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The role of bacterial diversity on the antibiotic and herbicide biodegradation in agricultural soils

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Thesis presented to obtain the degree of Doctor in Science:
Area: Agricultural Microbiobiology

Piracicaba
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“Quem consegue manter-se firme em pé
Não tem medo da crise quando sofre
Todo mundo no peito tem um cofre
Como um banco central pra guardar fé
A hipótese de ser faz quem não é
Trabalhar noite, dia, mês e ano
Sonhar alto, rezar e fazer plano
E ir à luta buscando o que não há
'Vez em quando' tem um que chega lá”
[...]

“Muito além do que pode tem quem vá
Sem receio dos ermos do além
Encontrar quem ta indo a vida vem
Esperando quem custa Deus está
Quem insiste num sonho é quem se dá
E quando nota o que fez se sente ufano
De início ninguém é veterano
Ninguém nasce sabendo o que defende
Mas depois de errar muito a gente aprende”.

~ Raimundo Nonato e Nonato Costa, Pensamento Positivo

CONTENTS

RESUMO

O papel da diversidade bacteriana na biodegradação de antibiótico e herbicida em solos agrícolas

O microbioma do solo é essencial para o fornecimento de serviços ecossistêmicos. A relação entre diversidade microbiana e serviços ecossistêmicos é bem elucidada para funções coletivas do microbioma do solo (decomposição de matéria orgânica e assimilação de carbono). Contudo, esta relação ainda permanece pouco elucidada para funções mais específicas (degradação de pesticidas e outros xenobióticos). Aqui, foram utilizadas a abordagem da diluição para extinção, a radiorespirometria e o sequenciamento alto rendimento do gene 16S rRNA para avaliar o papel da depleção da diversidade bacteriana na dissipação do antibiótico sulfadiazina (SDZ) e herbicida atrazina (ATZ), que são amplamente usados na suinocultura e agricultura, respectivamente. O microcosmo da SDZ foi montado utilizando solos sem (S1) e com histórico de aplicação de esterco suíno (S2). Enquanto o microcosmo da ATZ foi montado usando Solo de Pastagem (GS, sem histórico de aplicação de ATZ) e Solo Cultivado (CS, com histórico de aplicação de ATZ). A aplicação de esterco promoveu aumentos no pH e nos teores de carbono orgânico e macronutrientes, bem como impactou a estrutura e diversidade da comunidade bacteriana do solo. A depleção da diversidade bacteriana reduziu a mineralização da SDZ (^{14}C-CO_2) e a formação de resíduo não extraível (NER), mas o NER se recuperou após 42 d de incubação. Surpreendentemente, o solo natural não adubado (NS-S1) apresentou taxa de dissipação da SDZ mais rápida (DT_{90} = 2,0 versus 21 d) e teve um grande número de famílias bacterianas envolvidas nas principais vias de dissipação da SDZ (^{14}C-CO_2 e principalmente NER), tais como Isosphaeraceae, Ktedonobacteraceae, Acidobacteriaceae_Subgroup_1, Micromonosporaceae e Sphingobacteriaceae. De forma similar, o uso da terra e longo período de aplicação da atrazina promoveu mudanças nos atributos físicos e químicos do solo, na estrutura, diversidade e composição da comunidade bacteriana, bem como em sua capacidade para dissipar a atrazina. Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Acidobacteria, Verrucomicrobia, Planctomycetes e Gemmatimonadetes foram os filos dominantes ambos os solos (GS e CS). A mineralização da atrazina foi reduzida em 12 vezes quando comparada aos piores cenários de perda da diversidade bacteriana. O longo histórico de aplicação de atrazina resultou em maior eficiência da comunidade bacteriana na dissipação do herbicida. A perda da diversidade bacteriana refletiu na redução da complexidade, classificação das espécies (especialistas, generalistas e muito raras), além de aumentos na abundância de funções relacionadas à obtenção de transformações de energia e nitrogênio. Doze gêneros bacterianos (*Ralstonia*, *Nitrospira*, *Hirschia*, *MND1*, *Candidatus_Koribacter*, *OM27_clade*, *Arenimonas*, *Occallatibacter*, *Bryobacter*, *UTBCD1*, *Ellin6067* e *Crenobacter*) se correlacionaram com a mineralização da atrazina, sugerindo a seleção desses gêneros como potenciais degradadores de atrazina. A formação de NER foi a rota primária de dissipação da SDZ e com maior impacto da depleção da diversidade bacteriana. Enquanto a mineralização foi a rota primária de dissipação da ATZ no solo cultivado (CS). A perda da diversidade bacteriana resultou em comunidades bacterianas simplificadas, maior desbalanço na ocupação de nichos, bem como maior abundância de funções associadas obtenção de energia.

Palavras-chave: Comunidade bacteriana, Sulfonamidas, S-triazinas, Dissipação de pesticidas, Diluição para extinção

ABSTRACT

The role of bacterial diversity on the antibiotic and herbicide biodegradation in agricultural soils

Soil microbiome is essential for providing ecosystem services. The relationship between microbial diversity and ecosystem services is well elucidated for soil microbiome collective functions (i.e. organic matter decomposition and carbon assimilation). However, this relationship remains poorly understood for more specific functions (pesticides and o other xenobiotics degradation). Here, the dilution-to-extinction approach, radiorespirometry and high-throughput sequencing of 16S rRNA gene were used to assess the role of bacterial diversity depletion in the dissipation of antibiotic sulfadiazine (SDZ) and herbicide atrazine (ATZ), which are widely used in pig farming and agriculture, respectively. SDZ microcosm was assembled using paralel soils without (S1) and with (S2) a long-term of swine manure application. While the ATZ microcosm was assembled using Pasture Soil (GS, without long-term of ATZ application) and Cultivated Soil (CS, with long-term of ATZ application). Swine manure application promoted increases in pH and organic carbon and macronutrient contents, as well as impacted the soil bacterial community structure and diversity. The bacterial diversity depletion reduced SDZ mineralization (^{14}C-CO_2) and non-extractable residue (NER) formation, but NER recovered after 42 d of incubation. Surprisingly, the non-manured soil (treatment NS-S1) had a faster SDZ dissipation rate (DT_{90} = 2.0 versus 21 d) and had a large number of bacterial families involved in the main SDZ dissipation pathways (^{14}C-CO_2 and mainly NER), such as Isosphaeraceae, Ktedonobacteraceae, Acidobacteriaceae_Subgroup_1, Micromonosporaceae and Sphingobacteriaceae. Similarly, land use and the long-term of atrazine application promoted changes in the soil physico-chemical attributes, bacterial community structure, diversity and composition, as well as in its capacity to dissipate atrazine. Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Acidobacteria, Verrucomicrobia, Planctomycetes and Gemmatimonadetes were the dominant phyla in both soils (GS and CS). Atrazine minerazation was reduced by 12-fold when compared to the worst scenarios of bacterial diversity depletion. Long-term atrazine application resulted in greater bacterial community efficiency in herbicide dissipation. Bacterial diversity depletion reflected in the reduction of complexity, classification of species (specialists, generalists and very rare), in addition to increases in the abundance of functions related to obtaining energy and nitrogen transformations. Twelve bacterial genera (*Ralstonia, Nitrospira, Hirschia, MND1, Candidatus_Koribacter, OM27_clade, Arenimonas, Occallatibacter, Bryobacter, UTBCD1, Ellin6067* and *Crenobacter*) correlated with atrazine mineralization, suggesting the selection of these genera as potential atrazine degraders. The NER formation was the SDZ primary dissipation route and had the greatest impact of the bacterial diversity depletion. While mineralization was the ATZ primary dissipation route in cultivated soil (CS). The bacterial diversity depletion resulted in simplified bacterial communities, greater imbalance in the niches occupation, as well as higher abundance of functions associated with obtaining energy.

Keywords: Bacterial community, Sulfonamides, S-triazines, Pesticide dissipation, Dilution-to-extinction

1. SOIL MICROBIAL DIVERSITY AND ITS ROLE IN PESTICIDE BIODEGRADATION

The great challenge of agriculture has been to reconcile efficiency in land use and productivity increase with sustainable agricultural practices that promote biodiversity and ecosystem services conservation (Daam et al., 2019; Landis, 2016). The modern agriculture raised after green revolution, which brought a huge range of technological packages and allowed for large-scale production. In this scenario, agrochemicals (mineral fertilizers and pesticides) are inserted, which have become crucial in agricultural practices worldwide and contributed decisively to the expression of the crops productivity through nutrients supplementation (fertilization) as well as pests, weeds, and diseases control (Feld et al., 2015; Malhotra et al., 2015).

Agrochemical use has contributed to ensure food production and reduce hunger in the world. However, there is a strong discussion in civil society and the scientific community regarding environmental problems and risks to human health associated with the agrochemical intensive and indiscriminate use (Ortiz-Hernández et al., 2013; Rodríguez et al., 2020). For pesticides, only a small fraction of the amount applied reaches the target organism. It is estimated that about 0.1 to 3.0% of the applied dose reaches the target (pests, weeds, disease agents etc.) and the remaining amount reaches the most distinct environmental compartments, such as water bodies, soils, sediments, and soil biota (Gill and Garg, 2014).

Soil microorganisms play crucial roles for maintenance and functioning of the soil ecosystem, such as biogeochemical cycles, organic matter decomposition, nitrogen biological fixation, nutrient solubilization, disease suppression, and degradation of pesticide and other xenobiotics (Daam et al., 2019; Jacobsen and Hjelmsø, 2014). The importance of microbial diversity has been elucidated for collective ecosystem functions, especially involved in the carbon and nitrogen cycles (Maron et al., 2018; Philippot et al., 2013). However, the functional role of soil microbiota is underestimated for more restricted ecosystem functions, such as the pesticide biodegradation (de Souza et al., 2017; Hernandez-Raquet et al., 2013).

The literature shows controversial results for collective functions due to their high functional redundancy in the environment, which does not allow obtaining consistent results for communities having low microbial diversity (Kirk et al., 2004; Naeem and Li, 1997; Reed and Martiny, 2007) since when a microbial group is depleted within the community another one assumes its functional role (de Souza et al., 2021). However, several research works have shown a direct relationship between microbial diversity and pesticide degradation. In general,

environments with higher microbial diversity have greater capacity to degrade pesticides, thus decreasing its persistence (Cravo-Laureau et al., 2011; de Souza et al., 2021).

Microbial diversity relations to ecosystem functions can be assessed by different approaches. Studies with defined mixture of microbial species showed that samples with higher diversity increased biomass (Naeem and Li, 1997). However, in these studies, only the cultivable fraction of the whole microbial community was assessed, which corresponds to just over 1-5% of the total microbial diversity, which is not representative of natural environments (Naeem and Li, 1997; Peter et al., 2011). Other studies come from the artificial modification of the soil microbial diversity by "dilution-to-extinction" approach used to inoculate sterile samples, which demonstrate that collective ecosystem functions were little affected by diversity depletion (Allison and Martiny, 2008; Peter et al., 2011), but that those functions performed by more specific microbial groups can be strongly impacted (Cravo-Laureau et al., 2011; de Souza et al., 2017).

Several soil functions have been impacted by microbial diversity depletion. For examples, low diversity microbial communities reduced up to 5-fold the soil denitrification rates (Jung et al., 2016; Philippot et al., 2013). In addition, soils with low bacterial diversity were more susceptible to pathogens invasion (Van Elsas et al., 2012). Jung et al. (2016) reported that soil microbial diversity depletion increased the microbial community's ability to degrade diesel in microcosm experiments, but it affected the N cycle. Maron et al. (2018) observed that loss in microbial diversity impacted carbon cycle in soils, highlighting the importance of understanding how it impacts soil functioning in face of the global changes in terrestrial ecosystems.

In our previous study, chlorothalonil biodegradation was strongly affected by soil bacterial diversity loss and its mineralization was ~20-fold lower than the control treatment whereas its degradation was completely hindered at 10^{-1} dilutions (Souza et al., 2017). Phenanthrene mineralization rate was also strongly affected by microbial diversity loss (Hernandez-Raquet et al., 2013). At disturbance levels above 10^{-6}, the ability to degrade the xenobiotic was partially or totally impaired, like observed for the biodegradation of polycyclic aromatic hydrocarbons (PAHs) (Cravo-Laureau et al., 2011). Cook et al. (2006) reported that depleted microbial communities were not capable to degrade polyoxyethylene 10 lauryl ether (POL-10). It is important to point out that most studies have evaluated the effects of pesticide use on soil microbiota, but the impacts of soil microbial diversity on pesticide degradation are poorly understood (Feld et al., 2015; Jacobsen and Hjelmsø, 2014).

Pesticide models: sulfadiazine and atrazine

Sulfadiazine

The presence of antibiotic compounds in the environment is a concerning subject to environmental and health agencies, especially due to the increase in the population of antibiotic resistant microbes (Kümmerer, 2009; Sukul et al., 2008; Tappe et al., 2013). Antibiotic residues reach agricultural soils mainly through fresh animal manure, sewage sludge, and biosolid applications, but also through release of animal wastes (feces and urine) during grazing (Cycoń et al., 2019). Sulfadiazine [4-animo-N-2-pyrimidinyl-benzenesulfonamide] (SDZ) (Figure 1) is one of the main drugs for therapeutic and prophylactic use in the animal production chain. In Brazil, it is administered mainly in poultry and pig farming (Regitano and Leal, 2010). After use, only about 10 to 60% of its molecules are metabolized in the animal's digestive tract, the remainder being eliminated with feces and urine (Tappe et al., 2013). In other words, SDZ can reach the soil directly via animal excrement during pasture or indirectly via raw manure aplication as an organic fertilizer (Shi et al., 2019; Yeom et al., 2017), thus becoming a point source of soil and water contamination and favoring antibiotic resistance genes dispersion (Mulla et al., 2016; Tappe et al., 2013). SDZ mineralization rate is often low (< 2%) (Kreuzig and Höltge, 2005; Schmidt et al., 2008), but may range from 12 to 32% in soils incubated with manure for 300 d (Tappe et al., 2013).

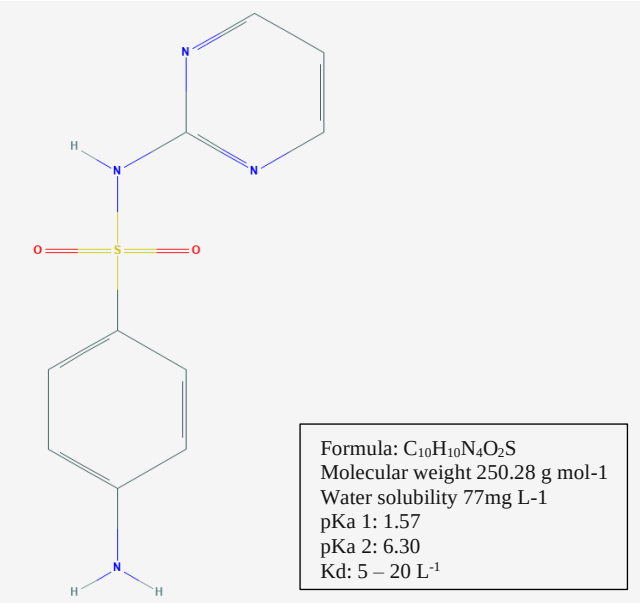

Figure 1. Chemical structure of sulfaziazine [4-animo-N-2-pyrimidinyl-benzenesulfonamide] (SDZ) (PucChem 2021a; Reia et al., 2020; Sukul et al., 2008).

Sulfadiazine and its metabolites (4-hydroxysulfadiazine and N-acetyl-sulfadiazine) tend to be quite stable in both fresh and composted manure (Lamshöft et al., 2010, 2007; Tappe et al., 2013). In addition, a large fraction of the amount applied (> 50%) remains as non-extractable residues in the soil (Förster et al., 2009), despite its low to moderate sorption potential (Leal et al., 2013; Reia et al., 2020), explaining its fast dissipation rate under temperate conditions (Förster et al., 2009). However, less is known about the sulfadiazine biodegradation in tropical conditions, as well as how soil microbial diversity can impact its dissipation.

Atrazine

Weed management in agricultural areas and pastures often demands high inputs of herbicides (Grube et al., 2011). In Brazil, glyphosate, 2,4-D, and s-triazines (atrazine, ametrine, and simazine) are the most consumed herbicides (Carmo et al., 2013), which were extensively studied in terms of environmental behavior (Carmo et al., 2013; Sene et al., 2010). However, most studies have not considered the importance of the soil microbiome in their biodegradation (de Souza et al., 2017). Pesticides environmental fate studies often evaluate their sorption/desorption, leaching, runoff, and dissipation rates (Mendes et al., 2017), but rarely assess the role of soil microbial communities on their degradation (de Souza et al., 2021).

Atrazine [2-Chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] (ATZ) (Figure 2) is a selective herbicide, used to control broadleaf weeds in corn, sugarcane, and sorghum (Aguiar et al., 2020; Dechene et al., 2014). It has high mobility in soils due to its high solubility in water (33.0 mg L^{-1}) and low sorption to soils (K_d = 2.6 L kg^{-1} and K_{OC} = 100 L kg^{-1}) (Carmo et al., 2013), and its half-life can vary from 20 to > 365 d in soils depending on environmental conditions and soil type, but microorganisms are responsible for most of its degradation (Jablonowski et al., 2010; Martinazzo et al., 2010). Moreover, atrazine and its dealkylated (deethylatrazine, deisopropylatrazine, and deisopropyldeethylatrazine) and deaminated (N-isopropylammelide, N-ethylammelide) metabolites are toxic, carcinogenic, and endocrine disruptors (Lopes et al., 2020; Singh et al., 2018).

