)glycine; Chemical formula: $C_3H_8NPO_5$) (**Figure 6**.) was first synthesized in 1950, but it did not raise attention in the first ten years. After the molecule was transferred to another laboratory (Aldrich Chemical Co.), Monsanto Company re-discovered it during their investigation associated with aminomethylphosphonic acid (AMPA) (SZÉKÁCS & DARVAS, 2012). It was patented in 1971 (HTTP9). The principle of glyphosate herbicidal action is to inhibit the activity of the enzyme named 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in plants, which is involved in the plant's nitrogen metabolism process. By inhibiting the activity of this enzyme, glyphosate disrupts the biosynthesis of aromatic amino acids in plants, leading to abnormal growth and eventual death (ORCARAY et al., 2010).

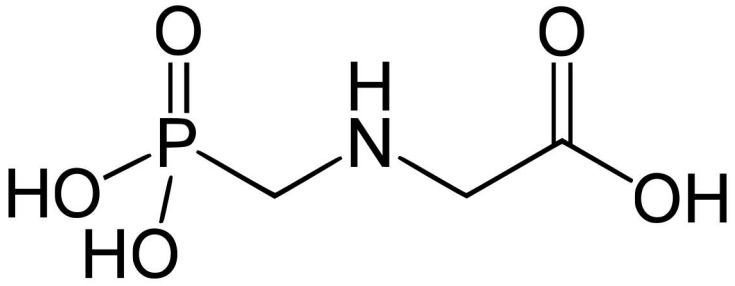

Figure 6. The chemical structure of glyphosate (HTTP10)

Glyphosate is an active ingredient that can be effectively used against weeds; it has become

one of the most popular ingredients of herbicides since the 1990s because of its high effectiveness. Another advantage was that there have been only a few reports proving that glyphosate would be toxic to animals; moreover, once glyphosate is spread into the natural environment, it quickly deactivates. In recent decades, the usage of glyphosate has been rapidly increasing due to the expansion of crop cultivation, leading to the gradual rise of weeds with resistance to glyphosate (HELANDER et al., 2012).

The previous conclusion, that glyphosate and glyphosate-based herbicides (GBHs) pose no risk to the natural environment and humans (WILLIAMS et al., 2000), has been continuously challenged by researchers with different opinions over the past 30 years. Based on the reports from the European Food Safety Authority (EFSA) and the Environmental Protection Agency (EPA), glyphosate and its metabolite aminomethylphosphonic acid (AMPA, CH_6NPO_3) may be a threat to the natural environment and humans. According to the scientific evidence, they could cause cancer, be toxic to the liver cells, damage the DNA of human beings, and act as endocrine disruptors (GASNIER et al., 2009).

2.6.1.2 The introduction of glyphosate-based herbicides and their additives

Herbicides are usually commercially available as formulated compounds, containing not only the active ingredient, but several additives and co-formulants as well. The active ingredient in GBHs is the glyphosate, and the additive could be various formulations. The main aim of using additives in herbicides is to enhance their efficacy and to improve absorption. Among these additives, polyethoxylated tallow amine (POEA) compounds are commonly used as surfactants in GBHs. POE-15 is a specific form of POEA that contains 15 ethoxylate units. This compound was widely used in formulations, but played a more important role in the adverse effects of GBHs than previously assumed. In the study by MESNAGE et al. (2013) POE-15 showed stronger toxic effects on human cells compared to glyphosate alone. The researchers observed that the degree of toxicity was more closely linked to the amount of POE-15 present, rather than the glyphosate content, suggesting that the so-called “inert” additives may in fact be key contributors to the overall toxicity of GBHs (MESNAGE et al., 2013).

2.6.1.3 The usage of glyphosate and glyphosate-based herbicides

To protect and promote agricultural products, the herbicides applied in agriculture and horticulture increased continuously in terms of variety and quantity. Glyphosate-based herbicides are the most widely used herbicides in the world (GARCÍA et al., 2022). According to the data from the Food and Agriculture Organization (FAO), the global application of herbicides was 1.50 million tons in 2011 and increased by 21% to reach 1.94 million tons in 2022 (HTTP11), however, the actual amount of global herbicide usage is estimated to be triple than the data collected by FAO (MAGGI et al., 2019). The overproduction and overly intensive use of glyphosate-based herbicides (GBHs) has become a severe problem. Only in the United States of America (USA) over 1.6 billion kilograms of glyphosate-active ingredients have been used from 1974 to 2016, which covers 19% of the estimated global glyphosate-active ingredients' usage (BENBROOK, 2016). It was reported that the usage of GBH has increased sharply after the genetically engineered glyphosate-tolerant crops were introduced in 1996. Until 2019, the annual usage of glyphosate-based herbicides had reached 700,000 tons (TANG et al., 2019) and in our days more than 2000 GBHs are available on the global market (KLÁTYIK et al., 2023).

Despite the concerning trends, the globally published data on glyphosate and GBH use are still relatively scarce. However, with increased attention to environmental monitoring and increased regulation, public data on glyphosate use are continuously improving (BENBROOK, 2016).

2.6.1.4 Glyphosate and glyphosate-based herbicides (GBHs) in the environment

Glyphosate performs well in water solubility (11.6 g/L at 25°C) (SZÉKÁCS & DARVAS, 2012). Therefore, according to scientific reports, glyphosate and GBH residues can be found in the water and soil in the natural environment (HAYNES et al., 2000). Moreover, glyphosate and GBH residues (such as AMPA) are the most common pesticides that are found in European soils. SILVA et al. (2019) found that less than 1/5 of the tested European agricultural topsoil remains uncontaminated by glyphosate; the highest concentration was 2.05 mg/kg. Another research performed in Brazil revealed, that after glyphosate treatment on agricultural lands, the glyphosate concentration in runoff water can be measured between 1.24 and 6.1 mg/L (LIMA et al., 2023). It is important to note that glyphosate and GBH residues found in the environment

rarely occur as solo contaminants; they are often present alongside with other pesticides and their metabolites. According to SILVA et al. (2019), the most common combinations detected in the tested topsoil were glyphosate with AMPA and glyphosate with AMPA and phthalimide.

Besides the acute and chronic biological effects of glyphosate and GBHs, such as the recently revealed cytotoxic, carcinogenic, teratogenic, and endocrine-disrupting effects (MEFTAUL et al., 2020; TÓTH et al., 2020; VAN BRUGGEN et al., 2018), researchers recently expanded to include its role in fostering antibiotic resistance (DA COSTA et al., 2022; LIAO et al., 2021). The investigations into the effects of glyphosate and GBHs on antibiotic resistance are concerning not only at the microbial ecosystem level but also on specific bacteria such as *E. coli* (STAUB et al., 2012) and *Salmonella* spp. (KURENBACH et al., 2015, KURENBACH et al., 2018). Still, one of the most studied members of environmentally relevant, opportunistic pathogen species *P. aeruginosa* has not been deeply investigated in these terms.

In the upcoming section, bacterial species *P. aeruginosa* and its effect on environmental health will be introduced in detail.

2.7 *Pseudomonas aeruginosa*, an opportunistic pathogen

2.7.1.1 General description of species *P. aeruginosa*

In 1882, Carle Gessard (France) isolated the first strain of *Pseudomonas aeruginosa* from wound infections of injured soldiers (MOORE & FLAWS, 2011). Over the past 140 years, the species has been proved to be one of the most common opportunistic Gram-negative bacteria that can be isolated from both the natural and clinical environments. It is a non-spore-forming rod with 0.5–1 μm width and 1-5 μm length and is motile due to its polar flagellum (HTTP12). *P. aeruginosa* is an aerobic – facultatively anaerobic bacterium that can survive and grow in oxygenated environments but is also capable of utilizing nitrate for anaerobic respiration to facilitate its growth (FRANGIPANI et al., 2008). *P. aeruginosa* can grow within a temperature range of 4 °C to 42 °C, and 37 °C is the optimal temperature for its growth (DIGGLE & WHITELEY, 2020). As species *P. aeruginosa* can utilize a variety of compounds as carbon or nitrogen sources, it can grow well on a wide range of media (LABAUVE & WARGO, 2012). *P. aeruginosa* can be found in various natural environments, such as water, soil, and rhizosphere

(ARAOS, 2020).

2.7.2 **Virulence of *P. aeruginosa***

P. aeruginosa is an opportunistic pathogen that might infect plants, animals, and humans if the host's immune system is deficient or weak. In clinical surroundings, it is a widely known opportunistic pathogen and a leading cause of nosocomial infections, especially in intensive care units (ICUs) (LISTER et al., 2009). *P. aeruginosa* infections affect more than 2 million patients every year (CROSS et al., 1983), and in 2019, the estimated deaths caused by this bacterial species were more than 500,000 across 11 different types of infection (GBD, 2019). Half of the lethal infections were associated with antimicrobial resistance (ARC, 2022). Patients who get cancer, burns, injuries (like skin, eyes, etc.), Acquired Immunodeficiency Syndrome (AIDS), and pulmonary diseases like cystic fibrosis (CF), or chronic obstructive pulmonary disease (COPD) are more likely to be infected by *P. aeruginosa* (QIN et al., 2022). In the environment, it is a major opportunistic pathogen in animals, including fish (ALI et al., 2021) such as *Oreochromis niloticus* and *Clarias gariepinus* (ALGAMMAL et al., 2020) . Environment and other non-clinical settings are increasingly reported as potential reservoirs of *P. aeruginosa* (KASZAB et al., 2021), but to reveal the genetic relations between environmental and clinical strains, phylogenetic investigations, such as pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), or multilocus sequence typing (MLST) are proposed (KASZAB et al., 2015).

Virulence of *P. aeruginosa* is determined by the interplay of several virulence factors that can be divided into three major categories, namely structural elements (e.g., type IV pili, flagella and secretion systems), secreted factors such as exotoxins and exoenzymes, and factors related to bacterial cell-to-cell interaction (quorum sensing, biofilm formation) (LIAO et al., 2022). The major virulence mechanisms of species *P. aeruginosa* are summarized in **Figure 7**.

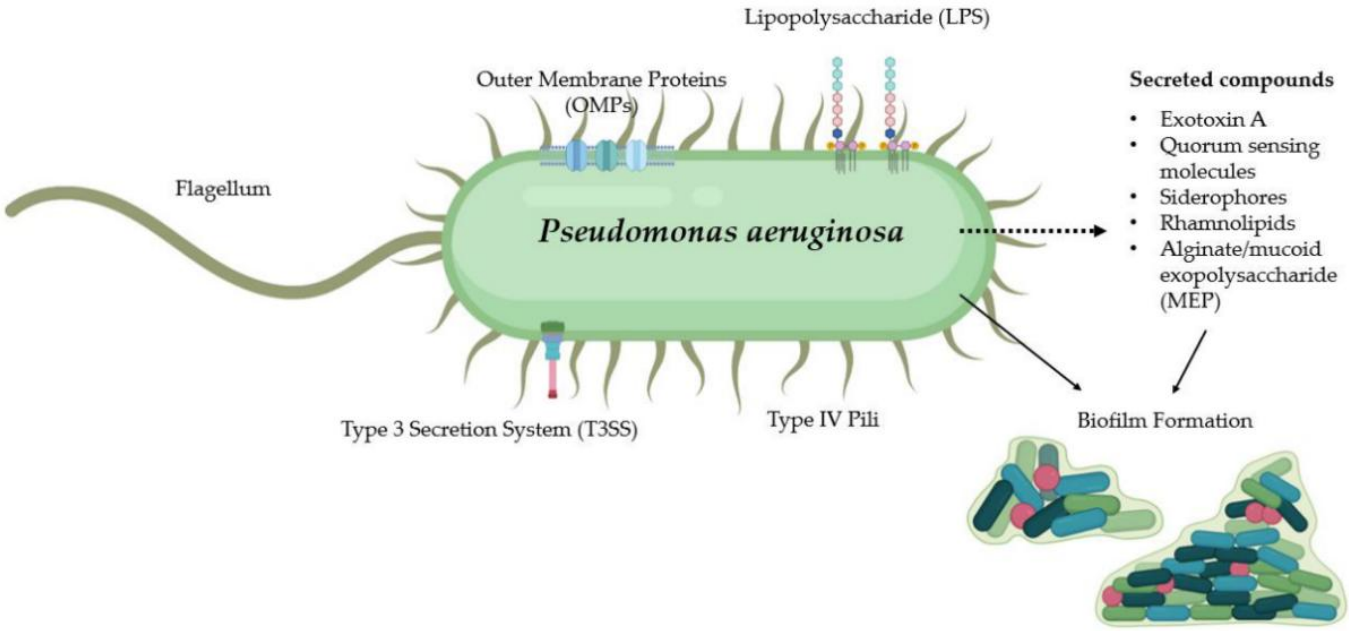

Figure 7. Virulence factors with a key role in the pathogenesis of *P. aeruginosa* (KILLOUGH et al., 2022).

2.7.3 Survival mechanisms of *Pseudomonas aeruginosa*

Compared to other environmentally relevant bacteria, *P. aeruginosa* can survive and proliferate in various environments and can adapt to extremely adverse conditions (ELABED et al., 2019). One reason for this capability is the large genome size (~5–7 Mbp), which enhances the spread, metabolism, and growth performance of the strains (KLOCKGETHER et al., 2011). Furthermore, the regulatory genes controlling biochemical capabilities enable the adaptation to different environments (STOVER et al., 2000) . The second characteristic is the ability of *P. aeruginosa* to form biofilms that enable the cells to adhere to each other and to various biotic and abiotic surfaces (REICHHARDT, 2023). Compared to the free-floating (planktonic) state, although in biofilm form, *P. aeruginosa* usually has lower virulence and is irremovable, it becomes more stable and can survive in adverse environments (LEID, 2009; OLSEN, 2015; TART & WOZNIAK, 2008) . Moreover, biofilm formations are considered as hot spots of antimicrobial resistance (FLORES-VARGAS et al., 2021).

2.7.4 Antibiotic resistance of *Pseudomonas aeruginosa*

P. aeruginosa is capable of resisting various antibiotics: the mechanisms, similarly to other bacterial species, can be classified as intrinsic resistance mechanisms and acquired resistance mechanisms, such as plasmid-mediated resistance (horizontal gene transfer). Moreover, we can

distinguish the so-called adaptive resistance mechanisms and quorum sensing (QS)-dependent antibiotic resistance, as major contributors in the antimicrobial resistance of *P. aeruginosa* (LISTER et al., 2009).

