ESCOLA DE CIÊNCIAS DA SAÚDE E DA VIDA
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E EVOLUÇÃO DA BIODIVERSIDADE
MESTRADO EM BIOLOGIA EM ECOLOGIA E EVOLUÇÃO DA BIODIVERSIDADE

CAROLINA SILVEIRA DE OLVEIRA DA SILVA

ANÁLISE DE ISOLADOS BACTERIANOS ORIUNDOS DE AMOSTRAS DE ÁGUA DO SISTEMA AQUÍFERO GUARANI EM RELAÇÃO À SUA SUSCETIBILIDADE A AGROTÓXICOS E ANTIMICROBIANOS

Porto Alegre
2022

PÓS-GRADUAÇÃO - *STRICTO SENSU*

Pontifícia Universidade Católica
do Rio Grande do Sul

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

ESCOLA DE CIÊNCIAS DA SAÚDE E DA VIDA

PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E EVOLUÇÃO DA BIODIVERSIDADE

MESTRADO EM ECOLOGIA E EVOLUÇÃO DA BIODIVERSIDADE

ANÁLISE DE ISOLADOS BACTERIANOS ORIUNDOS DE AMOSTRAS DE ÁGUA DO SISTEMA AQUÍFERO GUARANI EM RELAÇÃO À SUA SUSCETIBILIDADE A AGROTÓXICOS E ANTIMICROBIANOS

Carolina Silveira de Oliveira da Silva

Orientadora: Drª. Renata Medina da Silva

Coorientadora: Drª. Sílvia Dias de Oliveira

DISSERTAÇÃO DE MESTRADO

PORTO ALEGRE – RS – BRASIL

2022

CAROLINA SILVEIRA DE OLIVEIRA DA SILVA

ANÁLISE DE ISOLADOS BACTERIANOS ORIUNDOS DE AMOSTRAS DE ÁGUA DO SISTEMA AQUÍFERO GUARANI EM RELAÇÃO À SUA SUSCETIBILIDADE A AGROTÓXICOS E ANTIMICROBIANOS

Dissertação apresentada como requisito para obtenção do grau de Mestre pelo Programa de Pós-Graduação em Ecologia e Evolução da Biodiversidade da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientadora: Drª. Renata Medina da Silva

Coorientadora: Drª. Sílvia Dias de Oliveira

Porto Alegre

2022

SUMÁRIO

1. RELAÇÃO DE FIGURAS

2. RELAÇÃO DE TABELAS

3. DEDICATÓRIA E AGRADECIMENTOS

Dedico este trabalho à minha família, que sempre me apoiou e incentivou a realizá-lo, sempre se mostrando extremamente pacientes durante os momentos de dificuldade. A vocês, também, meus mais sinceros agradecimentos.

Agradeço à minha orientadora, Drª. Renata Medina da Silva, pela confiança e cooperação imprescindíveis para a realização desse trabalho. Saiba que sua paixão pela pesquisa sempre me serviu como eterna fonte de inspiração.

À minha co-orientadora, Drª. Sílvia Dias de Oliveira, por agregar os mais valiosos conhecimentos.

À Drª. Audrey Menegaz Proença cujo conhecimento e paciência se mostraram essenciais para a finalização desse trabalho.

Aos colegas do Laboratório de Imunologia e Microbiologia (PUCRS) e pesquisadores do Instituto do Petróleo e dos Recursos Naturais (IPR/PUCRS) por toda ajuda, troca de conhecimentos e experiências, e incomparável apoio emocional até as últimas etapas.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), à Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) e ao Instituto do Petróleo e dos Recursos Naturais (IPR/PUCRS) pelo apoio científico e financeiro.

4. RESUMO

O uso de agrotóxicos em atividades rurais gera impacto no meio ambiente, sendo possível encontrar indícios de contaminação por esses compostos em amostras de diferentes ambientes aquáticos. Há relatos de adaptações de bactérias à presença de agrotóxicos e de resistência cruzada entre essas moléculas e antimicrobianos. O Sistema Aquífero Guarani (SAG) é um dos mais importantes sistemas hidrostáticos da porção sul da América do Sul. Como parte de um projeto anterior, 23 isolados bacterianos foram obtidos de amostras de água do SAG de três regiões de intensa agricultura do estado do Rio Grande do Sul (RS, Brasil). O presente estudo investigou a suscetibilidade desses isolados bacterianos a pesticidas e antimicrobianos. A maioria dos isolados foi identificada como pertencente aos gêneros *Bacillus*, *Lysinibacillus* ou *Pseudomonas*. Também foi possível observar um exemplar de *Enterococcus*, *Leuconostoc* e *Staphylococcus*. Todos os isolados foram expostos a um gradiente de concentração de herbicida à base de ácido diclorofenoxiacético (1,2 µg/mL, 1,5 µg/mL e 1,2 µg/mL) e herbicida à base de glifosato (4 µg/mL, 6 µg/mL e 8 µg/mL), separadamente, por 45 h a 25ºC. Alíquotas de 5 h, 20 h, 30 h e 45 h dos tratamentos foram analisadas para estimativa de sobrevivência (contagens de unidades formadoras de colônia por mL). Os isolados apresentaram resposta bastante heterogênea aos tratamentos com herbicidas, dentre os quais 19 foram tolerantes ou altamente tolerantes a pelo menos uma concentração de um herbicida. Os isolados de Nova Palma (Quarta Colônia) mostraram um valor médio de sobrevivência máxima (SM) a ambos os herbicidas significativamente superior ao de doutras regiões. Além disso, o tipo resposta dos isolados a ambos os herbicidas mostrou uma correlação positiva significativa. Os 13 isolados que foram identificados como *Bacillus*, *Lysinibacillus*, *Pseudomonas* e *Enterococcus* também foram submetidos a testes de suscetibilidade a antimicrobianos, utilizando os métodos de disco difusão e microdiluição. Destes, 7 apresentaram resistência a pelo menos um fármaco, muitos destes com valores de MIC acima do valor de corte para resistência. Os isolados de *Pseudomonas* foram os que apresentaram resistência ao maior número de antimicrobianos testados. Integrando os dados, os isolados com o maior e o menor índice de múltipla resistência a antibióticos (MRA) apresentaram valores altos e baixos de SM aos herbicidas, respectivamente. Uma análise de componentes principais também mostrou que o padrão de resposta aos antimicrobianos parece estar relacionado às características taxonômicas dos isolados, o que não foi observado para a resposta aos herbicidas. Nossos dados indicam que herbicidas e antimicrobianos podem favorecer a propagação de algumas populações bacterianas do SAG e prejudicar a manutenção de outras. Desta forma, estes dados servem como evidências de que tais estressores estão potencialmente alterando a estrutura das comunidades microbianas em ambientes naturais. Eles também destacam a importância de estudar os microrganismos desses ambientes como potenciais indicadores e/ou remediadores de impacto ambiental derivado de atividades humanas.

Palavras-chave: Tolerância microbiana; Resistência Microbiana; Ecologia Microbiana; Poluição aquática; Pesticidas; Antimicrobianos

5. ABSTRACT

Analysis of bacterial isolates from water samples of the Guarani Aquifer System regarding their susceptibility to agrochemicals and antimicrobials

The use of pesticides in rural activities induces an impact on the environment, and it is possible to find evidence of contamination by these compounds in samples from different aquatic environments. There are reports of adaptations of bacteria to the presence of pesticides and of cross-resistance between these compounds and antimicrobial drugs. The Guarani Aquifer System (GAS) is one of the most important hydrostatic systems in the southern portion of South America. As part of a previous project, 23 bacterial isolates were obtained from GAS water samples from three agriculture-intensive regions of the state of Rio Grande do Sul (RS, Brazil). The present study investigated the susceptibility of these bacterial isolates to pesticides and antimicrobials. Most isolates were identified as belonging to the genera *Bacillus*, *Lysinibacillus* or *Pseudomonas*. It was also possible to observe one representative of *Enterococcus*, *Leuconostoc* and *Staphylococcus*. They were all exposed to a concentration gradient of 2,4-Dichlorophenoxyacetic acid-based herbicide (1.2 µg/mL, 1.5 µg/mL and 1.2 µg/mL) and glyphosate-based herbicide (4 µg/mL, 6 µg/mL and 8 µg/mL), separately, for 45 h at 25ºC. Aliquots from 5 h, 20 h, 30 h and 45 h treatments were analyzed for survival estimation (colony-forming unit/mL counts). The isolates presented a very heterogeneous response to the herbicides' treatments, among which 19 were tolerant or highly tolerant to at least one concentration of one herbicide. The isolates from Nova Palma (Quarta Colônia) showed mean values of maximum survival (MS) for both herbicides significantly higher than those from other regions. Furthermore, the response of isolates to both herbicides showed a significant positive correlation. The 13 isolates that were identified as *Bacillus*, *Lysinibacillu*s, *Pseudomonas* and *Enterococcus* were also submitted to antimicrobial susceptibility tests, using disk diffusion and microdilution methods. Among these, 7 presented resistance to at least one drug, many of which presented MIC values above the breakpoint value for resistance. The *Pseudomonas* isolates showed resistance to the highest number of antimicrobials tested. Integrating the data, the isolates with the highest and the lowest index of multiple antibiotic resistance (MAR) showed high and low values of MS to herbicides, respectively. A principal component analysis also showed that the pattern of response to antimicrobials seemed to be related to the taxonomic characteristics of the isolates, which was not observed for the response to herbicides. Our data indicate that herbicides and antimicrobials may favor the propagation of some GAS bacterial populations, harming the maintenance of others. In this context, these data serve as evidence that such stressors are potentially altering the structure of microbial communities in natural environments. They also highlight the importance of studying the microorganisms in these environments as potential indicators and/or remediators of environmental impact from human activities.

Keywords: Microbial tolerance; Microbial Resistance; Microbial Ecology; Aquatic pollution; Pesticides; Antimicrobials

6. APRESENTAÇÃO

Dado ao crescente uso de compostos químicos na agricultura e pecuária e na emergente crise de bactérias multirresistentes, esta dissertação de mestrado se propôs a analisar o perfil de suscetibilidade aos herbicidas Roundup® (à base de glifosato) e DEZ® (à base de 2,4-D), bem como a antimicrobianos, de bactérias isoladas a partir de amostras de poços irrigados com água do Sistema Aquífero Guarani (SAG), localizados na região de Alegrete, Candelária e Quarta Colônia, no Rio Grande do Sul (RS). Tais amostras foram obtidas como parte do projeto "Mapeamento em Subsuperfície do Aquífero Guarani", desenvolvido pelo Instituto do Petróleo e dos Recursos Naturais (IPR) em 2018.

Além de serem os agrotóxicos mais comercializados no Brasil (1), o glifosato e o 2,4-D estão dentre os herbicidas mais utilizados na parte central e leste da região da depressão central do RS (2), região de origem das amostras de água do SAG, a partir das quais os isolados bacterianos usados neste trabalho foram obtidos. De acordo com o IBGE, estas regiões possuem uma população de habitantes estimada em 31.475 (Candelária), 63 mil (Quarta Colônia) e 72.490 (Alegrete) (3). Dentre os cultivares, destaca-se o cultivo de arroz (3), especialmente nas regiões de Alegrete e Candelária.

Em 27 de agosto de 2019**,** o Ato nº 58 alterou as classificações toxicológicas dos agrotóxicos no Brasil, em relação ao Decreto n° 4074, de 04 de janeiro de 2002, que regulava tal classificação até então. Com esta alteração, a maioria dos agrotóxicos passou da categoria "altamente tóxico" para "pouco tóxico". O 2,4-D passou de Classe I – "extremamente tóxico" para a Categoria 4 – "produto pouco tóxico". O Glifosato foi de Classe III – "medianamente tóxico" para a Categoria 5 – "produto improvável de causar dano agudo". Esta flexibilização para uso de agrotóxicos no país auxiliou o Brasil a se manter na posição de maior consumidor de agrotóxicos do mundo, posição que lidera desde 2008 (4). Neste sentido, o Brasil se configura como um país com um alto potencial de apresentar inúmeros problemas decorrentes da presença de agrotóxicos no ambiente, muitos dos quais ainda são desconhecidos.

Esta dissertação, apresentada no formato de um manuscrito, é iniciada com uma introdução sobre o uso de pesticidas no Brasil e no mundo, os mecanismos de ação de ambos os herbicidas utilizados nesta pesquisa, assim como o seu comportamento no ambiente. Além disso, também é abordado o conceito de resistência bacteriana a antimicrobianos e os principais mecanismos que levam bactérias a desenvolverem esse fenótipo. Dentre as possíveis causas, é salientada a contaminação ambiental por pesticidas e antimicrobianos, com enfoque em reservatórios d'água doce. A seção metodológica descreve como foi realizada a recuperação

dos isolados bacterianos utilizados neste estudo, a sua identificação taxonômica através de métodos moleculares, assim como os testes de suscetibilidade a herbicidas e antimicrobianos. Em seguida, são apresentados os resultados obtidos através dos métodos previamente descritos. Inicialmente é relatado que identificamos bactérias como pertencendo aos gêneros *Bacillus* e *Lysinibacillus*, *Pseudomonas*, *Enterococcus*, *Staphylococcus* e *Leuconostoc*, enquanto uma parcela dos isolados não foi possível de ser identificada. Em termos gerais, a maior parte dos isolados se mostrou tolerante ou altamente tolerante aos herbicidas, enquanto uma alta porcentagem também apresentou resistência aos antimicrobianos testados. Uma relação entre a resposta frente a estes dois tipos de estressores também foi avaliada para os isolados. Finalmente, a última seção representa a discussão levantada com base nestes resultados corroborados e/ou comparados com estudos semelhantes encontrados na literatura.

O manuscrito desta dissertação é pretendido ser submetido ao periódico *Applied and Environmental Microbiology*® (fator de impacto 4.792), já encontrando-se nas regras de formatação estipuladas pela revista.

REFERÊNCIAS:

1. ANVISA (2018) RELATÓRIO DE ATIVIDADES DA GERÊNCIA GERAL DE TOXICOLOGIA 2017: Principais ações, resultados e perspectivas. Brasília
2. Talha-Mar Soluções Ambientais (2010) Levantamento do Uso e da Criticidade dos Agrotóxicos Usados no Estado do Rio Grande do Sul. Centro de Vigilância em Saúde da Secretaria da Saúde - CEVS/SES
3. IBGE - Instituto Brasileiro de Geografia e Estatística. <https://cidades.ibge.gov.br/brasil/rs/> Acesso em: 27/04/2021
4. Pignati WA, Lima FAN de S e, Lara SS, *et al.* (2017) Distribuição espacial do uso de agrotóxicos no Brasil: uma ferramenta para a Vigilância em Saúde. Ciência & Saúde Coletiva 22:3281–3293. https://doi.org/10.1590/1413-812320172210.17742017

7. MANUSCRITO

Herbicide and antibacterial drugs susceptibility profile of bacterial isolates from the Guarani Aquifer System

Carolina Silveira de Oliveira Silva[1], Sílvia Dias de Oliveira[1], Audrey Menegaz Proença[1,2], Eduarda Vargas Abati[1], Mariana Rosa dos Reis[1], Letícia Marconatto[2], Cássio Stein Moura[3+], Renata Medina-Silva[1,2*]

[1]Pontifical Catholic University of Rio Grande do Sul, PUCRS, School of Health and Life Sciences, Laboratory of Immunology and Microbiology, Porto Alegre, Brazil

[2]Pontifical Catholic University of Rio Grande do Sul, PUCRS, Institute of Petroleum and Natural Resources, Geobiology Group, Porto Alegre, Brazil

[3]Pontifical Catholic University of Rio Grande do Sul, PUCRS, Institute of Petroleum and Natural Resources, Interdisciplinary Group of Applied Geophysics, Porto Alegre, Brazil.
[+]Present affiliation: Geofísica Stein (www.geostein.org), Porto Alegre, Brazil.

[*] **Corresponding author**:

Dr. Renata Medina-Silva
renata.medina@pucrs.br
Av. Ipiranga, 6681, Prédio 96J, Sala 607, Porto Alegre, RS, Brazil,

Keywords: Microbial tolerance; Microbial Resistance; Microbial Ecology; Aquatic pollution; Pesticides; Antimicrobials

Abstract

Contamination of water bodies by substances used in human activities, such as pesticides and antibiotics, is an environmental problem of deep concern on a global scale. This study investigated the susceptibility to pesticides and antimicrobials of 23 bacterial isolates from water samples of the Guarani Aquifer System (GAS) - one of the most important hydrostatic systems in the southern portion of South America- from three agriculture-intensive regions of the state of RS (Brazil). They were exposed to a concentration gradient of 2,4-D-based herbicide and glyphosate-based herbicide, separately, for 45 h at 25ºC. Those that were identified as *Bacillus*, *Lysinibacillu*s, *Pseudomonas* and *Enterococcus* were also submitted to antimicrobial susceptibility tests. In the herbicides' treatments 19 isolates were tolerant or highly tolerant to at least one concentration of one chemical, with significant positive correlation between maximum survival values of both treatments. From the 13 isolates tested for antimicrobials, 7 presented resistance to at least one drug. Also, the isolates with the highest index of multiple antibiotic resistance showed high values of maximum survival to herbicides. The response to antimicrobials seemed to be related to isolates' taxonomy, which was not observed for the response to herbicides. Our study was the first to raise data about the susceptibility to herbicides and antimicrobials of bacterial isolates from an aquifer, indicating that these chemicals may interfere in population dynamics of bacterial species in their environment. They also highlight the importance of studying microbes from unexplored environments as potential indicators and/or remediators of environmental contaminants, in line with the One Health principle.