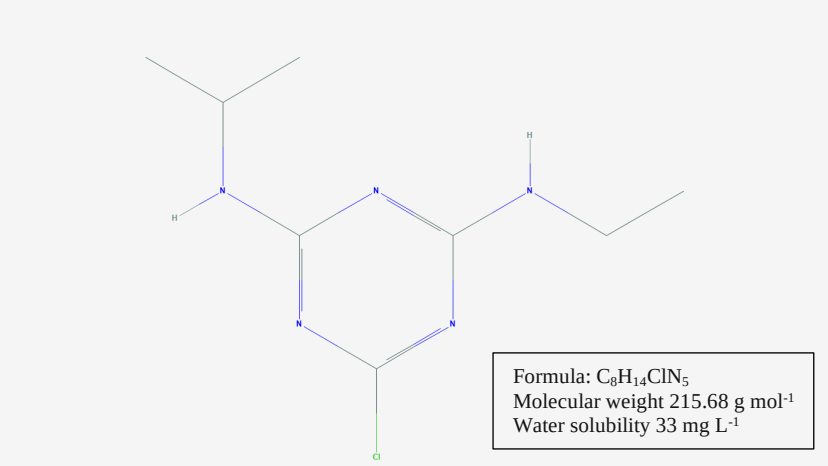

Figure 2. Chemical structure of Atrazine [2-Chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] (ATZ) (PubChem, 2021b).

Atrazine dissipation in the environment is directly correlated with soil biota (Hsieh et al., 2020; Yale et al., 2017). Several microbial strains have been isolated and identified for their high capacity to mineralize atrazine, using the herbicide molecule as a single source of carbon or nitrogen (Singh and Singh, 2014). Efficient bacterial isolates were prospected from soils with long history of herbicide application (Cao et al., 2021; Li et al., 2020), such as *Pseudomonas* spp., *Agrobacterium* spp., *Alcaligenes* spp., *Arthrobacter* spp., *Aminobacter* spp., *Bacillus* spp., *Comamonas* spp., *Enterobacter* spp., *Klebsiella* spp., *Nocardia* spp., *Pseudaminobacter* spp., *Rhodococcus* spp., *Rhizobium* spp., *Stenotrophomonas* spp., and *Xanthomonas* Spp. (Singh et al., 2018). However, atrazine's biodegradation has been limited to the use of bacterial strains in the culture medium, not considering the interaction of the herbicide with the soil matrix and the soil bacterial community. Thus, the role of bacterial diversity in the biodegradation of atrazine is unknown, but it should have less deleterious effects on the soil microbial community than antibiotics and fungicides due to its mode of action destined to plants.

General objectives and hypothesis

This thesis aims to evaluate how bacterial diversity depletion affects biodegradation of the antibiotic sulfadiazine and the herbicide atrazine in soils having long term application history. We assessed how changes in the alpha diversity, composition, and structure of soil bacterial communities impact these contaminants environmental behavior, especially their mineralization and dissipation rates. We hypothesized that sulfadiazine and atrazine biodegradation is strongly reduced in soils having depleted bacterial diversity, but it will depend

on their application history and the presence of adapted microorganisms, as well as on the chemical nature of the contaminant (antibiotic or herbicide).

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2. SULFADIAZINE DISSIPATION AS A FUNCTION OF SOIL BACTERIAL DIVERSITY [(a)]

Abstract

Antibiotic residues in the environment are concerning since results in dispersion of resistance genes. Their degradation is often closely related to microbiome metabolism. However, the impacts of soil bacterial community on sulfadiazine (SDZ) dissipation remains unclear, mainly in tropical soils. Our main goals were to evaluate effects of long-term swine manure application on soil bacterial structure as well as effects of soil microbial diversity depletion on SDZ dissipation, using "dilution-to-extinction approach" and ^{14}C-SDZ. Manure application affected several soil attributes, such as pH, organic carbon (OC), and macronutrient contents as well as bacterial community structure and diversity. Even minor bacterial diversity depletion impacted SDZ mineralization and non-extractible residue (NER) formation rates, but NER recovered along 42 d likely due to soil diversity recovery. However, this period may be enough to spread resistance genes into the environment. Surprisingly, the non-manured natural soil (NS-S1) showed faster SDZ dissipation rate (DT_{90} = 2.0 versus 21 d) and had a great number of bacterial families involved in major SDZ dissipation pathways (mineralization and mainly NER), such as Isosphaeraceae, Ktedonobacteraceae, Acidobacteriaceae_(Subgroup_1), Micromonosporaceae, and Sphingobacteriaceae. This result is unique and contrasts our hypothesis that long-term manured soils would present adaptive advantages and, consequently, have higher SDZ dissipation rates. The literature suggests instantaneous chemical degradation of SDZ in acidic soils responsible to the fast formation of NER. Our results show that if chemical degradation happens, it is soon followed by microbial metabolism (biodegradation) performed by a pool of bacteria and the newly formed metabolites should favors NER formation since SDZ presented low sorption. It also showed that SDZ mineralization is a low redundancy function.

Keywords: SA, antibiotic, degradation, ecosystem services, biodiversity depletion.

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Introduction

About 2.0 x 10^5 t y^{-1} of antibiotics are used in human and veterinary medicine (Liu et al., 2020c). Global antibiotic consumption, expressed in defined daily dose (ddd), increased from 21.1 billion (2010) to 34.8 billion (2015), and should reach 128 billion until 2030 (Hu et al., 2020). Sulfonamides (SAs) are the most widely used class of antibiotics in veterinary medicine; it is estimated that about 2.0 x 10^4 t are introduced into the biosphere yearly and that 50-75% are used in veterinary medicine (Gao et al., 2019b; Liu et al., 2020c). SAs are the third most employed class of antibiotics in Brazil. The country consumed 715 t in 2013, with an expected increase of 41% until 2030 (up to ~ 1.0 x 10^3 t y^{-1}) (Van Boeckel et al., 2015, 2017).

Antibiotic residues reach agricultural soils through fresh animal manure, sewage sludge, and biosolid applications as well as animal waste (feces and urine) releases during grazing (Cycoń et al., 2019). Animal manures are the main entry route of SAs into the environment since only 10-40% of the administered dose is metabolized in the body and the remainder is eliminated in the feces and urine (Conde-Cid et al., 2018). Using animal manure and biosolids as fertilizers is a sustainable agricultural practice widely recommended by promoting proper disposal and nutrient cycling that are crucial to protect the environment and to promote crop development (Hu et al., 2017; Edesi et al., 2020). However, the presence of antibiotic residues may contaminate soils and water resources, thus risking animal and human health (Cycoń et al., 2019). Also, resistance genes spread is concerning since emergence of microbial strains tolerant to different classes of antibiotics has hampered infectious disease treatments all over the world (Zhang et al., 2017).

Sulfadiazine [4-animo-N-2-pyrimidinyl-benzenesulfonamide] (SDZ) is one of the most commonly used veterinary antibiotic in the world (Carvalho and Santos, 2016; Van Boeckel et al., 2017; Conde-Cid et al., 2018) to treat and inhibit bacterial infections (Tappe et al., 2013), but most administered SDZ is excreted in animal feces and urine. A study using 14C-SDZ showed that only 4% of SDZ applied rate remained in the pig body after 10 d whereas 44% was detected in the manure as the parent substance (^{14}C-SDZ), besides 4-hydroxysulfadiazine (4-OH-SDZ) and N-acetyl-sulfadiazine (Ac-SDZ) (Lamshöft et al., 2007).

SDZ is considered to have high mobility and is found in various environmental compartments, such as swine manures [300 to 198,000 µg kg^{-1}] (dry base) (Cycoń et al., 2017), waters [0.01 to 12 µg L^{-1}], and soils [4 to 760 µg kg^{-1}] (Adamek et al., 2016; Biošić et al., 2017). Overall, SAs present low sorption to soils (Kd = 0.4 to 14 L kg^{-1}) (Cycoń et al., 2019), but SDZ sorption can range from low (in most cases) to high (especially in highly weathered tropical

soils) (Förster et al., 2009; Leal et al., 2013). The unexpected high sorption behavior for SDZ is often associated to its ionization and specific interaction mainly with soil organic matter and metal (Fe and Al) hydrous oxides in the clay fraction (Förster et al., 2009; Reia et al., 2020). Most studies targeting antibiotics considers mainly detection and spread of resistance genes (Chen et al., 2019a, 2019b), but information on their environmental dissipation is limited. In soils, SDZ dissipation is fast primarily due to formation of non-extractable residues (NER), a mechanism that is often carried out by soil microbes but can also be chemical (Förster et al., 2009; Reia et al., 2020). However, little is known about the roles of the soil microbial community structure and diversity on SDZ degradation, which represent a critical gap for understanding its behavior in the environment. Soil microbial population is an important antibiotic as well as other xenobiotic degraders (Daam et al., 2019).

Current research has focused on understanding the role of microbial diversity on ecosystem functions (Griffin et al., 2017; Glick, 2018; Maron et al., 2018; Trivedi et al., 2019), providing valuable information on soil microbiome contribution (Van Elsas et al., 2012; Philippot et al., 2013). The role of soil microorganisms was elucidated for global ecosystem functions, such as those related to carbon and nitrogen cycles (Cardinale et al., 2006, 2011), which have high functional redundancy in the environment, but little is known for more specific ecosystem functions, such as for degradation of pesticides and other organic pollutants (Cravo-Laureau et al., 2011; Hernandez-Raquet et al., 2013). However, it is known that soil microbial diversity plays a crucial role on pesticide degradation in soils (Satish et al., 2017; Daam et al., 2019) and that microbial diversity depletion, as could be a result of certain agricultural soil management practices, can compromise pesticide degradation, resulting in its accumulation in soils (Souza et al., 2017). Soil microbial diversity can also act as an important biological barrier hampering spread of resistant genes to antibiotics (Chen et al., 2019b).

In this context, we hypothesized that: i) soil microbial structure as well as its diversity depletion impacts SDZ dissipation (mineralization and other routes of metabolism) and ii) SDZ dissipation is faster in soils with history of swine manure application due to its impact on soil microbial structure, which should present more adapted soil microbial population. Therefore, our main goal was to evaluate how soil bacterial structure and diversity depletion affect SDZ dissipation in parallel field soils without and with history of swine manure application. For this, the experimental microcosms were set up using the “dilution-to-extinction approach”, which involves soil microbial community extraction and dilutions, and further reinoculation in the same sterile soil (Wertz et al., 2006; Van Elsas et al., 2012). This artificial modification in the microbial community allows to mimic the impact of anthropogenic interferences or

disturbances on soil diversity and its functioning, such as nutrient cycling, disease suppression, and pollutant degradation (Mendes et al., 2011; Peter et al., 2011; Philippot et al., 2013; Souza et al., 2017).

Material and methods

SDZ and reagents

Radiolabeled (^{14}C, uniformly labeled on the phenyl ring, specific activity = 3.54 MBq mg^{-1}, and purity ≥ 99%) (Izotop, Budapest, Hungary) and analytical standard (purity ≥ 99%) (Merck, Darmstadt, Germany) SDZ molecules were used. The ^{14}C-molecule was used to trace SDZ dissipation whereas the analytical standard-SDZ was used to trace soil microbial community structure and diversity. All other reagents (acetone, methanol, and ethyl acetate) were HPLC analytical grade.

Soil samples

Soil samples [S1 (22°58'29.4"S and 47°37'52.0"W, without manure application) and S2 (22°58'30.0"S and 47°38'06.7"W, with 12-y history of swine manure application] were collected from the upper 0-10 cm layers, on a farm located in the Mombuca city, São Paulo state, Brazil. The farm has two sheds that hold ~4,000 animals, generating ~8 t d^{-1} of manure that are stored in a pond and further applied to sugarcane areas. Samplings were composed of 10 sub-samples that were properly homogenized, sieved (2 mm mesh), and stored at 4 ºC. Subsamples of 500 g of each soil were air-dried to determine their physical and chemical attributes (Table 1).

Table 1. Physical and chemical soil attributes as well as sorption (K_d and K_{oc}) and dissipation (DT_{50} and DT_{90}) of sulfadiazine in parallel soils without (S1) and with (S2) swine manure application.

Soil attributes	Soil S1 Typic Hapludult	Soil S2 Typic Hapludalf
Sand (g kg^{-1})	319	596
Silt (g kg^{-1})	531	304
Clay (g kg^{-1})	150	100
OM (g kg^{-1})	6	10
pH ($CaCl_2$)	4.2	5.2
P (mg dm^{-3})	10	103
K ($mmol_c$ dm^{-3})	1.8	2.4
Ca ($mmol_c$ dm^{-3})	16	18
Mg (mmolc dm^{-3})	5	17
BS ($mmol_c$ dm^{-3})	22.8	37.4
CEC ($mmol_c$ dm^{-3})	81	57
V (%)	28	65
m (%)	52	0
Sorption		
$K_{d\ app}$ (L kg^{-1})	0.92	1.52
$K_{oc\ app}$ (L kg^{-1})	264	262
DT_{50} (d)		
NS	0.9	0.9
D1	2.1	1.7
D3	2.8	1.8
D6	3.1	2.6
SS	2.8	2.6
DT_{90} (d)		
NS	2.0	21
D1	20	41
D3	21	> 42
D6	> 42	> 42
SS	> 42	39

OM = organic matter; BS = base summation; CEC = cation exchange capacity; V = base saturation; m = Al saturation. K_d^{app} = apparent sorption coefficient: $K_{oc}^{app} = K_d^{app}$ normalized to organic carbon; and DT_{50} and DT_{90} = time to reach dissipation of 50 and 90%. NS = natural soil; D1, D3, and D6 = 10^{-1}; 10^{-3}, and 10^{-6} dilutions; and SS = sterile soil.

SDZ sorption

Sorption test was carried out according to OECD method 106 (OECD, 2000), with small modifications (Reia et al., 2020). ^{14}C-SDZ stock solution (160 Bq mL^{-1}) was prepared in 0.005 mol L^{-1} of $CaCl_2$. The assay was carried out using 1:2 soil to solution ratio (5 g:10 mL), in duplicates. Soil suspensions (slurries) were shaken on a horizontal shaker (190 rpm for 48 h) and then centrifuged for 15 min. Then, 1 mL-aliquots of the supernatant was taken to determine soil solution concentration at equilibrium (C_e). The sorbed concentration (S) was calculated by

the difference between initial concentration (C_i) and C_e, considering the soil to solution ratio. The apparent sorption coefficient was calculated as $K_{dapp} = S / C_e$, and then normalized to the organic carbon content (OC) of the soils [Kocapp = K_{dapp} x 100 / OC (%)] (Table 1).

Soil bacterial community assay

Subsamples (25 g, dry mass) of the natural (NS), sterilized (autoclaved at 120°C, 1 atm, for 3 cycles of 60 min) (SS), and diluted (D1, D3, and D6) soils were placed into biometer flasks, in triplicates. "Dilution-to-extinction approach" was used to simulate soil microbial diversity depletion by inoculating sterilized soils with diluted soil suspensions (D1 = 10^{-1}, D3 = 10^{-3}, and D6 = 10^{-6}) (Wertz et al., 2006; Van Elsas et al., 2012). Then, soil moisture was adjusted to 60% of maximum water holding capacity and the flasks were let incubate for 15 d to allow perturbed microbial community to structure (Van Elsas et al., 2012; Souza et al., 2017). Afterwards, analytical grade SDZ solution was applied at ~100 µg kg^{-1} and the flasks were incubated in a semi-dark room, at 25 ± 1°C for 42 d. At 0, 2, and 42 d after SDZ application, soil subsamples were removed to evaluate diversity and structure of the bacterial community by sequencing the 16S rRNA gene.

Soil-DNA was extracted using a commercial kit DNeasy Powerlyzer Power Soil (Qiagen, Hilden, Germany). Its quality and integrity were verified on a 1 % agarose gel, quantified with Qubit Fluorometer, and stored at -20ºC. Further, the DNA was prepared in two stages: i) the 16S rRNA gene was amplified by the 16S-515F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3') and 16S-806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3') primers and modified with pre-adapters. PCR reaction had a final volume of 25 µL, containing 3.0 µL of PCR Buffer, 2.5 µL of $MgCl_2$ (50 mM), 2.0 µL of dNTPs (2.5 mM), 0.1 µL of each primer, 0.3 µL Taq DNA polymerase (0.05 U μL^{-1}), 16 µL of milli-Q water, and 1.0 µL of the template DNA (approx. 50 ng). Amplification was performed at 95 ºC for 3 min, followed by 35 cycles at 94 ºC for 45s, 50 ºC for 60 s, 72 ºC for 90 s and final extension of 10 min at 72 ºC (Caporaso et al., 2011). The amplicons were confirmed by electrophoresis on a 1% agarose gel, resulting in a ~430 bp fragment and purified using AMPure XP beads (Beckman Coulter, Brea, USA); ii) the purified-DNA was subjected to a new PCR reaction to bind barcodes from the Nextera XT kit (Illumina, San Diego, USA).