P. aeruginosa can resist to the commonly used antibiotics in different ways: first, due to the alterations of the outer membrane, it has a selective capability to hinder the transfer of the antibiotic molecules or to pump out the antibiotic molecules using an efflux system. Second, *P. aeruginosa* can produce enzymes to cause alterations in the drug molecules to inhibit their binding to the target site or to completely inactivate them. Third, with the production of quorum-sensing signaling molecules, biofilm formation starts to build a physical barrier between the bacterial cells and the antibiotics. Fourth and last, *P. aeruginosa* is capable of acquiring resistance genes or of modifying its gene set with mutations; therefore, it can develop resistance or even multidrug resistance to a range of antibiotics. **Figure 8.** shows the major described antibiotic resistance mechanisms of *P. aeruginosa* (QIN et al., 2022).

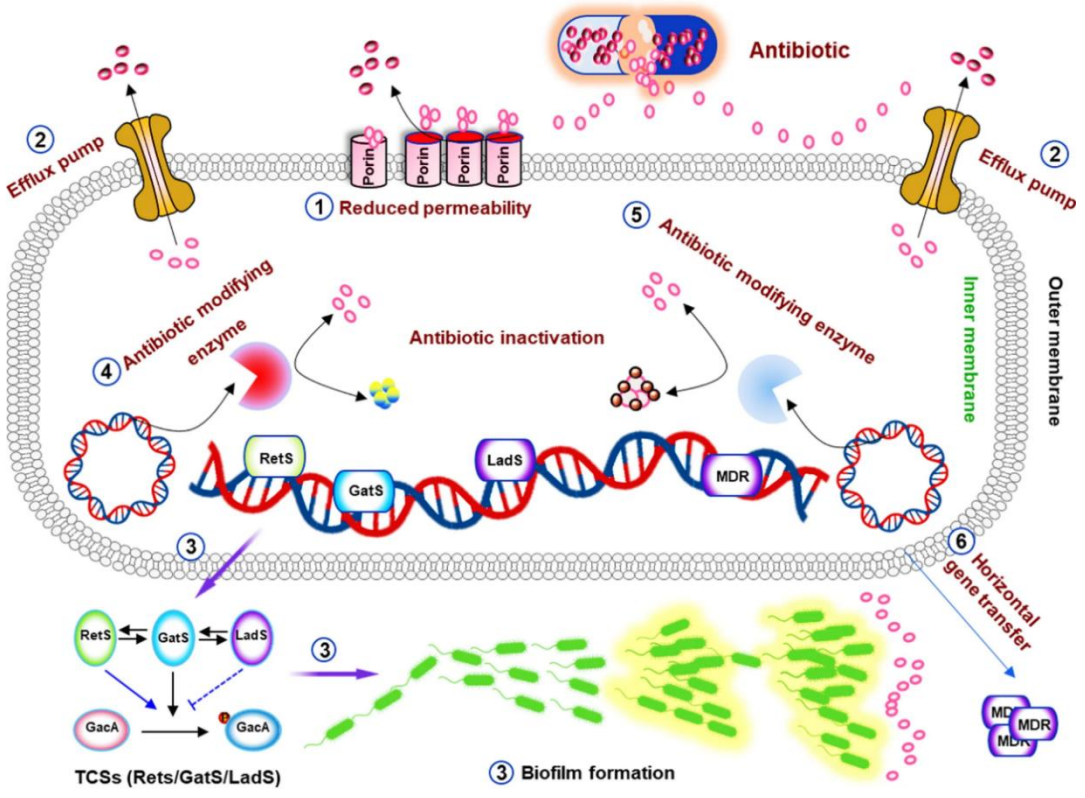

Figure 8. Mechanisms of antibiotic resistance of *P. aeruginosa* (QIN et al., 2022).
① outer membrane permeability, ② efflux systems, ③ biofilm-mediated resistance, ④ antibiotic-modifying enzymes, ⑤ antibiotic-inactivating enzymes, ⑥ mutations and acquisition of resistance genes

In recent decades, the antibiotic resistance of *P. aeruginosa* has become a global public health threat due to its increasing ability to develop resistance to multiple antibiotics (KOTHARI et al., 2023) . The rise of bacterial resistance urged the need for international consensus and standardized terminology to classify the main types of non-susceptibility. According to the scientific literature, multidrug-resistant (MDR) *P. aeruginosa* is defined as resistant to at least one antimicrobial agent in at least three antimicrobial classes. Extensively drug-resistant (XDR) *P. aeruginosa* are non-susceptible to at least one agent of multiple classes of antimicrobials, with only two or fewer effective classes remaining, while pandrug-resistant (PDR) *P. aeruginosa* is non-susceptible to all available antimicrobial classes including the key antibiotics of each antimicrobial group (MAGIORAKOS et al., 2012).

Based on the report of the European Centre for Disease Prevention and Control (ECDC), 32% of *P. aeruginosa* isolated in the European Union's countries were resistant to at least one of the experimental antibiotics tested (carbapenems, ceftazidime, piperacillin-tazobactam, aminoglycosides, and fluoroquinolones), and 19% of *P. aeruginosa* were resistant to two or more antibiotics (SPAGNOLO et al., 2021).

Based on the above-discussed scientific literature, the investigation on the virulence, antimicrobial resistance and phylogeny of environmental *P. aeruginosa* is an increasingly important issue that should be deeply analyzed in accordance with the One Health approach.

According to the available sources, there are still gaps in knowledge in the following fields:

- Are pesticides, such as the globally used glyphosate and GBHs acting as potential drivers of antimicrobial resistance in the case of *P. aeruginosa*?
- Can we develop a novel, combined methodology to examine the actual virulence features (invasive and cytotoxic) of environmental and antibiotic resistant *P. aeruginosa*?

To answer these scientific questions, laboratory experiments of environmental and clinical reference strains of *P. aeruginosa* were set as it is comprehensively described in the upcoming Materials and Methods section.

3 MATERIALS AND METHODS

3.1 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* (JIANG et al., 2021, data not shown). 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 AMR.

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

3.1.1 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* in the experiments

Strain	Collection	Reference	Source
HF234	MATE	HÁHN et al., 2022	Surface water, Hungary
P66	MATE	KASZAB et al., 2010	Hydrocarbon-contaminated groundwater, Hungary
ATCC 27853	NCAIM	MEDEIROS et al., 1971	Clinical (isolated from blood)
ATCC 10145	NCAIM	SKERMAN et al., 1989	Type strain, unknown source
ATCC 15442	NCAIM	WANG et al., 2014	Water bottle in the animal

			room

Based on previous experiments (as it can be seen from **Supplementary Table 1.**), 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 (SPILKER et al., 2004).

3.1.2 Examined pesticides

To evaluate the AMR-inducing effect of glyphosate [(N-(phosphonomethyl) glycine) with chemical formula: $C_3H_8NO_5P$, 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(CH_2CH_2O)m-H (CH_2CH_2O)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)

3.1.3 Preliminary screening of AMR inducing effect of pesticides

3.1.3.1 Testing the antibiotic resistance inducing effect using pre-exposure

Preliminary tests were applied to see if the five selected commercial GBHs (**Table 2.**) can have effects on a chosen, representative isolate of *P. aeruginosa* (HF234) (**Table 1.**) concerning its growth and antibiotic resistance if we use **pre-exposure**.

A volume of 45 mL of Luria–Bertani (LB) medium, containing 10.0 g of tryptone, 5.0 g of yeast extract, and 9.0 g of NaCl in 1000 mL of distilled water, was utilized for the experiment (LABAUVE & WARGO, 2012). The inoculation was carried out using 5.0 mL of an overnight bacterial suspension, adjusted to an optical density of $OD_{600} = 0.60 \pm 0.02$. The recommended dilution range of GBHs, according to the manufacturer's instructions, is from 0.2 to 3.5 v/v% concentration for agricultural and household applications. In order to match this range, the target concentration was set to be 0.5 v/v%, which was equivalent to 1.8–2.8 g/L of glyphosate acid, a concentration similar to the measured glyphosate levels found in runoff waters (1.24 – 6.1 mg/L) after agricultural activities (LIMA et al., 2023).

The prepared media were incubated on a horizontal rotary shaker for 72 hours at 28 °C. During the period of incubation, regular measurements of OD_{600} values were taken, and the growth curves based on the OD_{600} values were used to evaluate the sublethal effects under the selected GBH concentration. Mueller–Hinton agar (Merck Ltd., Germany) without GBHs was used to test the antibiotic resistance at 0h and 72h, respectively, to evaluate the effect of treatment on AMR. Liofilchem MIC test strips were applied to confirm the Minimal Inhibitory Concentrations (MICs), the lowest mg/L concentrations of antibiotics (breakpoints) that are inhibiting the growth of the test organism. The tested antibiotic agents were cefepime, piperacillin, gentamicin, ciprofloxacin, colistin, doripenem, meropenem, imipenem, representing six antibiotic classes (the same antibiotics that are listed in **Supplementary Table 1.**). The measurement followed the recommendations of the European Committee on Antimicrobial Susceptibility Testing, incubation parameters were 24 h at 35 °C (HTTP13) and the evaluation based on the comparison to the breakpoint interpretation tables of The European Committee on

Antimicrobial Susceptibility Testing (EUCAST).

All the experiments in our study were in triplicate, and the data were statistically analyzed by using Two-way ANOVA and post-hoc Tukey test for multiple comparisons at a significance level of 0.05 ($p < 0.05$). With this method, the differences in MIC value between non-treated and pre-exposed cultures could be determined.

3.1.3.2 Testing the antibiotic resistance inducing effect using co-exposure

To assess the effects of **co-exposure**, three different GBHs ('Roundup Mega', 'Dominator Extra 608 SL', 'Gladiator 480 SL'), which caused significant modification of MICs after 72h during pre-exposure assays, were repeatedly tested. The overnight strains of *P. aeruginosa* (listed in Table 1) without pre-exposure were used, and their suspensions were spread directly onto Mueller–Hinton agar containing 0.5 v/v% GBHs. Antibiotic resistance was tested in the same way as it was described in the above section.

3.1.4 Microplate chequerboard assay

According to the results of the preliminary screening assays using pre-exposure and co-exposure with GBHs), a microdilution checkerboard test was performed following a previously described method of FRATINI et al. (2017), with minor modifications. According to the method, the resistance-inducing effect of GBHs (Dominator Extra 608 SL, Fozat 480, Gladiator 480 SL, Roundup Mega, Total – see in Table 2.), glyphosate, and its formerly used co-formulant POE(15) was assessed in a quantitative way with imipenem, an antibiotic chosen during the preliminary testing phase. The assay was performed in triplicates with freshly prepared solutions of glyphosate, GBHs and antibiotics, as well as overnight bacterial suspensions.

To perform the 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, on the other hand, 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. The experimental setting is visualized at **Figure 9.**

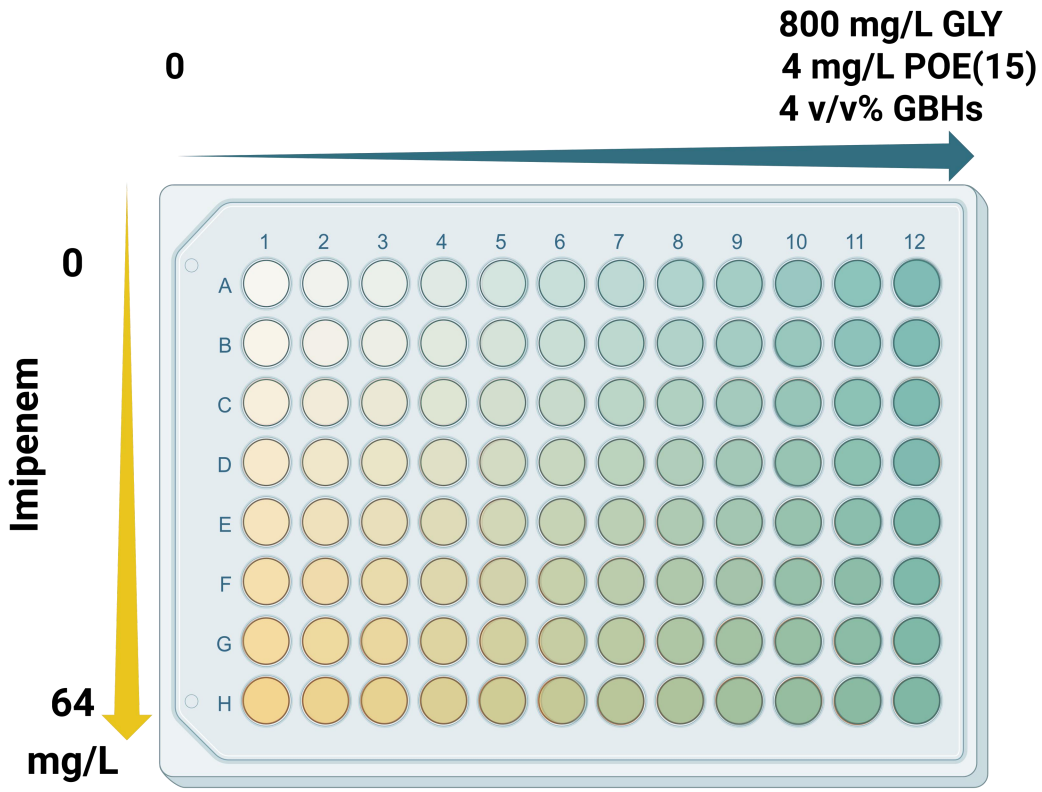

Figure 9. The experimental setting of the microplate chequerboard assay (created in Biorender by Kaszab E.)

After setting the concentrations of the test materials, each well of the microplate was supplemented with 50 µL of overnight *P. aeruginosa* bacterial suspensions of the 5 test strains (optical density of $OD_{600} = 0.60 \pm 0.02$), and to make the final volume 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 and a speed of 350 rpm in a microplate shaker thermostat (PST-60HL-4, BioSan, Latvia). To measure the inhibitory effect 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.

3.1.5 **Data analysis and statistics**

After performing the experiments, GraphPad Prism 7 software, version 7.00 (GraphPad Software Inc., San Diego, USA) was used to analyze the raw data. The three times repeated microplate measurements were averaged for statistical analysis. The absorbance data were first presented as average values, and then they were visually represented using heat maps. In order to assess the combined effects of imipenem and the glyphosate-related chemicals under investigation, the Fractional Inhibition Concentration Index (FICI) was calculated using the following mathematical expression (FALEIRO & MIGUEL, 2013), 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 determined based on the minimal inhibitory concentration (MIC) and fractional inhibitory concentration (FIC). It is used to quantify the interaction between two chemicals (e.g., antimicrobials).