INTRODUCTION

Since 2008, Brazil occupies the first position in the ranking of pesticide consumption in the world (1). The use of pesticides in rural activities impacts the environment, and it is possible to find evidence of contamination by these substances in samples from water reservoirs and other aquatic environments (2–5). Among the most used herbicides in Brazil (6), glyphosate and 2,4-dichlorophenoxyacetic acid (or 2,4-D) are broadly applied to a wide variety of crops in southern Brazil (7).

Glyphosate is an aminophosphonate analogous to the natural amino acid glycine, occupying its place in protein synthesis. First sold commercially in 1974, it has become the most common and intensively used herbicide in the world, registered to control weeds in a variety of agricultural and non-agricultural environments. In Brazil, more than thirty

formulations of the herbicide glyphosate are registered and marketed, including glyphosate-sesquisodium patented by Monsanto and sold as Round-up® (8). Glyphosate acts by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase), which is not present in mammalian systems. However, in addition to occurring in plants, which are the target organisms of glyphosate, this enzyme is also present in bacteria, fungi, algae and protists of the Apicomplexa group (9). In this context, there are studies that indicate different levels of sensitivity to glyphosate of the EPSP synthase enzyme of microbial species from these taxonomic groups (10–12).

The herbicide 2,4-D is a synthetic auxin derived from phenoxyacetic acid and registered for commercial use since the 1940s (13). Due to its commercial formulation in the form of salts, amine and ester, 2,4-D becomes a rapidly metabolized compound and is classified as biodegradable, not persisting on the water surface (14). However, a study reported that 2,4-D residues can promote changes in the structure of microbial communities in the soil (15), indicating that it should not be considered an inert and harmless compound to ecosystems.

Moreover, according to Kurenbach *et al*. (2015), herbicides have the ability to change the response of microorganisms to antibiotics. Simultaneous exposure of populations of *Escherichia coli* and *Salmonella enterica* subsp. *enterica* sv. Typhimurium to commercial herbicides and antibiotics of different classes promoted changes in the susceptibility of these populations to antimicrobials (16). Studies have demonstrated that the adaptive response of microbial populations generated through exposure to pesticides depends on the combination of bacterial strain, antibiotics and pesticides used during the experiment. These variations have also been noticed for commercial pesticide formulations (16–18).

Bacterial resistance to antimicrobials is the ability of bacteria to resist the effects of antibiotics or biocides that are intended to kill or control them (19). Variation in responses to antimicrobials can be caused by genetic or physiological differences between bacteria. Innate resistance may also depend on expressed or repressed genes, resulting in increased efflux or decreased influx of antimicrobials and, consequently, reduced intracellular concentrations of these drugs. Resistance mediated by changes in gene expression is also known as adaptive response, which acclimates bacteria to the environment (20, 21). It can be triggered by antimicrobials and other stressors, or even by environmental factors (22).

Antimicrobial contamination in water bodies due to agriculture is recognized as a growing problem worldwide (23, 24). Residues of antimicrobial drugs and other pollutants, even in low inhibitory concentrations, in the environment impose selective pressure on bacterial

populations, which results in the prevalence of resistant bacteria (25). Aquatic environments disseminate not only antimicrobial-resistant bacteria, but also transport resistance genes, which can induce significant genetic changes into bacterial populations in natural ecosystems. Thus, in such systems, environmental bacteria may act as an unlimited source of genetic elements that can act as vectors of resistance genes, which can reach pathogenic organisms, leading to an increased risk for human health (26, 27). A study that evaluated bacterial isolates from seawater identified that over 90% of them were resistant to more than one antibiotic and 20% to at least five different antimicrobials (28). However, there are no similar studies in the literature for microbial communities or isolates from continental water reservoirs.

Since antibiotics have been introduced into agricultural environments and spread through water bodies, some endemic bacterial populations resistant to these drugs may have eventually been selected (29). In parallel, some studies report that the use of herbicides leads to the accumulation of these components in the environment, which can impact microbial communities in different ecosystems by selecting herbicide-tolerant populations (15, 30, 31). Based on this, studies that evaluate herbicide-tolerant and/or antibiotic-resistant bacterial isolates derived from aquatic environments s are still scarce. Regarding microorganisms from underground water systems, like aquifers, no record in the literature was detected.

The Guarani Aquifer System (GAS) is one of the most important hydrostatic systems in the southern portion of South America. The Paraná River Basin, responsible for housing the GAS, is the most important hydrogeological province in Brazil. It has about 45% of the underground water reserves of the entire national territory and, due to its ability to store and release large amounts of water, it is a source of water for family consumption, industry, and agriculture (25). Chemical analyzes performed on water samples from the GAS indicated the presence of the pesticide 2,4-D (32), among other chemical compounds (33). These results indicate that this underground water storage system may be suffering anthropic impact.

In this context, this study analyzed bacterial isolates from water samples of the GAS along three regions of Rio Grande do Sul (RS), in relation to their susceptibility to pesticides and antibiotics. We detected several isolates with tolerance or resistance to glyphosate and/or 2,4-D, and also antibiotic-resistant ones.

METHODS

Origin of Bacterial Isolates

Bacterial isolates were previously obtained from water samples collected aseptically from

artesian wells connected to the GAS at different points from three regions - Candelária, Alegrete and Quarta Colônia - in the state of RS (Brazil) by the Institute of Petroleum and Natural Resources (IPR), in 2018 (29). The sampling sites (wells) identification, depth, region and coordinates (in Universal Transverse Mercator, UTM) (32), as well as the number of bacterial isolates obtained from each one, are indicated in Table 1. The isolates were preserved in 30% glycerol and stored in freezers at -80ºC.

For the present analyses, a total of 23 isolates were recovered in BHI (Brain Heart Infusion) broth, at 28 ºC, for 24 to 48 h. These cultures then were plated on BHI agar and incubated at 28 ºC for 24 to 48 h for colony isolation. The colonies were analyzed under light microcopy (1000x) after Gram staining to confirm the isolate purity and morphology. For all herbicide treatments and antimicrobial susceptibility analyses, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 33019 and *Enterococcus faecalis* ATCC 29212 were used as reference strains.

Table 1: Sampling sites of the Guarani Aquifer System water samples from which the bacterial isolates were previously obtained. The names, well depth, coordinates (in UTM) and number of bacterial isolates of each site, as well as their region and city in Rio Grande do Sul state (Brazil), are indicated.

Region	City	Sampling Sites				Nº of isolates
		Name (abbreviation)	Well depth (meters)	UTM X	UTM Y	
Candelária	Candelária	Candelária (C)	91	326,940	6,716,545	4
		Várzea do Botucaraí (VB)	60	328,378	6,690,715	3
Alegrete	Alegrete	Caverá 1 (CAV1)	104	641,697	6,679,200	1
		Caverá 2 (CAV2)	112	639,680	6,679,013	3
		Capivari (CAP)	123	601,401	6,696,673	6
Quarta Colônia	Faxinal do Soturno	Gruta Sítio Alto (GRU)	40	253,331	6,731,263	1
		Gruta dos Mellos (MEL)	100	257,279	6,728,510	2
	Nova Palma	Caemborá (CAE)	140	277,021	6,737,934	1
		Riacho Felis (RF)	60	275,426	6,740,767	2

Source of sampling sites' data: Soares e*t al*., 2019 (33).

Taxonomic Identification of Bacterial Isolates

For taxonomic identification of bacterial isolates, DNA was extracted using the QIAamp® DNA Stool Mini Kit (50) (Qiagen). The complete sequence of small ribosomal subunit rRNA (16S) gene was amplified through polymerase chain reaction (PCR) using the

following primers: 9 forward (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1542 reverse (5′ AGA AAG GAG GTG ATC CAG CC 3′) (34). Amplification was performed in a 50 µL mixture, consisting of 1.5 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of each dNTP, 1 U Platinum *Taq* DNA polymerase, 1X PCR reaction buffer, and approximately 10 ng of genomic DNA. PCR conditions used were the following: an initial activation at 94 °C for 2 min and 25 cycles of 45 s at 94 °C, 45 s at 55 °C, and 60 s at 72 °C, followed by an extension at 72 °C for 3 min. The reaction products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega) and sequenced by the capillary method by ACTGene Análises Moleculares (Nova Alvorada, RS, Brazil). The forward and reverse sequencing reads were assembled and trimmed into single contigs using the software DNA Sequence Assembler version 5.15.0 (*Phred quality score cutoff of* < 20). Contigs were then aligned against the National Center for Biotechnology Information (NCBI) database through the Basic Local Alignment Search Tool (BLAST®). Similar and reference sequences were downloaded from the NCBI database to perform a phylogenetic analysis. Sequences were further aligned using the ClustalW tool incorporated in MEGA X (35). Phylogenetic analyses were performed using the Phylogeny Tool on MEGA X. The phylogenetic trees were constructed using the maximum likelihood method and the Tamura-Nei model (36). Statistical significance was measured by 1,500 bootstrap replications. The 16S rRNA sequences from isolates that were taxonomically identified were deposited in the NCBI database: C9 (OM949960), C14 (OM949967), CAE1 (OM949968), CAP2 (OM949990), CAV19 (OM952179), CAV211 (OM952208), GRU33 (OM952259), MEL33 (OM952437), VB1 (OM952920) and VB4 (OM952921).

Herbicide Treatments

Bacteria were grown in BHI broth (at 25 °C during 24-48h) and the optical density of cultures were measured at a wavelength of 600 nm using a spectrophotometer. The cultures were washed with 0.9% saline to remove growth medium. Bacterial suspensions were prepared in 0.9% saline in a cell density equivalent to the 0.5 McFarland standard. The isolates were exposed to a concentration gradient of glyphosate and 2,4-D in microdilution tests in 96-well plates (26,27). Nominal concentrations were used to perform this test. For glyphosate, the treatment concentrations were 4 µg/mL, 6 µg/mL, and 8 µg/mL; and for 2,4-D, the concentrations were 1.2 µg/mL, 1.5 µg/mL and 1.2 µg/mL. These concentrations were equivalent to 10x the maximum permitted values (MPV) for glyphosate and 2,4-D, stipulated by the Brazilian National Council for the Environment (CONAMA) Resolution No. 396 (2008)

(37), which provides guidelines for the groundwater chemical parameters. The control group was exposed to sterile 0.9% saline solution. Four different exposure times were chosen for dilution and seeding of aliquots: 5 h, 20 h, 35 h, and 45 h. The control group and treatments were diluted at 10^{-3}, 10^{-4} and 10^{-5}, drop-plated (10 µL) in triplicate on BHI agar, and cultured at 28 ºC for 24 h, for subsequent colony count and estimation of colony-forming units per mL (CFU/mL). Data were expressed in semi-log survival curves. Differences were considered significant when at least one $\log_{10}$ of variation (considering the standard deviation) was observed in relation to the control without herbicides (100% survival). There are no standard criteria for considering bacteria sensitive, tolerant or resistant to herbicides, since different studies use distinct parameters to classify bacterial species or isolates regarding this profile. In our experiments we considered that the isolates that presented significant increased survival compared to the control along the herbicides' treatments were distinguished as "highly tolerant", similarly as previous studies did (30, 31, 38–40). Moreover, those isolates that showed significant decreased survival were considered sensitive, and those that had no significant differences compared to the control were considered tolerant. From the survival curves, the average value of maximum survival (MS) (along with MS treatment time and concentration) to both herbicides of all isolates were selected as a parameter to be used for different comparison analysis among isolates.

Antimicrobial Susceptibility Tests

The antibiotic susceptibility testing was performed for isolates that we properly identified at least to genus level, by Kirby-Bauer disk diffusion method according to Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST) (41) guidelines using commercial antimicrobial disks. BrCAST is officially recognized by European Committee on Antimicrobial Susceptibility Testing (EUCAST) as a national committee, following its standards and parameters. For those that presented resistance to at least two antimicrobial drugs we calculated the multiple antibiotic resistance (MAR) index (42, 43). MAR index, when applied to a single isolate, is defined as *a/b*, where *a* represents the number of antibiotics to which the isolate was resistant, and *b* represents the number of antibiotics to which the isolate was exposed (42).

Minimum inhibitory concentrations (MICs) were determined for representatives of different classes of antibiotics, with isolates that were detected as resistant in the disk diffusion tests. *Pseudomonas* isolates were tested for ceftazidime (cephalosporin), tobramycin (aminoglycoside) and aztreonam (monobactam). The *Bacillaceae* family were tested for

meropenem (carbapenem), erythromycin (macrolide), clindamycin (lincosamide) and vancomycin (glycopeptide). MICs were determined by microdilution method according to BrCAST standards. The breakpoints for resistance adopted were those established by the BrCAST guidelines.

Statistical Analysis

Statistical analyses were performed using R (version 3.6.1) (44). MS values of all isolates were $\log_{10}$ transformed and Shapiro-Wilk normality test was applied ($p < 0.05$). Levene's test for homogeneity of variance ($p < 0.05$), was used to verify to MS variances of isolates grouped by region. To compare MS values of isolates grouped by regions, *one-way* ANOVA ($p < 0.05$) and the Tukey HSD (Honestly Significant Difference) post-hoc test ($p < 0.05$) were applied. A linear regression analysis ($p < 0.05$) was employed between MS data on Glyphosate and 2,4-D. The Pearson correlation test was also applied for these data ($p < 0.05$). MS values were $\log_{10}$ transformed prior to analysis and visualization to ensure normality. A Principal Coordinate Analysis (PCA) with data scaling was performed using R package "vegan". Five dimensions were investigated, consisting of $\log_{10}$-transformed MS data from glyphosate and 2,4-D treatments, and the diameters of inhibition zones from disk diffusion tests of three broad-spectrum antibiotics. Diameters of inhibition zones were centered at zero using the resistance cutoff value for each antibiotic/isolate pair, such that positive values indicated susceptibility and negative values indicated resistance. This transformation was also applied prior to heatmap visualization. A Permutation test (PERMANOVA) was applied for the PCA analysis ($p < 0.05$).

Statistical analyses were performed using R (version 3.6.1) (44). MS values of all isolates were $\log_{10}$ transformed and Shapiro-Wilk normality test was applied ($p < 0.05$). Levene's test for homogeneity of variance ($p < 0.05$) was used to verify to MS variances of isolates grouped by region. To compare MS values of isolates grouped by regions, *one-way* ANOVA ($p < 0.05$) and the Tukey HSD (Honestly Significant Difference) post-hoc test ($p < 0.05$) were applied. A linear regression analysis ($p < 0.05$) was employed between MS data on Glyphosate and 2,4-D. The Pearson correlation test was also applied for these data ($p < 0.05$). MS values were $\log_{10}$ transformed prior to analysis and visualization to ensure normality. A Principal Coordinate Analysis (PCA) with data scaling was performed using R package "vegan". Five dimensions were investigated, consisting of $\log_{10}$-transformed MS data from glyphosate and 2,4-D treatments, and the diameters of inhibition zones from disk diffusion tests of three broad-

spectrum antibiotics. Diameters of inhibition zones were centered at zero using the resistance cutoff value for each antibiotic/isolate pair, such that positive values indicated susceptibility and negative values indicated resistance. This transformation was also applied prior to heatmap visualization. A Permutation test (PERMANOVA) was applied for the PCA analysis ($p < 0.05$).

RESULTS

Taxonomic Identification of Isolates

We performed the taxonomic identification of 23 isolates from the GAS, based on the sequencing of 16S rRNA gene and on phylogenetic analyses (Figure 1). Among the isolates, nine presented a phylogenetic relationship with genera from the *Bacillaceae* family. From these, five (C15, GRU33, MEL33, RF3121, and VB1) showed similarity with *Lysinibacillus* strains sequences, most belonging to the *L. fusiformis* species. Three other isolates (C11, MEL13, and RF311) also grouped within this *Lysinibacillus* clade, but with a very low bootstrap value, which cannot support their taxonomic identification. Moreover, four isolates (C14, CAV19, CAV23, and VB5) formed another clade, in this case with *Bacillus cereus* and *B. subtilis* strains sequences. Apart from the *Bacillaceae* isolates, three bacteria grouped within a clade with reference sequences of *Pseudomonas* genus, each one with close phylogenetic relationship with distinct species of this genus: *P. rhodesiae* (CAP2), *P. protegens* (C9) and *P. koreensis* (VB4). Also, the isolate CAP5 grouped with *Enterococcus faecalis* and *E. hirae* strains, CAV211 within a *Leuconostoc mesenteroideis* and *L. pseudomesenteroides* clade, and CAE1 with *Staphylococcus* strains (more related to *S. warneri*), all supported by moderate to high bootstrap values.