PCR reaction consisted: 3.0 µL of PCR buffer, 2.5 µL of $MgCl_2$ (50 mM), 2.0 µL of dNTPs (2.5 mM), 5 µL adapter (barcode), 0.3 µL of Taq DNA polymerase (0.05 U μL^{-1}), 17.2 µL milli-Q water, and 15 µL of the previous reaction product (final volume of 50 µL).

Amplification conditions were: 95 °C for 3 min, 5 cycles of 95 °C for 45 s, 57 °C for 1 min 45s, 72 °C for 1 min, followed by a final extension of 72 °C for 4 min. The samples were purified, and the target fragment confirmed by agarose gel electrophoresis (1%). Amplicon library was quantified on the Qubit Fluorometer (Thermo Fisher Scientific, Waltham, USA) to standardize equimolar amounts of DNA. The V4-region of the 16S rRNA gene was amplified and sequenced on Illumina MiSeq platform, using MiSeq Reagent Kit V3 600-cycles (Illumina, San Diego, USA) according manufacturer's specifications.

SDZ dissipation assay

This study was performed exactly on the same way as the previous experiment, except that radiolabeled ^{14}C-SDZ (even at the same application rate, corresponding to ~7.9 kBq flask^{-1}) and duplicates were used to evaluate dissipation according to OECD method 307 (OECD, 2002). At 2, 7, 14, 21, 28, 35, and 42 d, ^{14}C-CO_2 evolved (mineralization) was trapped into 10 mL of 0.2 mol L^{-1} NaOH and an aliquot of 1 mL was taken and mixed with 10 mL scintillating solution (Ultima Gold, PerkinElmer, Massachusetts, USA) to quantify radioactivity by liquid scintillation (LS) (TRI-CARB 2910 Scintillator, PerkinElmer, Massachusetts, USA). At 0, 2, 7, 21, and 42 d, the soil was transferred to Teflon flasks (250-mL) and extracted twice with 50 mL methanol; the slurries were horizontally shaken at 150 rpm for 9 h (1st extraction), followed by 12 h (2nd extraction), centrifuged at 4200 rpm for 15 min (10 °C), and 1 mL aliquot of the supernatant taken to quantify radioactivity by LS.

The extracts were collected (Scott flasks), rotavaporated, resuspended in 3-mL methanol, and an aliquot of 100 µL was applied to thin-layer chromatography (TLC) plate, which was developed in ethyl acetate to identify the presence of the parent compound (SDZ) and its metabolites, thus determining DT_{50} and DT_{90}. Afterwards, the soil slurry was oven-dried at 40 ºC, macerated in a porcelain mill, and an aliquot of 0.2 g was taken and combusted in a Biological Oxidizer (Biological Oxidizer OX-500, R.J. Harvey Instrument Corporation, New York, USA) to determine NER by LS.

Data analysis

Sequencing data analysis was implemented using the packages DADA2 (Callahan et al., 2016), PHYLOSEQ (McMurdie and Holmes, 2013) and GGPLOT2 (Wickham, 2016). Briefly, the multiplexed reads were assigned to sample metadata. Then, FASTQ files were filtered to remove primers, adapters, and barcodes. $Q < 30$ was used to remove low-quality sequences, resulting in sequence sizes of 2 x 260 bp.

Paired-ends sequences were reassembled, using DADA2 learnErros algorithm to correct errors in the read assembles for each sample. After this stage, the sequences were de-replicated (denoising) to obtain unique sequences and their abundance. After adding paired-end reads, the chimeras were removed and the sequences were assigned to each Amplicon Sequencing Variants (ASVs).

ASVs were used as input for sequence alignments, using SILVA 132 database as reference in the DADA2 assign taxonomy function (Callahan et al., 2016). The generated taxonomy was imported to the PHYLOSEQ package (McMurdie and Holmes, 2013), which has tools in its routine for analyzing phylogeny data, available in the VEGAN, ADE4, APE, and PICANTE packages. In addition, GGPLOT2 package was used for graphic processing. ASVs of the same species were agglomerated and evaluated up to the genera level.

Data were also subjected to analysis of non-metric multidimensional scaling analysis (NMDS), based on the Bray-Curtis distance metric (Buttigieg and Ramette, 2014). Bacterial community similarity analysis (ANOSIM) was implemented using PAST, version 4.1. Alpha diversity (Shannon and Simpson indexes) was estimated and contrasted using Tukey test ($p < 0.05$). Spearman's correlation was used to evaluate the influence of soil bacterial community composition on either SDZ mineralization or formation of NER ($P_{value} < 0.05$), the main routes of SDZ dissipation.

Results

Soil physical and chemical attributes

Swine manure application promoted significant changes in the physical and chemical attributes of the soils. The manured soil (S2) showed higher OM, OC, P, K^+, Ca_2^+, and Mg_2^+ and sand contents as well as higher pH and base saturation (V%) than S1 (Table 1).

Soil bacterial community diversity

The natural soil with history of swine manure application (NS-S2) showed initially higher bacterial diversity than that without history (NS-S1) (H = 4.43 versus 3.74 and 1-D = 0.98 versus 0.94, respectively) (Figure 1). The diversity indexes significantly decreased after artificial modifications of the soils, either by bacterial community dilution (mainly for D6) or soil sterilization (H decreased from 3.74 to 1.35 for S1 and from 4.43 to 1.92 for S2) (Figure 1). However, bacterial community diversity recovered along with incubation time. At 42 d after SDZ application, H ranged from 3.75 to 2.76 for S1 and from 4.07 to 2.99 for S2; and bacterial

diversities for D1 and D3 were statistically equaled to that of natural soils (NS-S1 and NS-S2) (Figure 1).

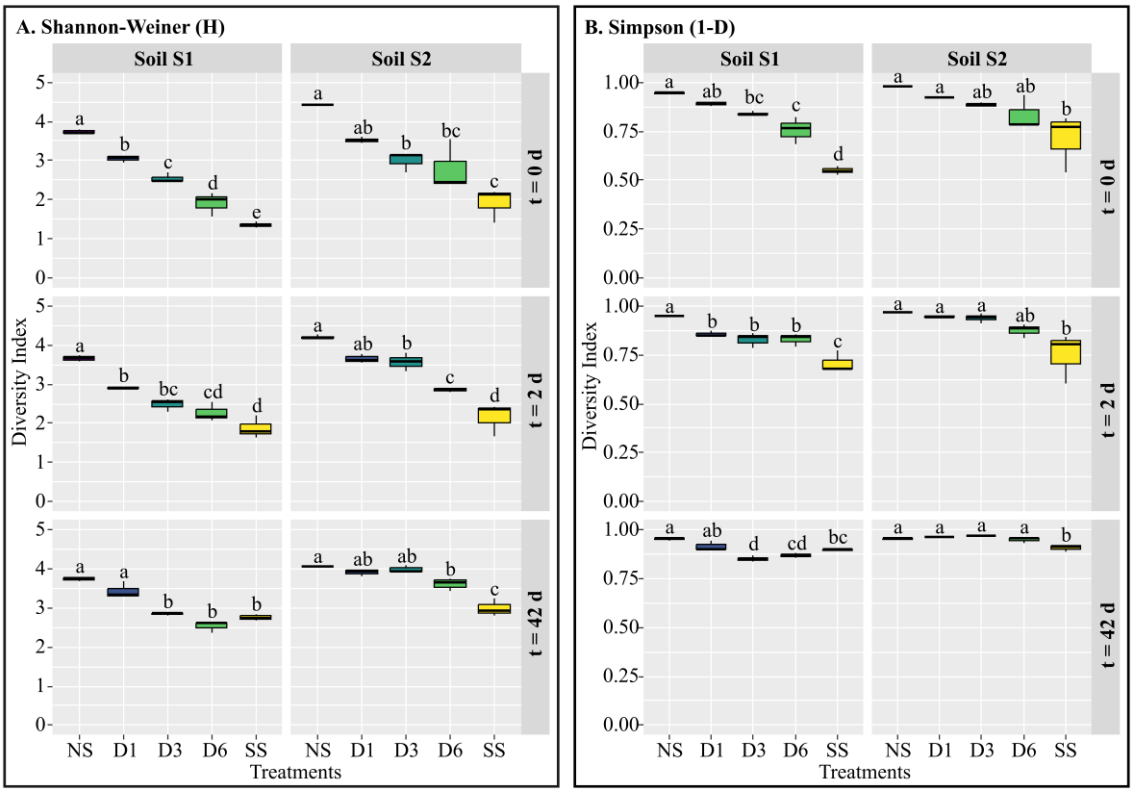

Figure 1. Boxplots of Shannon-Weiner (H) (A) and Simpson (1-D) (B) diversity indexes in parallel soils without (S1) and with (S2) swine manure history at 0, 2, and 42 d after sulfadiazine application (n = 3). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil. Different letters indicate statistical difference by Tukey ($p < 0.05$).

Structure and composition of soil bacterial community

DNA sequences were generated covering 45,669 reads per sample (merged and non-chimera). The amount of 388 ASVs were annotated (Q > 32), which allowed identifying 17 phyla, 31 classes, 87 orders, 163 families, and 288 genera of bacteria (Figs. S1 and S2, Table S1).

The NMDS in association with one-way ANOSIM showed that soil bacterial community could be grouped in three distinct clusters by soil, as follows: C1 and C4 = NS; C2 and C5 = D1 and D3; C3 and C6 = D6 and SS for S1 and S2, respectively (Figure 2, Table S2). Soil bacterial community structure of S1 differed from S2 (Figure 2B). In addition, the bacterial community structure of the natural soils (C1 and C4) also differed from their respective diluted

or sterilized soils (C2, C3, C5, and C6) (Table S2). However, C2 and C3 for S1 (R_{ANOSIN} = 0.59) and C5 and C6 for S2 (R_{ANOSIN} = 0.25) overlaps, mainly due to the diversity recovery of the most perturbed soils (D6 and SS) with incubation time (at 42 d); diversity recovery was more prominent in the manured soil (S2).

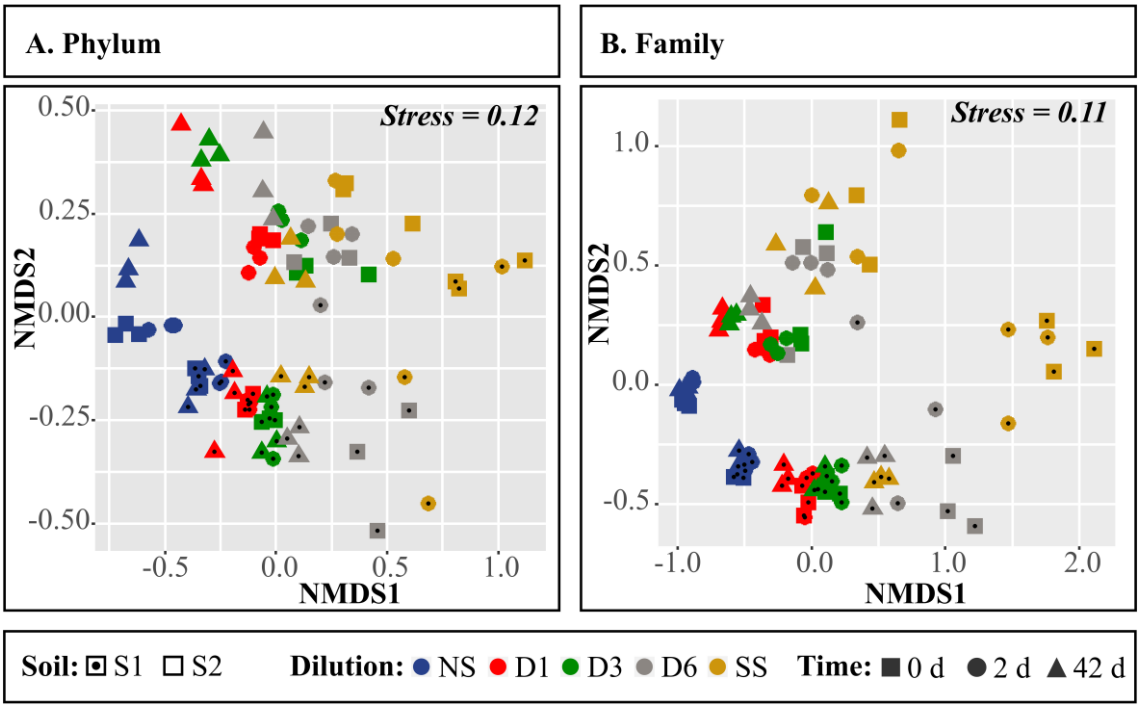

Figure 2. Non-metric multidimensional scaling of the bacterial community at phylum (A) and family (B) levels in parallel soils without (S1) and with (S2) swine manure history at 0, 2, and 42 d after sulfadiazine application (n = 3). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

The most abundant phyla for both soils were Proteobacteria, Chloroflexi, Actinobacteria, Bacteroidetes, Planctomycetes, Acidobacteria, and Firmicutes (totalizing > 90% of the sequences). Deinococcus-Thermus was detected only in S1, whereas Dependentiae and Patescibacteria only in S2 (Figure 3A). Acidobacteria were suppressed from diluted and sterilized soils, presenting much lower diversity, but Proteobacteria abundance increased, mainly for S2. In addition, Actinobacteria were suppressed from sterilized soils, mainly for S1 (Figure 3 A).

For NS-S1, the most abundant families were Sphingomonadaceae, Ktedonobacteraceae, Micromonosporaceae, Acidobacteriaceae_(Subgroup_1), Caldiliceaceae, and Burkholderiaceae. In this soil, Rickettsiaceae abundance increased in the artificially perturbed treatments, mainly in the SS, whereas Mycobacteriaceae abundance increased for D6 and

Burkholderiaceae abundance increased for D1 and D3 (Figure 3B). For NS-S2, the most abundant families initially were Acidobacteriaceae_(Subgroup_1), Sphingomonadaceae, Sphingobacteriaceae, Alicyclobacillaceae, Ktedonobacteraceae, and Micromonosporaceae; and Gaiellacea abundance increased with incubation time ($t \geq 2$ d). However, Gaiellacea was suppressed whereas Rickettsiaceae abundance increased in the artificially perturbed treatments (Figure 3B). For S1 and S2, Acidobacteriaceae_(Subgroup_1) was also suppressed in the perturbed treatments.

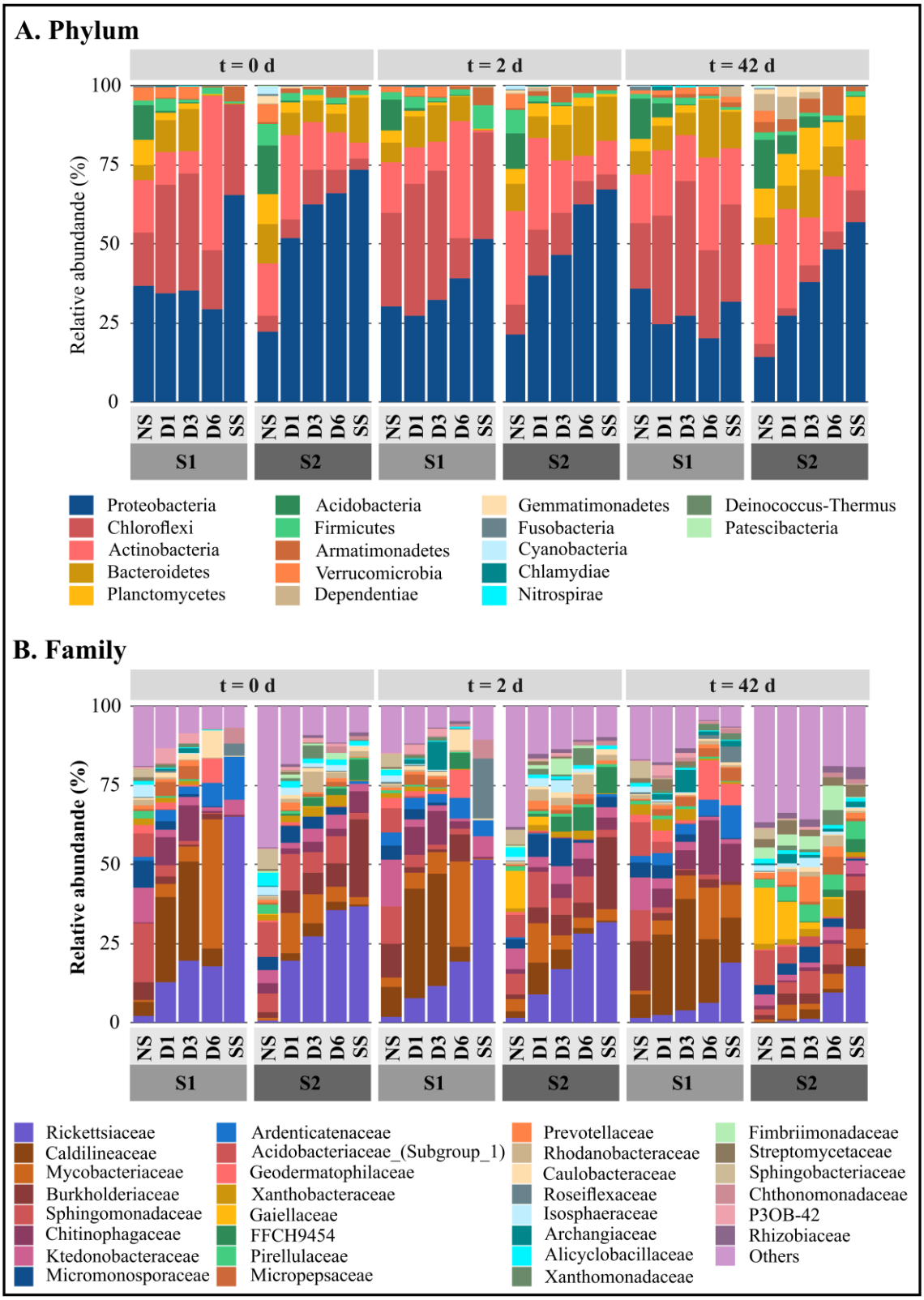

Figure 3. Relative abundance of the bacterial communities at phylum (A) and family (B) levels in parallel soils without (S1) and with (S2) swine manure history at 0, 2, and 42 d after sulfadiazine application (n = 3). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

SDZ dissipation

SDZ mineralization was low (≤ 7.5% of the applied amount at 42 d) and manure application did not affect its rate (Figure 4). However, mineralization was ~2 to 3 times higher in the NSs (6.8-7.5%) than in the perturbed soils (D1, D3, D6 and SS), corresponding to 2.5-3.2% and 2.8-3.3% of the applied radioactivity (AR) in S1 and S2, respectively (Figure 4).