The evaluation categories of FICI are as follows (ODDS, 2003):

FICI < 0.5 synergism

0.5 < FICI < 4.0 indifference

4.0 ≤ FICI antagonism

To reveal the differences between the effect of glyphosate/POE (15)/GBHs on the tested antibiotic (imipenem) and to exclude the variance of the individual strains, the absorbance values of the five examined *P. aeruginosa* strains were averaged and compared to their respective imipenem control samples (containing bacterial suspension and imipenem). For statistical analysis, a two-way ANOVA was performed, followed by Dunnett's multiple comparisons tests. 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.

3.2 Development of a zebrafish (*Danio rerio*) microinjection model for the *in vivo* virulence of environmental *Pseudomonas aeruginosa*

In the next experimental setting, another important scientific question was analyzed, which could clarify the actual virulence (cytotoxic or invasive feature) of environmental *P. aeruginosa* strains. As it was summarized in the Literature review section, the available techniques to determine the virulence of environmental *P. aeruginosa* do not always provide sufficient information about both the invasive and cytotoxic features of a given strain, because they are usually using only one exposure route. 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, which 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.

3.2.1 Selection of bacterial strains for virulence testing

In order to develop and validate the new virulence model, a diverse set of 15 *P. aeruginosa* strains were chosen (**Table 3.**). Environmental and clinical *P. aeruginosa* 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 (SPILKER et al., 2004; ATZÉL et al., 2008).

These *P. aeruginosa* strains were fully characterized in previous studies and were chosen, because they varied in origin, phenotypic and genetic traits, multilocus sequence types (MLSTs[1]),

[1]MLST a technique used to classify bacterial strains based on the sequences of internal fragments of multiple DNA sequences of housekeeping genes, is applicable to reveal the genetic distance between *P. aeruginosa* strains of various origin. Using PUBMLST, the public database for molecular typing and microbial genome diversity (HTTP14), closely related strains can be identified and categorized into sequence types (STs) (MAIDEN et al., 1998).

antibiotic resistance profiles, virulence factors, biofilm-forming ability, motility, and *in vivo* virulence as previously determined using the *Galleria mellonella* (wax moth) model (KASZAB et al., 2021). The detailed characteristics of the tested strains are summarized in **Supplementary Table 2**.

Table 3. The list of selected 15 *P. aeruginosa* strains

Designation	Collection	Origin
ATCC10145	NCAIM	Type strain, unknown source
ATCC 15442*	NCAIM	Water bottle in animal room
ATCC 27853	NCAIM	Clinical
KPS-3	MATE	Clinical, Hungary
P9	MATE	Hydrocarbon contaminated groundwater, Hungary
P14**	MATE	Hydrocarbon contaminated soil, Hungary
P18	MATE	Hydrocarbon contaminated soil, Hungary
P43	MATE	Hydrocarbon contaminated groundwater, Hungary
P66**	MATE	Hydrocarbon contaminated groundwater, Hungary
P69	MATE	Hydrocarbon contaminated soil, Hungary
P114	MATE	Compost, Hungary
P135	MATE	Hydrocarbon contaminated soil, Hungary
P144	MATE	Hydrocarbon contaminated soil, Hungary
P164	MATE	Sewage, Hungary
P177	MATE	Hydrocarbon contaminate groundwater, Hungary

*Non-virulent strain used as negative control; **Strains used for optimization

The environmental strain ATCC15442 was used as a negative control due to its non-pathogenic nature (WANG et al., 2014). 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, highlighted in **Supplementary Table 2.**), which represented virulent and avirulent features based on the previous *G. mellonella* virulence assay (KASZAB et al., 2021).

3.2.2 Preparation of *P. aeruginosa* bacterial suspensions

Bacterial strains were grown in Luria-Bertani (LB) broth (composition was previously described in 3.1.3.1. section), because LB was previously confirmed to be safe (non-toxic) to zebrafish larvae (GARAI et al., 2021) the test organism that was planned to be involved in the

newly developed virulence assay. Overnight cultures of the *P. aeruginosa* strains were diluted with fresh LB until they reached an optical density of OD_{600} = 0.60 ± 0.02. These stock cultures were then further diluted to achieve the desired infectious dose.

3.2.3 Maintenance of zebrafish and egg collection

Zebrafish were kept and bred according to the general protocol of the MATE zebrafish lab. The laboratory-bred AB strain of zebrafish was breeding within groups (one group contains 30 females and 30 males) 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 (CSENKI et al., 2019). The laying of individual pairs was delayed over time to ensure a continuous supply of single-celled embryos.

3.2.4 Microinjection of zebrafish embryos

To determine the best 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 (CSENKI et al., 2019).

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). The initial bacterial suspension was serially diluted to achieve the required concentrations in the final injection volume. The experimental settings

during the optimization phase are summarized in **Table 4.**

Table 4. 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}	$2.4\cdot\times 10^{-1}$	
	150 µM (1.77 nL)	8.4×10^{1}	$8.4\cdot\times 10^{0}$ *	$8.4\cdot\times 10^{-1}$	
	200 µM (4.17 nL)	2.0×10^{2}	$2.0\cdot\times 10^{1}$	$2.0\cdot\times 10^{0}$	
Yolk (Y)	100 µM (0.52 nL)		$2.4\cdot\times 10^{0}$	$2.4\cdot\times 10^{-1}$	$2.4\cdot\times 10^{-2}$
	150 µM (1.77 nL)		$8.4\cdot\times 10^{0}$ *	$8.4\cdot\times 10^{-1}$	$8.4\cdot\times 10^{-2}$
	200 µM (4.17 nL)		$2.0\cdot\times 10^{1}$	$2.0\cdot\times 10^{0}$	$2.0\cdot\times 10^{-1}$

* Minimum infectious dose (MID)

For the microinjection, three different levels of bacterial dilutions (**Table 4**.) were used to represent the minimum infective dose (MID) of *P. aeruginosa* in the final injection volume, that is 10^2 CFU (LIZEWSKI et al., 2002; ROSER et al., 2014). Additionally, one lower (10^{-1}), and one higher (10^{-3}) level of dilutions were used. During yolk injection, lower bacterial concentrations (10^{-2}-10^{-4}) were used compared to perivitelline space injection to get an insight into sublethal symptoms.

As the zebrafish's chorionic pore size (0.77 µm) (CHEN et al., 2020) is smaller than the described size of *P. aeruginosa* cells, the cross infection between the embryos was prevented until the hatching phase (starting at 72h). Two hours after injection, solidified and/or unfertilized eggs were discarded, and the developing embryos were transferred in groups to media containing sterilized E3 (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, and 0.16 mM $MgSO_4$). The microinjected embryos were incubated at 25 ± 1 °C in a Memmert thermostat to ensure optimal temperatures for both test organisms. Treatments in preliminary testing phase were conducted in three replicates with ten embryos each (n = 30).

The size of the droplets used for microinjection is critical for the survival of zebrafish larvae after treatment, as well as affecting the infective dose of the tested strain. For fish embryos, the maximum injection volume that does not negatively affect larval survival in both treated and control groups is 10% of the total yolk volume (WALKER et al., 1992), which is about 4.2 nL (equivalent to a 200 µm diameter droplet) for zebrafish larvae (SCHUBERT et al., 2014). The

test volume has been thoroughly tested in previous experiments with no apparent deaths or developmental disorders (CSENKI et al., 2019; GARAI et al., 2020), so it was considered safe for use. To avoid adverse effects on the test organism, in addition to this maximum volume, two smaller droplet sizes were used in the optimization phase, that are still easily adjustable, namely 100 µm in diameter (0.52 nL) and 150 µm in diameter (1.77 nL). Injection volumes were validated for dimensional stability (CSENKI et al., 2019).

In summary, to optimize the new infection protocol's parameters, the examined environmental strains (P66 and P14) and the uninoculated control medium (LB) were analyzed using two routes of infection (PV, Y), three selected droplet sizes, and the chosen bacterial concentrations (altogether 27 different settings). The sublethal symptoms were recorded every 24 hours until hatching (72 hours). Each embryo received only one type of treatment, so a total of 81 endpoints were used. To verify *P. aeruginosa* infection, dead embryos were analyzed by re-isolating the infectious strain, as it was suggested (XIE et al., 2014).

The flowchart of the optimization protocol for microinjection is shown in **Figure 10**. As it can be seen the collected zebrafish embryos were microinjected into the yolk (Y) and the perivitelline space (PV) with different drop sizes and relevant volumes (100µm, 150µm, and 200µm) of the test material. After microinjection, the embryos were incubated under controlled conditions to allow the infection to progress. Mortality and any morphological changes were recorded at 24h/48h/72h to determine the optimal time for reading.

After optimization stage with the examined two *P. aeruginosa* strains (P14 and P66), results were evaluated, and the ideal bacterial density, injection volume, drop size, and incubation time were determined. In the next step, the optimized microinjection protocol was repeatedly conducted, but the infection was performed only with the chosen droplet size, respective volume, bacterial concentrations and incubation time. Treatments in this validation phase were conducted in five replicates with ten embryos each (n = 50) using 15 *P. aeruginosa* strains.

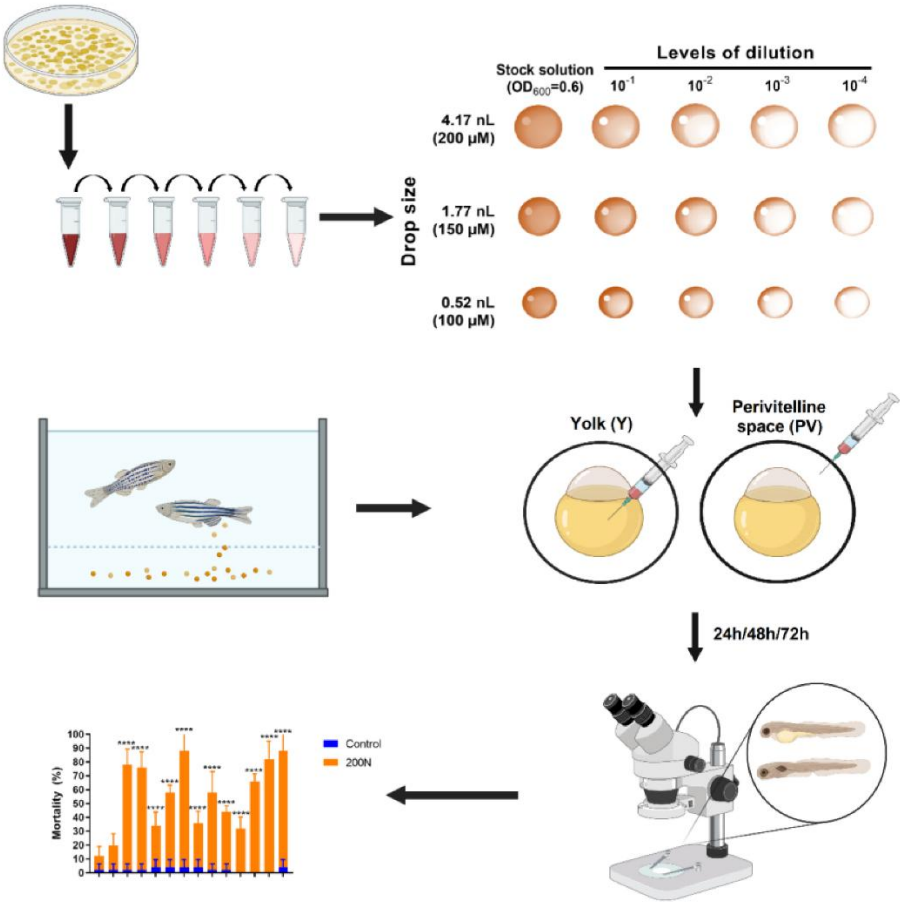

Figure 10. The flowchart of the preliminary testing protocol for the optimization of microinjection. Image was created in Biorender.com by CSENKI-BAKOS Zs. (KASZAB & JIANG et al., 2023)

3.2.5 Experimental endpoints

During both the optimization and validation phases, mortality and sublethal symptoms of the 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* (VELIKOVA et al., 2016) 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%).

Regarding sublethal symptoms, pericardial edema, vitelline edema, caudal deformity, craniofacial deformity, and disorganized abnormal embryonic shape were defined as sublethal

endpoints (CSENKI et al., 2023). To document sublethal symptoms, stereomicroscopes (Leica M205 FA, Leica DFC 7000T camera, Leica Application Suite X, Leica Microsystems GmbH, Wetzlar, Germany) were used, and digital images of transverse larvae were captured at 30x magnification.

3.2.6 **Statistical analysis**

Normality of data was analyzed in R software version 4.2.1. (HTTP15) with the Shapiro-Wilk normality test (ROYSTON, 1982). The homogeneity of variance was tested by the Bartlett test (BARTLETT, 1937). 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. (HTTP15), the ordinary one-way ANOVA was used, and then the Dunnett multiple comparison test was performed, with a confidence interval of 95%.

4 RESULTS

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

Using the methodology described in section 3.1, 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 pre-exposure and co-exposure schemes.

4.1.1 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 F**igure 11**. As it was a preliminary screening stage, glyphosate and POE(15) were not investigated, and only one *P. aeruginosa* strain was involved.

Figure 11. contains A, B, and C subfigures. Figure 11/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%. As shown in Figure 11/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 any significant inhibitory effect compared to the non-treated control.

Figure 11/B is a bar graph that displays the Minimum Inhibitory Concentration (MIC) values of the tested antibiotics on HF234 after 72 hours of pre-exposure to the GBHs. The MIC is the lowest concentration of an antibiotic to prevent the growth of bacteria. The alterations between the non-treated and pre-treated culture’s MIC values show the effects of GBHs on the susceptibility of the examined bacterial strain. Based on the results, after 0.5% GBH pre-exposure, the MIC values of piperacillin and gentamicin increased 1.5-2.0x compared to the

control group, although the observed results were not statistically significant. Meanwhile, it was observed that the MIC value of imipenem exhibited a notable increase ranging from 2 to 32 times, compared to the non-treated control. 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. At the same time, meropenem and doripenem, the tested antibiotics of the same class (carbapenems), remained without any significant changes in their MIC values.