Moreover, Figure 1 also illustrates five isolates (CAP3, CAP6, CAP7, CAP10, and CAV28) that did not show any close phylogenetic relationship with the reference sequences employed in this analysis, and formed together a distinct clade, in which they showed to share high similarity. Most of these bacteria were isolated from the same collection site (CAP, see Table 1), which may in part explain their phylogenetic proximity.

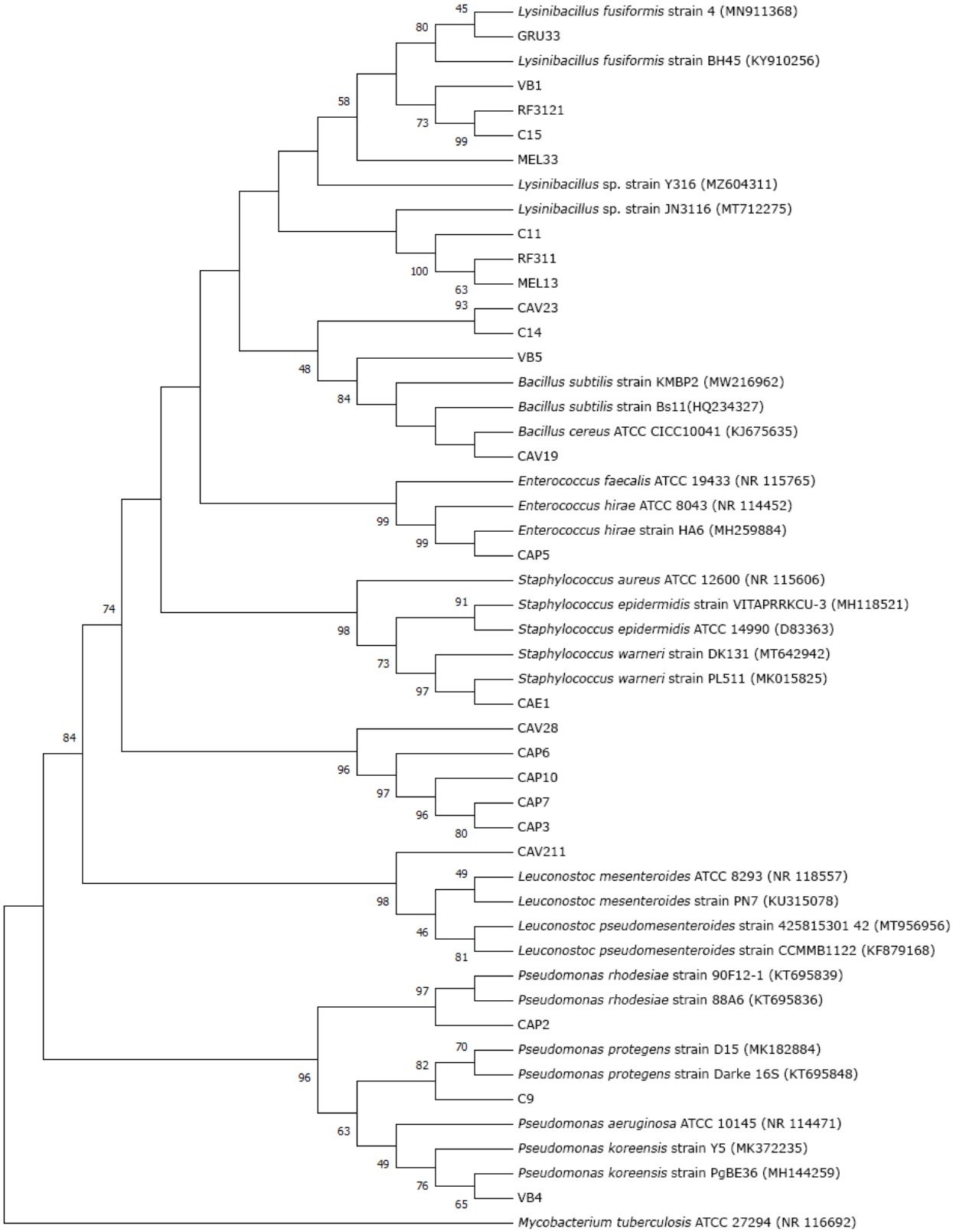


Figure 1 Taxonomic identification of the 23 bacterial isolates. Phylogenetic analyses were performed with reference sequences obtained from the Nucleotide BLAST® database. Phylogenetic trees were constructed using the maximum likelihood method and Tamura-Nei model based on 16S rRNA gene sequences. Bootstrap percentages based on 1500 replications are shown at branch points (values bellow 45 were cutoff).

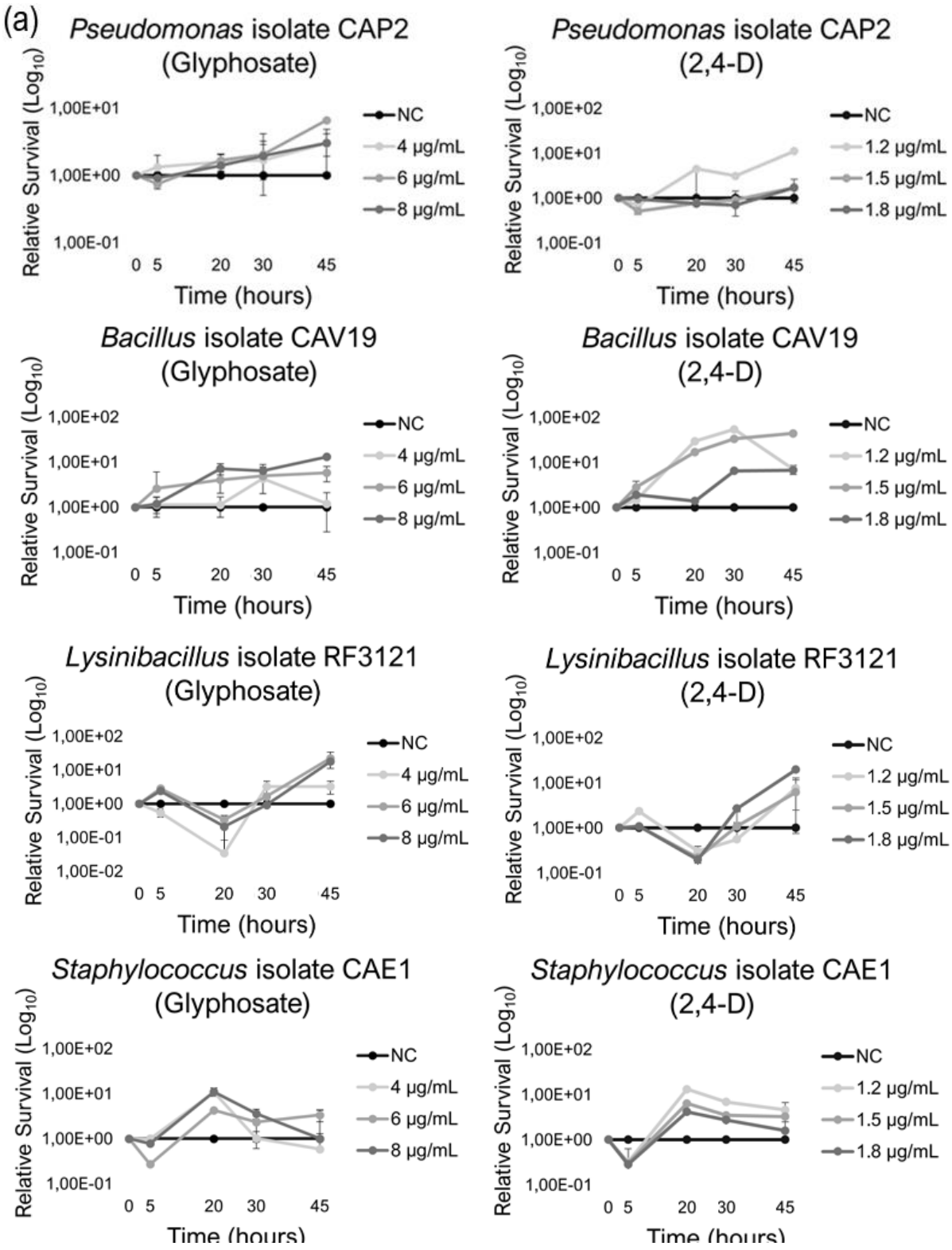
Herbicide Treatments

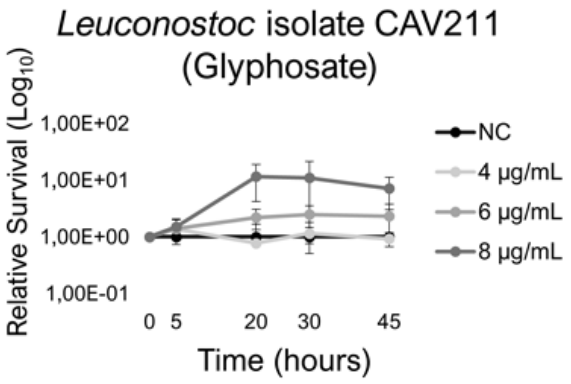
All the 23 bacterial isolates from the GAS were analyzed for survival after exposure to different concentrations of glyphosate-based herbicide (Roundup®) and 2,4-D-based herbicide (DEZ®) for 45 h. CFU/mL counts relative to control without herbicides were recorded and semi-log survival curves were prepared. It was possible to observe highly heterogeneous responses among isolates, and for some of them, differences were also detected between the two herbicide treatments. Moreover, a concentration-response or time-response relationship was rarely observed, even for the reference strains. Nevertheless, differently from the reference strains, that showed significant decreased survival (susceptibility) compared to the control, the majority of environmental isolates were tolerant (no significant differences compared to the control) or highly tolerant (significant increased survival compared to the control) in most concentrations of both herbicides, along the treatment period.

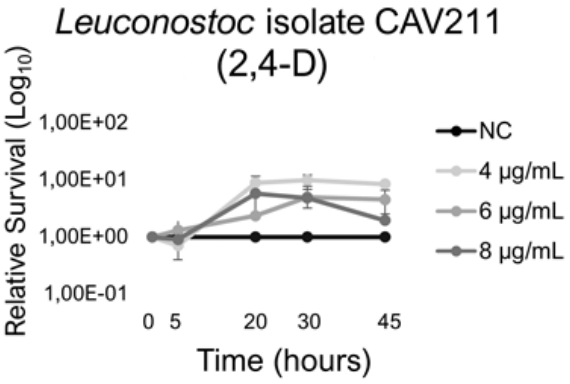
From the 15 isolates that were identified taxonomically, six demonstrated tolerance to the herbicides (Supplementary Figure 1a). The other nine isolates presented distinguished significant responses, which are illustrated in Figure 2. *Pseudomonas* CAP2 had a slight growth promotion by the different concentrations of glyphosate along time and a significant high tolerance to the lowest concentration of 2,4-D at 45 h. *Bacillus* CAV19 presented a significant growth promotion in most concentrations of both herbicides, reaching the maximum survival value when treated with the lowest concentration of 2,4-D in 30 h. Among *Lysinibacillus* isolates, RF3121 presented a peculiar behavior, with a tendency to be sensitive at 20 h, but with further growth promotion and high tolerance at 45 h, which was similar for most concentrations of both herbicides. Moreover, *Staphylococcus* CAE1 showed tendency to tolerate all treatments, with a point of high tolerance at 20 h, for some concentrations of both herbicides, whereas the isolate CAV211, identified as *Leuconostoc*, presented a tendency to efficiently grow when treated with both herbicides, showing high tolerance in the highest concentration of glyphosate (Figure 2a).

Differently from this tolerance/high tolerance pattern, *Bacillus* C14 tended to be sensitive at all concentrations over time, significantly at 20 h and 45 h for both herbicides, but mainly for 2,4-D (Figure 2b). *Bacillus* CAV23 presented a similar behavior, but solely do glyphosate. Also, *Lysinibacillus* GRU33 was sensitive to both herbicides at 45 h. The *Enterococcus* CAP5 also showed a tendency to be sensitive to both herbicides, with significant survival decrease in 45 h for glyphosate and along all treatment for 2,4-D. The reference strains *B. cereus* ATCC 33019 and *E. faecalis* ATCC 29212 also showed a significant sensitivity response to both

herbicides. *P. aeruginosa* ATCC 27853 presented a distinct behavior, with significant sensitivity at 5 h and further growth promotion and tolerance, for all concentrations of both herbicides (Figure 2b).

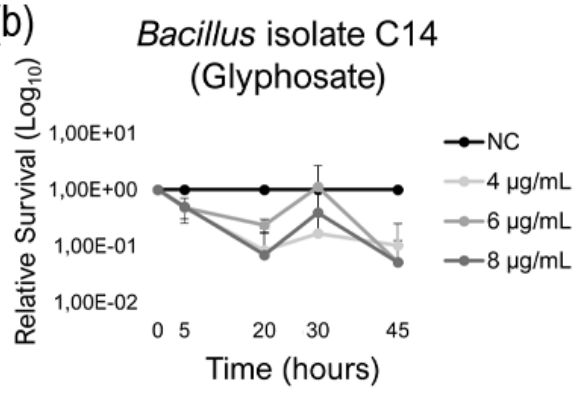


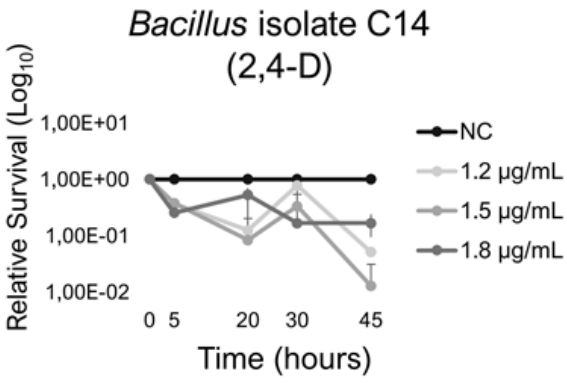




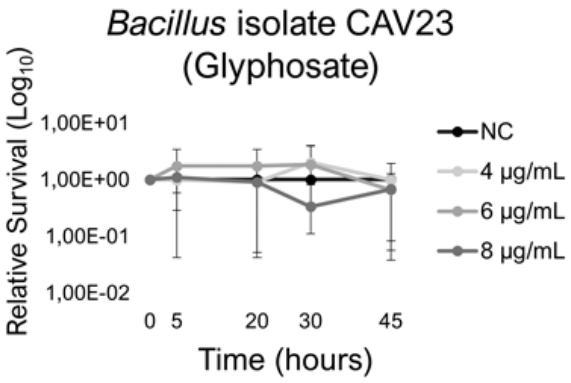
324

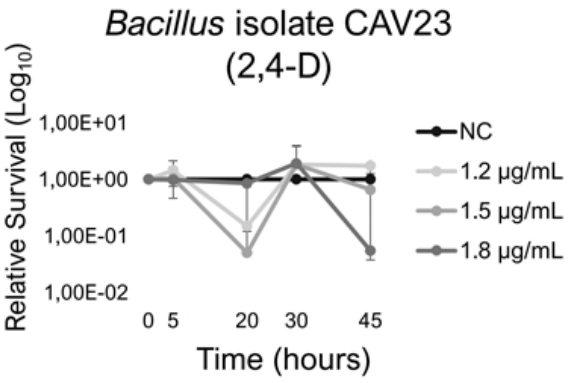
(b)