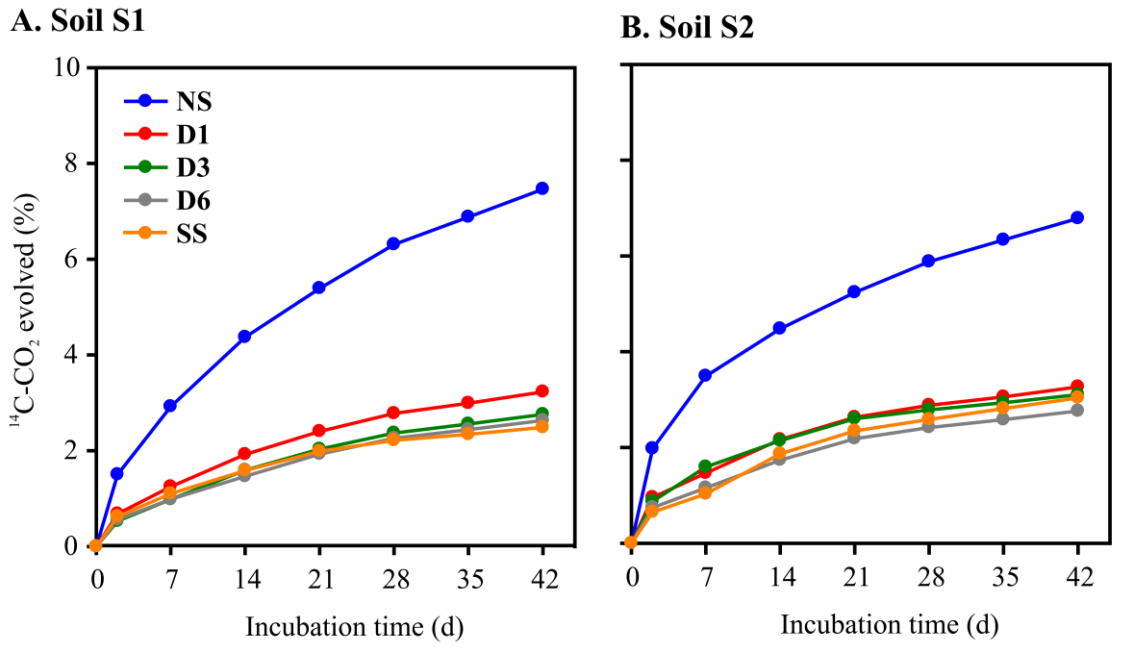

Figure 4. Mineralization rate of ^{14}C-sulfadiazine in parallel soils without (S1) (A) and with (S2) (B) swine manure history up to 42 d of sulfadiazine application (n = 2). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

For both soils, the initial amounts of ^{14}C-extracted residues corresponded to 82-85% of the AR, which dropped considerably after 2 d of incubation, especially in the natural soils (corresponding to 12 and 28% of the AR in the NS-S1 and NS-S2, respectively) (Figure 5). At the end of the experiment (42 d), the extracted amounts corresponded to 3-18 and 12-40% of the AR in the S1 and S2, respectively. The formation of ^{14}C-NERs was faster in the NSs, being most abrupt in the NS-S1 (corresponding to 87 and 72% of the AR in the NS-S1 and NS-S2, respectively) (Figure 6). At 42 d, this fraction corresponded to 84-90 and 75-85% of the AR in S1 and S2, respectively, except for D3-S2 (57%).

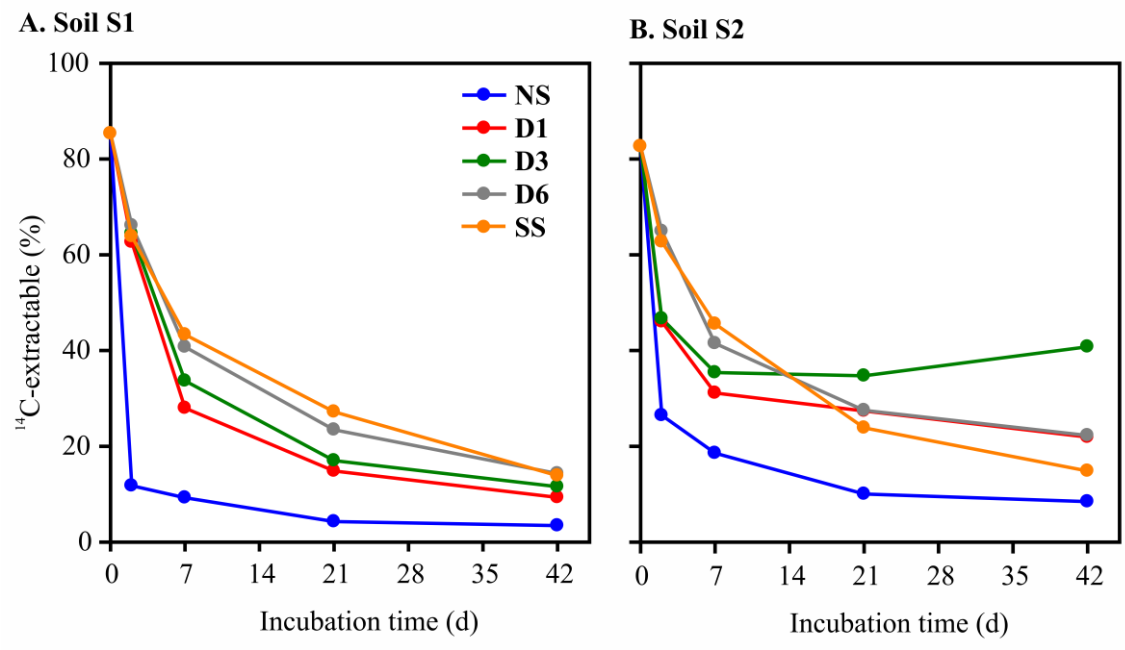

Figure 5. Extractable ^{14}C-sulfadiazine residues in parallel soils without (S1) (A) and with (S2) (B) swine manure history up to 42 d after sulfadiazine application (n = 2). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

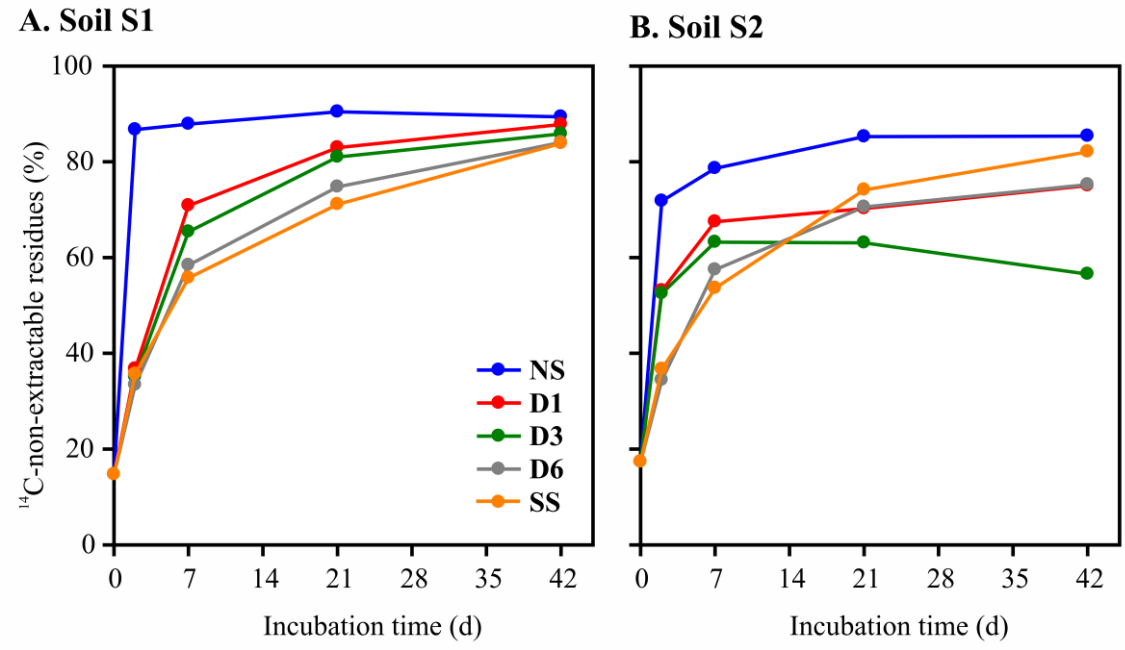

Figure 6. ^{14}C-non-extractable residues (NER) in parallel soils without (S1) (A) and with (S2) (B) swine manure history up to 42 d after sulfadiazine application (n = 2). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

SDZ showed fast dissipation in all treatments ($DT_{50} \leq 3.1$ d), but it was more accentuated in the NSs (DT_{50} = 0.9 d in both S1 and S2) (Table 1). Nevertheless, DT_{90} was lower in the NS without manure application history (DT_{90} = 2.0 and 21 d for NS-S1 and NS-S2, respectively). Three metabolites (M) resulted from SDZ degradation: M1 (Rf = 0.2), M2 (Rf = 0.5), and M3 (Rf = 0.6) (Figure 7). M1 was quickly detected from 2 up to 7 d of incubation, especially in the perturbed treatments having lower diversity (M1 corresponded to 10% and 20% of the AR to S1 and S2, respectively). M2 and M3 were detected previously [after 7 d, at low concentration (< 5% of the AR)] and were completely dissipated up to 21 d in S1. In S2, they were detected later (at 42 d) and at much higher amounts. M3 was most abundant in D3-S2, corresponding to 16% of the AR, in which the NER atypically decreased at latter incubation periods (Figure 5).

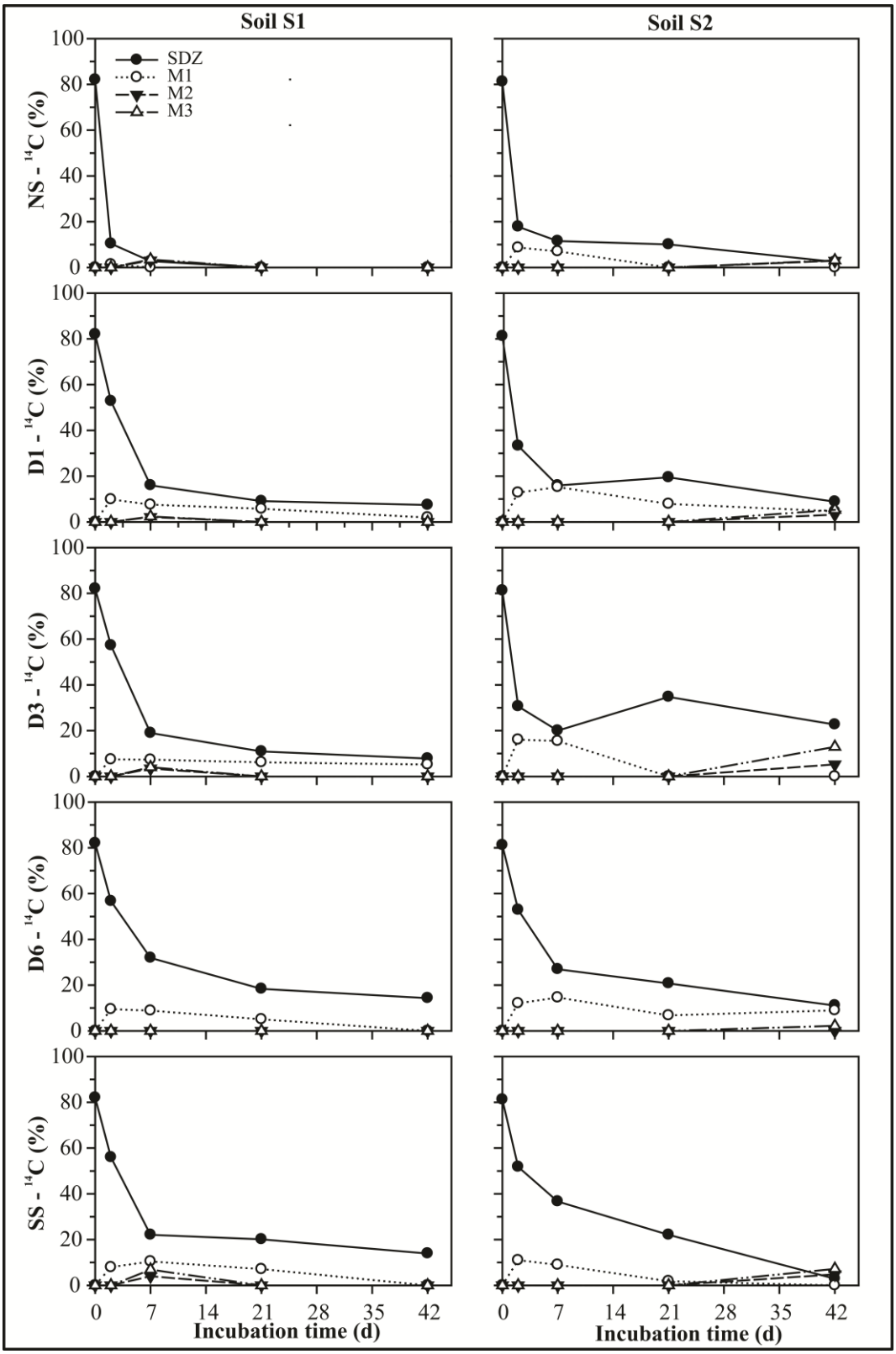

Figure 7. ^{14}C-sulfadiazine and its metabolites (M1, M2, and M3) in parallel soils without (S1) (A) and with (S2) (B) swine manure history up to 42 d after sulfadiazine application (n = 2). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

Soil bacterial community related to SDZ dissipation

Despite its lower diversity, S1 has a much great number of bacterial families correlated with the main routes of SDZ dissipation, such as CO_2 evolution and NER formation [$R > 0.50$ and $p < 0.01$] with the main pathways (Figure 8). The families showing the most effective roles were Isosphaeraceae ($R_{CO2} = 0.89$ and $R_{NER} = 0.97$), Ktedonobacteraceae ($R_{CO2} = 0.88$ and $R_{NER} = 0.94$), Acidobacteriaceae_(Subgroup_1) ($R_{CO2} = 0,88$ and $R_{NER} = 0.93$), Micromonosporaceae ($R_{CO2} = 0.87$ and $R_{NER} = 0.83$), Sphingobacteriaceae ($R_{CO2} = 0.78$ and $R_{NER} = 0.76$), Burkholderiaceae ($R_{CO2} = 0.69$ and $R_{NER} = 0.68$), Sphingomonadaceae ($R_{CO2} = 0.66$ and $R_{NER} = 0.71$), and Streptomycetaceae ($R_{CO2} = 0.64$ and $R_{NER} = 0.68$). Overall, their abundances tended to increase with the incubation time (up to 42 d) (Figure 3B). Conversely, Rickettsiaceae, Mycobacteriaceae, Chitinophagaceae, Ardenticatenaceae, Geodermatophilaceae, Pirellulaceae, Alicyclobacillaceae, Xanthomonadaceae and Chthonomonadaceae) had their abundances decreased with incubation time; therefore, showed negative correlations with CO_2 and NER (Figure 8). For S2, a much smaller number of bacterial families were correlated with SDZ dissipation, mainly with mineralization. These families comprehended Acidobacteriaceae_(Subgroup_1) ($R_{CO2} = 0.88$), Sphingobacteriaceae ($R_{CO2} = 0.85$), Geodermatophilaceae ($R_{CO2} = 0.68$), Gaiellaceae ($R_{CO2} = 0.68$), Roseiflexaceae ($R_{CO2} = 0.63$), Ardenticatenaceae ($R_{CO2} = 0.63$), and P3OB-42 ($R_{CO2} = 0.45$) (Figure 8).