Figure 11/C is illustrating the inhibition zones of imipenem, one of the antibiotics tested, after pre-incubation of HF234 strain to different GBHs.

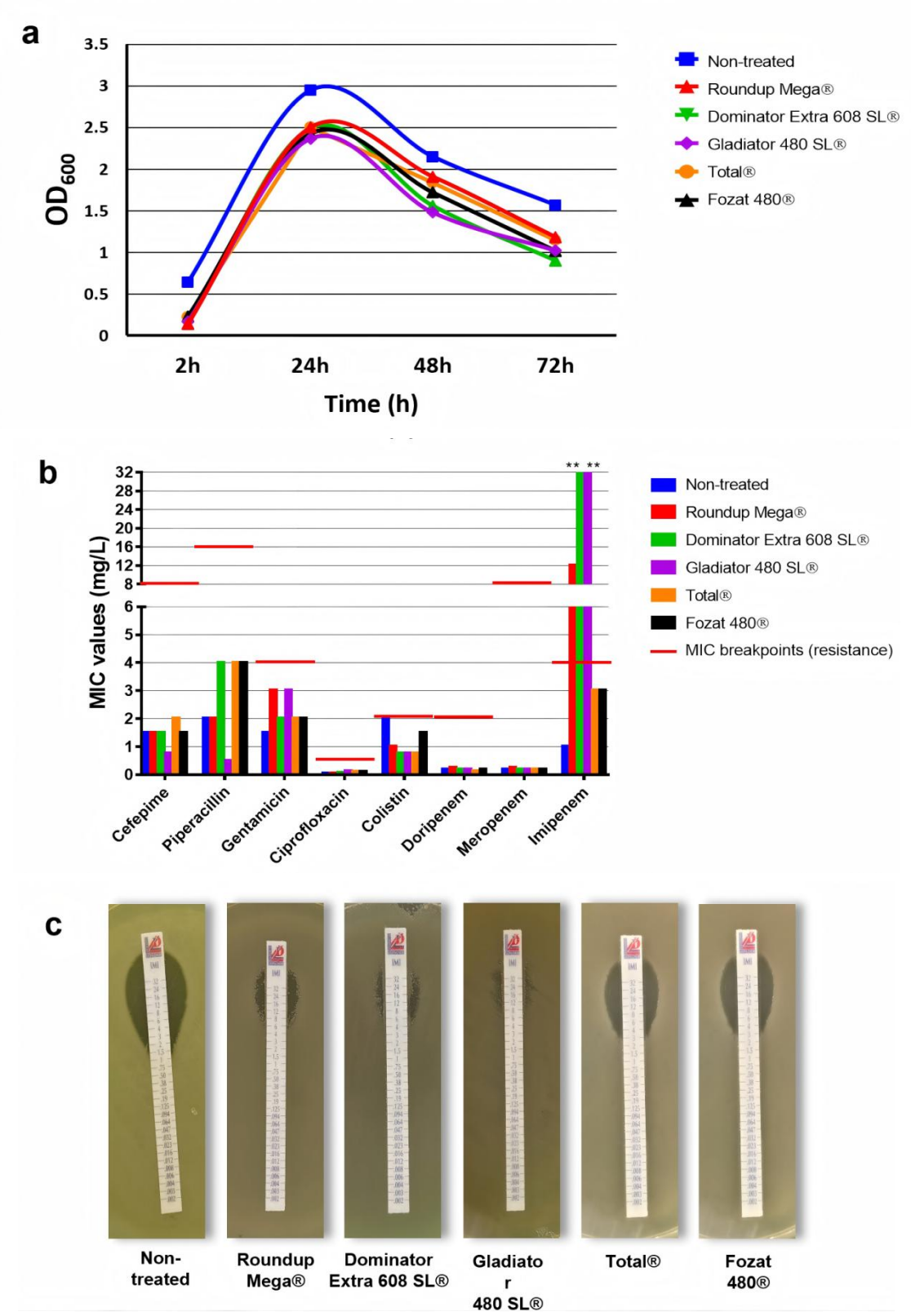

Figure 11. Pre-exposure of *P. aeruginosa* HF234 using 0.5 v/v% GBHs (averaged values obtained from three technical replicates). (a) The optical density (OD_{600}) of broth cultures which was treated with GBHs for 72 h. (b) MIC values of HF234 cultures after 72 h pre-exposure, plated on GBH-free agar 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. (HÁHN et al., 2022)

4.1.2 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.

In the co-exposure assay, the effect of GBHs was measured using all the five distinct *P. aeruginosa* strains outlined in Table 1. Results of the co-exposure experiment are summarized in **Table 5**. Our results showed that MIC values of imipenem under co-exposure with the tested three GBHs increased from 0.75-3 to 4-32 mg/L concentrations that is equal or above the EUCAST breakpoint of resistance. The alterations in MIC values of imipenem were the most pronounced in the case of Dominator Extra 608 SL and Gladiator 480 SL..

Table 5. Minimal inhibitory concentrations (MIC values) of imipenem on *P. aeruginosa* strains during co-exposure (without pre-exposure) plated on 0.5 v/v% GBHs containing Mueller-Hinton plates

Antibiotic	**GBH (0.5 v/v%) content of the plates**	**MIC values of imipenem on *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) (HTTP16)

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

4.1.3 Results of 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 3.1.4), 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 also 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 12**. showing the averaged absorbance values of three replicate measurements as a heatmap in case of each strain. This figure shows the interactions between imipenem and various concentrations of glyphosate, POE (15), and GBHs on *P. aeruginosa* strains. Relative fold growth (CEBALLOS-GARZON et al., 2022) is shown by blue bars. The color intensity in the heatmap corresponds to the absorbance values, and darker shades mean a more intensive bacterial growth. This visualization shows the strain-dependent effect of co-exposure.

The averaged differences (%) of absorbance values of *P. aeruginosa* strains under co-exposure compared with the absorbance values of the relevant solo imipenem exposition are summarized in **Figure 13**. Each bar represents the averaged absorbance values of five *P. aeruginosa* strains co-exposed to imipenem and test materials (glyphosate, GBHs, POE(15)) 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). This visualization illustrates the strain-independent effect of co-exposure.

The drug interactions between imipenem and glyphosate/POE(15)/GBHs were assessed using the FIC index. As mentioned, the FIC index determines whether the combined effect of the tested chemicals is synergistic, antagonistic, or additive (FALEIRO & MIGUEL, 2013; ODDS, 2003) . The values of the FIC index calculated in this study are presented in **Supplementary Table 3**.

It can be seen from Figure 12., 13., and Supplementary Table 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* at any point of this concentration range. Based on our results for imipenem alone, at higher concentrations (16-64 mg/L), the absorbance values were generally lower, so the solo antibiotic treatment inhibited the growth of the test strains.

When combined with glyphosate, even at low concentrations of imipenem, the absorbance values increased, suggesting that glyphosate reduces the inhibitory effect of imipenem: the MIC

values of imipenem increased from 1-3 mg/L to 32-64 mg/L. According to the results of Figure 12., among all the strains tested, the strongest antagonistic effect was determined in the case of *P. aeruginosa* ATTC10145. The negative correlation was more pronounced at higher glyphosate concentrations (400-800 mg/L).

Regarding FIC index determination, the high FICI value (18.6) of glyphosate versus imipenem clearly indicates an antagonistic effect, which means that there is a significant decrease in the effectiveness of imipenem when combined with glyphosate, irrespective to the bacterial strains (Figure 13.). In other words, the resistance of *P. aeruginosa* to imipenem increased.

As for POE(15), a slight absorbance increase 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 different types of GBHs.

'Total', that was non-toxic to the examined *P. aeruginosa* strains in the examined concentration range, and clearly induced resistance to imipenem with a significant increase in absorbance (up to 192 %). Depending on the tested bacterial strain, a simultaneous decrease in imipenem MIC values was determined (4-64 mg/L). The calculation of the FIC index proved the antagonistic effect with a FICI value of 12.8 (see **Supplementary Table 3.**).

Upon further analysis from Supplementary Table 3, it was found that '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 other 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-exposure 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 formulas slightly increased the tolerance to imipenem compared to the solo antibiotic treatment.

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’ exhibited a more 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 MIC levels reaching up to 64 mg/L.

To summarize, the microplate chequerboard assay revealed that the major interactions between imipenem and glyphosate was “antagonism”, with POE(15) was “indifference”, while with GBHs, depending on the cytotoxicity of the examined formula, were “antagonism” or “indifference”.

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

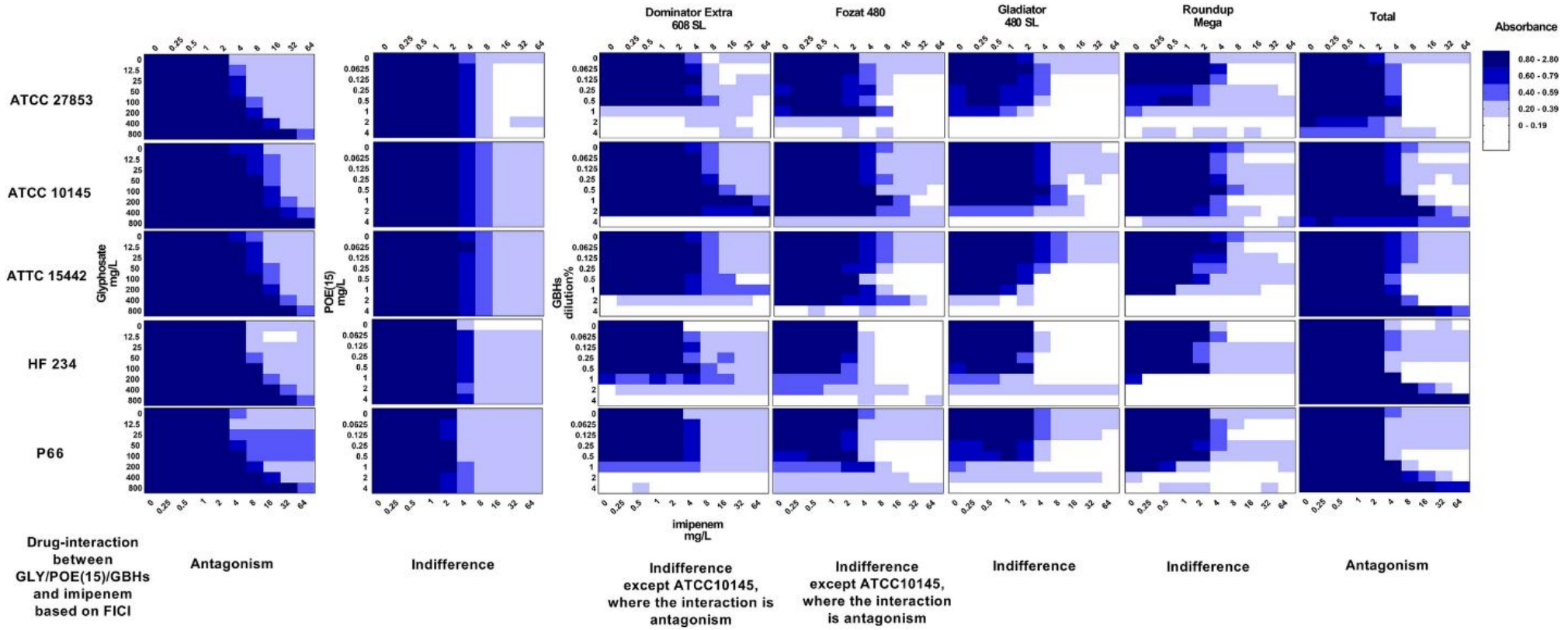

Figure 12. Heatmap of the averaged absorbance values of each tested *P. aeruginosa* strains (ATCC 27853, ATCC 10145, ATCC 15442, HF234, P66) during co-exposure with different concentrations of glyphosate, POE(15), GBHs and imipenem. The X-axis represents imipenem concentration ranging from 0 to 64 mg/L, 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%)

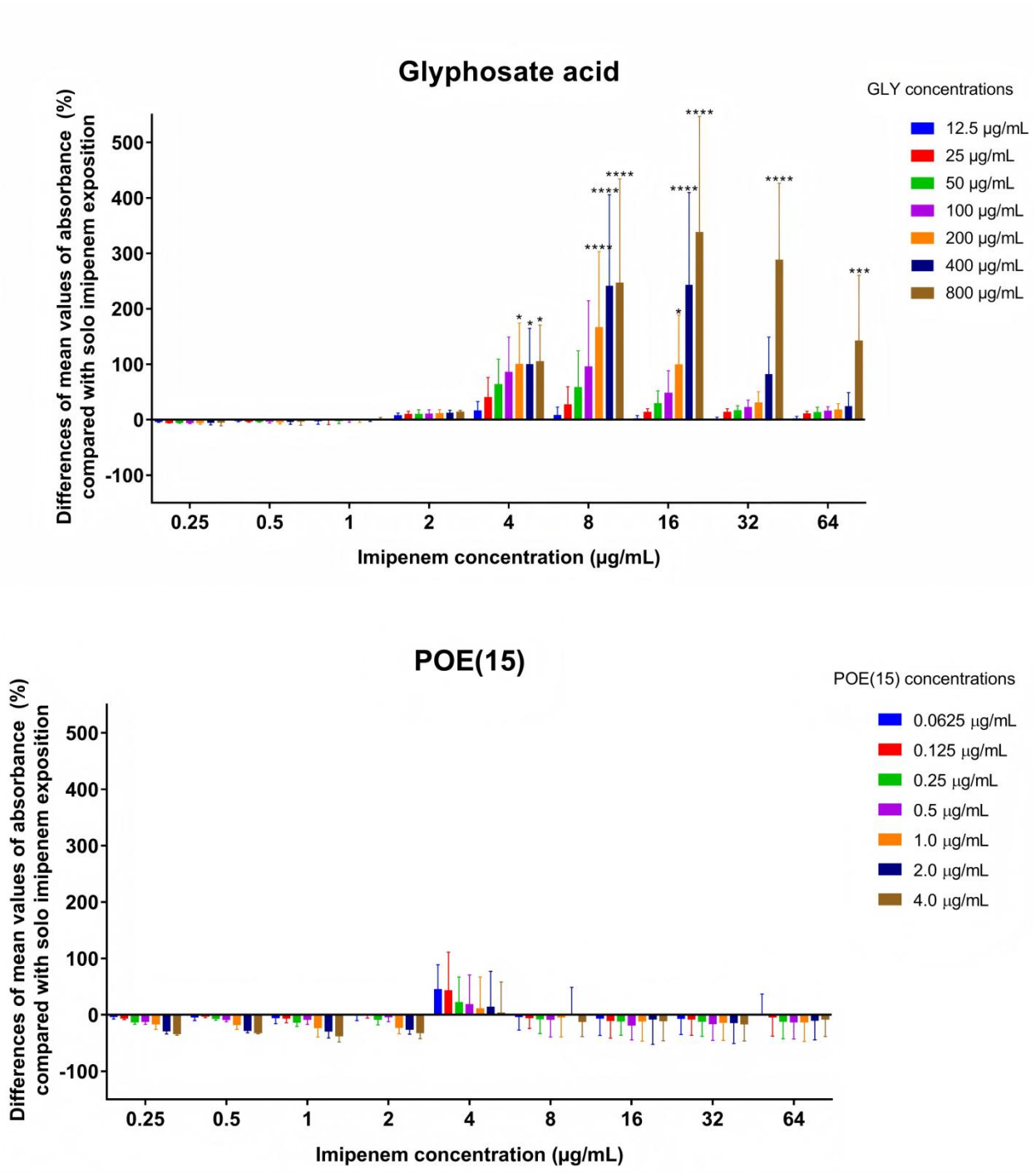

Figure 13. Differences in averaged absorbance of the five *P. aeruginosa* strains (ATCC 27853, ATCC 10145, ATCC 15442, HF234, P66) during co-exposure to the combination of test materials (glyphosate, POE(15), GBHs) and imipenem compared to solo imipenem exposure with the same concentration (expressed in %). Statistical analysis: Two-way ANOVA and Dunnett's multiple comparisons tests. *, **, *** and **** are significantly different from solo imipenem values ($p < 0.0332$, $p < 0.0021$, $p < 0.0002$ and $p < 0.0001$, respectively).