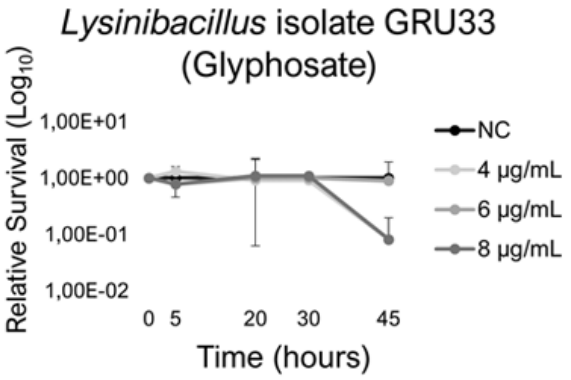


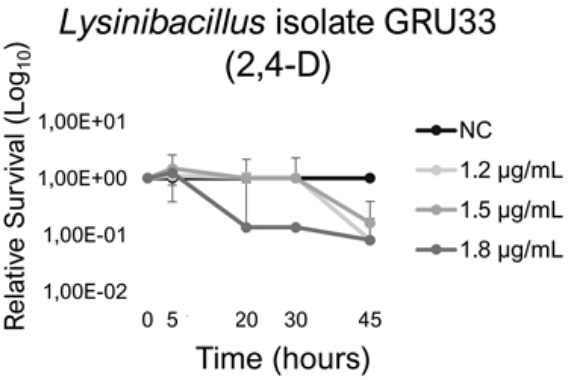
325





326





327
328
329

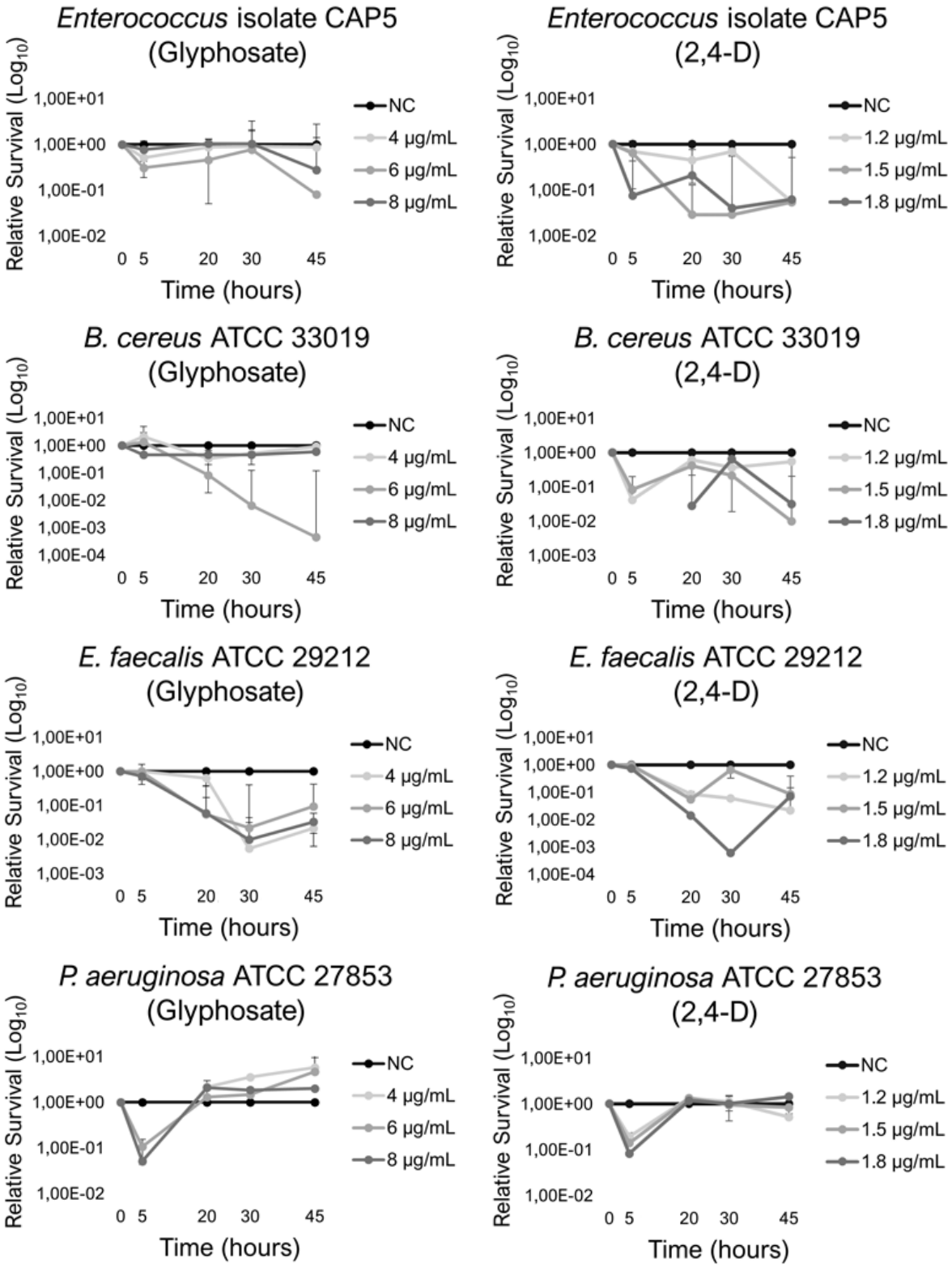
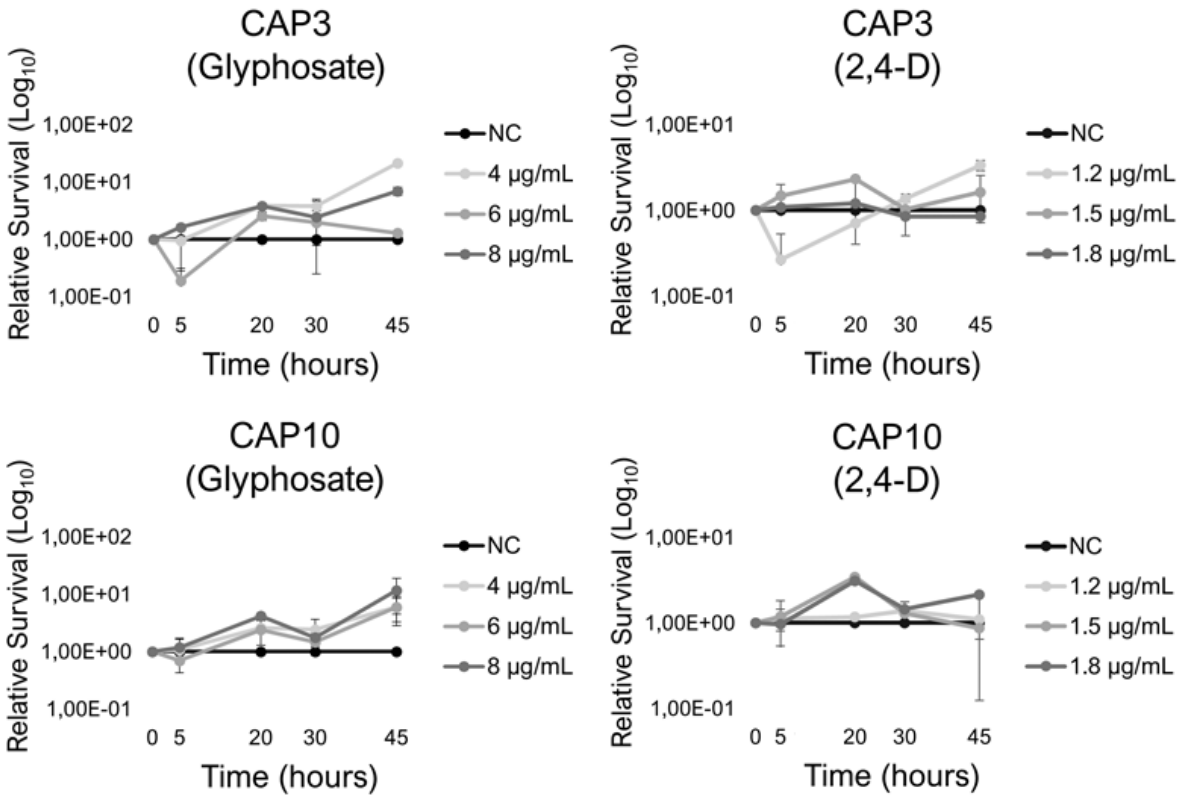


Figure 2 Relative survival to glyphosate and 2,4-D, in semi-log curves, of the taxonomically identified isolates: (a) isolates that presented high tolerance in at least one concentration of one herbicide along the treatments; (b) isolates that presented a sensitivity response to the herbicides and the reference strains. Differences were considered significant when at least one $\log_{10}$ of variation (considering the standard deviation) was observed in relation to the control without herbicides (NC).

Regarding the eight isolates that did not have a clear taxonomic identification (the unsupported *Lysinibacillus* isolates and those from the distinguished clade) no specific pattern of response to the herbicides was observed. Nevertheless, more than a half of these bacteria (isolates CAP3, CAP10, CAV28, MEL13, and RF311) were highly tolerant to at least one concentration of one herbicide (Figure 3) and the other three were tolerant to these chemicals (Supplementary Figure 1b). Only CAV28 presented a sensitivity response, which was uniquely to 6 µg/mL glyphosate at 30 and 45 h. Moreover, among these isolates, MEL13 showed tolerance in initial exposure times, which was followed by the highest tolerance response to both herbicides among all 23 isolates. For 2,4-D treatment, this highly tolerant behavior occurred under a time-response pattern, with survival values slightly decreasing with increasing concentrations (Figure 3).



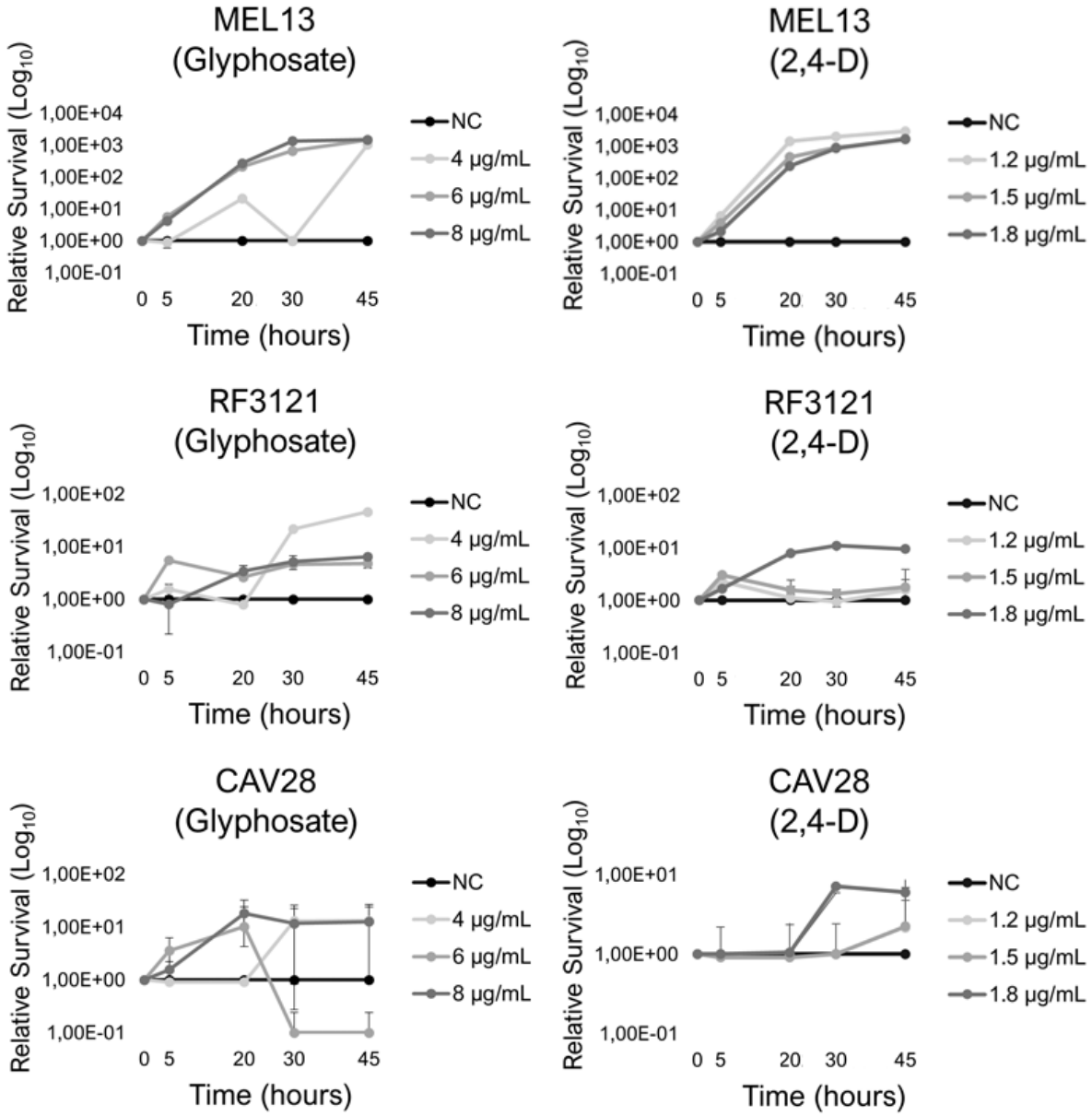


Figure 3 Relative survival to glyphosate and 2,4-D, in semi-log curves, of the unidentified and unsupported isolates. All these isolates presented mostly a highly tolerant behavior to the herbicides along the treatments. Differences were considered significant when at least one $\log_{10}$ of variation (considering the standard deviation) was observed in relation to the control without herbicides (NC).

From the survival experiments, the average values of MS were extracted for comparisons among isolates and reference strains (Table 2). Most of the isolates had their MS values at 30 or 45 min-treatment, and especially for glyphosate, at the maximum concentration tested. The MS values were then $\log_{10}$ transformed and used to compare isolates. The Shapiro-Wilk test indicated a normal distribution of $\log_{10}$-transformed MS values from treatments with glyphosate ($W = 0.9532$; $p = 0.2957$) and 2,4-D ($W = 0.9647$; $p = 0.5159$), and the Levene's test pointed that MS variances were homogeneous for both herbicides (glyphosate: $F = 0.6189$, $p = 0.6542$;

2,4-D: F = 1.1962, p = 0.3431). Figure 4 shows the log10-transformed MS average values of isolates grouped by their sampling region: Candelária, Alegrete and Quarta Colônia (which is subdivided in Nova Palma and Faxinal do Soturno subregions). Nova Palma and Alegrete presented the highest MS averages for both herbicides. For glyphosate, *one-way* ANOVA test indicated significant differences among regions´ MS (F = 4.451, p = 0.0098), and the Tukey post-hoc test distinguished that Nova Palma was different from Candelária (p = 0.018), Faxinal do Soturno (p = 0.037) and reference strains (p = 0.031) (Figure 4a). For 2,4-D, ANOVA also detected significant differences among groups (F = 3.664, p = 0.021), but the Tukey test showed that only Nova Palma and the reference strains differed significantly (p = 0.016) (Figure 4b).

Table 2: Maximum survival (MS) values (along with MS time and concentration) extracted from the survival curves to the both herbicides glyphosate and 2,4-D of all Guarani Aquifer System isolates and reference strains.