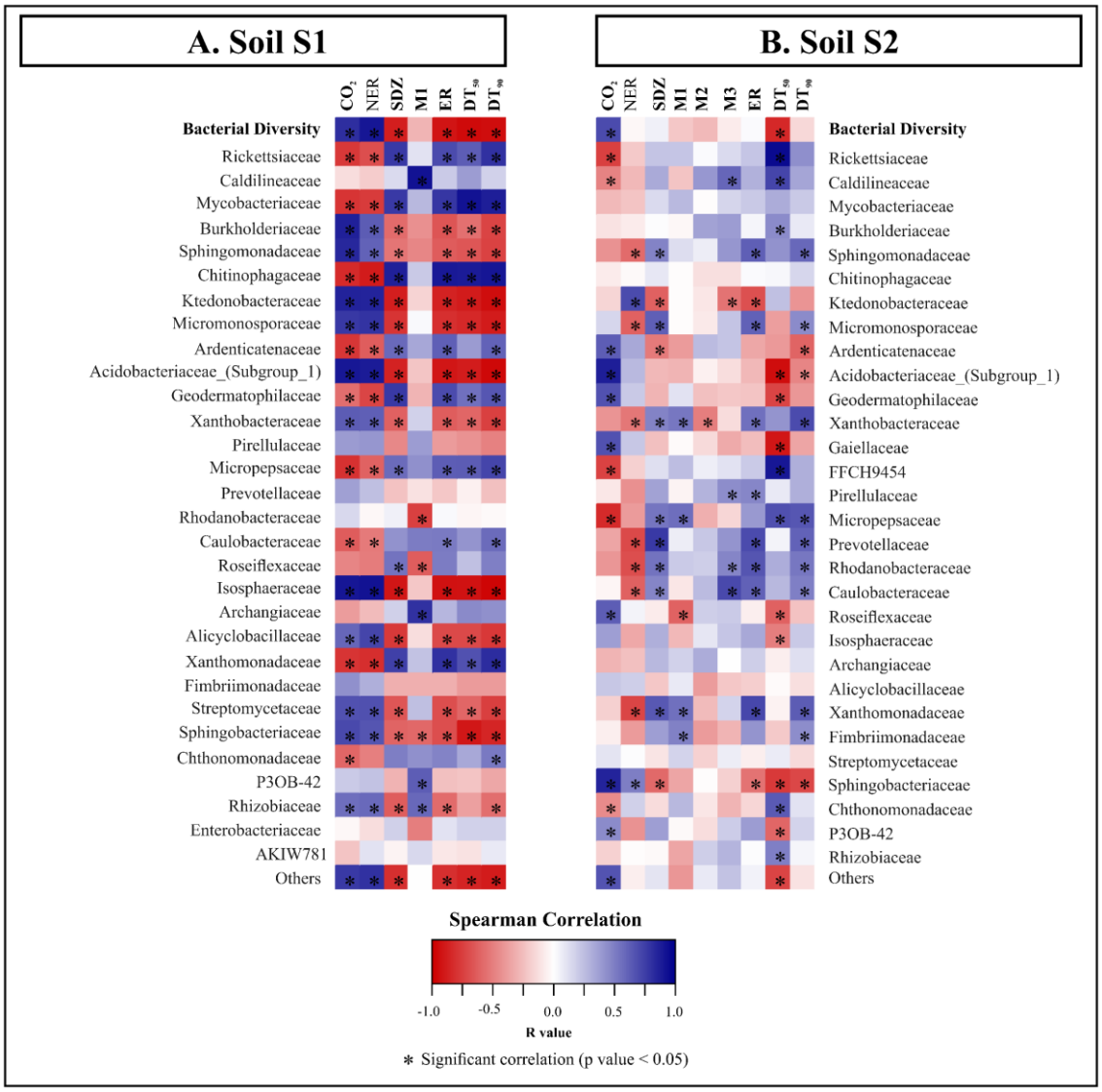

Figure 8. Correlation between bacterial families and pathways of ^{14}C-sulfadiazine dissipation in parallel soils without (S1) (A) and with (S2) (B) swine manure history up to 42 d after sulfadiazine application. CO_2 = mineralization; NER = non-extractable residues; SDZ = sulfadiazine dissipation; M1, M2, M3 = metabolites, DT_{50} and DT_{90} = time to reach dissipation of 50 and 90%.

Discussion

Swine manure amendments for 12 consecutive years enhanced pH (from 4.2 to 5.2) as well as OM (from 6 to 10 g kg^{-1}) and macronutrient contents of the soil. Animal manure can have potentially toxic elements, such as heavy metals or antibiotic residues, but they also have high nutrient contents that can be recycled and used by soil microorganisms and plants (Hammesfahr et al., 2011; Li et al., 2020). Hammesfahr et al. (2011) showed that swine manure application not only enhanced nutrient contents but also modified the fatty acid profile and

increased enzymatic activity and soil basal respiration, regardless of SDZ presence in the manure.

Bacterial communities in the natural soil (NS-S1 and NS-S2) showed higher diversity indexes than the artificially perturbed soil microbial communities, either by dilution or sterilization (Figure1), as previously observed (Van Elsas et al., 2012; Trivedi et al., 2019; Yu et al., 2019). Soil microbial communities are largely responsive to environmental disturbances (Fierer and Jackson, 2006; Trivedi et al., 2019) and act as important indicator of soil functions (Delgado-Baquerizo et al., 2016; Bünemann et al., 2018), affecting mainly those having lower degree of environmental redundancy, such as degradation of recalcitrant organic pollutants (Cravo-Laureau et al., 2011). However, the bacterial community of perturbed soils showed gradual diversity recovery along with the experiment (42 d), mainly for the least diluted groups (D1 and D3) (Figure 1), showing that although disruptions on soil microbial community diversity were drastic, they were only transient.

Swine manuring also affected the structure of soil bacterial community (Figure 2). Organic residue amendments often affect composition and structure of soil microbes (Ozlu et al., 2019; Wang et al., 2020) due to the presences of distinct sources of C and other nutrients (Liu et al., 2020a), autochthonous microorganisms (Rieke et al., 2018), and hazardous trace elements and other xenobiotics, such as pesticides and pharmaceutical drugs (Cycoń et al., 2019). Diversity of the manured soil (NS-S2) was slightly higher. Liu et al. (2020b) observed that bacterial diversity decreased in swine manured soil (+ nitrogen fertilizer), which was attributed to the high manure application rates. In addition, the soil bacterial community could be segregated into clusters according to their disturbance levels: NS (low disturbance), D1-D3 (intermediate disturbance) and D6-SS (high disturbance) (Figure 1 and Table S2). The "dilution-to-extinction approach" is not capable to predict structure and composition of the inoculated community (Yan et al., 2015), but this artificial modification seems to mimic properly anthropogenic perturbations on the ecosystem functioning (Peter et al., 2011; Van Elsas et al., 2012).

The most abundant phyla on both natural soils were Proteobacteria (~ 40%) > Chloroflexi (~ 18%) > Actinobacteria (~ 17%) > Bacteroidetes (~ 9%) > Planctomycetes (~ 4%) > Acidobacteria (~ 3%) > Firmicutes (~ 2%), which echoes literature data (Rieke et al., 2018; Li et al., 2020). Agricultural soil bacterial community depends on spatial and temporal variations as well as on management practices (Trivedi et al., 2016) and often shows different combinations of Proteobacteria, Verrucomicrobia, Acidobacteria, Chloroflexi, and Actinobacteria (Delgado-Baquerizo et al., 2018; Pershina et al., 2018; Jiao et al., 2019; Trivedi

et al., 2019; Tan et al., 2020), representing 60-90% of the total abundance (Delgado-Baquerizo et al., 2018). Firmicutes and Bacteroidetes prevail in swine manure, representing 70% of the total abundance (Lu et al., 2014).

Proteobacteria and Chloroflexi are adapted to oligotrophic environments (Kersters et al., 2006; Whitman et al., 2008), such as in the NS-S1. Proteobacteria hosts a wide diversity of bacteria that act on C and N cycles (Kersters et al., 2006), but they can also bioremediate pesticide-contaminated sites, especially by Gammaproteobacteria (Pseudomonas sp., Aerobacter sp., Acinetobacter sp., Moraxella sp., and Plesiomonas sp.), Betaproteobacteria (Burkholderia sp. and Neisseria sp.), Alphaproteobacteria (Sphingomonas sp.) (Doolotkeldieva et al., 2018; Castro-Gutiérrez et al., 2020). Conversely, Actinobacteria and Bacteroidetes were more abundant in NS-S2. They are copiotrophic bacteria, effective in degrading organic matter (Naether et al., 2012). Actinobacteria is also linked to the production of antibiotic and other xenobiotic-degrading extracellular enzymes (Zhang et al., 2019), whereas Acidobacteria are often associated with C-cycle (Naether et al., 2012). SDZ application seemed only to have a secondary impact on the microbial community structure, but it is a bacteriostatic molecule (Qin et al., 2020) capable to impact mainly bacteria from non-adapted environments (Cycoń et al., 2019).

Dilution and sterilization resulted in bacterial diversity depletion and increased relative abundances of Proteobacteria, Chloroflexi, Actinobacteria, Bacteroidetes, and Planctomycetes, but reduced that of Acidobacteria (Figure 3A). Deinococcus-Thermus occurred only in S1, while Dependentiae and Patescibacteria occurred only in S2 (Figure 3A), but lower abundance. Diversity depletion by dilution favors the most abundant groups and eliminates the rare groups (Wertz et al., 2006, 2007; Souza et al., 2017), which is corroborated by the higher abundance of Chloroflexi, Actinobacteria, and Bacteroidetes in S1 and the higher abundance of Proteobacteria in S2 (Figure 3A). The conversion of native forests to monoculture systems promoted similar changes on soil microbial community also favoring the most abundant groups (Peter et al., 2011; Landis, 2016). These changes may have a strong impact on the ecosystem services (Cardinale et al., 2011; Philippot et al., 2013), affecting biodegradation of microplastics, petroleum derivatives, pesticides, and antibiotics (Jacobsen and Hjelmsø, 2014; Qin et al., 2020) that require specialized microbial groups (Cravo-Laureau et al., 2011; Feld et al., 2015).

SDZ mineralization was low (≤ 7.5%) and little affected by swine manure application (Figure 4) but was near twice the values found in the literature (Kreuzig and Höltge, 2005; Schmidt et al., 2008; Förster et al., 2009; Reia et al., 2020). Its mineralization was ~2-3 times

lower in the perturbed treatments (D1, D3, D6 and SS) (Figure 4). Chlorothalonil mineralization rate was ~20 times lower in the worst scenario of the diversity depletion (D6) than in the NS (= 12% of AR) (Souza et al., 2017). Hernandez-Raquet et al. (2013) reported that phenanthrene mineralization was greater at lower (10^{-1} and 10^{-3}) (95-100%) than at intermediate (10-5) (60%) to low (10^{-8}) (0%) diversity dilution levels. Agricultural practices usually simplify (deplete) soil microbial diversity (Landis, 2016; Andreote and Silva, 2017), which can compromise vital ecosystem functions as already mentioned. Long term swine manure application did not seem to affect SDZ mineralization, but soil microbial diversity depletion seemed to affect the abundance of SDZ degraders impacting its mineralization. It could favor its accumulation (Vryzas, 2018) and subsequent dissemination of resistance genes in the environment (Chen et al., 2019b).

Despite its slow mineralization, SDZ dissipation was extremely fast in all treatments ($DT_{50} \leq 3.1$ d) primarily due to the fast formation of NERs, especially in the natural soils (Kreuzig and Höltge, 2005). Similar results have been reported for other tropical soils ($DT_{50} <$ 2.6 and $DT_{90} = 6.3$ d) (Reia et al., 2020), which were different from those for temperate soils [$DT_{50} = 24$ d (Förster et al., 2009) and $DT_{50} > 216$ d (Engelhardt et al., 2015)]. Unexpectedly, NS-S2 showed slower SDZ dissipation rate ($DT_{90} = 20$ versus 0.9 d) (Table 1) despite its higher OC content, sorption potential, soil microbial diversity, and theoretically more adapted soil microbes. In soils, SDZ and other SAs biodegradation is carried out by co-metabolism (Sukul et al., 2008; Ricken et al., 2017; Reis et al., 2018), although *Microbacterium* (Topp et al., 2013) and *Arthrobacter* (Deng et al., 2016) are capable to utilize SAs as sole carbon and energy sources in culture medium. Therefore, SDZ biodegradation should be enhanced and not hindered in the manured soil, having higher content of readily available organic material. Conversely, manure application can enhance soil pH and dissolved organic C contents, which hamper sorption due to enhanced molecule deprotonation and co-solvency, respectively, and subsequently decrease NER formation (Engelhardt et al., 2015). In parallel, NS-S1 has lower pH (4.2 versus 5.2) that should favor SDZ hydrolysis to sulfanilic acid (Sukul and Spiteller, 2006). Hu et al. (2020) argued that accelerated degradation at lower pH can be attributed to SDZ enhanced electrostatic attraction to microbial cell membrane as well as to its enhanced hydrolysis. Despite its low sorption potential ($Kd << 5$ L kg^{-1}), SDZ bioavailability on both soils was limited by the fast formation of NER. Bioavailability is essential to soil microbes foster substrate breakdown (Jacobsen and Hjelmsø, 2014; Souza et al., 2017), but certain bioavailable molecules, such as antibiotics, may have deleterious effects on soil microbial community inhibiting its degrading ability (Cycoń et al., 2019; Qin et al., 2020).

SDZ dissipation was faster in the natural than in the perturbed soils, suggesting that SDZ degradation was at least partially driven by soil microbes (Yang et al., 2009; Mulla et al., 2016). For acidic tropical soils, our recent work proposed that SDZ degradation should initially involve a chemical step due to the "instantaneous" formation of an unknown metabolite, having the same TLC-retardation factor as M1 (Reia et al., 2020). Here, M1 was detected afterwards, i.e. 2 d after SDZ application, likely because the soils were sandier and had much lower clay contents (≤ 15%), which suggest much lower contents of Fe, Mn, and Al hydrous oxides that are believed to act as "catalysts" of SDZ chemical reaction (breakdown). SDZ degradation pathways involve mainly hydroxylation and acetylation reactions (Hu et al., 2020), forming mainly mono-hydroxylated sulfadiazine (likely 4-OH-SDZ) (Deng et al., 2018), but also N-acetyl-SDZ (Ac-SDZ) and N-acetyl-4-hydroxy-SDZ (N-Ac-4-OH-SDZ) (Chen and Xie, 2018; Hu et al., 2020). Often, the 4-OH-SDZ is the most quickly formed and most frequently detected metabolite (Lamshöft et al., 2007; Sukul et al., 2008; Förster et al., 2009; Hammesfahr et al., 2011). It has greater sorption potential (Sukul et al., 2008; Lamshöft et al., 2010) and is more deleterious to soil microbiota (Hammesfahr et al., 2011) than SDZ itself. The acetylated forms are often reverted to SDZ, via deacetylation (Lamshöft et al., 2007; Schmidt et al., 2008).

The families correlating better with the two major dissipation pathways for SDZ were: for S1) Isosphaeraceae ~ Ktedonobacteraceae ~ Acidobacteriaceae_(Subgroup_1) ~ Micromonosporaceae > Sphingobacteriaceae > Burkholderiaceae ~ Sphingomonadaceae ~ Streptomycetaceae; and for S2) Acidobacteriaceae_(Subgroup_1) ~ Sphingobacteriaceae > Geodermatophilaceae ~ Gaiellaceae ~ Roseiflexaceae ~ Ardenticatenaceae > P3OB-42, which have several bacterial strains described as SA degraders (Islas-Espinoza et al., 2012; Deng et al., 2016, 2018; Mulla et al., 2016). Thus, different bacterial families were correlated with SDZ dissipation in each soil, except for Acidobacteriaceae_(Subgroup_1) and Sphingobacteriaceae. They belong mainly to Proteobacteria, Actinobacteria, and Acidobacteria phyla that are often referred as SA degraders (Mulla et al., 2016; Reis et al., 2018; Hu et al., 2020). Moreover, S1 showed a greater number of families positively correlated with either mineralization (12 versus 8 families) or NER formation (12 versus 2 families) paths, which was translated into slightly higher SDZ dissipation rate. This result is unique and contrasts our hypothesis that long-term manured soils would present adaptive advantages and, consequently, have higher SDZ biodegradation rates.