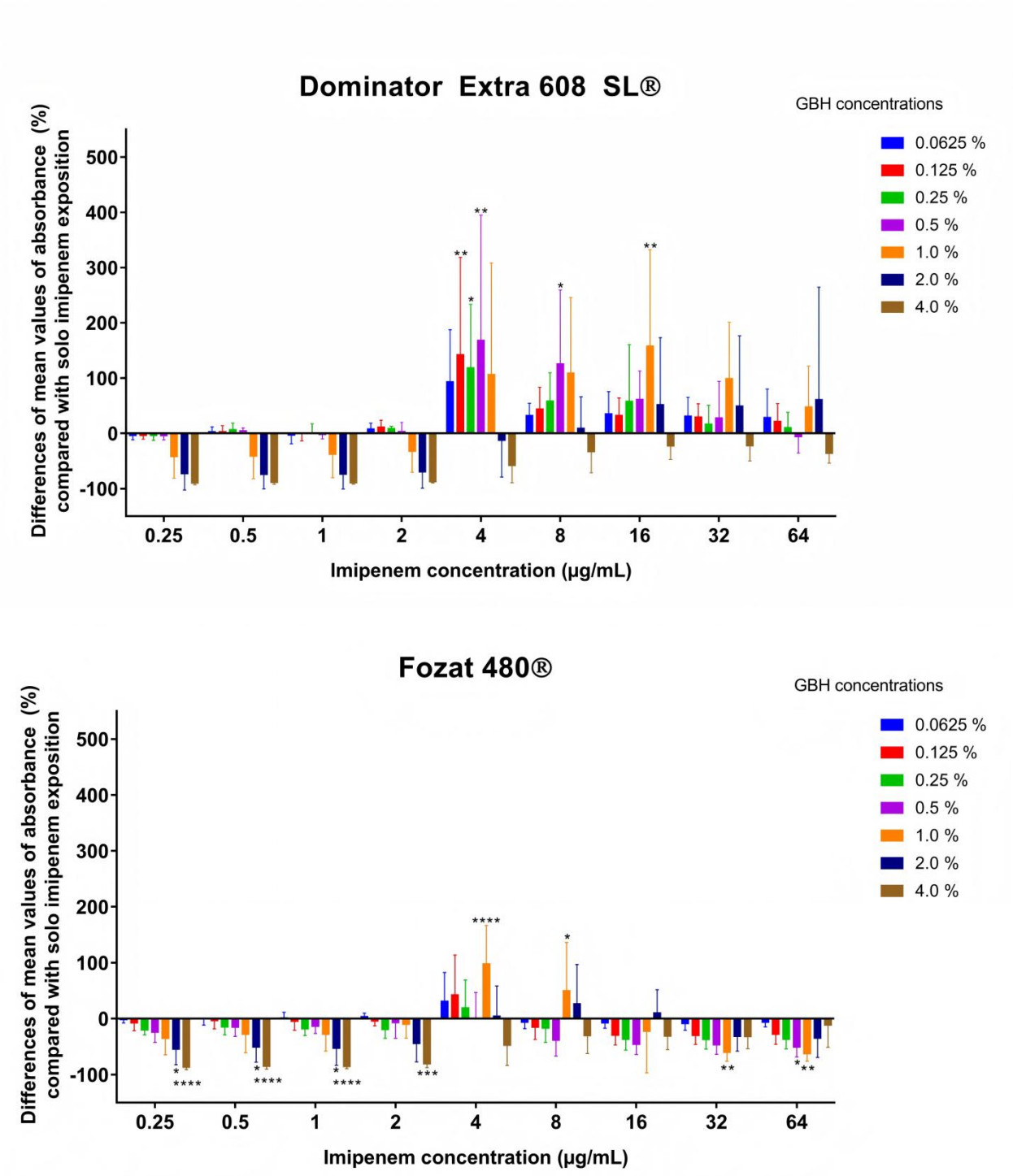

Figure 13. (cont.) Differences in averaged absorbance of the five *P. aeruginosa* strains (ATCC 27853, ATCC 10145, ATCC 15442, HF234, P66) during co-exposure to the combination of test materials (glyphosate, POE(15), GBHs) and imipenem compared to solo imipenem exposure with the same concentration (expressed in %).
Two-way ANOVA and Dunnett's multiple comparisons tests. *, **, *** and **** are significantly different from solo imipenem values ($p < 0.0332$, $p < 0.0021$, $p < 0.0002$ and $p < 0.0001$, respectively).

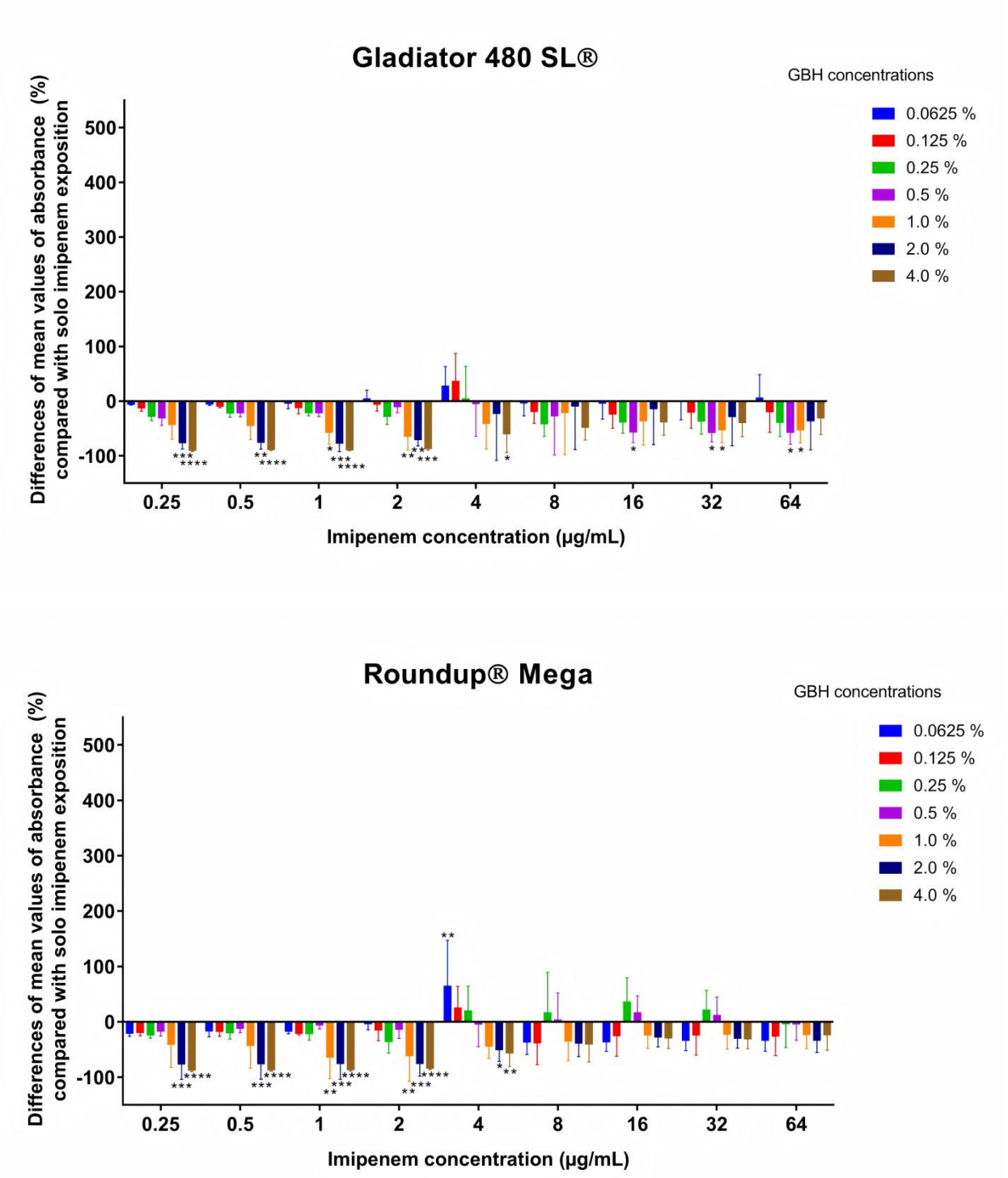

Figure 13. (cont.) Differences in averaged absorbance of the five *P. aeruginosa* strains (ATCC 27853, ATCC 10145, ATCC 15442, HF234, P66) during co-exposure to the combination of test materials (glyphosate, POE(15), GBHs) and imipenem compared to solo imipenem exposure with the same concentration (expressed in %).
Two-way ANOVA and Dunnett's multiple comparisons tests. *, **, *** and **** are significantly different from solo imipenem values ($p < 0.0332$, $p < 0.0021$, $p < 0.0002$ and $p < 0.0001$, respectively).

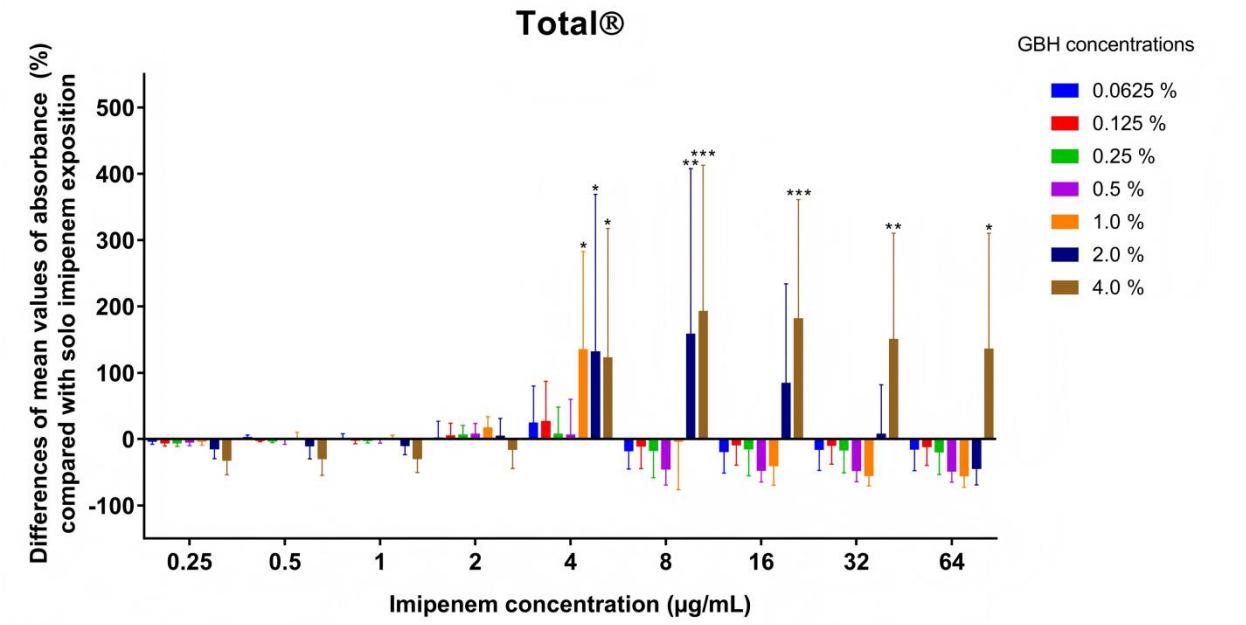

Figure 13. (cont.) Differences in averaged absorbance of the five *P. aeruginosa* strains (ATCC 27853, ATCC 10145, ATCC 15442, HF234, P66) during co-exposure to the combination of test materials (glyphosate, POE(15), GBHs) and imipenem compared to solo imipenem exposure with the same concentration (expressed in %). Two-way ANOVA and Dunnett's multiple comparisons tests. *, **, *** and **** are significantly different from solo imipenem values ($p < 0.0332$, $p < 0.0021$, $p < 0.0002$ and $p < 0.0001$, respectively).

4.2 Evaluating the zebrafish (*Danio rerio*) microinjection virulence model and optimizing the model

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.

4.2.1 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 3.2.4). 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 *P. aeruginosa* strains (P66 and P14) used for optimization 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 (KASZAB et al., 2021).

4.2.1.1 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. The relevant results are shown as histograms in **Figure 14.**, which illustrates the mortality rates of zebrafish embryos injected with *P. aeruginosa* strains at the 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.