Identification	***Isolate***	***Glyphosate survival***			***2,4-D survival***		
		MS	***MS Time (hours)***	***MS concentration (mM)***	***MS***	***MS Time (hours)***	***MS concentration (mM)***
Lysinibacillus	C15	7.42E+00	20	8	5.81E+00	45	1.2
	GRU33	1.30E+00	5	4	1.48E+00	5	1.5
	MEL33	2.72E+00	30	6	5.56E+00	30	1.2
	RF3121	2.22E+01	45	6	1.97E+01	45	1.8
Bacillus	VB1	3.52E+00	45	8	2.71E+00	20	1.2
	CAV19	1.30E+01	45	8	5.42E+01	30	1.2
	CAV23	2.00E+00	30	4	1.92E+00	30	1.8
	C14	1.11E+00	30	6	7.77E-01	30	1.2
	VB5	2.12E+00	45	8	2.25E+00	20	1.8
Pseudomonas	CAP2	6.58E+00	45	6	1.11E+01	45	1.2
	C9	6.32E+00	20	4	3.22E+00	45	1.8
	VB4	2.04E+00	30	8	2.15E+00	45	1.2
Enterococcus	CAP5	1.04E+00	20/30	8	6.92E-01	5	1.2
Staphylococcus	CAE1	1.10E+01	20	8	1.31E+01	20	1.2
Leuconostoc	CAV211	1.15E+01	20	8	9.07E+00	30	1.2
Unidentified	CAP3	2.14E+01	45	4	3.34E+00	45	1.2
	CAP6	3.44E+00	45	8	1.35E+00	30	1.2
	CAP7	3.47E+00	45	8	3.37E+00	45	1.8
	CAP10	1.16E+01	45	8	3.45E+00	20	1.5
	CAV28	1.82E+01	20	8	7.13E+00	30	1.8
Unsupported *Lysinibacillus*	C11	1.71E+00	30	8	1.76E+00	30	1.2/1.8
	RF311	4.57E+01	45	4	1.11E+01	30	1.8
	MEL13	1.49E+03	45	8	2.94E+03	45	1.2
Bacillus cereus ATCC 33019		2.13E+00	5	4	6.46E-01	30	1.8

Pseudomonas aeruginosa ATCC 27853	5.75E+00	45	4	1.44E+00	45	1.8
Enterococcus faecalis ATCC 29212	1.01E+00	5	4	9.85E-01	5	1.5

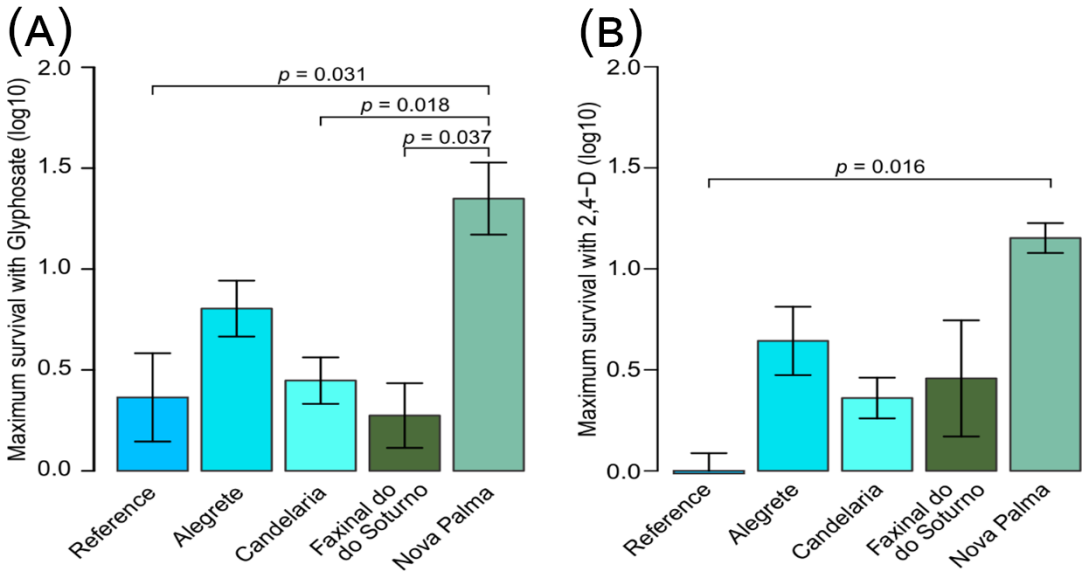


Figure 4 Average values of log10-transformed Maximum Survival (MS) of isolates from the Guarani Aquifer System in presence of herbicides, according to region of origin. Error bars = mean ± SEM. Quarta Colônia was subdivided into its subregions, Nova Palma and Faxinal do Soturno. (a) MS with glyphosate showed significant difference among regions (*one-way* ANOVA, $F = 4.451$, $p = 0.0098$), which was detected in a post-hoc test (Tukey HSD), with Nova Palma significantly different from Candelária ($p = 0.018$), Faxinal do Soturno ($p = 0.037$) and reference strains ($p = 0.031$). (b) For 2,4-D, significant difference among groups was also observed (*one-way* ANOVA, $F = 0.3664$, $p = 0.021$), in which Nova Palma differed only from the reference strains (Tukey HSD, $p = 0.016$)

We also performed a linear regression analysis using MS data (normalized by $\log_{10}$) from both glyphosate and 2,4-D treatments combined, indicating the 23 GAS isolates (plus three reference strains) by region of origin (Figure 5a) and genera (Figure 5b). The regression was significant ($R^2 = 0.801$, $a = 0.998$, $p < 0.001$), as well as the Pearson correlation test that was also applied for this analysis ($t = 10.078$, $p < 0.001$, *correlation coefficient* = 0.899). These data indicates that most isolates tended to present similar responses when treated separately with glyphosate and 2,4-D. Moreover, this analysis indicated that no pattern of behavior was detected for the isolates regarding their region or genera.

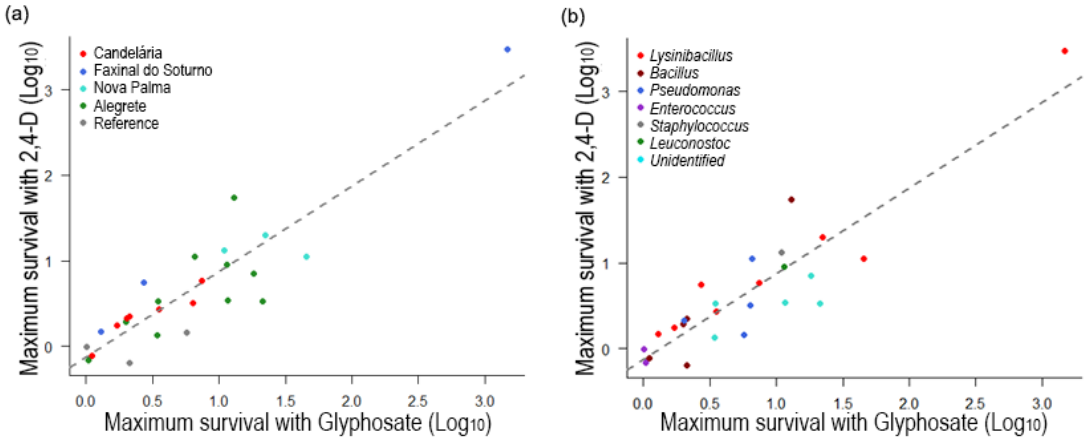


Figure 5 Linear regressions between maximum survival (MS) values with Glyphosate and 2,4-D treatments. Each point represents an isolate (23 from Guarani Aquifer System and three reference strains), with different colors indicating their region of origin (a) or their genera (b). Data were normalized by $\log_{10}$ to group large values and distribute small values ($R^2 = 0.801$, $a = 0.998$, $p < 0.001$). The outlier isolate in the upper right does not change the pattern, and the regression is still significant without it. Pearson correlation test was also applied ($t = 10.078$, $p < 0.001$, *correlation coefficient* = 0.899).

Antimicrobial Susceptibility

The antimicrobial susceptibility profiles of 13 isolates identified as *Lysinibacillus*, *Bacillus*, *Pseudomonas* and *Enterococcus* are summarized in Table 3. The isolates with identification of *Staphylococcus* and *Leuconostoc* were not included in these tests. *Staphylococcus* is described as strongly related to human microbiota or infections, and rarely associated to microbial communities from aquatic environments (45). *Leuconostoc* is a genus native to plants, and it can also be found in silage and fermented food products (46), also described as potentially pathogenic to humans (47), without reports as occurring frequently in aquatic environments.

We first determined their susceptibility profile to antimicrobials using the Kirby-Bauer disk diffusion method. Those that were detected as resistant were further submitted to minimal inhibitory concentration (MIC) tests of antimicrobials of different classes.

Table 3: Susceptibility to antimicrobials profiles of 13 isolates identified as *Lysinibacillus*, *Bacillus*, *Pseudomonas* or *Enterococcus* and reference strains. For the diffusion disk test, the antibiotics to which isolates were resistant are indicated. The minimum inhibitory concentration (MIC) of the antibiotics that were tested for this purpose are indicated for the isolates that showed resistance in the disk diffusion test. The multiple antibiotic resistance (MAR) index is indicated for isolates that were resistant to at least two antibiotics.

Identification	***Isolate***	***Susceptibility profile (disk diffusion test)***	***MIC (µg/mL)***							*MAR index*
			MER	*CAZ*	*ERI*	*TOB*	*CLI*	*ATM*	*VAN*	
Lysinibacillus	C15	ERY, CLI, IPM, LZD, VAN	—	—	2	—	4	—	8	0.625
	GRU33	—	—	—	—	—	—	—	—	—
	MEL33	—	—	—	—	—	—	—	—	—
	RF3121	ERY, LZD, IPM, MEM, VAN	8	—	8	—	—	—	4	0.625
Bacillus	VB1	CLI, IPM	—	—	—	—	4	—	4	0.25
	CAV19	—	—	—	—	—	—	—	—	—
	CAV23	—	—	—	—	—	—	—	—	—
	C14	MEM, VAN	16	—	—	—	—	—	8	0.25
	VB5	IPM	—	—	—	—	—	—	—	—
	CAP2	ATM	—	—	—	—	—	32	—	—
Pseudomonas	C9	CAZ, FEP, TOB, ATM, LVX	—	8	—	2	—	64	—	0.45
	VB4	AMK, ATM, CAZ, FEP, GEN, TOB	—	8	—	2	—	32	—	0.54
Enterococcus	CAP5	—	—	—	—	—	—	—	—	—
Bacillus cereus ATCC 33019		—	—	—	—	—	—	—	—	—
Pseudomonas aeruginosa ATCC 27853		—	—	—	—	—	—	—	—	—
Enterococcus faecalis ATCC 29212		—	—	—	—	—	—	—	—	—

Antimicrobials: AMK: amikacin; **ATM**: aztreonam; **CAZ**: ceftazidime; **CLI**: clindamycin; **ERY**: erythromycin; **FEP**: cefepime; **GEN**: gentamicin; **IPM**: imipenem; **LZD**: linezolid; **LVX**: levofloxacin; **MEM**: meropenem; **TOB**: tobramycin; **VAN**: vancomycin. Sensitive (—).

To illustrate the results from the disk diffusion test for *Pseudomonas* and *Bacillacea*e isolates, heatmaps were prepared based on the diameters of inhibition zone, in which positive values indicated susceptibility and negative values indicated resistance (Figure 6).

All the isolates identified as *Pseudomonas* were resistant to at least one of the 11 tested antimicrobials in the disk diffusion tests, in which isolates C9 and VB4 were resistant to five and six antimicrobials, respectively, with similar (but not identical) resistance profiles (Table 3, Figure 6a). *Pseudomonas* was the genus that presented resistance to the highest number of drugs (a total of seven antimicrobials for the three isolates), with a MAR index of 0.45 for C9

and 0.54 for VB4. Moreover, the MIC values observed for ceftazidime and tobramycin (8 µg/mL and 2 µg/mL, respectively), were the same for both C9 and VB4 isolates, which were both at the breakpoint values to consider them as resistant. For aztreonam, all MIC results remained above the breakpoint value, reaching the maximum of 64 µg/mL for C9 and 32 µg/mL for the other two isolates.

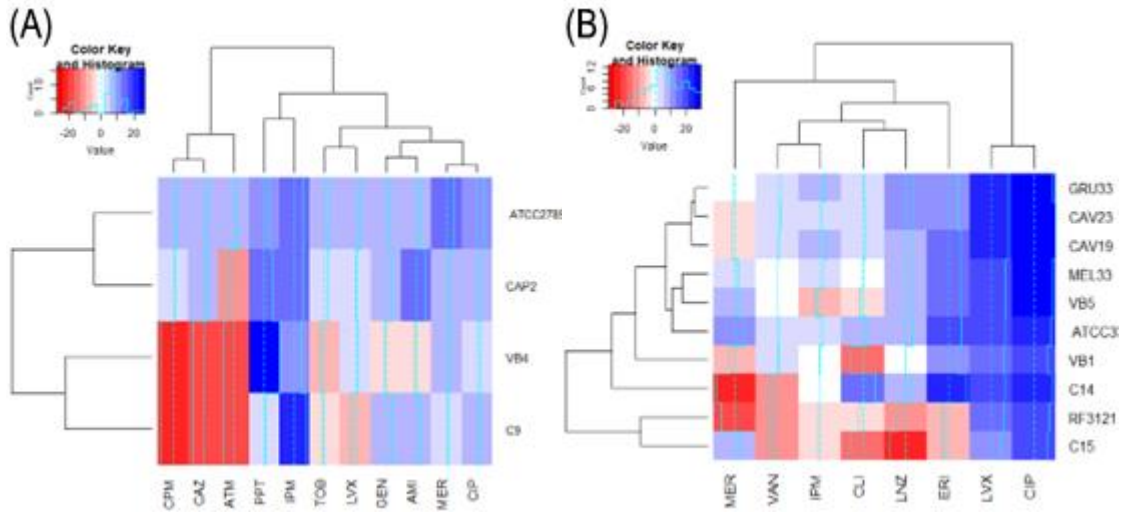


Figure 6 Heatmaps illustrating the results from the disk diffusion test. (a) Isolates from *Pseudomonas* genus; (b) Isolates from *Bacillaceae* family. Diameters of inhibition zone were centered at zero using the resistance cutoff value for each antibiotic/strain, such that positive values (blue) indicated susceptibility and negative values (red) indicated resistance.

Isolates from *Bacillaceae* family showed a heterogeneous response to antimicrobials, with resistance to a total of six drugs among five isolates (Table 3, Figure 6b). Isolates C15 and RF3121 (*Lysinibacillus*) presented the profile of resistance to the largest number of drugs in this family, each resistant to a different group of five antimicrobials. Moreover, as the number of antimicrobials tested for *Bacillaceae* (a total of eight) were lower compared to *Pseudomonas* isolates, the MAR index of both C15 and RF3121 was the highest among all resistant isolates, reaching 0.625. Among the other three *Lysinibacillus* isolates, VB1 presented resistance to two antimicrobials (MAR index of 0.25) and the other two were sensitive to all drugs tested. The MIC values for the antimicrobials tested were very heterogeneous among these isolates, in all cases above the resistance breakpoints. From the four *Bacillus* isolates, VB5 was resistant to only one antimicrobial (imipenem), and C14 to meropenem and vancomycin (the latter with a MAR index of 0.25), whereas the other two isolates were 100% sensitive. Nevertheless, for both drugs tested in C14, the MIC values were above the resistance breakpoints for these antimicrobials.

We also evaluated antimicrobial susceptibility of the *Enterococcus* isolate CAP5, which showed to be sensitive for all six drugs tested. Moreover, all reference strains (*B. cereus* ATCC 33019, *E. faecalis* ATCC 29212 and *P. aeruginosa* ATCC 27853) presented sensitivity to all antimicrobials tested (Table 3, Figure 6).

Tolerance to Herbicides and Resistance to Antimicrobials

The linear regression between normalized MS data on glyphosate and 2,4-D (Figure 5) was used to include the MAR index of isolates that were resistant to at least two antimicrobials (Figure 7). There were no general tendencies to highlight from this analysis, however, it was possible to observe that the two isolates with the highest MAR indices (the yellow ones) presented high values of MS for both herbicides, whereas those with minor MAR indices (the purple ones) had lower MS measures.

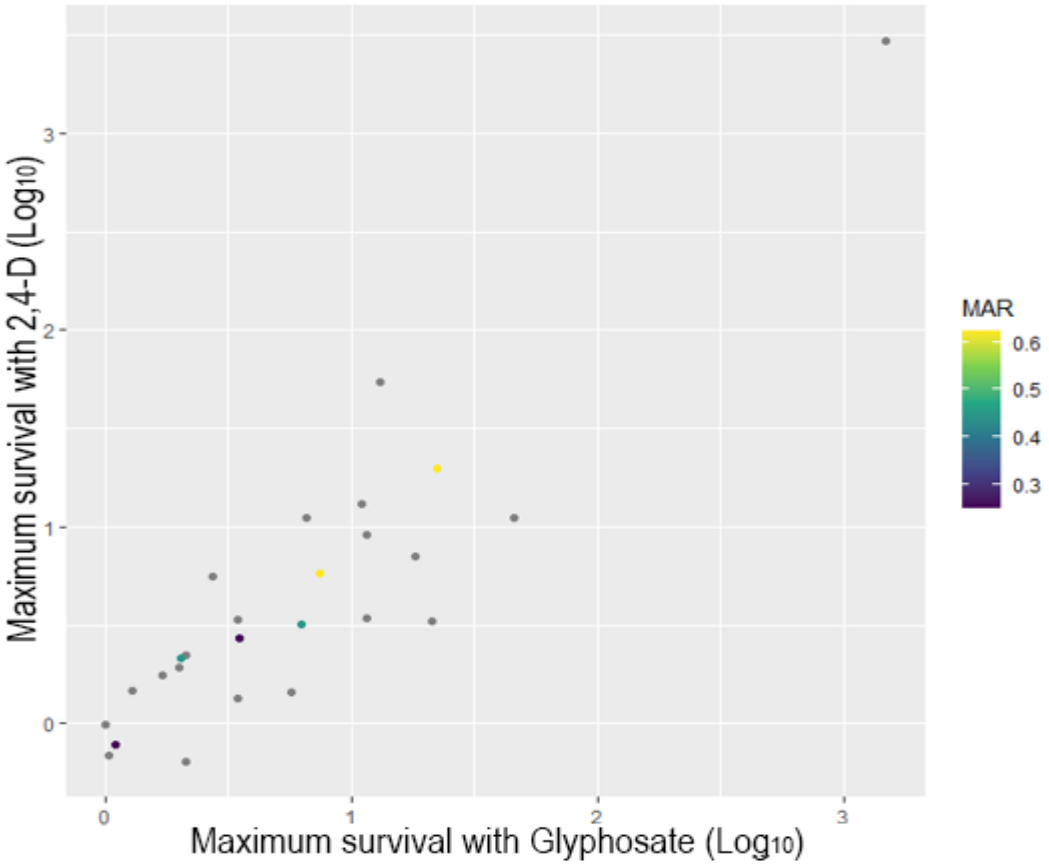


Figure 7 Linear regression between maximum survival (MS) data on glyphosate and 2,4-D (Figure 5) along with calculated multiple antibiotic resistance (MAR) index. Each point represents an isolate (23 from Guarani Aquifer System and three reference strains), with purple to yellow colors indicating the MAR index from 0.25 to 0.65. Isolates in gray did not have MAR index calculated (were sensitive to all, or resistant to one, or not tested for antimicrobials). Data normalization, linear regression and Pearson correlation information are the same presented for Figure 5.