Two biodegradation pathways are known for SAs (Chen and Xie, 2018), involving Sad ABC genes. Sad A and Sad B encode two flavin-dependent monooxygenases that promote deacetylation via ipso-hydroxylation of the aromatic SA ring, while Sad C gene encodes a flavin

reductase that promotes molecule breakdown (Reis et al., 2018; Chen et al., 2019a). Sad ABC genes were mostly found in bacteria grown in culture medium containing SAs (Ricken et al., 2013, 2017), belonging mainly to the Proteobacteria and Actinobacteria phyla (Chen et al., 2019a). SA-degrading genes absence in complex environments, such as soils, suggests this ecosystem function is very sensitive to the composition and interactions within microbial community of the soil (Reis et al., 2018). SDZ mineralization and NER formation rates were impacted even at the lowest disturbance degree (D1). Acidobacteria depletion may have contributed to lower SDZ dissipation rate in the perturbed soils since its members are efficient degraders of recalcitrant organic compounds in the environment (Fierer et al., 2007; Gao et al., 2019a). However, SAs are not apparently degraded by this group (Kielak et al., 2016). NER fraction increased in the perturbed soils with incubation time, reaching values close to the natural soils after 42 d likely due to the recovery of bacterial community diversity in both soils, showing that disturbance effects were transient.

SDZ dissipation rates were quite similar in both natural soils despite their completely distinct soil microbial community structure (only DT_{90} was higher for NS-S2, but not DT50). The effects of artificial modifications, even for the sterilized soils, were transitory regarding NER formation but not SDZ mineralization. The main route of SDZ dissipation was formation of NER (despite its low sorption), not mineralization. These results suggest that SDZ mineralization has low functional redundancy. SDZ immediate transformation in acidic soils should involve primarily chemical reactions (Reia et al., 2020), but our data suggest that microbial degradation should take place soon after.

Conclusions

Long-term swine manure application changed completely soil bacterial community structure, but only slightly enhanced its diversity. The most abundant phyla in both natural soils were Proteobacteria, Chloroflexi, Actinobacteria, Bacteroidetes, Planctomycetes, Acidobacteria, and Firmicutes, whereas Proteobacteria, Actinobacteria, and Chloroflexi predominated in the perturbed soils. Despite these abrupt changes, SDZ dissipation was quite similar in the manured and non-manured soils and NER was the primary route of SDZ dissipation. As a matter of fact, SDZ dissipation was somewhat slower in the manured soil at longer run (DT_{90} = 20 versus 2.1 d) that was supposed to have more adapted soil microbes, thus contradicting our hypothesis. The non-amended soil had a greater number of bacterial families that correlated with NER formation and SDZ mineralization, such as Isosphaeraceae, Ktedonobacteraceae, Acidobacteriaceae_(Subgroup_1), Micromonosporaceae, and

Sphingobacteriaceae. However, soil bacterial diversity depletion decreased SDZ mineralization (~2-3 times) and initial formation of NER (~1.5-2.5 times), but NER improved considerably up to 42 d likely due to recover of the soil microbial diversity. Therefore, these results novel by showing that: i) SDZ mineralization is a low redundancy soil function, but it is not the case for NER, ii) long term manure application decreased SDZ dissipation, iii) if SDZ chemical degradation does happen instantaneously, its biodegradation immediately followed it, iv) SDZ biodegradation is favored by a pool of soil bacteria and the newly formed metabolites should favors NER formation since SDZ presented low sorption, and v) depletion in soil bacterial diversity due to soil perturbation is only a transient effect, and most SDZ dissipation recovers up to 42 d. However, this period may be enough to spread resistance genes into the environment.

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Supplementary Material

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Sulfadiazine dissipation as a function of soil bacterial diversity☆

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ABSTRACT

Antibiotic residues in the environment are concerning since results in dispersion of resistance genes. Their degradation is often closely related to microbial metabolism. However, the impacts of soil bacterial community on sulfadiazine (SDZ) dissipation remains unclear, mainly in tropical soils. Our main goals were to evaluate effects of long-term swine manure application on soil bacterial structure as well as effects of soil microbial diversity depletion on SDZ dissipation, using "extinction dilution approach" and ^{14}C-SDZ. Manure application affected several soil attributes, such as pH, organic carbon (OC), and macronutrient contents as well as bacterial community structure and diversity. Even minor bacterial diversity depletion impacted SDZ mineralization and non-extractible residue (NER) formation rates, but NER recovered along 42 d likely due to soil diversity recovery. However, this period may be enough to spread resistance genes into the environment. Surprisingly, the non-manured natural soil (NS–S1) showed faster SDZ dissipation rate (DT_{90} = 2.0 versus 21 d) and had a great number of bacterial families involved in major SDZ dissipation pathways (mineralization and mainly NER), such as *Isosphaeraceae, Ktedonobacteraceae, Acidobacteriaceae_(Subgroup_1), Micromonosporaceae, and Sphingobacteriaceae*. This result is unique and contrasts our hypothesis that long-term manured soils would present adaptive advantages and, consequently, have higher SDZ dissipation rates. The literature suggests instantaneous chemical degradation of SDZ in acidic soils responsible to the fast formation of NER. Our results show that if chemical degradation happens, it is soon followed by microbial metabolism (biodegradation) performed by a pool of bacteria and the newly formed metabolites should favors NER formation since SDZ presented low sorption. It also showed that SDZ mineralization is a low redundancy function.

Depletion on the soil bacterial diversity reduced mineralization (2–3 times) and dissipation rates of sulfadiazine and its dissipation was lower in the log-term manured soil.

1. Introduction

About 2.0 × 10^5 t y^{-1} of antibiotics are used in human and veterinary medicine (Liu et al., 2020c). Global antibiotic consumption, expressed in defined daily dose (ddd), increased from 21.1 billion (2010) to 34.8 billion (2015), and should reach 128 billion until 2030 (Hu et al., 2020). Sulfonamides (SAs) are the most widely used class of antibiotics in veterinary medicine; it is estimated that about 2.0 × 10^4 t are introduced into the biosphere yearly and that 50–75% are used in veterinary medicine (Gao et al., 2019b; Liu et al., 2020c). SAs are the third most employed class of antibiotics in Brazil. The country consumed 715 t in 2013, with an expected increase of 41% until 2030 (up to ~ 1.0 × 10^3 t y^{-1}) (Van Boeckel et al., 2015, 2017).

Antibiotic residues reach agricultural soils through fresh animal manure, sewage sludge, and biosolid applications as well as animal waste (feces and urine) releases during grazing (Cycoń et al., 2019). Animal manures are the main entry route of SAs into the environment since only 10–40% of the administered dose is metabolized in the body and the remainder is eliminated in the feces and

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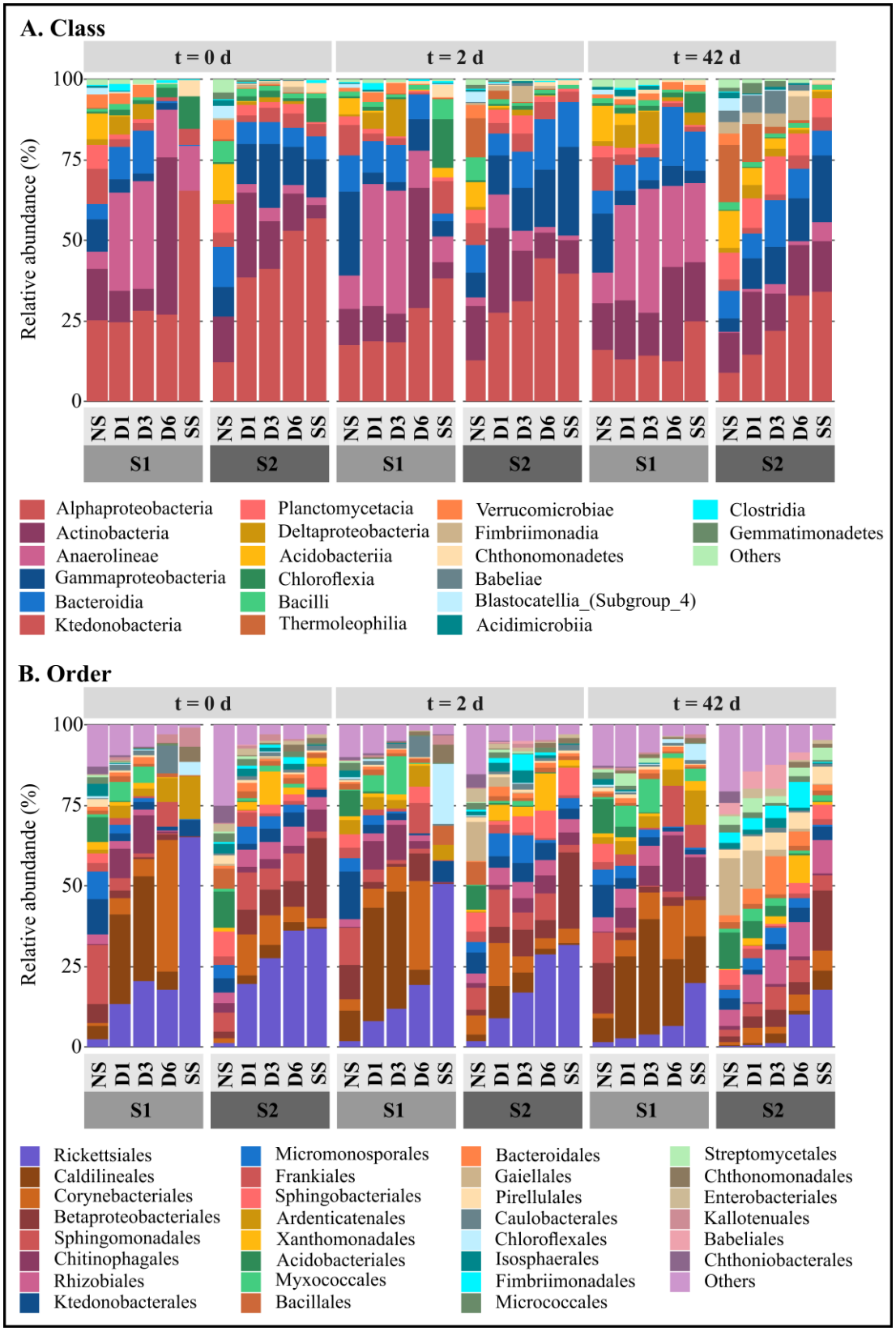

Figure S1. Relative abundance of the bacterial communities at class (A) and order (B) levels in parallel soils without (S1) and with (S2) swine manure history at 0, 2, and 42 d after sulfadiazine application (n = 3). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

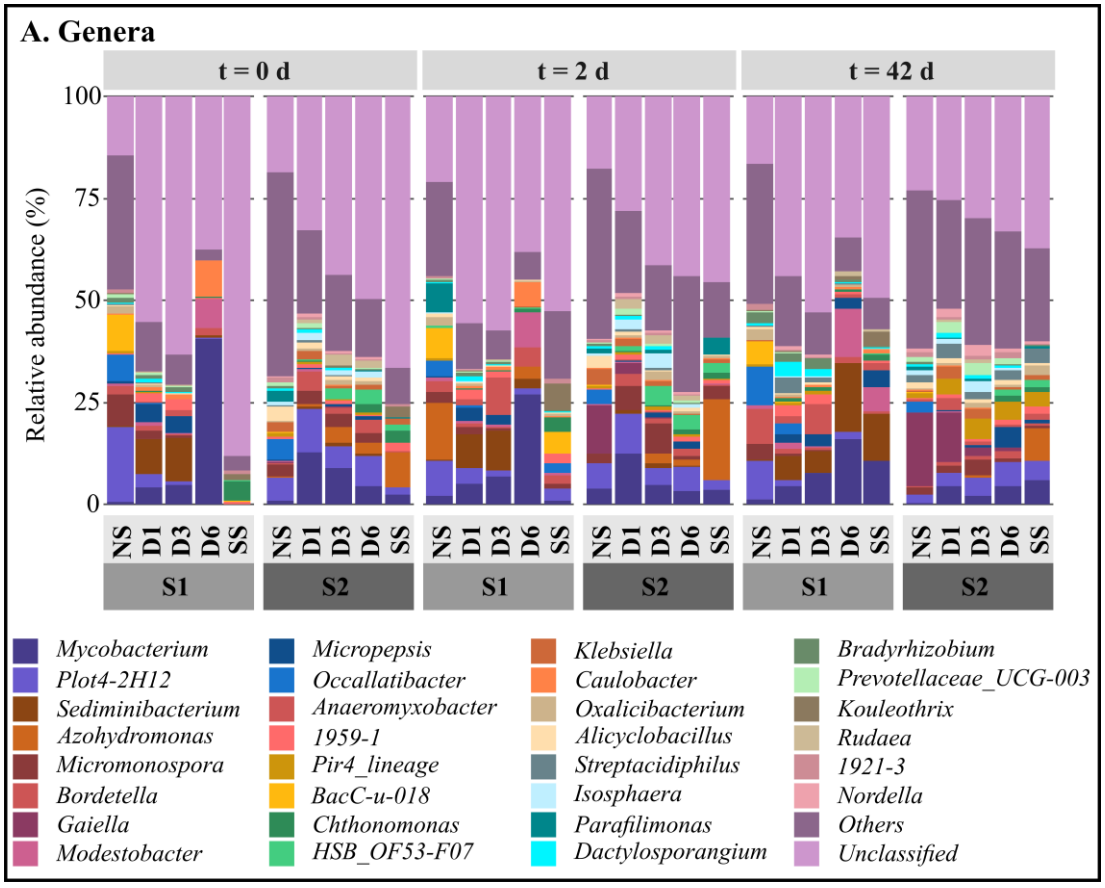

Figure S2. Relative abundance of the bacterial communities at genera level in parallel soils without (S1) and with (S2) swine manure history at 0, 2, and 42 d after sulfadiazine application (n = 3). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

Table S1. 16S rRNA sequencing in parallel soils without (S1) and with (S2) swine manure history at 0, 2, and 42 d after sulfadiazine application (n = 3).

Soil	Treatments (n = 3)	Paired end reads (raw)	denoised Forward	denoised Reverse	Merge and non-chimera	Quality Score (Q)
Time = 0 d after sulfadiazine application						
S1	NS	97,437	48,237	46,340	33,914	32.2
	D1	106,461	52,663	51,966	42,941	32.6
	D3	114,249	56,520	56,008	46,801	32.3
	D6	111,700	55,262	54,964	48,311	32.4
	SS	101,725	50,326	50,132	44,679	31.9
S2	NS	96,421	47,704	44,712	31,085	32.4
	D1	105,299	52,116	50,910	42,115	32.4
	D3	95,955	47,467	46,913	39,552	32.4
	D6	90,360	44,718	44,303	40,141	32.8
	SS	94,415	46,744	46,442	43,389	32.8
Time = 2 d after sulfadiazine application						
S1	NS	97,626	48,294	46,353	33,582	32.2
	D1	121,579	60,121	59,577	50,672	32.5
	D3	121,579	60,121	59,577	50,672	32.5
	D6	121,015	59,857	59,571	52,506	32.5
	SS	96,408	47,726	47,466	44,026	32.6
S2	NS	115,694	57,247	54,522	38,052	32.2
	D1	138,995	68,789	67,365	57,937	32.9
	D3	114,122	56,468	55,569	47,920	32.8
	D6	134,098	66,348	65,944	59,144	32.7
	SS	103,950	51,445	51,222	45,252	32.2
Time = 42 d after sulfadiazine application						
S1	NS	125,899	62,318	60,297	48,310	32.5
	D1	125,301	62,002	61,048	51,668	32.6
	D3	113,037	55,953	54,482	43,164	32.1
	D6	124,217	61,505	61,070	51,731	32.1
	SS	138,995	68,793	68,443	59,105	32.4
S2	NS	107,018	52,983	50,164	36,896	32.3
	D1	109,105	54,027	52,218	41,694	32.1
	D3	113,037	55,953	54,482	43,164	32.1
	D6	121,874	60,301	59,616	52,697	32.7
	SS	109,749	54,326	53,964	48,966	32.7
Total		3,367,321	1,666,334	1,635,642	1,370,084	---
Median		112,368	55,608	54,482	44,965	32.4
Mean		112,244	55,544	54,521	45,669	32.4

NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

Table S2. Treatment clusters according to analysis of similarity (ANOSIM) based on *Bray-Curtis* distance among soil bacterial communities of parallel soil without (S1) and with (S2) swine manure application.

Clusters	R_{values} ANOSIM		
	Soil S1		
	C1	C2	C3
C1	-	1.00*	0.77*
C2	-	-	0.59*
C3	-	-	-
	Soil S2		
	C4	C5	C6
C4	-	0.72*	0.80*
C5	-	-	0.25*
C6	-	-	-

Clusters C1 and C4 = NS; C2 and C5 = D1-D3; C3 and C6 = D6-SS for S1 and S2, respectively. R_{value} indicate similarity degree; $R_{value} < 0.50$ = cluster overlapping; $0.50 < R_{value} < 0.75$ = individual clusters with certain overlapping; $R_{value} > 0.75$ = no overlapping. (* $p < 0.002$).