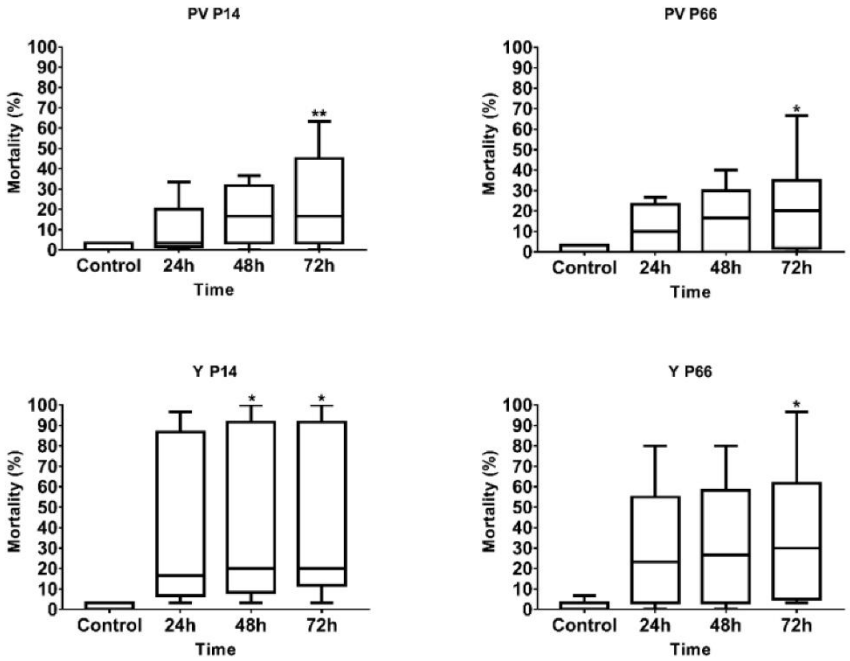

Figure 14. Mortality results of the examined *P. aeruginosa* strains during optimization of zebrafish microinjection model with different times of incubations (24, 48 and 72 h). Mean values were analyzed with One-way ANOVA followed by Dunne's multiple-comparison test at 95% confidence interval.
P-value: 0.1234 (ns), 0.0332 (*), 0.0021 (**)
PV—perivitelline injection; Y—yolk injection.

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

In the next step, different tenfold dilutions of the bacterial stock solution (10^{-1} to 10^{-4}) were tested to determine the optimal bacterial concentration for microinjection. **Figure 15.** 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 15, mortality rates at the 10^{-1} dilution level to both strains (P14 and P66), with the PV microinjection route, were statistically 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, indicating these dilutions were too low to induce significant mortality. 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, suggesting insufficient bacterial concentration. Thus, the optimal bacterial dilutions were determined to be 10^{-1} for PV injection and 10^{-2} for Y injection.

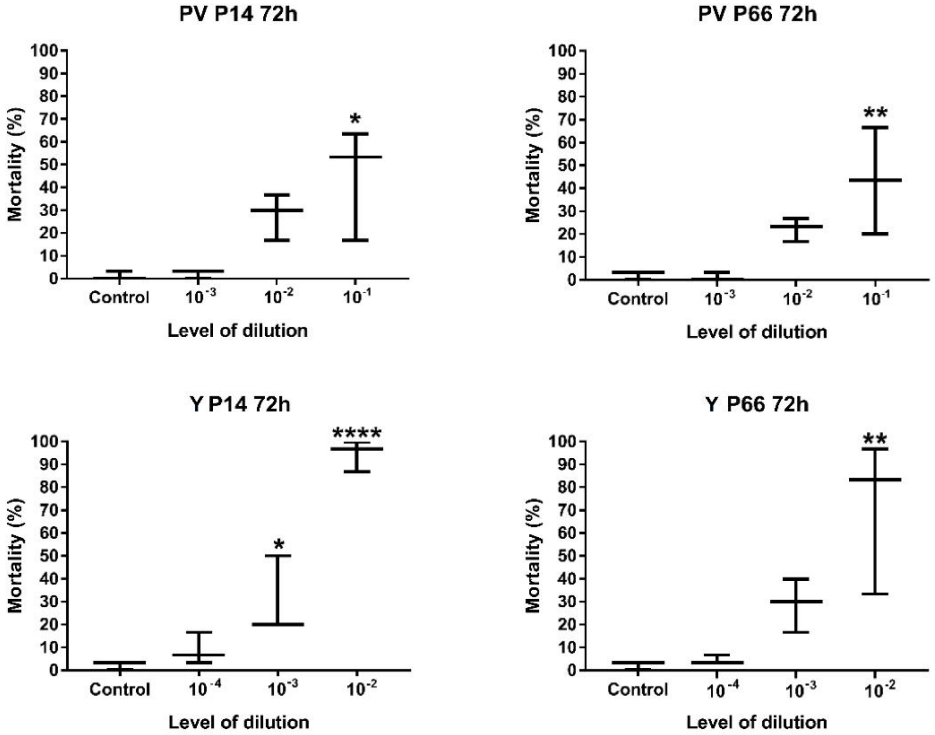

Figure 15. Mortality results of the examined *P. aeruginosa* strains during optimization of zebrafish microinjection model with different levels dilutions of bacterial strains (10^{-1}–10^{-4}). Mean values were analyzed with One-way ANOVA followed by Dunnett's multiple-comparison test at 95% confidence interval.
P-value: 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0001 (****)
PV—perivitelline injection; Y—yolk injection.

4.2.1.3 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 16.**, 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 according to general requirements for virulence testing, only one drop size was selected for the finalized microinjection method. 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.

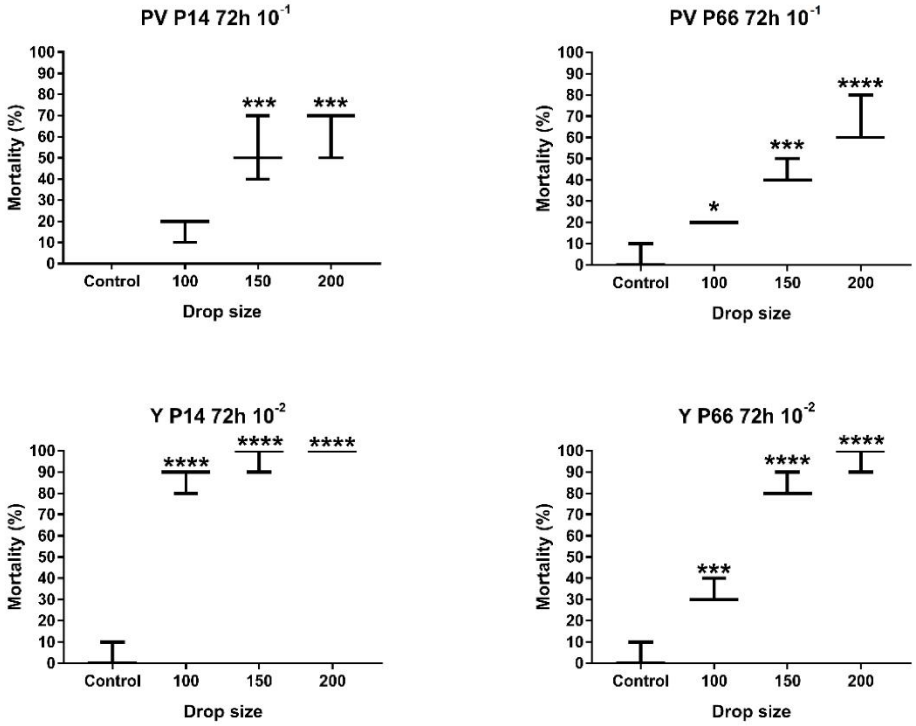

Figure 16. Mortality results of the examined *P. aeruginosa* strains during optimization of the zebrafish microinjection model with different drop sizes (100, 150, 200 µL). Mean values were analyzed with one-way ANOVA followed by Dunnett's multiple-comparison test at a 95% confidence interval.
P-value: 0.1234 (ns), 0.0332 (*), 0.0002 (***), 0.0001 (****).
PV—perivitelline injection; Y—yolk injection.

4.2.1.4 Development of the recommended 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.

4.2.2 Validation of the developed microinjection method with a set of *P. aeruginosa* isolates

The optimized infection protocol was validated by testing with 15 different environmental and clinical strains of *P. aeruginosa*, with various phenotypic, genetic, and phylogenetic characteristics. **Figure 17.** represents the examined *P. aeruginosa* strains across their phylogenetic tree obtained by Multilocus Sequence Typing (MLST) and their phenotypic virulence (mortality) obtained by the newly developed, combined microinjection method in

zebrafish embryos. Both environmental and clinical strains of *P. aeruginosa* showed a wide range of virulence, which proves the capacity of the applied method to differentiate *P. aeruginosa* strains as it can be seen in the distribution (avirulent, weakly virulent, moderately virulent virulent) in Figure 17. 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. As shown in Figure 17, there are significant differences between the examined strains that can be linked to the MLST profiles. For example, strain P14 and P18, based on their Sequence Types (STs) are phylogenetically close to each other, and they showed a high virulence to zebrafish embryos. This result is supporting the theory that several virulence characteristics can be correlated with the population structure of *P. aeruginosa* (FRESCHI et al., 2019) and suggests that the virulence characteristics of *P. aeruginosa* can be highly strain-specific. Meanwhile, no significant connection was found between the range of phenotypic antibiotic resistance and the *in vivo* virulence of the examined strains.

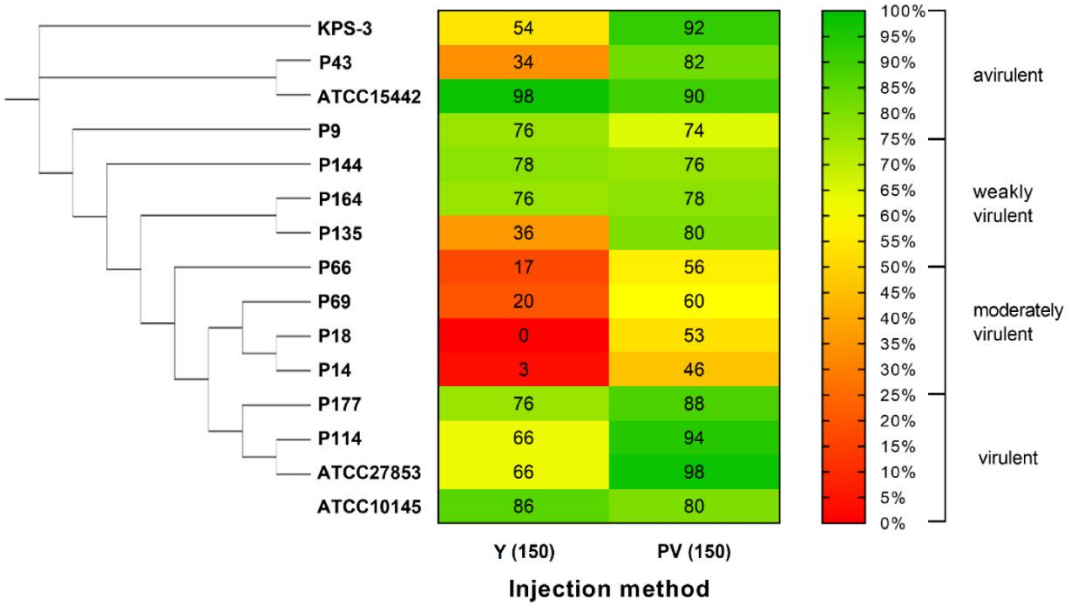

Figure 17. Virulence of the examined environmental and clinical *P. aeruginosa* strains using the combined microinjection virulence model and the spread of the *in vivo* virulence (mortality) across the phylogenetic tree obtained based on the multilocus sequence types (STs) of the examined strains (ATCC10145 was not classified). Y—yolk injection; PV—perivitelline injection. Avirulent: survival rate of 75–100%; weakly virulent: survival rate of 50–74%; moderately virulent: survival rate of 25–49%; virulent: survival rate of 0–24%.

4.2.3 Sublethal symptoms detected at the end of incubation

In addition to the embryo mortality results, sublethal symptoms of the surviving embryos

were also observed at the end of incubation. Based on the visual inspection of the embryos, although all member of the same group was 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 of the newly developed virulence protocol.

Figure 18. visualizes the main types of malformations detected at the end of the experiment. Previously, symptoms mentioned in the literature for bacterial infections were natal sepsis, tachycardia, edema, and vascular leakage (KEIJ et al., 2021). In our study, yolk and pericardial edema were the most significant malformations observed. 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 18. 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. (KASZAB & JIANG et al., 2023)

4.2.4 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.

In this newly developed 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.

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 19**.

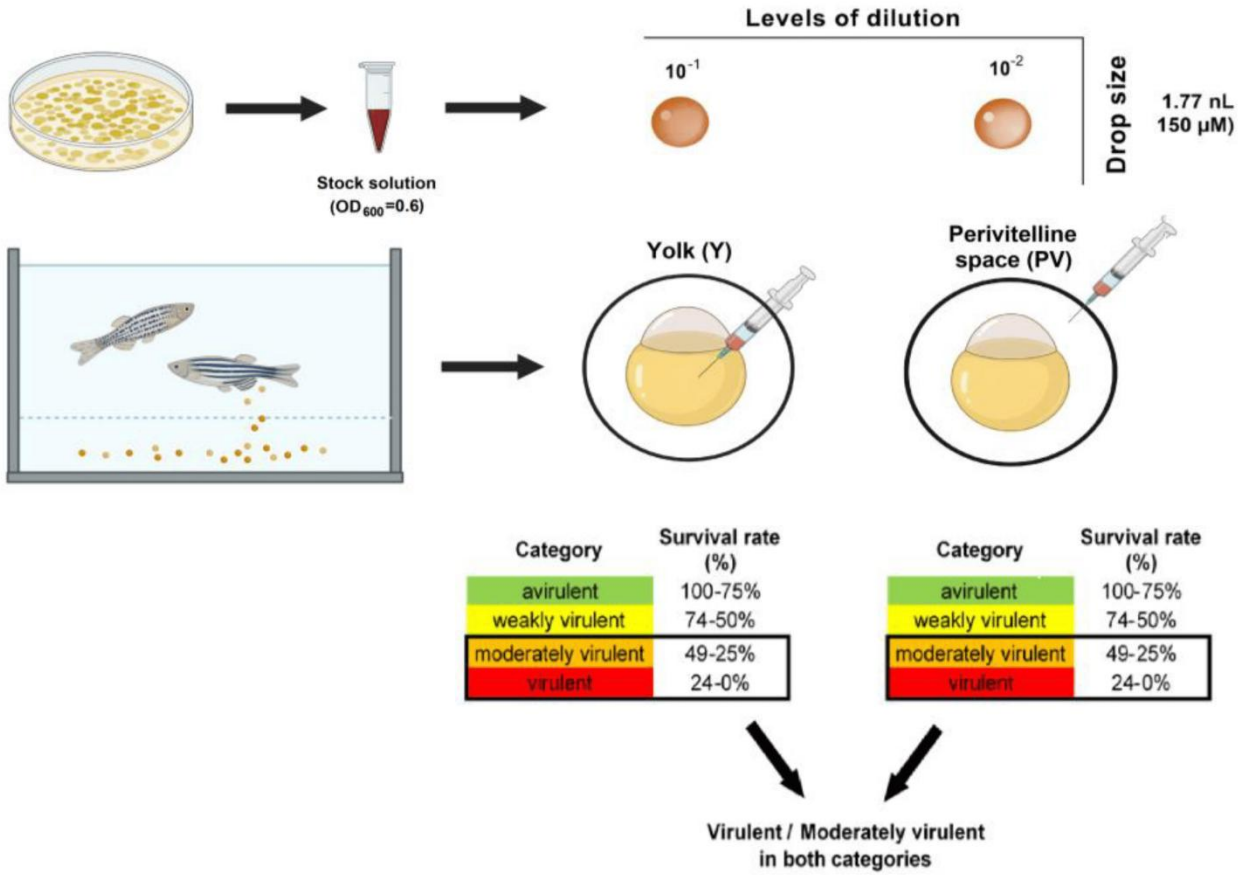

Figure 19. The recommended flowchart of the newly developed virulence infection model using zebrafish

larvae to evaluate the virulence of *P. aeruginosa*.