To verify a possible relationship between high tolerance to herbicides and resistance to antimicrobials among the GAS isolates (and reference strains), we performed a PCA with data scaling using log_{10}-transformed MS values from survival experiments along with the diameter of the inhibition zones from the disk diffusion tests (Figure 8). The results from this analysis explained 88.27% of the total variance among data. It indicated that 51.09% of the variance was explained by the first principal component (PC1), which produced a separation among isolates that coincided with their taxonomy, as the *Bacillaceae* genera formed a distinct group from a *Pseudomonas* cluster, and also from *Enterococcus* isolates. The second principal component (PC2) explained 37.19% of the variance, mostly indicating the variability within these groups. The arrows in figure 8 represent the direction of each of the five axes included in this analysis. As the arrows are mostly aligned with axes x and y, we can infer that PC1 may explain the variance promoted by differences in antibiotic resistance, whereas PC2 represent the variance among isolates regarding tolerance to herbicides. They also indicate that IPM resistance data is opposed to that from CIP and LVX. It was possible to observe that antibiotic resistance was clearly distinct between *Bacillaceae* cluster and the other isolates, whereas the herbicides' tolerance did not show any tendency related to taxa, but indicated that the least and the most tolerant isolates were within the *Bacillaceae* group. Moreover, it was not possible to observe a general relationship between tolerance to herbicides and resistance to antibiotics in any of the bacterial taxa, but in both *Bacillaceae* and *Pseudomonas* clusters some isolates presented both properties.

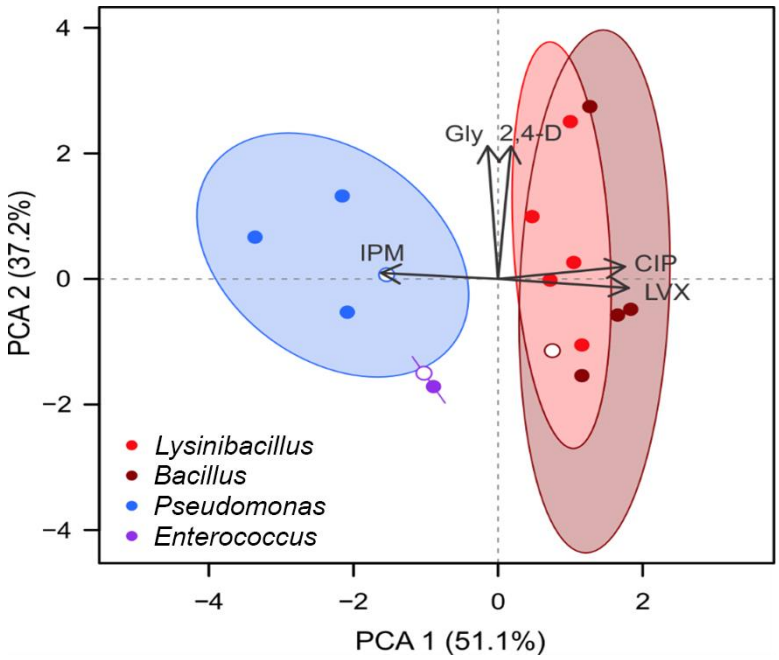


Figure 8 Principal component analysis (PCA) with data scaling showing distances among isolates according to maximum survival (MS) and antibiotic sensitivity. The analysis comprised five axes: $\log_{10}$-transformed MS data for glyphosate and 2,4-D, as well as diameters of inhibition zones for the broad-spectrum antibiotics imipenem (IPM), ciprofloxacin (CIP) and levofloxacin (LVX). Blank circles indicate the reference strains for each group. The ellipses were calculated with 95% confidence intervals (PERMANOVA test, $p < 0.05$); the *Enterococcus* ellipse is a straight line, as there are only two isolates of this genus. The arrows represent the direction of each of the five axes of this analysis.

DISCUSSION

This study evaluated the susceptibility of bacterial isolates from the GAS to herbicides and antimicrobials. The 15 isolates that had an attributed taxonomy were identified into six different genera. Most isolates (nine) were detected as belonging to the family *Bacillaceae* (genera *Bacillus* and *Lysinibacillus*). Species from the *Bacillaceae* family are described as widespread, occurring very frequently in aquatic environments (48), and easily cultivable from different sources. Moreover, several species (including those from *Bacillus* and *Lysinibacillus*) present the ability to form spores, which occurs as a natural defense of the bacteria to disturbs and in diverse ecological niches (49, 50). This may, in part, explain the high frequency of isolates from this taxonomic group in our study.

The genus with the second largest number of isolates detected was *Pseudomonas*, which is also described as widespread, and very abundant in aquatic environments (51, 52). Furthermore, three other genera appeared on our analysis: *Enterococcus*, *Staphylococcus* and *Leuconostoc*. Whereas *Staphylococcus* is commonly associated with mucous membranes or

appears as a skin commensal (45), and *Leuconostoc* is usually found in plants and fermented food (46), there are several species of *Enterococcus* that can frequently be found in nature (53). Even though *Staphylococcus* and *Leuconostoc* are not as abundant as the other detected genera in aquatic environments, these bacteria can also be found in such habitats. Moreover, *Staphylococcus* and *Leuconostoc* are genus of interest for being potential pathogens (47, 54).

The high percentage of herbicide-tolerant and highly tolerant isolates indicate that most of these bacteria may have metabolic abilities that enable them to cope with the presence of glyphosate and/or 2,4-D in their environment. These results thus may indicate that at least part of the bacteria of the GAS are not negatively impacted by the presence of these compounds in the environment. However, part of our isolates presented significant decrease or (mainly) increase in survival when exposed to these agrochemicals, which may be considered as an important issue regarding the structure of microbial communities in this aquatic environments, as previously discussed (55). Additionally, the regression (and correlation) analysis performed with MS data from treatments with glyphosate and 2,4-D was significant, indicating a correlation of response (sensitivity, tolerance or high tolerance) between both agrochemicals' treatments.

Similar results have already been described in studies on the ability of bacteria not only to tolerate the presence but also to use pesticides as a nutrient source. Several strains of different genera were tested against a variety of pesticides. Gravina *et al.* (2017) evaluated the tolerance of *E. coli* to the herbicide paraquat due to antioxidative responses (56). The bacterium *Pantoea ananatis*, isolated from agricultural soil, resisted and grew in the presence of the selective herbicide mesotrione (57). Glyphosate-based herbicides and their metabolites are degraded in different environmental matrices in contaminated niches via bacterial enrichment approach (58–60). 2.4-D mitigation strategies also rely on bacterial metabolic pathways to use and degrade this compound (61). Moreover, species of *Pseudomonas* were reported as presenting the ability to use glyphosate as a nutrient source for their growth (62, 63) and *Lysinibacillus* have also been described as capable of biodegrading different herbicides (62, 64, 65).

Our data indicated that one *Pseudomonas* isolate (CAP2), from the Alegrete region, presented a tendency to grow in the glyphosate treatment, and also a significant increase in survival under 2,4-D exposure. The other two *Pseudomonas* isolates (both from the Candelaria region) presented a different response (a regular tolerance) to these herbicides. Even without significant differences between MS values of Alegrete and Candelária, the sampling region may have some influence on these differences. More importantly, each of these three isolates showed

close relationship with distinct *Pseudomonas* species in the phylogeny, which may better explain their metabolic differences.

Furthermore, the isolate MEL13 (unsupported *Lysinibacillus*) presented the most efficient growth among all isolates, along both herbicides´ treatments, in a concentration and time-response manner for 2,4-D. Distinguishingly, this isolate was obtained from the sampling site MEL (Quarta Colônia region), which was the only one that presented water samples with previous detection of at least one herbicide (2,4-D), in maximum concentrations of 0.004 µg/mL (32). Even detected in much lower concentration than we tested *in vitro*, it is possible that the presence of 2,4-D in in sub-lethal doses in this aquifer may have contributed to chances in the susceptibility pattern to this herbicide, and also to other stressors (like antibiotics), of bacteria from GAS environment, as previously reported in *in vitro* experiments (16). All these results indicate that at least some of the isolates we tested may be adapted to use glyphosate and/or 2,4-D as nutrient source for their populational growth, pointing to an ecological concern regarding the ecology of water ecosystems. Nevertheless, it is important to note that the other isolate from site MEL, the MEL33 (identified as *Lysinibacillus*), did not present this kind of response, showing solely tolerance to both herbicides. However, our data with MS values from all isolates indicated significant differences among their region of origin regarding this parameter, especially for Nova Palma isolates under Glyphosate treatment. These results - including the whole heterogeneity we observed among all isolates, even from the same region - reinforces that the microbial response to these herbicides may be influenced by environmental factors, but also by species or even strain specific traits, as previous studies also reported (17, 66–69).

The literature does not report any objective criteria or parameters to determine when bacterial species or isolates can be considered tolerant or resistant to herbicides, and these two terms are often (but not always) used as synonyms. Nevertheless, some authors made suggestions regarding this issue. Bellinaso *et al*. (2003) proposed that “tolerance and resistance against pesticides in general is attributed due to physiological changes that induce microbial metabolism to follow a new metabolic pathway that help organisms to bypass a biochemical reaction which could otherwise be inhibited by some specific pesticides” (38). Additionally, the resistance could be due to emergence of mutations inherited by different strains of microbes (70). Curutiu *et al*. (2017) propose that the occurrence of tolerance or resistance to herbicides among bacteria is perhaps a unique feature, which is regulated both genetically and physiologically. Some also suggest the idea that microbial strains that have developed

609 resistance to pesticides are capable of frequently degrading them (67), or use “resistance” to
610 refer to the ability of bacteria to grow in the presence of herbicides, irrespective of duration of
611 treatment (31). Some studies (30, 38, 40, 66) used some of these biochemical and genetic
612 criteria to define bacteria as resistant, strongly tolerant, highly tolerant or hyper tolerant to
613 herbicides, whereas some others solely considered the bacterial tolerance to the maximum
614 concentration tested of the herbicides to define or select them as resistant to these chemicals
615 (67–69). As our survival tests were performed in 0.9% saline, and thus did not contain any
616 nutritional source except the tested herbicides, the isolates that presented significantly increased
617 survival (the “highly tolerants”, like MEL13), might have utilized these herbicides as a sole
618 nutrient/energy source, through some biodegradation process. Thus, based on the criteria of
619 “growing” or “degrading” ability, we could define these isolates as highly tolerant (or even
620 resistant) against the tested herbicides. Moreover, this ability of degrading herbicides revealed
621 a biotechnological potential for such bacteria, which can be further applied to bioremediation
622 processes adapted (not only, but mainly) to aquatic environments. Our data also demonstrated
623 that many of the isolates had their MS values at the maximum concentrations tested of the
624 herbicides, which could be another indicative of the use of these molecules as a source of
625 nutrients.

626 Most of the isolates tested in this study were resistant to at least one antibiotic and the
627 MIC values detected for most antimicrobials were above the breakpoint for resistance.
628 *Pseudomonas* was the genus that showed resistance to the highest number of antimicrobials
629 tested. Nevertheless, *Lysinibacillus* isolates presented the highest MAR indices (0.625), which
630 is also an important parameter to be considered. In aquatic environments of huge volumes of
631 water, such as aquifers, we did not expect to find bacteria resistant to antibiotics to which they
632 initially have no intrinsic resistance. However, as the collection sites were located in areas of
633 high agricultural and livestock labor, these activities may be a source of chemicals (including
634 antimicrobials) impacting the surrounding environments, potentially inducing changes in the
635 susceptibility profile of native bacterial strains (71). Even antibiotics occurring in low
636 concentrations in terrestrial and aquatic environments (72), it is already reported that the spread
637 of faecal material (via sewage effluents and animal waste) has contributed to contamination
638 with antibiotic resistant bacteria and resistance genes in almost the entire planet, including
639 freshwater systems (72–75). Furthermore, the passage of antibiotic resistance genes and
640 mechanisms in the environment can promote an increment in the resistome content, which
641 would lead to an increaed number of bacteria within a community expressing resistance to one

or several antibiotics (76). It may also occur in groundwater reservoirs, such aquifers, since they maintain permanent connections with surface water and terrestrial environments (77).

Low susceptibility to antimicrobials was observed in *P. aeruginosa* from environmental origin (78). Conversely, studies with environmental and patient-derived isolates of *P. aeruginosa* found that environmental isolates were significantly more susceptible to antibiotics than patient-derived isolates (79, 80). Nevertheless, the environment plays a crucial a role both in evolution and transmission of resistance (72), and it's not possible to predict where and under what circumstances the critical steps for antibiotic resistance will occur and what new forms of resistance will appear (81). Moreover, all *Pseudomonas* isolates in our study showed to be resistant to aztreonam (the only antibiotic to which an entire clade of bacteria showed resistance). Resistance to aztreonam by environmental *Pseudomonas* strains was reported by Luczkiewicz *et al*. (2015), in which the majority of isolates were from wastewater and marine coastal zone (82).

It has been described that herbicide application can potentially contribute to antimicrobial resistance (71). According to Kurenbach *et al*. (2017), the simultaneous exposure of populations of *E. coli* and *S.* Typhimurium to commercial herbicides and antibiotics of different classes promoted a change in the susceptibility of these populations to antimicrobials (18). When we included the MAR indices in the regression analysis of both herbicide MS values, it was possible to observe that the isolates with the highest and lowest MAR indices seamed to present increased and decreased MS values, respectively. Nevertheless, it is important to note that the antibiotics tested were not the same for all bacteria and that eight of these isolates (unidentified or unsupported) were not tested for any antimicrobial. So, conclusions from this analysis may be taken with caution.

Moreover, the PCA result pointed to a phylogenetic-related pattern of response to antibiotics, independently on the region of origin of the isolates. This taxon-related pattern was not observed for the isolates' response to herbicides. As we previously discussed, it has already been reported that at least *Pseudomonas* and *Lysinibacillus* present the ability to tolerate and even use herbicide as a nutrient source (60, 62–65). Studies using different taxonomic groups against a single herbicide or set of herbicides along with the susceptibility profile test to antimicrobials are still scarce. Our data also indicated that it was not possible to observe a general relationship between tolerance to herbicides and resistance to antibiotics in any of the bacterial taxa studied, but showed that both *Bacillaceae* and *Pseudomonas* clusters presented some isolates with both properties. Moreover, the combined tolerance to herbicides and

antibiotic resistance did not seem to depend on region/ collection sites. However, it does not indicate that environmental factors may have an irrelevant importance on the bacteria's response to these chemicals. In fact, since our data revealed a pattern of tolerance or resistance to herbicides and antimicrobials for most isolates, we can infer that this may be the pattern for at least some groups of bacteria in the microbial communities from different regions the Guarani Aquifer.

Most studies that evaluated the impact of herbicides on microorganisms, or the tolerance of microbial species to these agrochemicals, analyzed bacterial isolates from soil, especially from rhizospheric microbiota (38, 67–71). Even when species from aquatic environments were tested, the methods applied were biochemical (56, 57, 70, 83, 84) or survival/growth curves based on optical densities (56, 57). Other studies used the maximum concentration of herbicides that the isolates tolerated to evaluate them regarding this feature (67–69). As far as we could search, our study was the first to raise data on susceptibility to herbicides of bacterial isolates from an aquifer environment, employing survival measures based on CFU/mL counts. Moreover, recent studies used herbicides only in sub-lethal concentrations to verify if it changed antibiotics MIC values in these isolates (17, 66, 71, 85). Differently from those, we analyzed the bacterial response to herbicides and antibiotics independently, without any pre-induction, thus analyzing the *in vitro* spontaneous responses of the isolates to these biocides. Nevertheless, it is important to note that abiotic factors in their original environment may induce variabilities on the response we observed in our *in vitro* tests, as already reported in previous studies (66, 69). Shahid & Khan (2018) even propose that may be impossible to generalize the elements that influence toxicity or tolerance of agrochemicals, not only in natural environments, but also along the different *in vitro* tests already performed by different studies (69).

Aquifers are huge reservoirs of groundwater, considered one of the most import sources of safe freshwater for human consumption. Biocide-susceptible organisms in natural environments are of benefit to human society as an ecosystem service, either locally, in the short-term control of target species, and globally, since a broadly susceptible community of microbes (including pathogens) represents option values for future generations to treat infectious disease and manage pest outbreaks (55). This study detected that most isolates from the GAS presented tolerance or high tolerance to glyphosate and/2,4-D, and resistance to at least one antimicrobial. Concerning the context of the One Health principle, there is a great need to manage susceptibility to antibiotics and pesticides as one valuable strategy for environmental sustainability and human health (55, 86). In this context, our results raised

relevant data, pointing to the importance of characterizing microbes from unexplored environments as potential indicators of human environmental impact and/or remediators of contaminants.