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3. LONG-TERM ATRAZINE APPLICATION SELECTS A SOIL BACTERIAL COMMUNITY MORE EFFICIENT IN PROMOTING ITS BIODEGRADATION

Abstract

Atrazine (ATZ) is one of the most widely used herbicides in the world with about 7 x 10^4 – 9 x 10^4 tons y^{-1} of active ingredient. ATZ and its metabolites persistence in soil and water has been of great concern since these substances are carcinogenic and endocrine disruptors. The main route of atrazine degradation involves soil microorganisms, with several bacterial and fungal strains efficient to herbicide dissipation in vitro. To date, there are no studies considering the role of soil bacterial diversity on the atrazine biodegradation in soil without and with long-term of herbicide use. Here, we assessed how bacterial diversity depletion affect atrazine dissipation in the grazed soil (GS, without long-term of atrazine application) and cultivated soil (CS, with long-term of atrazine application). We used the "dilution-to-extinction" approach to artificially simulate bacterial diversity loss, followed by radiorespirometry assays and 16S rRNA high throughput sequencing. The Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Acidobacteria, Verrucomicrobia, Planctomycetes, and Gemmatimonadetes were the dominant phyla in both soils (CS and GS). The atrazine mineralization was reduced 10-fold in relation to the worst scenarios of bacterial diversity depletion. Long-term of atrazine application has resulted in greater community efficiency in dissipating the herbicide. The bacterial diversity loss reflected a reduction in complexity, species classification (specialists, generalists and too rare), in addition to increases in the abundance of functions related to obtaining energy and nitrogen transformations. Twelve bacterial genera (*Ralstonia*, *Nitrospira*, *Hirschia*, *MND1*, *Candidatus_Koribacter*, *OM27_clade*, *Arenimonas*, *Occallatibacter*, *Bryobacter*, *UTBCD1*, *Ellin6067* and *Crenobacter*) correlated with atrazine mineralization, suggesting the selection of these genera as potential atrazine-degraders. The bacterial communities of soil with a long-term of atrazine application were less impacted by the of diversity depletion and showed a higher number of microbial groups associated with the herbicide biodegradation.

Keywords: Bacterial diversity; S-triazine herbicides; Adapted soils; High-throughput sequencing; Herbicide dissipation

Introduction

Atrazine [2-chloro-4-ethylamino-6-isopropylamino) -1,3,5-triazine] (ATZ) is the second most widely used herbicide in the world (~7 x 10^4 – 9 x 10^4 tons of active ingredient is consumed per year) (Fan et al., 2020; Henn et al., 2020; Khatoon and Rai, 2020). It is often used to control pre-emergence and post-emergence of weeds in corn, millet, sugarcane, and sorghum (Aguiar et al., 2020; Leal et al., 2019). ATZ belongs to the chemical group of chlorinated S-triazines and is formed by an aromatic heterocyclic ring alternating carbon and nitrogen atoms, besides to the chlorine substituent (Henn et al., 2020; Pertile et al., 2021; Singh et al., 2018). This herbicide molecule has high mobility in the soil profile, which is the result of its high solubility in water (33.0 mg L^{-1}) and low to moderate sorption to soil particles (Kd = 2,6 L kg^{-1} e KOC = 100 L kg^{-1}) (Lopes et al., 2020; Zhang et al., 2019; Zhao et al., 2018).

In the soil, atrazine is a compound considered persistent with a half-life ranging from 40 to more than 365 d (Jablonowski et al., 2010; Singh et al., 2018). Similar to other halogenated aromatic compounds, atrazine and its dealkylated (deethylatrazine, deisopropylatrazine, and deisopropyldeethylatrazine) and deaminated (N-isopropylammelide, N-ethylammelide) metabolites are toxic, carcinogenic and endocrine disruptors (Lopes et al., 2020; Singh et al., 2018). The ATZ dissipation in the environment is directly correlated with the degradation capacity of the soil microbiome (Hsieh et al., 2020; Yale et al., 2017).

ATZ is biodegraded by three major pathways, one is purely hydrolytic while the others are mixed (hydrolytic-oxidative) (Singh et al., 2018). In the first, ATZ is used as the sole N source and its aromatic ring is finally cleaved releasing CO2. Briefly, ATZ is hydrolyzed to hydroxyatrazine (HA) (atzA gene) that is degraded to N-isopropylammelide (atzB gene) and then transformed to cyanuric acid (CA) (atzC gene). CA is cleaved to biuret (atzD gene) that is converted to allophanate (atzE gene) and then completely mineralized to CO2 and NH3 (atzF gene) (de Souza et al., 1996; Hsieh et al., 2020). In the second, ATZ is N-dealkylated into deethylatrazine or deisopropylatrazine, which is further dealkylated into deisopropyldeethylatrazine or hydrolyzed to CA. In the third, ATZ is hydrolyzed to HA that is broken-down and forms N-isopropylammelide or N-ethylammelide as intermediate metabolites (Solomon et al., 2013; Topp et al., 2000), which are further converted into CA (Yang et al., 2010). Apart of the metabolic pathway, CA is often attacked by cyanuric acid amidohydrolase, biuret amidohydrolase, and allophanate hydrolase releasing CO_2 and NH_3 (Hsieh et al., 2020; Singh et al., 2018).

Major atrazine biodegradation studies have focused on the isolation and enrichment of bacterial and fungal strains with the potential to degrade herbicide molecule in vitro (Jacobsen and Hjelmsø, 2014; Singh and Singh, 2014; Singh et al., 2018). Other ATZ degradation data were obtained through kinetic studies, which often disregard the role of soil microbial diversity. To date, there are no robust approaches considering how the diversity and composition taxonomy of bacterial communities can affect the ATZ dissipation in the soil.

Soil microbiota plays a key role in maintaining the energy and nutrient flow in the ecosystem (Cardinale et al., 2011; Coleman and Whitman, 2005; Philippot et al., 2013). The relationship between microbial biodiversity and the ecosystem functioning has received enormous attention in recent decades (Gamfeldt and Roger, 2017; Trivedi et al., 2019). The central issue in this study field involves how the decline of species diversity impacts the ecosystem functioning and services (Cardinale et al., 2002; Wertz et al., 2006). In fact, the microbial diversity role is well understood for the ecosystem collective functions, such as the organic matter decomposition, biomass accumulation and nutrient cycling (de Souza et al., 2021; Yang et al., 2021).

The microbial diversity depletion has shown lesser impact on these collective functions, due to the high degree of functional redundancy within soil microbial communities. In contrast, more specific functions - with a lower degree of functional redundancy, such as pesticides and other pollutants biodegradation – are strongly impacted by the loss of soil bacterial diversity (Cravo-Laureau et al., 2011; Hernandez-Raquet et al., 2013).

In our previous studies using the “dilution-to-extinction” approach, we showed that the degradation of chlorothalonil (fungicide) was reduced ~ 20-fold in relation to the largest soil bacterial diversity depletion (10^{-6}), while the degradation of sulfadiazine (antibiotic) was reduced by ~ 3-fold in relation to the worst scenarios of soil bacterial diversity decline (10^{-6} and sterile soil) (de Souza et al., 2021). However, we do not know the extent of the effect of bacterial diversity depletion on the herbicide biodegradation (atrazine), which is likely to have a less deleterious effect on the soil microbial community than antibiotics and fungicides.

In this context, we hypothesize that: i) the effect of microbial diversity depletion on the atrazine degradation is less in soils with long-term of herbicide application; ii) The ability to dissipate atrazine is extremely sensitive to microbial diversity loss, especially in soils that have received few herbicide applications; iii) soil bacterial communities adapted to the atrazine application are more resilient to microbial diversity disturbance.

Therefore, the objective of this research was to evaluate how the bacterial diversity depletion in the soils without and with long-term application impacts the biodegradation and

dissipation of the atrazine herbicide. For this, we employ the “dilution-to-extinction” technique (Van Elsas et al., 2012; Wertz et al., 2006) and simulate different disturbances in the microbial diversity of the soil (light, intermediate and extreme) and analyze atrazine dissipation and bacterial communities using radiometric technique and high-throughput sequencing approach.

Material and Methods

Soil samples collection

Soil samples were collected in the 0-10 cm depth layer, on a farm located in the Sinop city, Mato Grosso state, Brazil. The local climate is Aw (warm and humid tropical), according to Köppen’s classification system, showing an annual rainfall of 1974,47 mm, concentrated between October and April and average temperature of 24.7º C, with 19.0 ºC and 32.6 ºC of minimum and maximum temperatures, respectivily (Mota et al., 2013; Souza et al., 2013). Soil samples were collected in Brazilian Cerrado, whick is wide agribusiness frontiers. Grazed Soil sample (GS, 11°52’45” S 55°22’54” W) was collected in pasture area without atrazine application. While Cultivated Soil sample (CS, 11°53’15” S 55°22’48” W) was collected in agricultural area (no-till system) under corn and soybean rotation and with 7 years of atrazine application.

The sampling procedure consisted of collecting 10 sub-samples per area, which were subsequently homogenized in a collection bucket, forming a composite sample representative of the area (~35 kg of soil per area). After collection, a portion of 500 g of each soil was used to determine the physical and chemical properties at the Department of Soil Science / ESALQ-USP. Soil samples remainder were taken to Laboratory of Soil Microbiology at ESALQ-USP, an aliquots ~ 2 kg of soil were immediately stored in the ultra-freezer (-80 ºC). The other soil fraction was air dried and sieved in a 2 mm mesh and stored in the cold room (2–4 ºC) until the microcosm assembly. Soils textural class, as well as its physical and chemical properties are shown in table 1.

Table 1. Physical and chemical attributes, sorption parameters and DT_{50} values in the Grazed Soil (GS) without long-term of atrazine application and Cultivated Soil (CS) with long-term of atrazine application.

[1] **Physical and chemical attributes**	[2] **Typic Hapludox**	
	Grazed Soil (GS)	**Cultivated Soil (CS)**
[3] Sand (g kg^{-1})	680	710
Silt (g kg^{-1})	282	260
Clay (g kg^{-1})	38	30
[4] OM (g kg^{-1})	16	43
[5] OC (g kg^{-1})	9.3	24.9
[6] pH ($CaCl_2$)	4.3	5.1
[7] P (mg dm^{-3})	4	12
K ($mmol_c$ dm^{-3})	< 0.9	2.4
Ca ($mmol_c$ dm^{-3})	3	33
Mg (mmolc dm^{-3})	3	20
[8] SB ($mmol_c$ dm^{-3})	6.6	55.4
[9] CEC ($mmol_c$ dm^{-3})	40.6	89.4
[10] V (%)	16	64
[11] m (%)	48	0
[12] Sorption parameters		
$K_{d\ app}$ (L kg^{-1})	0.74	2.96
$K_{oc\ app}$ (L kg^{-1})	80.2	118.8
[13] DT_{50} (d)		
NS	22	4
D1	26.5	7.8
D3	27	11
D6	37	17.3
SS	44	25.8

[1] Soil properties determined according to Van Raij et al. (1987) and EMBRAPA (1997); [2] Soil classification according to USDA (1999); [3] granulometry determined by a Buyoucos densimeter; [4] organic matter; [5] organic carbon; [6] hydrogen potential in $CaCl_2$; [7] P, K, Ca, and Mg determined by ion exchange resin method; [8] Sum of bases; [9] cation exchange capacity; [10] saturation by base; [11] saturation by aluminum. [12] Sorption parameters determined according to OECD (2000): $K_{d\ app}$ = Apparent sorption coefficient; $K_{oc\ app}$ = Apparent partition coefficient normalized by organic carbon; [13] degradation rate of 50 percent of the target compound (half-life), expressed in days. NS = Natural Soil; D1, D3, and D6 = dilutions 10^{-1}, 10^{-3} and 10^{-6}; SS = sterile soil.

Atrazine and reagents

For microcosm, the atrazine radiolabeled molecule (uniformly labeled on the aromatic ring, specific activity = 5920 MBq $mmol^{-1}$, and purity ≥ 99%) (Hhubb American Radiolabeled Chemicals, St. Louis, Missouri, USA) and atrazine analytical standard (purity ≥ 99%) (Merck, Darmstadt, Germany) were used. All reagents used had an analytical grade of HPLC. The extraction of the pesticide was carried out with methanol. The mobile phase for elution of the TLC plates consisted of chloroform, acetone, acetic acid, and water (50: 30:15: 5, v/v/v/v) (Peixoto et al., 2000).

Atrazine sorption assay

Sorption test was carried out according to the batch equilibrium methodology – OECD Method 106 (OECD, 2000). The ^{14}C-ATZ stock solution (174 Bq mL^{-1}) was prepared in 0.005 mol L^{-1} of $CaCl_2$ solution. Assay was carried out using the ratio 1:2 - soil: radiolabelled solution (5 g of dry soil:10 mL of ^{14}C-ATZ in $CaCl_2$ solution). The suspensions were subjected to agitation on a horizontal shaker at 120 rpm for 6 h and centrifuged at 3,000 rpm for 15 min (Inoue et al., 2006). Then, 1 mL of the supernatant used to determine the equilibrium concentration (C_e) by liquid scintillation. Sorbet concentration (S) was determined by difference between the initial concentration (C_i) and equilibrium concentration (C_e). While apparent sorption coefficient was calculated as $K_{d\,app} = S/C_e$ and expressed in L kg^{-1}. In addition, the partition coefficient was determined by normalization of $K_{d\,app}$ by soils organic carbon content (OC) according to equation $K_{OC\,app} = K_{d\,app}$ x 100/OC (%), also expressed in L kg^{-1} (Table 1).

Soil bacterial community microcosm

Soil bacterial community microcosm was assembled using the "dilution-to-extinction approach" (Van Elsas et al., 2012; Wertz et al., 2006). For this, an initial soil suspension of 10% (m/v) was used to generate the different microbial community dilutions. Sub-samples of 25 g soils were weighed and sterilized in an autoclave (120 °C, 1.0 atm, for 3 cycles of 60 min). Samples of natural soil (NS), sterile soil (SS) and dilutions D1, D3 and D6 (in triplicate) were transferred to biometric flasks. Then, the sterilized soil samples were inoculated with soil suspension dilutions D1 = 10^{-1}, D = 10^{-3} and D6 = 10^{-6} and soil moisture was adjusted to 60%

of maximum water holding capacity. Biometric flasks were incubated in dark semi-dark room at 25 ± 1°C for 15 d to allow perturbed microbial community to structure (de Souza et al., 2021, 2017; Van Elsas et al., 2012).

Afterwards, ATZ analytical grade solution was applied at dose of 1.92 mg kg^{-1} and the flasks were incubated in a semi-dark room, at 25 ± 1 °C for 70 d. At 0, 14, and 70 d after atrazine application, soil subsamples were collected to evaluate diversity, composition, and structure of the bacterial community by sequencing the 16 S rRNA gene.

Soil DNA was extracted using a commercial DNeasy Powerlyzer Power Soil Kit (Qiagen, Hilden, Germany). DNA quality and integrity were checked using agarose gel (1%, m/v) electrophoresis and quantified in a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, USA), and then stored at -20 ºC. DNA sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, USA), targeting the V4 region of the 16S rRNA (Caporaso et al., 2011). DNA clone library was built based in the MiSeq Reagent Kit V3 600-cycles (Illumina, San Diego, USA). DNA was prepared in two steps: at first, the 16S rRNA gene was amplified using 16S-515F (5'–TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG–3') and 16S-806R (5'–GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG–3') primers, modified with pre-adapters. PCR reaction comprised 12,5µL of 2x PCR Ultra Mix (PCR BioSystems), 0,5 µL of each primer (10 mM), 1 µL of template-DNA and ultra-pure water for final volume = 25 µL. Amplification was performed at 94 ºC for 3 min (DNA strand denaturation), followed by 30 cycles at 94 ºC for 30 s, 50 ºC for 30 s, 72 ºC for 30s, and a final extension of 10 min at 72ºC (Caporaso et al., 2011). 16S rRNA amplicons were confirmed by agarose gel electrophoresis, resulting in a ~ 390 bp fragment. The amplified DNA was purified using AMPure XP beads (Beckman Coulter, Brea, USA). At second, the purified DNA was subjected to other PCR reaction to link the barcodes according to Nextera XT Kit (Illumina, San Diego, USA). PCR reaction comprised 12,5µL of 2x PCR Ultra Mix (PCR BioSystems), 2,5 µL of each adapter (Nextera XT Index 1 Primers (N7XX barcode) and Nextera XT Index 2 Primers (S5XX), 2,5 µL of previous reaction product and ultra-pure water for final volume = 25 µL. Which was performed under the following conditions: 95 °C for 3 min, 8 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; followed by a final extension of 72 °C for 5 min. At reaction end, purified DNA amplification was evaluated by agarose gel electrophoresis. All purified amplicons were pooled at equal molar amount and the final concentration of the library was determined using a SYBR green quantitative PCR assay with primers specific to the Illumina adapters Kapa (KAPA Biosystems, Boston, Massachusetts, USA). The sample pool (2 nM) was denatured with 0.1 N NaOH, then diluted to 20 pM and combined with 20%

denatured 20 pM PhiX, prepared following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform, using a 2 x 300 cycle V3 kit, following standard Illumina sequencing protocols. The sequenced DNA covered ~45,000 reads per sample and had paired-end reads of ~290bp.

Atrazine biodegradation assay

Atrazine biodegradation assay was performed according to OECD method 307 (OECD, 2002), using the radiorespirometry technique. For this, biometric flasks containing 25 g of soil (in duplicate) and different microbial diversity levels (NS, D1, D3, D6, and SS) received an atrazine dose of ~ 1.92 mg kg^{-1} (radioactivity ~ 5.9 kBq $flask^{-1}$). Subsequently, the humidity was adjusted to 60% of the maximum water retention capacity and the flasks were incubated in a semi-dark room at a temperature of 25 ± 1 °C.