Our results were published in *Antibiotics* (Q1, impact factor: 4.3) in 2023 (KASZAB & JIANG et al., 2023).

5 DISCUSSION

5.1 Glyphosate and glyphosate-based herbicides (GBHs) induce phenotypic imipenem resistance in *Pseudomonas aeruginosa*

P. aeruginosa frequently displays resistance to numerous types of antibiotics and therapeutic agents, posing a challenge during infection due to the difficulty in finding effective treatment options (DIGGLE & WHITELEY, 2020; WOOD et al., 2023) . Therefore, it is extremely worrying that environmental pollution (LARSSON & FACH, 2022) is increasingly reported to induce antimicrobial resistance.

Glyphosate is the most extensively used herbicide worldwide (GARCÍA et al., 2022), therefore has continually attracted scientific and public attention. Inadequate regulation has led to the frequent use of glyphosate and the increasing concentration of glyphosate residues in the environment, which may cause significant changes in the microbial communities, including their antibiotic resistance (LIAO et al., 2021; DA COSTA et al., 2022).

However, attention in terms of antibiotic resistance has not been sufficiently paid to pathogenic *P. aeruginosa*, which is capable of tolerating (RAOULT et al., 2021) and metabolizing glyphosate as a sole source of phosphorus or nitrogen, leading to its complete degradation within 96 hours (HOODAJI et al., 2012) . This phenomenon of bacteria using glyphosate to enhance their survival strategies may include antibiotic resistance mechanisms; therefore, the genetic and metabolic pathways associated with glyphosate and their effect on antibiotic resistance of *P. aeruginosa* needs to be deeply investigated as it was with other pathogenic species such as *E. coli* (STAUB et al., 2012) and *Salmonella* strains (KURENBACH et al., 2015, KURENBACH et al., 2018).

In our experiments, the effects of glyphosate on the susceptibility of *P. aeruginosa* to the antibiotic imipenem was evaluated. Pre-exposure, co-exposure and the microplate checkerboard method was used to test whether resistance to imipenem in *P. aeruginosa* increased as a result of exposure to glyphosate. The chosen methods helped us to observe the interactions between antibiotics and the tested chemicals (glyphosate, POE(15) and GBHs) and to see how their

interactions affect the antibiotic sensitivity of *P. aeruginosa*. It was found that when *P. aeruginosa* strains were exposed to glyphosate, their resistance to imipenem increased significantly in a concentration-dependent manner. This means that the higher the concentration of glyphosate, the greater the resistance of *P. aeruginosa* to imipenem. Furthermore, four environmental and one clinical *P. aeruginosa* strains were tested, and it was found that these strains were tolerant to GBHs from 1 to 4 v/v% (equivalent to 3.6–14.4 g/L glyphosate acid, depending on the type of formulation) concentrations. This range of doses is commonly used in agriculture and horticulture based on DUKE & POWLES (2008). Phenotypic resistance of *P. aeruginosa* strains under GBH co-exposure was detected to increase against imipenem and reached MIC concentrations in the range of 4 to 64 mg/L, while the breakpoint of resistance to imipenem is 4 mg/L (HTTP16). As for the effects of five different GBHs, the POE(15) free, IPA-salt containing the 'Total' solution significantly increased the MIC of all the five tested *P. aeruginosa* strains when co-exposed to imipenem. Two other herbicides, 'Fozat 480' and 'Dominator Extra 608 SL' (containing IPA salt and DMA salt, respectively), reduced the susceptibility to imipenem in only one strain, ATCC 10145. This result suggests that the effect was not universal in all strains tested and the type of glyphosate salt was not the determining factor in the observed changes in *P. aeruginosa* susceptibility to imipenem. Similar tests were done by Kurenbach et al., on *Salmonella* spp. and *E. coli* (KURENBACH et al., 2017), where GBHs were used at concentrations to reach 200–3,000 mg/L active ingredients of glyphosate. According to their results, bacteria exposed to different formulations of herbicides showed different antibiotic resistance alterations. In another study, glyphosate and the glyphosate-containing 'Roundup' herbicide were found to make *E. coli* and *S. Typhimurium* resistant to kanamycin and ciprofloxacin (KURENBACH et al., 2015). This also suggests that herbicides can affect the resistance of bacteria.

The phenomenon of antibiotic resistance caused by herbicide formulations should be taken into serious account because the herbicide active component in food is regulated and maximum residue levels (MRLs) are set by national and international bodies, such as the Food and Agriculture Organization's Codex Alimentarius Commission (CAC), but many potential residues, or additives in urban and rural environments have not been controlled (KURENBACH et al., 2017).

In addition to the antibiotic resistance inducing effects of herbicides, various reports

summarizing the role of other compounds in bacterial resistance. Studies have shown that non-steroidal anti-inflammatory drugs such as salicylate and other chemotactic obstacle avoidance agents can induce non-hereditary resistance to antibiotics in *E. coli* (VERMA et al., 2018).

Non-hereditary resistance refers to the bacteria that can induce resistance by adjusting some physiological mechanism instead of being heritable variation (ROSNER, 1985). In our study, a similar pattern was observed that resistance to imipenem in *P. aeruginosa* was associated with glyphosate and GBHs exposure. This resistance may be achieved through a mechanism called the "efflux or permeability-related mechanism", as was suggested in the case of *E. coli* (KURENBACH et al., 2015). In other words, this mechanism can pump antibiotics out of the cell, reducing the effectiveness of the drug (LI et al., 2015) or disable the intake of antibiotics through porin channels. This resistance may also be achieved through general cellular response mechanisms against external potential stress that does not require changing the structure of the bacteria's target for a particular antibiotic (PÖPPE et al., 2020). It's important to understand this mechanism of action and can help the supervision and decision-making of antibiotics.

Imipenem, meropenem and doripenem all belong to the carbapenem class of antibiotics. In clinical settings, the resistance of *P. aeruginosa* to this class of antibiotics (carbapenems) can be divided into three distinct phenotypes: imipenem-resistant-meropenem-susceptible type I (IRMS), meropenem-resistant-imipenem-susceptible type II (MRIS), and imipenem-resistant-meropenem-resistant type III (IRMR). It is described that IRMS is resistant to imipenem but sensitive to meropenem. This resistance is usually due to multiple mutations in the bacterial outer membrane protein oprD, resulting in the porin downregulation (PRAGASAM et al., 2016), thereby reducing the passage of antibiotics into the bacteria. At the same time, MRIS is resistant to meropenem but sensitive to imipenem. This resistance is usually due to overexpression of the mexAB-oprM efflux operon in clinical *P. aeruginosa* strains, which pumps antibiotics out of the cell. The last one, IRMR is resistant to both imipenem and meropenem. This resistance is commonly seen in strains that carry the plasmid-mediated carbapenemase gene, and the carbapenemases can directly inactivate these antibiotics (PRAGASAM et al., 2016).

In our experiments, the *P. aeruginosa* (both clinical and environmental) showed resistance to imipenem after pre-exposure and co-exposure to antibiotics and GBHs, while remaining

susceptible to meropenem and doripenem. This suggests that there may be mechanisms related to the regulation of oprD porins, similar to those described in the IRMS phenotype (PRAGASAM et al., 2016) . In addition, it may also involve the general mechanisms of bacteria against environmental stress, which do not necessarily involve changes in specific targeted structures (PÖPPE et al., 2020) . These possibilities and the molecular mechanisms of the glyphosate and GBH-induced imipenem resistance in *P. aeruginosa* are yet to be investigated.

5.2 Evaluating the *in vivo* virulence of environmental *Pseudomonas aeruginosa* using a zebrafish (*Danio rerio*) microinjection model

In recent years, researchers have developed numerous models to study the hazard of *P. aeruginosa*, a type of bacteria that can cause infections, especially in people with weakened immune systems. To investigate how *P. aeruginosa* behaves and causes disease, a variety of experimental hosts have been used to assess its virulence, such as rodents (KASZAB et al., 2015; KUKAVICA-IBRULJ et al., 2008; VAN HEECKEREN & SCHLUCHTER, 2002) , *Drosophila melanogaster* (FAUVARQUE et al., 2002) , *Caenorhabditis elegans* (TAN et al., 1999a; TAN et al., 1999b) , *Galleria mellonella* (MIYATA et al., 2003) and zebrafish (*Danio rerio*) (CLATWORTHY et al., 2009; KUMAR et al., 2018; NOGARET et al., 2021; ROCKER et al., 2015) . These organisms were selected because they share important physiological and genetic features with humans, making them valuable models for understanding human disease. Among these models, zebrafish larvae are particularly noteworthy. One of the main reasons they are favored in research is the ethical considerations. According to European laws, zebrafish larvae that have not yet reached the stage where they can eat independently are not considered the same as laboratory animals (HTTP17) . Therefore, zebrafish embryos younger than 120 hours post-fertilization (hpf) have fewer ethical concerns and regulations in their use for experiments are not strict (BONDUE et al., 2023). Zebrafish larvae offer several practical advantages as well. Similar to *C. elegans* and *D. melanogaster*, zebrafish require less maintenance, are less costly to keep, and the experiments using them can be conducted quickly and can handle a large number of samples at once. At the same time, zebrafish has the advantage of being a vertebrate model. This makes zebrafish larvae an efficient and cost-effective option for testing the virulence of *P. aeruginosa* (BOSE & GHOSH, 2015; WOOD et al., 2023).

The newly developed virulence testing protocol for *P. aeruginosa* has numerous advantages and broaden the scope of virulence evaluation not only for species *P. aeruginosa*, but for other fast-growing, environmentally relevant pathogens as well. This protocol is designed to be completed within just 72 hours, that is a short testing period compared to other vertebrate models. Moreover, the protocol requires only two levels of bacterial dilutions (10^{-1} dilution for PV injections, and 10^{-2} dilution for Y injections) in a very small volume, which simplifies the preparation and execution of the tests and decreases the biohazard and the amount of hazardous waste during performance. An innovative aspect of this protocol is the use of two parallel exposition pathways, which allows the testing of both the invasive and cytotoxic features of *P. aeruginosa*, moreover, we can analyze the sublethal symptoms. In general, this optimal method provides a more comprehensive assessment of the *in vivo* virulence features of *P. aeruginosa* by evaluating the interactions with the host cells.

During the optimization of the test protocol, it was observed that injecting directly into the yolk (Y) usually caused higher mortality than injecting into the perivitellinar space (PV). This was notable even though a lower number bacteria were used for the yolk injections. The higher mortality from yolk injections (Y) is likely because the yolk feeds the developing embryo and directly exposes the embryo's vital resources to the bacteria. Therefore, direct injection into this critical area (Y) probably leads to rapidly emerging and more severe infections compared to the injection into the PV (KAMLER, 2008; MORAN, 2007) . The observation about the higher mortality in yolk injections (Y) was further confirmed by testing 15 different strains of *P. aeruginosa* from environmental and clinical sources. It was found that yolk injections (Y) consistently caused more deaths in the zebrafish larvae compared to perivitelline injections (PV).

Compared to previous studies, it was found that virulent of the same strain can be different depending on the virulence model used. For instance, strain P14 exhibited different levels of virulence between the *Galleria mellonella* model and the zebrafish larvae model (KASZAB et al., 2021). In the wax moth model, it showed very low mortality (indicating low virulence), but in the zebrafish model, it was found to be more virulent, particularly when injected directly into the yolk. Similarly, the strain P66 also showed differences in its virulence feature between the two models. While it was highly virulent in the *Galleria mellonella* model, its impact in the zebrafish model varied depending on the exposition route. These differences indicating the host-dependent virulence features of *P. aeruginosa* strains. Another study about the comparison of zebrafish

larvae microinjection and *G. mellonella* virulence testing on *K. pneumoniae* showed host-dependent differences as well, which was explained by the infection dynamics of the strains examined, and the unique biological systems of the hosts. For example, zebrafish and wax moths have different immune responses, body temperatures, and cellular environments and all those factors may affect the process of infection (ZHANG et al., 2019).

In the validation phase of the newly developed virulence assay, 15 different *P. aeruginosa* strains with different phylogenetic profiles were used. MLST (Multilocus Sequence Typing) analysis of the examined *P. aeruginosa* strains showed that clinical and environmental *P. aeruginosa* strains belonging to closely related sequence types (STs) tended to have similar virulence patterns against zebrafish larvae, regardless of their antibiotic sensitivity. This may suggest that the genetic structure of the bacteria, reflected in their STs, contributes to their virulence characteristics (MAIDEN et al., 1998; URWIN & MAIDEN, 2003) . The MLST analysis further verified that the population structure of *P. aeruginosa* may also play a role in the virulence performance of a given strain (FRESCHI et al., 2019).