Acknowledgments

This study is one of the MS degree requirements for CSOS at the Pontifical Catholic University of Rio Grande do Sul (PUCRS). We thank CNPq (Brazilian National Council for Scientific and Technological Development) for the of master's scholarship for CSOS, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) for financial support [Finance Code 001] and AMG post-doc scholarship. SDO is Research Career Awarded of the CNPq.

Funding

This work was supported by the National Counsel of Technological and Scientific Development (CNPq) (master's scholarship); and CAPES Pró-Alertas 24/2014 Program (financial support) and CAPES-PRINT (PUCRS) Program (post-doc scholarship), both from and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil).

Conflict of Interest

The authors declare they have no conflict of interest.

REFERENCES

1. Pignati WA, Lima FAN de S e, Lara SS de, Correa MLM, Barbosa JR, Leão LH da C, Pignatti MG. 2017. Distribuição espacial do uso de agrotóxicos no Brasil: uma ferramenta para a Vigilância em Saúde. Ciência & Saúde Coletiva 22:3281–3293.
2. Sousa AS, Duaví WC, Cavalcante RM, Milhome MAL, do Nascimento RF. 2015. Estimated Levels of Environmental Contamination and Health Risk Assessment for Herbicides and Insecticides in Surface Water of Ceará, Brazil. Bulletin of Environmental Contamination and Toxicology 2015 96:1 96:90–95.
3. Chiarello M, Graeff RN, Minetto L, Cemin G, Schneider VE, Moura S. 2017. Determination of pesticides in water and sediment by HPLC-HRMS and its relationship with the use and land occupation. Química Nova 40:158–165.

4. Vieira DC, Noldin JA, Deschamps FC, Resgalla C. 2016. Ecological risk analysis of pesticides used on irrigated rice crops in southern Brazil. Chemosphere 162:48–54.
5. Belo MS da SP, Pignati W, Dores EFG de C, Moreira JC, Peres F. 2012. Uso de agrotóxicos na produção de soja do estado do Mato Grosso: um estudo preliminar de riscos ocupacionais e ambientais. Revista Brasileira de Saúde Ocupacional 37:78–88.
6. ANVISA. 2018. RELATÓRIO DE ATIVIDADES DA GERÊNCIA GERAL DE TOXICOLOGIA 2017: Principais ações, resultados e perspectivas. Brasília.
7. Talha-Mar Soluções Ambientais. 2010. Levantamento do Uso e da Criticidade dos Agrotóxicos Usados no Estado do Rio Grande do Sul. Centro de Vigilância em Saúde da Secretaria da Saúde - CEVS/SES.
8. Possidônio De Amarante Junior O, Rodrigues TC, Santos D. 2002. GLIFOSATO: PROPRIEDADES, TOXICIDADE, USOS E LEGISLAÇÃOQuim. Nova.
9. Bentley R, Haslam E. 1990. The Shikimate Pathway — A Metabolic Tree with Many Branche. Critical Reviews in Biochemistry and Molecular Biology 25:307–384.
10. Cao G, Liu Y, Zhang S, Yang X, Chen R, Zhang Y, Lu W, Liu Y, Wang J, Lin M, Wang G. 2012. A Novel 5-Enolpyruvylshikimate-3-Phosphate Synthase Shows High Glyphosate Tolerance in Escherichia coli and Tobacco Plants. PLOS ONE 7:e38718-.
11. Schulz A, Krüper A, Amrhein N. 1985. Differential sensitivity of bacterial 5-enolpyruvylshikimate-3-phosphate synthases to the herbicide glyphosate. FEMS Microbiology Letters 28:297–301.
12. Priestman MA, Funke T, Singh IM, Crupper SS, Schönbrunn E. 2005. 5-Enolpyruvylshikimate-3-phosphate synthase from Staphylococcus aureus is insensitive to glyphosate. FEBS Letters 579:728–732.
13. Burns CJ, Swaen GMH. 2012. Review of 2,4-dichlorophenoxyacetic acid (2,4-D) biomonitoring and epidemiology. Critical Reviews in Toxicology 42:768–786.
14. Wilson RD, Geronimo J, Armbruster JA. 1997. 2,4-D dissipation in field soils after applications of 2,4-D dimethylamine salt and 2,4-D 2-ethylhexyl ester. Environmental Toxicology and Chemistry 16:1239–1246.

15. Aguiar LM, dos Santos JB, Barroso GM, Laia ML de, Gonçalves JF, da Costa VAM, Brito LA. 2020. Influence of 2,4-D residues on the soil microbial community and growth of tree species. International Journal of Phytoremediation 22:69–77.
16. Kurenbach B, Marjoshi D, Amábile-Cuevas CF, Ferguson GC, Godsoe W, Gibson P, Heinemann JA. 2015. Sublethal Exposure to Commercial Formulations of the Herbicides Dicamba, 2,4-Dichlorophenoxyacetic Acid, and Glyphosate Cause Changes in Antibiotic Susceptibility in Escherichia coli and Salmonella enterica serovar Typhimurium. mBio 6:1–9.
17. Kurenbach B, Hill AM, Godsoe W, van Hamelsveld S, Heinemann JA. 2018. Agrichemicals and antibiotics in combination increase antibiotic resistance evolution. PeerJ 6:e5801.
18. Kurenbach B, Gibson PS, Hill AM, Bitzer AS, Silby MW, Godsoe W, Heinemann JA. 2017. Herbicide ingredients change Salmonella enterica sv. Typhimurium and Escherichia coli antibiotic responses. Microbiology (United Kingdom) 163:1791–1801.
19. World Health Organization. 2022. Antimicrobial resistance.
20. Fernández L, Breidenstein EBM, Hancock REW. 2011. Creeping baselines and adaptive resistance to antibiotics. Drug Resistance Updates 14:1–21.
21. Fernández L, Hancock REW. 2012. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin Microbiol Rev 25:661–681.
22. Palmer AC, Kishony R. 2013. Understanding, predicting and manipulating the genotypic evolution of antibiotic resistance. Nature Reviews Genetics 14:243–248.
23. Araújo C, Torres C, Silva N, Carneiro C, Gonçalves A, Radhouani H, Correia S, da Costa PM, Paccheco R, Zarazaga M, Ruiz-Larrea F, Poeta P, Igrejas G. 2010. Vancomycin-resistant enterococci from Portuguese wastewater treatment plants. Journal of Basic Microbiology 50:605–609.
24. Novo A, Manaia CM. 2010. Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. Applied Microbiology and Biotechnology 87:1157–1166.
25. Ohore OE, Addo FG, Zhang S, Han N, Anim-Larbi K. 2019. Distribution and relationship between antimicrobial resistance genes and heavy metals in surface sediments of Taihu Lake, China. Journal of Environmental Sciences 77:323–

335.
26. Resende JA, da Silva VL, Diniz CG. 2020. Aquatic environments in the one health context: Modulating the antimicrobial resistance phenomenon. Acta Limnologica Brasiliensia 32.
27. Yang Y, Song W, Lin H, Wang W, Du L, Xing W. 2018. Antibiotics and antibiotic resistance genes in global lakes: A review and meta-analysis. Environment International 116:60–73.
28. Yang J, Wang C, Shu C, Liu L, Geng J, Hu S, Feng J. 2013. Marine Sediment Bacteria Harbor Antibiotic Resistance Genes Highly Similar to Those Found in Human Pathogens. Microbial Ecology 65:975–981.
29. Rangasamy K, Athiappan M, Devarajan N, Samykannu G, Parray JA, Aruljothi KN, Shameem N, Alqarawi AA, Hashem A, Abd_Allah EF. 2018. Pesticide degrading natural multidrug resistance bacterial flora. Microbial Pathogenesis 114:304–310.
30. Mohanty S, Jena H. 2019. Degradation kinetics and mechanistic study on herbicide bioremediation using hyper butachlor-tolerant Pseudomonas putida G3. Process Safety and Environmental Protection 125.
31. Pileggi M, Pileggi SAV, Sadowsky MJ. 2020. Herbicide bioremediation: from strains to bacterial communities. Heliyon 6.
32. Soares GB, Basso NR de S, Moura CS. 2019. Caracterização hidroquímica de águas subterrâneas aliada ao uso de óxido de grafeno reduzido para adsorção de herbicidas. Porto Alegre.
33. García Moreno W, Soares G, Pires J, Sotelo D, Moura C. 2021. Hydrogeochemical and Geophysical Evaluation of the Recharge Zone of Guarani Aquifer in Municipally of Candelária, Rio Grande do Sul, Brazil. Geotechnical and Geological Engineering 39.
34. Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Research 17:7843–7853.
35. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Molecular Biology and Evolution 35:1547–1549.
36. Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in

the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10:512–526.

37. CONAMA. 2008. Dispõe sobre a classificação e diretrizes ambientais para o enquadramento das águas subterrâneas e dá outras providências. Diário Oficial da União, Brasil.

38. Bellinaso MDL, Greer CW, Peralba M do C, Henriques JAP, Gaylarde CC. 2003. Biodegradation of the herbicide trifluralin by bacteria isolated from soil. FEMS Microbiology Ecology 43:191–194.

39. Curutiu C, Lazar V, Chifiriuc MC. 2017. Pesticides and antimicrobial resistance: from environmental compartments to animal and human infections, p. 373–392. *In* Grumezescu, AM (ed.), New Pesticides and Soil Sensors. Academic Press.

40. Olchanheski LR, Dourado MN, Beltrame FL, Zielinski AAF, Demiate IM, Pileggi SA v, Azevedo RA, Sadowsky MJ, Pileggi M. 2014. Mechanisms of Tolerance and High Degradation Capacity of the Herbicide Mesotrione by Escherichia coli Strain DH5-α. PLOS ONE 9:e99960-.

41. BrCAST. 2012. Tabelas de pontos de corte para interpretação de CIMs e diâmetros de halos, 3rd ed.

42. Krumperman PH. 1983. Multiple Antibiotic Resistance Indexing of Escherichia coli to Identify High-Risk Sources of Fecal Contamination of FoodstAPPLIED AND ENVIRONMENTAL MICROBIOLOGY.

43. Mandal M, Das SN, Mandal S. 2020. Principal component analysis exploring the association between antibiotic resistance and heavy metal tolerance of plasmid-bearing sewage wastewater bacteria of clinical relevance. Access Microbiology 2.

44. R Core Team. 2019. R: A Language and Environment for Statistical Computing. 3.6.1. Vienna, Austria.

45. Byrd AL, Belkaid Y, Segre JA. 2018. The human skin microbiome. Nature Reviews Microbiology 16:143–155.

46. Poulsen VK, Koza A, Al-Nakeeb K, Oeregaard G. 2020. Screening for texturing Leuconostoc and genomics behind polysaccharide production. FEMS Microbiology Letters 367:fnaa179.

47. Ino K, Nakase K, Suzuki K, Nakamura A, Fujieda A, Katayama N. 2016. Bacteremia due to Leuconostoc pseudomesenteroides in a Patient with Acute

Lymphoblastic Leukemia: Case Report and Review of the Literature . Case Reports in Hematology 2016:1–4.

48. Alcaraz LD, Moreno-Hagelsieb G, Eguiarte LE, Souza V, Herrera-Estrella L, Olmedo G. 2010. Understanding the evolutionary relationships and major traits of Bacillus through comparative genomics. BMC Genomics 11:332.

49. Carlin F. 2011. Origin of bacterial spores contaminating foods. Food Microbiology 28:177–182.

50. Gauvry E, Mathot A-G, Leguérinel I, Couvert O, Postollec F, Broussolle V, Coroller L. 2017. Knowledge of the physiology of spore-forming bacteria can explain the origin of spores in the food environment. Research in Microbiology 168:369–378.

51. Nair A v., Joseph N, Krishna K, Sneha KG, Tom N, Jangid K, Nair S. 2015. A comparative study of coastal and clinical isolates of pseudomonas aeruginosa. Brazilian Journal of Microbiology 46:725–734.

52. Silby MW, Winstanley C, Godfrey SAC, Levy SB, Jackson RW. 2011. Pseudomonas genomes: diverse and adaptable. FEMS Microbiology Reviews 35:652–680.

53. Byappanahalli MN, Nevers MB, Korajkic A, Staley ZR, Harwood VJ. 2012. Enterococci in the environment. Microbiol Mol Biol Rev 76:685–706.

54. Argemi X, Hansmann Y, Prola K, Prévost G. 2019. Coagulase-Negative Staphylococci Pathogenomics. Int J Mol Sci 20:1215.

55. Jørgensen PS, Aktipis A, Brown Z, Carrière Y, Downes S, Dunn RR, Epstein G, Frisvold GB, Hawthorne D, Gröhn YT, Gujar GT, Jasovský D, Klein EY, Klein F, Lhermie G, Mota-Sanchez D, Omoto C, Schlüter M, Scott HM, Wernli D, Carroll SP, project L with R. 2018. Antibiotic and pesticide susceptibility and the Anthropocene operating space. Nature Sustainability 1:632–641.

56. Gravina F, Dobrzanski T, Olchanheski LR, Galvão CW, Reche PM, Pileggi SA, Azevedo RA, Sadowsky MJ, Pileggi M. 2017. Metabolic Interference of sod gene mutations on catalase activity in Escherichia coli exposed to Gramoxone® (paraquat) herbicide. Ecotoxicol Environ Saf 139:89–96.

57. Prione LP, Olchanheski LR, Tullio LD, Santo BCE, Reche PM, Martins PF, Carvalho G, Demiate IM, Pileggi SA v, Dourado MN, Prestes RA, Sadowsky MJ, Azevedo RA, Pileggi M. 2016. GST activity and membrane lipid saturation

prevents mesotrione-induced cellular damage in Pantoea ananatis. AMB Express2016/09/13. 6:70.

58. Dick RE, Quinn JP. 1995. Glyphosate-degrading isolates from environmental samples: occurrence and pathways of degradation. Applied Microbiology and Biotechnology 43:545–550.

59. Ermakova IT, Shushkova T v, Sviridov A v, Zelenkova NF, Vinokurova NG, Baskunov BP, Leontievsky AA. 2017. Organophosphonates utilization by soil strains of Ochrobactrum anthropi and Achromobacter sp. Archives of Microbiology 199:665–675.

60. Singh S, Kumar V, Gill JPK, Datta S, Singh S, Dhaka V, Kapoor D, Wani AB, Dhanjal DS, Kumar M, Harikumar SL, Singh J. 2020. Herbicide Glyphosate: Toxicity and Microbial Degradation. Int J Environ Res Public Health 17:7519.

61. Kumar A, Trefault N, Olaniran AO. 2016. Microbial degradation of 2,4-dichlorophenoxyacetic acid: Insight into the enzymes and catabolic genes involved, their regulation and biotechnological implications. Critical Reviews in Microbiology 42:194–208.

62. Moore JK, Braymer HD, Larson AD. 1983. Isolation of a Pseudomonas sp. Which Utilizes the Phosphonate Herbicide GlyphosateAPPLIED AND ENVIRONMENTAL MICROBIOLOGY.

63. Singh S, Kumar V, Datta S, Wani AB, Dhanjal DS, Romero R, Singh J. 2020. Glyphosate uptake, translocation, resistance emergence in crops, analytical monitoring, toxicity and degradation: a review. Environmental Chemistry Letters 18:663–702.

64. Reyes-Cervantes A, Robles-Morales DL, Téllez-Jurado A, Huerta-Ochoa S, Jiménez-González A, Medina-Moreno SA. 2021. Evaluation in the performance of the biodegradation of herbicide diuron to high concentrations by Lysinibacillus fusiformis acclimatized by sequential batch culture. Journal of Environmental Management 291:112688.

65. Gaur VK, Bajaj A, Regar RK, Kamthan M, Jha RR, Srivastava JK, Manickam N. 2019. Rhamnolipid from a Lysinibacillus sphaericus strain IITR51 and its potential application for dissolution of hydrophobic pesticides. Bioresource Technology 272:19–25.

66. Brigitta K, Delphine M, F A-CC, C FG, William G, Paddy G, A HJ, J GS. 2015.

Sublethal Exposure to Commercial Formulations of the Herbicides Dicamba, 2,4-Dichlorophenoxyacetic Acid, and Glyphosate Cause Changes in Antibiotic Susceptibility in Escherichia coli and Salmonella enterica serovar Typhimurium. mBio 6:e00009-15.