Although molecular techniques are widely used to identify some specific virulence factors of *P. aeruginosa* (KASZAB et al., 2011; LIEW et al., 2019; WEI et al., 2020; WOLFGANG et al., 2003), these techniques often fail to fully reveal the behavior of the bacteria in the actual host. Therefore, animal models still play a significant role in virulence testing. The newly developed zebrafish embryo microinjection model provides a unique perspective: the advantage of the zebrafish microinjection model presented in our study was tested and validated using fully characterized *P. aeruginosa* strains, not only from strain collections and clinical settings but also from environmental sources. Therefore, future applications can be used not only for clinical purposes but also for environmental strains.

However, our approach has limitations as it is only optimized for fast-growing gram-negative bacteria (*P. aeruginosa*) with a limited number of clinical strains. Therefore, further validation may be required to evaluate whether the protocol is suitable for testing clinical strains, other (slow-growing) Gram-negative, Gram-positive bacterial species, or fungi.

6 CONCLUSIONS

In our days, environmental safety is harmed by several factors such as the increasing rate of pesticide use worldwide and the spread of antibiotic-resistant, virulent microorganisms such as the 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. It was 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, it is presumed 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 GBH formulations showed variances in the effects on imipenem sensitivity of *P. aeruginosa* which 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 antibiotics on the resistance of bacteria. It may help to prevent the problem of antibiotic resistance induced 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 utilizing zebrafish embryos by microinjection are important for evaluating the infectivity of different microorganisms, particularly in assessing both nosocomial and environmental isolates. Zebrafish embryos share certain similarities with higher vertebrates. Models take advantage of these immunological similarities and can help to 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. The results show that significant differences can be detected depending on the type of exposure, even in the case of the same *P. aeruginosa* strain. The virulence was generally evaluated as moderate, or high under Y injection, while PV injection generally resulted in lower mortality compared to Y injections.

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 3 days, which is very rapid. 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 analyzing antimicrobial resistance and virulence, which may help to combat *P. aeruginosa* infections in the future. The new scientific findings of my research are summarized as follows.

7 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.

8 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

9 SUMMARY

P. aeruginosa is a ubiquitous bacterial pathogen associated with severe infections and antimicrobial resistance. Therefore, understanding the environmental drivers of antibiotic resistance and quantifying the virulence of environmental *P. aeruginosa* strains are critically important.

Glyphosate is the active ingredient of many commercially available herbicides and is widely used in agriculture and horticulture. In recent years, many studies have demonstrated that glyphosate and glyphosate-based herbicides (GBHs) can affect non-targeted organisms in aquatic and terrestrial ecosystems, thus posing a potential threat to human and environmental health.

To understand whether glyphosate and GBHs affect the susceptibility of *Pseudomonas aeruginosa* to imipenem, a potent carbapenem type of antibiotic, and to explore the causes of bacterial resistance, the study investigated how glyphosate and GBH affect the resistance of *P. aeruginosa*. Environmental and clinical *P. aeruginosa* strains were exposed to glyphosate and GBHs solutions at different concentration levels. The induction of imipenem resistance in GBHs, glyphosate, and an additive, POE(15), was tested by microplate checkerboard dilution assay. To quantify their effects, the minimum inhibitory concentration (MIC) for imipenem was used as the baseline in this study, and the changes in resistance during pre-exposition and co-exposition to the test materials were recorded through phenotypic assays.

The resistance of *P. aeruginosa* to imipenem increased significantly when exposed to glyphosate and GBHs, and the resistance was concentration-dependent, but not to POE(15). The results of the FIC assay indicated that glyphosate and several GBHs had antagonistic effects on imipenem.

These results may provide reliable reference data for research in this field and highlight the need to be more cautious about the use of glyphosate and GBHs, because they can increase antibiotic resistance and pose potential risks to the environment and human health. Therefore, here are some suggestions:

1. It is recommended that public health and environmental authorities pay more attention to the possible risks related to herbicide use, especially in areas near hospitals, farms, or wastewater systems.

2. It may also be useful to monitor glyphosate residues together with antibiotic-resistant bacteria in the environment.

3. More in-depth research should be carried out to explore the mechanisms by which herbicides affect bacterial resistance to antibiotics.

Regarding the environmental risk of *P. aeruginosa*, besides antimicrobial resistance, determining the actual virulence of a given strain in a fast and reliable way is extremely important. *In vivo* virulence models play a key role in these terms; therefore, another goal of this research was to explore new ways to determine the virulence of *P. aeruginosa* in a fast and reliable way using zebrafish embryos as the test organism. In order to optimize the newly developed method, a series of optimization and validation tests were carried out.

In this study, zebrafish (*Danio rerio*) embryos were injected with *P. aeruginosa* in yolk (Y injection) and perivitelline (PV injection). The results of infection at two different injection pathways, the mortality, and phenotypic malformations of juveniles were observed and recorded, meanwhile, the genetic analysis of the bacterial strains was performed using previously determined phylogenetic information obtained by multi-locus sequence typing (MLST) technique.

Experiments have shown that even for the same strain, different injection pathways can lead to significantly different pathogenicity results. The virulence was generally moderate to high under Y injection, while PV injection generally resulted in lower virulence compared to Y injections. Through the results of MLST, it is further confirmed that the population structure of *P. aeruginosa* may also play a role in the virulence performance of a given strain.

Our results suggest that the zebrafish embryo injection model is an effective tool to evaluate the virulence of *P. aeruginosa*. The virulence results of two injection pathways of the zebrafish model were compared and combined with the MLST technique, which helped deepen the understanding of the virulence of the *P. aeruginosa* strain. The research provides a newly developed, combined virulence assay to examine the virulence of fast-growing environmental microorganisms, such as *P. aeruginosa* in an unexpensive, easy and reliable way. The novel method can be used for the rapid virulence testing of environmentally relevant opportunistic pathogens and support decision-making processes. Another possible application is to involve the methodology for the safety investigation of microbial products used in bioremediation, soil conditioning or as probiotics.

10 ACKNOWLEDGMENTS

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11 APPENDICES

11.1 References

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11.2 Supplementary data

11.2.1.1 Ethics statement

The Animal Protocol (2013) was approved under the Hungarian Government Regulation on animal experiments (40/2013 (II.4)). All studies were completed before the treated individuals (*Danio rerio* embryos) would have reached the free-feeding stage.

11.2.2 **Supplementary Table 1.**

The minimal inhibitory concentrations (MICs) of wild-type (non-treated) *P. aeruginosa* strains on Mueller-Hinton agar used in glyphosate/GBH/POE(15) testing

Antibiotics	**Group**	**MIC breakpoints of resistance (R >)***	**MIC values of *P. aeruginosa* strains (mg/L)**				
			ATCC 27853	**ATCC 10145**	**ATCC 15442**	**HF234**	**P66**
Cefepime	Cephalosporins	8	8	8	3	1.5	2
Piperacillin	Penicillins	16	4	4	3	4	8
Gentamicin	Aminoglycosides	4	1	**12**	1.5	2	0.75
Ciprofloxacin	Fluoroquinolones	0.5	0.75	0.38	0.09	0.064	0.13
Colistin	Polymyxins	2	**6**	**6**	**4**	**4**	**3**
Doripenem	Carbapenems	2	1	1	0.5	0.19	1
Meropenem	Carbapenems	8	1.5	1.5	0.75	0.125	0.38
Imipenem	Carbapenems	4	2	3	0.75	1	1

*EUCAST: Clinical breakpoints and dosing of antibiotics (HTTP16)
Non-susceptible strains according to the EUCAST breakpoint interpretation tables are highlighted in bold

11.2.3 **Supplementary Table 2.**

The phenotypic and molecular features of the *Pseudomonas aeruginosa* strains used during the development of the novel zebrafish micro virulence test (first published: KASZAB et al., 2021)

Designation	**Origin**	***G. mellonella* virulence (mortality%, 48h)**	**Multilocus sequence type**	**Virulence factors**							**Biofilm forming ability (48h)**	**Antibiotic resistance phenotype**	**Serotype**	**Swimming**	**Swarming**	**Twitching**
				exoS	***exoU***	***lasB***	***algD***	***aprA***	***plcH***	**hemolysis**						
ATCC10145	Type strain, unknown source	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ATCC 15442	Water bottle in animal room	n.d.	252	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ATCC 27853	Clinical	85% (virulent)	155	+	-	+	+	+	+	+	++	Sensitive (10/0)	n.d.	n.d.	n.d.	n.d.
KPS-3	Clinical	65% (moderately virulent)	253	-	+	+	+	+	+	+	+++	Sensitive (10/0)	n.d.	n.d.	n.d.	n.d.
P9	Hydrocarbon contaminated groundwater	25% (avirulent)	377	-	+	+	+	+	+	-	++	Resistant (10/1)	ONT	NM	NM	M
P14	**Hydrocarbon contaminated soil**	**5% (avirulent)**	**2586***	**-**	**+**	**+**	**+**	**+**	**+**	**+/-**	**+++**	**Resistant (10/5)**	**O3**	**HM**	**NM**	**M**
P18	Hydrocarbon contaminated soil	10% (avirulent)	3243*	-	+	+	+	+	+	+/-	+	Sensitive (10/0)	O3	M	M	M
P43	Hydrocarbon contaminated groundwater	10% (avirulent)	253	+	-	+	+	+	+	+	+	Multidrug resistant (10/6)	ONT	M	M	M
P66	**Hydrocarbon contaminated groundwater**	**95% (virulent)**	**455**	**+**	**-**	**+**	**+**	**+**	**+**	**+++**	**+++**	**Sensitive (10/0)**	**O11**	**HM**	**M**	**HM**
P69	Hydrocarbon contaminated soil	50% (weakly virulent)	439	-	+	+	+	+	+	++	-	Resistant (10/5)	O6	M	M	M
P114	Compost	85% (virulent)	3255*	+	-	+	+	+	+	+++	+	Resistant (10/4)	O1	HM	HM	HM
P135	Hydrocarbon contaminated soil	30% (weakly virulent)	3257*	-	-	+	+	+	+	+++	+	Intermediate (10/1)	O3	HM	M	HM
P144	Hydrocarbon contaminated soil	100% (virulent)	1411	-	+	+	-	-	-	+	-	Sensitive (10/0)	O1	HM	M	HM
P164	Sewage	100% (virulent)	3260*	+	-	+	+	+	-	++	-	Sensitive (10/0)	O11	M	NM	M
P177	Hydrocarbon contaminate groundwater	70% (moderately virulent)	3262*	+	-	+	+	+	+	++	+	Sensitive (10/0)	O6	HM	M	HM

bold: strains used for preliminary screening; n.d., no data; ; * unique sequence type; Virulence factors: + positive PCR; – negative PCR; Hemolysis on blood agar: - no hemolysis; + moderate hemolysis, ++ normal hemolysis, +++ intensive hemolysis; Biofilm-forming in a microtiter assay: – no biofilm producer; + weak biofilm producer; ++ moderate biofilm producer; +++ strong biofilm producer. Motility: HM – hypermotile; M – motile; NM – non-motile

11.2.4 **Supplementary Table 3.**

The determination of fractional inhibitory concentration index (FICI) of *P. aeruginosa* strains co-exposed to imipenem and glyphosate acid/POE(15)/ glyphosate-based herbicides.

	Imipenem (mg/L)					**Imipenem (mg/L)**					**Imipenem (mg/L)**					**Imipenem (mg/L)**					**Imipenem (mg/L)**					**Imipenem (mg/L)**				
	MIC_A					**MIC_A**					**MIC_A**					**MIC_A**					**MIC_A**					**MIC_A**				
	Glyphosate acid (mg/L)					**POE(15) (mg/L)**					**Dominator Extra 608 SL v/v%**					**Fozat 480 v/v%**					**Gladiator 480 SL v/v%**					**Roundup Mega v/v%**					**Total v/v%**				
	MIC_B					**MIC_B**					**MIC_B**					**MIC_B**					**MIC_B**					**MIC_B**				
	ATCC27853	ATCC10145	ATCC 15442	HF234	P66	ATCC27853	ATCC10145	ATCC 15442	HF234	P66	ATCC27853	ATCC10145	ATCC 15442	HF234	P66	ATCC27853	ATCC10145	ATCC 15442	HF234	P66	ATCC27853	ATCC10145	ATCC 15442	HF234	P66	ATCC27853	ATCC10145	ATCC 15442	HF234	P66	ATCC27853	ATCC10145	ATCC 15442	HF234	P66
MIC_A alone	2	2	2	4	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	1	2	2	2	2
MIC_A (in the presence of B)	32	>64	32	32	32	2	2	4	2	1	4	>64	4	4	2	4	8	2	2	2	2	4	2	2	2	2	4	4	2	2	4	16	16	>64	16
FIC_A	16	32	16	8	16	1	1	2	1	0.5	2	32	2	2	1	2	4	1	1	1	2	2	1	1	1	1	2	2	1	1	4	8	8	32	8
MIC_B alone	>800	>800	>800	>800	>800	>4	>4	>4	>4	>4	0.5	2	1	0.5	0.5	0.5	2.0	2.0	0.5	0.25	0.125	1.0	1.0	0.25	0.125	0.125	2.0	1.0	0.5	1,0	2.0	2.0	>4.0	>4.0	>4.0
MIC_B (in the presence of A)	>800	>800	>800	>800	>800	>4	>4	>4	>4	>4	0.0625	1.0	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	4.0	0.0625
FIC_B	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.125	0.5	0.0625	0.125	0.125	0.125	0.0313	0.0313	0.125	0.25	0.5	0.0625	0.0625	0.25	0.5	0.5	0.0313	0.0625	0.125	0.0625	0.0313	0.0313	0.0156	1.0	0.0156
FICI	17.0	33.0	17.0	9.0	17.0	2.0	2.0	3.0	2.0	1.5	2.125	32.5	2.062	2.125	1.125	2.125	4.0313	1.0313	1.125	1.25	2.5	2.0625	1.0625	1.25	1.5	1.5	2.0313	2.0625	1.125	1.0625	4.0313	8.0313	8.0156	33.0	8.0156
FICI (mean)	**18.6**					**2.1**					**1.9 (mean of four strains)**					**1.9 (mean of four strains)**					**1.6**					**1.5**					**12.8**				
FICI evaluation	**antagonism**					**indifference**					**indifference except ATCC10145, where FICI=32.5 - antagonism**					**indifference except ATCC10145, where FICI=4.03 - antagonism**					**indifference**					**indifference**					**antagonism**				

FICI < 0.5 – synergy; 0.5 < FICI < 4.0 – indifference; FICI ≥ 4.0 – antagonism