67. Shahid M, Khan M. 2017. Assessment of Glyphosate and Quizalofop Mediated Toxicity to Greengram [Vigna radiata (L.) Wilczek], Stress Abatement and Growth Promotion by Herbicide Tolerant Bradyrhizobium and Pseudomonas species. International Journal of Current Microbiology and Applied Sciences 6.
68. Shahid M, Khan MS. 2022. Tolerance of pesticides and antibiotics among beneficial soil microbes recovered from contaminated rhizosphere of edible crops. Current Research in Microbial Sciences 3:100091.
69. Shahid M, Khan MS. 2018. Glyphosate induced toxicity to chickpea plants and stress alleviation by herbicide tolerant phosphate solubilizing Burkholderia cepacia PSBB1 carrying multifarious plant growth promoting activities. 3 Biotech2018/02/14. 8:131.
70. Herman PL, Behrens M, Chakraborty S, Chrastil BM, Barycki J, Weeks DP. 2005. A Three-component Dicamba O-Demethylase from Pseudomonas maltophilia. Journal of Biological Chemistry 280:24759–24767.
71. Liao H, Li X, Yang Q, Bai Y, Cui P, Wen C, Liu C, Chen Z, Tang J, Che J, Yu Z, Geisen S, Zhou S, Friman V-P, Zhu Y-G. 2021. Herbicide Selection Promotes Antibiotic Resistance in Soil Microbiomes. Molecular Biology and Evolution 38:2337–2350.
72. Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, Bürgmann H, Sørum H, Norström M, Pons M-N, Kreuzinger N, Huovinen P, Stefani S, Schwartz T, Kisand V, Baquero F, Martinez JL. 2015. Tackling antibiotic resistance: the environmental framework. Nature Reviews Microbiology 13:310–317.
73. Chow LKM, Ghaly TM, Gillings MR. 2021. A survey of sub-inhibitory concentrations of antibiotics in the environment. Journal of Environmental Sciences 99:21–27.
74. Karkman A, Pärnänen K, Larsson DGJ. 2019. Fecal pollution can explain antibiotic resistance gene abundances in anthropogenically impacted environments. Nature Communications 10:80.

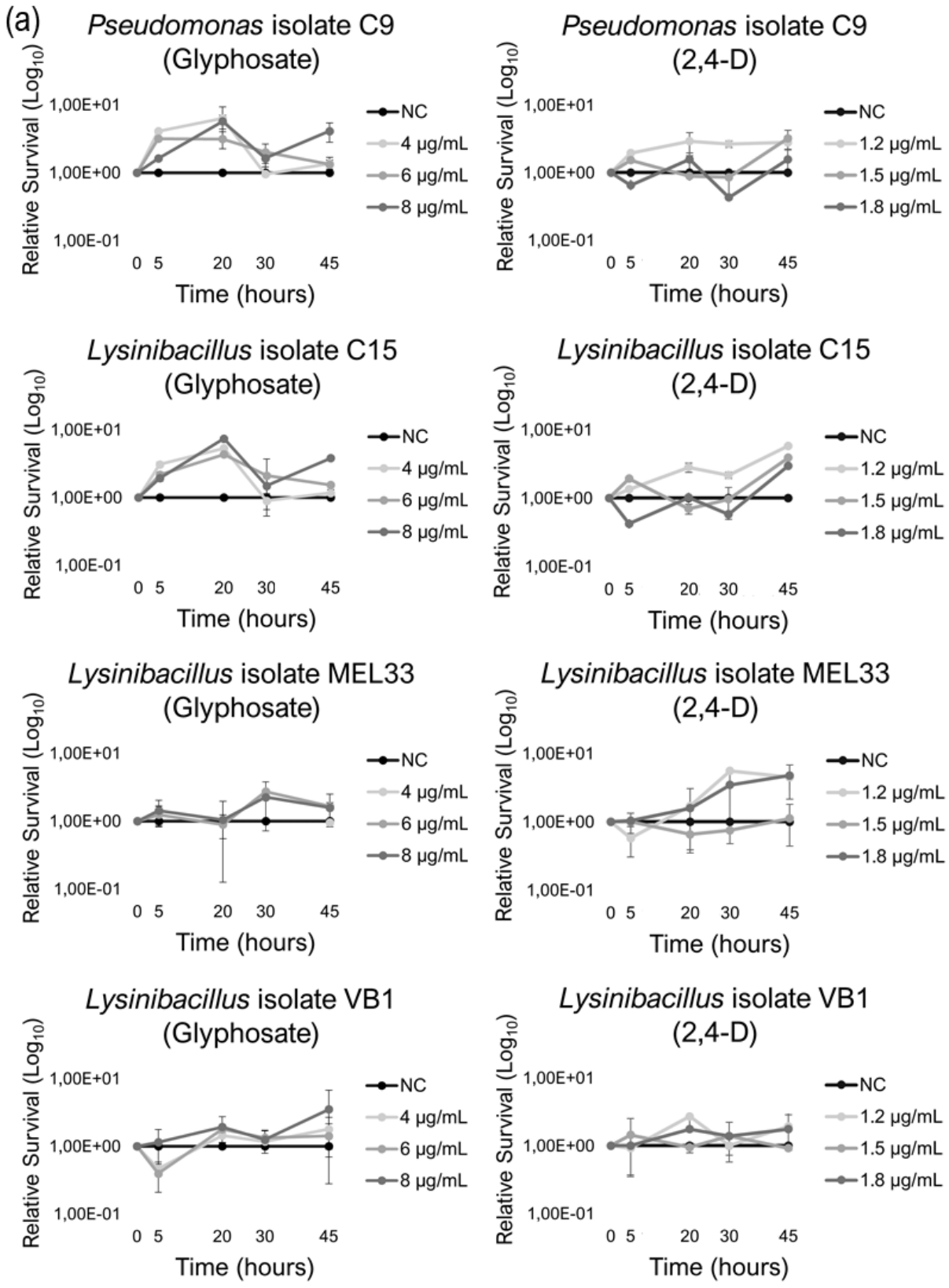
75. Pruden A, Arabi M, Storteboom HN. 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. Environmental Science and Technology 46:11541–11549.
76. Chen H, Liu C, Teng Y, Zhang Z, Chen Y, Yang Y. 2021. Environmental risk characterization and ecological process determination of bacterial antibiotic resistome in lake sediments. Environment International 147:106345.
77. Pulido-velazquez D, Sahuquillo A, Andreu J, Pulido-Velazquez M. 2007. An efficient conceptual model to simulate surface water body-aquifer interaction in Conjunctive Use Management Models. Water Resources Research 43.
78. Laborda P, Sanz-García F, Hernando-Amado S, Martínez JL. 2021. Pseudomonas aeruginosa: an antibiotic resilient pathogen with environmental origin. Current Opinion in Microbiology 64:125–132.
79. gholami S, Tabatabaei M, Sohrabi N. 2017. Comparison of biofilm formation and antibiotic resistance pattern of Pseudomonas aeruginosa in human and environmental isolates. Microbial Pathogenesis 109:94–98.
80. Ramsay KA, Wardell SJT, Patrick WM, Brockway B, Reid DW, Winstanley C, Bell SC, Lamont IL. 2019. Genomic and phenotypic comparison of environmental and patient-derived isolates of Pseudomonas aeruginosa suggest that antimicrobial resistance is rare within the environment. Journal of Medical Microbiology 68:1591–1595.
81. Larsson DGJ, Flach C-F. 2021. Antibiotic resistance in the environment. Nature Reviews Microbiology https://doi.org/10.1038/s41579-021-00649-x.
82. Luczkiewicz A, Kotlarska E, Artichowicz W, Tarasewicz K, Fudala-Ksiazek S. 2015. Antimicrobial resistance of Pseudomonas spp. isolated from wastewater and wastewater-impacted marine coastal zone. Environmental Science and Pollution Research International 22:19823.
83. Rovida AF da S, Costa G, Santos MI, Silva CR, Freitas PNN, Oliveira EP, Pileggi SAV, Olchanheski RL, Pileggi M. 2021. Herbicides Tolerance in a Pseudomonas Strain Is Associated With Metabolic Plasticity of Antioxidative Enzymes Regardless of Selection. Frontiers in Microbiology 12:1430.
84. Huete-Soto A, Castillo-González H, Masís-Mora M, Chin-Pampillo JS, Rodríguez-Rodríguez CE. 2017. Effects of oxytetracycline on the performance and activity of biomixtures: Removal of herbicides and mineralization of

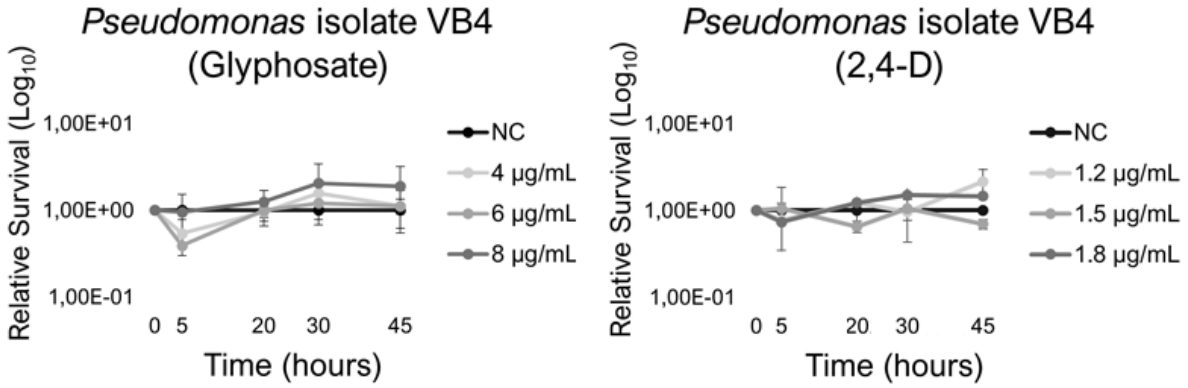
chlorpyrifos. J Hazard Mater 321:1–8.

85. Malagón-Rojas JN, Parra Barrera EL, Lagos L. 2020. From environment to clinic: The role of pesticides in antimicrobial resistance. Revista Panamericana de Salud Publica/Pan American Journal of Public Health 44.

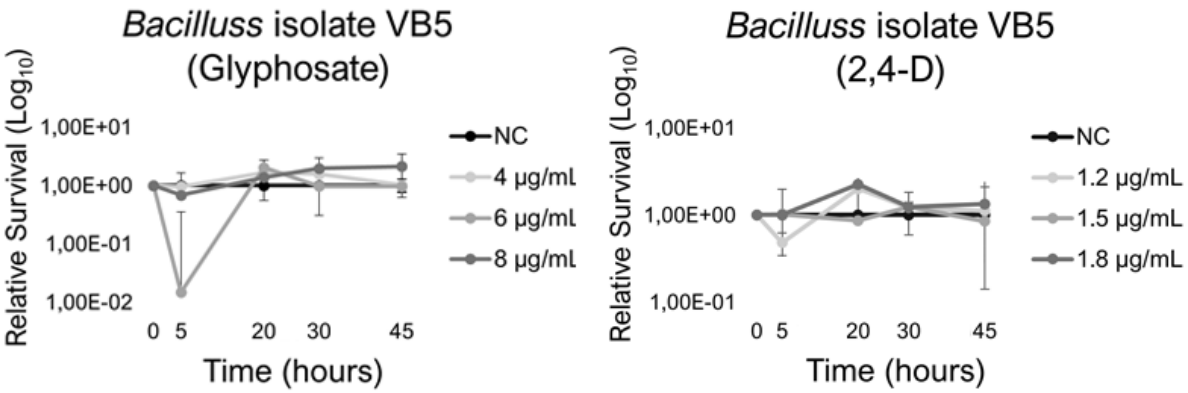
86. Humboldt-Dachroeden S, Mantovani A. 2021. Assessing Environmental Factors within the One Health Approach. Medicina 2021, Vol 57, Page 240 57:240.

Supplementary information

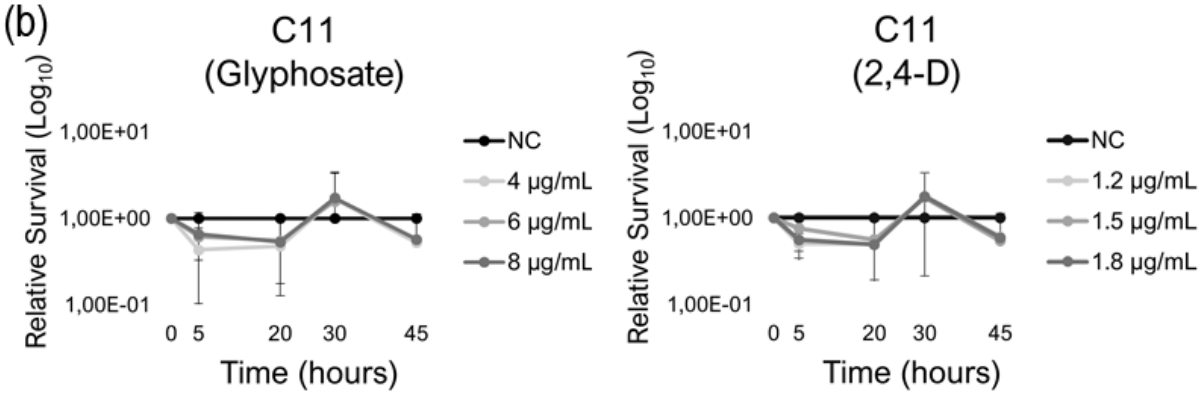




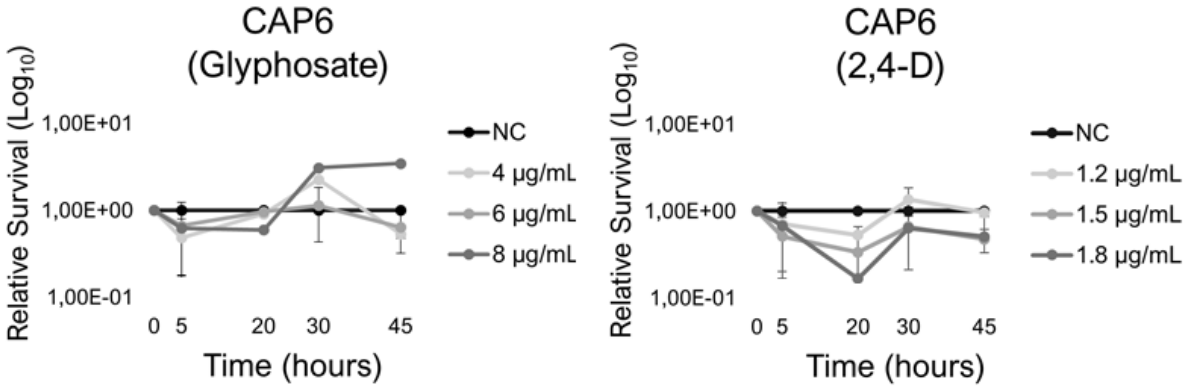
1018



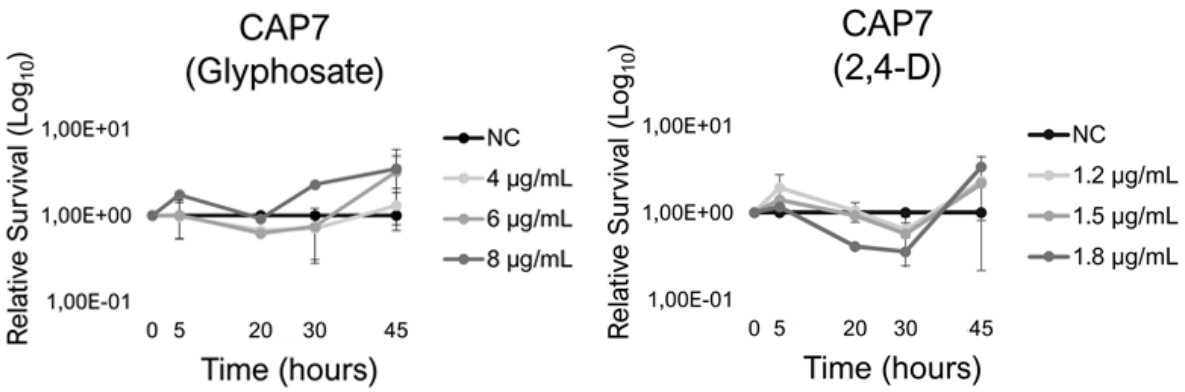
1019



1020



1021



Supplementary Figure 1 Relative survival to glyphosate and 2,4-D, in semi-log curves of isolates presented no significant responses to the herbicides along the treatments: (a) taxonomically identified isolates; (b) unidentified or unsupported isolates. Differences were considered significant when at least one $\log_{10}$ of variation (considering the standard deviation) was observed in relation to the control without herbicides (NC).

Pontifícia Universidade Católica do Rio Grande do Sul
Pró-Reitoria de Graduação
Av. Ipiranga, 6681 - Prédio 1 - 3º. andar
Porto Alegre - RS - Brasil
Fone: (51) 3320-3500 - Fax: (51) 3339-1564
E-mail: prograd@pucrs.br
Site: www.pucrs.br