The mineralization rate was determined weekly until 70 days after ^{14}C-ATZ application. ^{14}C-CO_2 released was trapped in 10 mL of 0.2 mol L^{-1} NaOH solution and the radioactivity was quantified by liquid scintillation into TRI-CARB 2910 scintillator (PerkinElmer, Massachusetts, USA).

^{14}C-ATZ and its residues extraction was carried out in the periods of 0, 7, 14, 21, 42 and 70 d after application. Atrazine and its residues were extracted in methanol (100%, v). In the period of 0 days, soil samples were extracted immediately after the application of ^{14}C-ATZ. For this, the soil samples were transferred to the teflon flasks (250 ml) and 50 ml of methanol were added. Soil slurries was subjected to stirring on a horizontal shaker at 220 rpm for 3 h. Afterwards, the flasks containing slurries were centrifuged at 4000 rpm for 15 min and at a temperature of 10 °C and 1 mL of the supernatant of each extract was transferred to a vial containing 10 mL of Ultima Gold scintillating solution (PerkinElmer, Massachusetts, USA) and the radioactivity was quantified by liquid scintillation. The extracts were collected in Scott flasks and concentrated by rotavapor, which then were resuspended in 3 mL of methanol. Atrazine and its metabolites presence in the extracts was verified by thin layer chromatography (TLC). For this, 100 µL of the concentrated extracts was applied to the TLC plate. The ATZ analytical standard was also applied. TLC plates was developed in solution of chloroform, acetone, acetic acid, and water (50:30:15:5, v/v/v/v) and retention factor (Rf) for atrazine and its metabolites was determined by plate reading in Cyclone One Plus (PerkinElmer, Massachusetts, USA). In addition, ^{14}C-ATZ degradation profile were using to estimate DT_{50} values.

Soil slurries were dried at 40 ºC in the forced air circulation oven. The dry soil samples were ground in a porcelain mill. Then, 0.2 g of soil in triplicate was subjected to combustion in the Biological Oxidizer (Biological Oxidizer OX-500, R.J. Harvey Instrument Corporation, New York, USA). The combustion gases were trapped in 10 mL of scintillating solution, ethanolamine, and methanol (7: 3: 2.5, v / v / v) and later counting the radioactivity by LSS.

Bioinformatic analysis

DNA sequences analysis was performed in the R Project environment, using the packages DADA2 (Callahan et al., 2016), PHYLOSEG (McMurdie and Holmes, 2013) and GGPLOT2 (Wickham, 2016). Briefly, the multiplexed sequences were assigned to the sample metadata table. Afterwards, the FASTQ files were subjected to filtering to remove primers, adapters, and barcodes. Cut-off score for removal of low-quality sequences was Q < 30, which resulted in sequences size of 2x250bp. The paired-end reads were reassembled using the DADA2 *learnErros algorithm* to predict the rate errors and apply the correction in the reads assembly for each sample. Then, the sequences were dereplicated (denoising) to obtain unique sequences and the abundance of each sequence in the sample. The paired-end sequences were merged, and the chimeras were removed.

Finally, the sequences were assigned to each ASVs (Amplicon Sequencing Variants). ASVs were used as input for the sequences alignment using the SILVA 132 database as a reference through the DADA2 *assignTaxonomy function* (Callahan et al., 2016). Taxonomy data generated in the DADA2 pipeline were imported into the PHYLOSEQ package (McMurdie and Holmes, 2013). PHYLOSEQ integrates a robust tool set for analyzing of ecological and phylogeny data what are available in VEGAN, ADE4, APE and PICANTE packages. In addition, GGPLOT2 package was used for graphic processing. ASVs were agglomerated and evaluated up to the phylum, class, order, family, and genera levels. ASV tables were using as input for non-metric multidimensional scaling (NMDS), PERMANOVA, co-occurrence networks, CLAM test, FAPROTAX and Spearman´s correlation test between bacterial composition and atrazine dissipation parameters.

Statistical analyses

The atrazine dissipation data (CO_2 evolved, extractable fraction, non-extractable residues, ATZ degradation and metabolites) were tabulated, and the mass balance was performed to verify the recovery rate of the applied radioactivity.

The alpha-diversity data were calculated from the ASVs table. After, they were subjected to the normality test, analysis of variance and Tukey's test (alpha 5%) for means comparison. Soil bacterial community structure was evaluated through non-metric multidimensional scaling analysis (NMDS), using the Bray-Curtis distance matrix (Buttigieg and Ramette, 2014). Multivariate Variance Permutation analysis (PERMANOVA) was applied to compare bacterial community groups in the different treatments (NS, D1, D3, D6, and SS) both soils (GS and CS) (Buttigieg and Ramette, 2014; Ramette, 2007).

The Bacterial absolute abundance matrix was used to implement the network analysis, which explored the complexity and connections between members of the bacterial community in the different treatments and soils (Khan et al., 2019; Pedrinho et al., 2020). The co-occurrence analysis was performed using the Spearman correlation values of the bacterial community composition data (Khan et al., 2019). For the network assembly, we used the correlation matrix with filter for $-0.75 \leq R_{SPEARMAN} \geq 0.75$ and P value ≤ 0.05. Networks were plotted in Gephi (Bastian et al., 2009). The nodes were filled at the phylum level. The edges represent the positive (blue) and negative (red) interactions between bacterial members. The nodes size represents betweenness centrality (Khan et al., 2019).

Multinominal Species Classification test (CLAM) for niche occupancy was used to calculate specialist, generalist, and rare species within each bacterial community (Chazdon et al., 2011). Meanwhile, the 16S rRNA sequences were assigned to FAPROTAX functional database (Louca et al., 2016) and the significant environmental functions in the treatment comparison groups were plotted in the STAMP Software (Parks et al., 2014). The soil bacterial community composition at family and genus levels was correlated to atrazine dissipation parameters by the Spearman correlation test and correlation matrix was plotted in R Core team version 4.0.

Results

Soil physical, chemical attributes and atrazine sorption coefficient

The physical and chemical attributes of grazed soil (GS) and Cultivated Soil (CS) are shown in table 1. CS presented higher values of pH, OM, OC, P, K, Ca, Mg, SB, CEC, and V

than G (Table 1). Atrazine sorption was extremely low in both soils. The $K_{d\ app}$ and $K_{oc\ app}$ values were 0.74 and 80.2 L Kg^{-1} and 2.96 and 118.8 L Kg^{-1}, respectively in GS and CS (Table 1).

Diversity, composition, and structure soil bacterial community

The artificial modification of the microbial community strongly affected alpha-diversity indexes in GS and CS (Figure 1). The CS-NS treatment (H = 4.15 and 1-D = 0.97) was slightly higher than the GS-NS (H = 4.00 and 1-D = 0.97). The diversity depletion effect was more accentuated in the GS treatments, having a greater impact on the diluted communities (D3, D6 and SS). After 70 d of incubation, the CS communities showed rapid recovery of alpha-diversity, especially in D1 and D3 treatments when compared to NS. On the other hand, the diversity gradient is still maintained in the GS communities after 70 d (Figure 1). At 70 d, Shannon-Weiner's diversity indexes ranged from 2.36 - 4.14 and 3.10 - 4.16, and Simpson's ranged from 0.83 - 0.98 and 0.87 - 0.97, respectively in GS and CS.

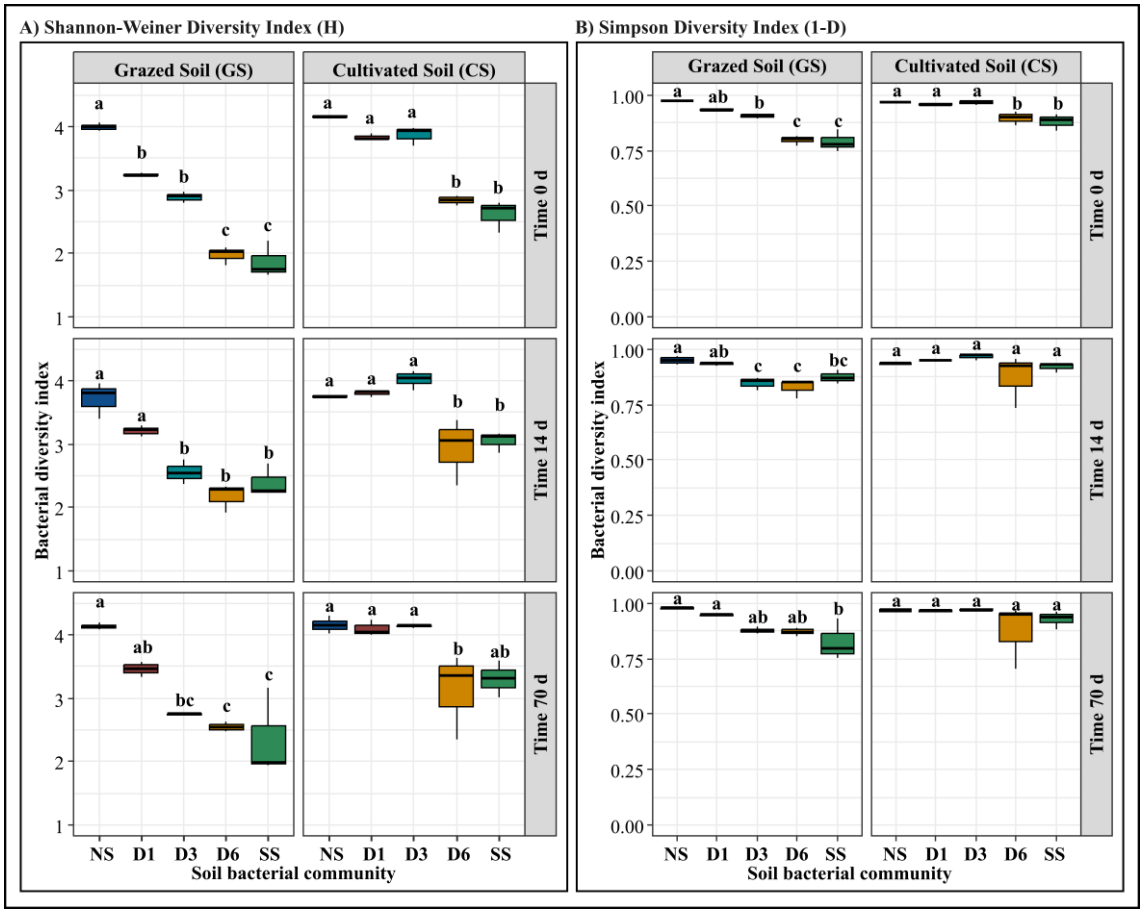

Figure 1. Boxplots of Shannon-Weiner (H) (A) and Simpson (1-D) (B) diversity indexes in the Grazed Soil (GS, without long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application) at 0, 14, and 70 d after atrazine application (n = 3). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil. Different letters indicate statistical difference by Tukey test (P value < 0.05).

The 16S rRNA gene sequencing yielded a coverage of merged and non-chimera sequences of 34,415 reads per sample and with 37.0 of Q Score average. Reads were assigned to 208 ASV, which were grouped into 14 phyla, 23 classes, 55 orders, 91 families and 149 genera (Figures S1 and S2; Table S1). The bacterial community composition at the taxonomic level of phylum and family is shown in figure 2. The most abundant phyla in both soils were Proteobacteria (~ 65.7%), Bacteroidetes (~ 9.9%), Firmicutes (~ 8.2%), Actinobacteria (~ 5.0%), Acidobacteria (~ 3.7%), Verrucomicrobia (~ 2.1%), Planctomycetes (~ 2.0%), and Gemmatimonadetes (~ 1.4%), which totaled ~ 98.2% of total community abundance (Figure 2A). Chloroflexi, Armatimonadetes, Dependentiae, Chlamydiae and Nitrospirae phyla did not occur in the communities of D6 and SS. As a result, Burkholderiaceae (~ 22.7%), Solirubrobacteraceae (~ 10.6%), Rhodanobacteraceae (~ 6.4%), Xanthobacteraceae (~ 6.1%), Rickettsiaceae (~ 5.3%), Chitinophagaceae (~ 4.4%), Micropepsaceae (~ 3.8%), Caulobacteraceae (~ 3.6%), Planococcaceae (~ 2.7%), Paenibacillaceae (~ 2.6%), Acidobacteriaceae_Subgroup_1 (~ 2.4%) were the most abundant families in bacterial community (Figure 2B).

The differential abundance Analysis of the bacterial community revealed a significant increase in the Rhodanobacteraceae, Sphingobacteriaceae, Micropepsaceae, Sphingomonadaceae, Caulobacteraceae abundances in the GS-D1 and GS-D3 and Burkholderiaceae, Rickettsiaceae and Caulobacteraceae in the GS-D6 and GS-SS in compared to NS-GS (Figure S3). While CS, a greater number of differential groups was observed into community, with a significant increase in Sphingomonadaceae, Chitinophagaceae, Planococcaceae, Micropepsaceae and Caulobacteraceae in the D1 and D3 bacterial communities. Beijerinckiaceae, Intrasporangiaceae, Rhizobiaceae, Caulobopepsaceae, Gemulaceae, Burkholderiaceae, Rickettsiaceae, and Rhodanobacteraceae presented differential occurrence at SS in relation to NS (Figure S3).

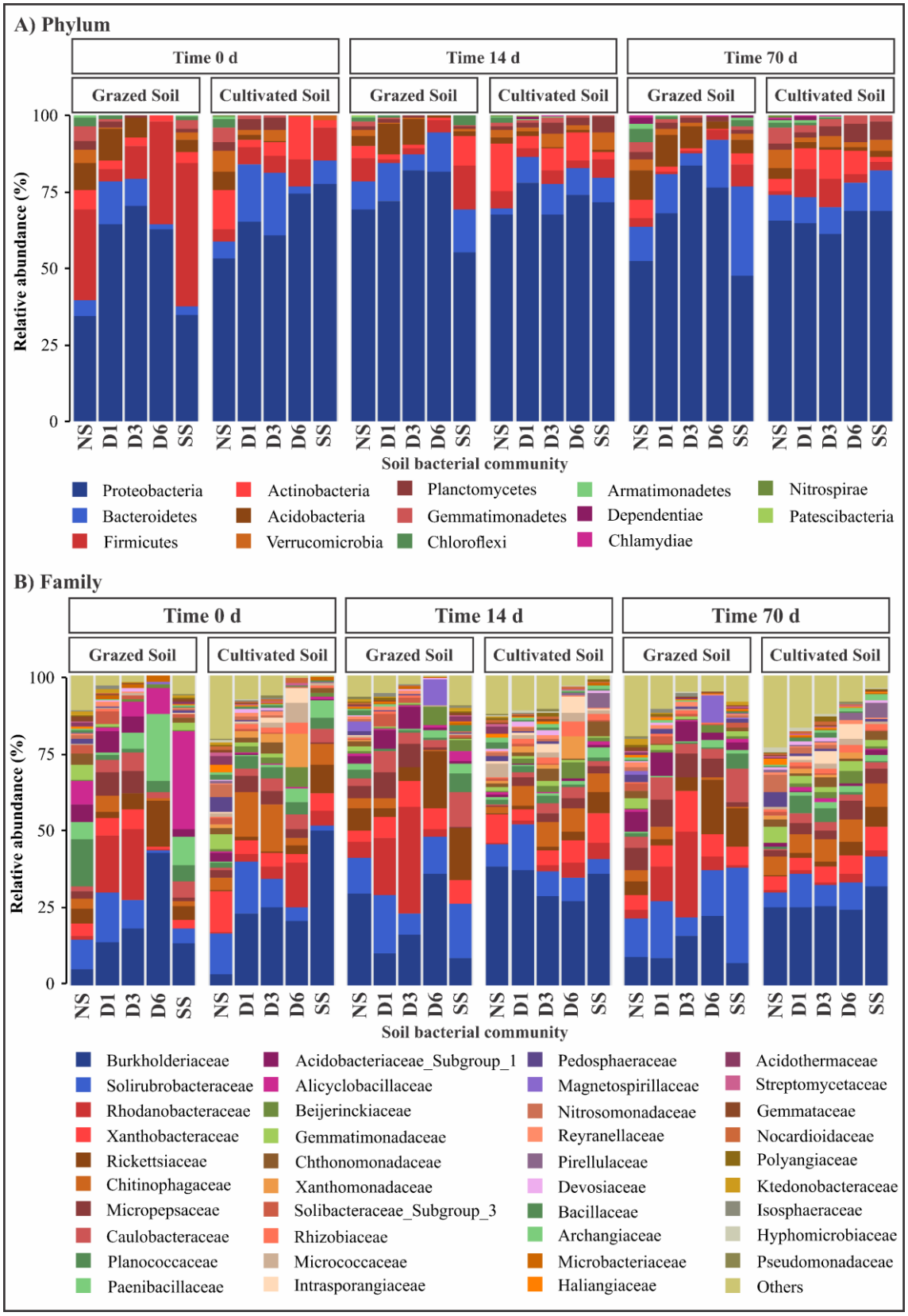

Figure 2. Relative abundance of the bacterial communities at phylum (A) and family (B) levels in the grazed soil (GS, without a long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application) at 0, 14, and 70 d of incubation (n = 3). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

The bacterial community structure was explored through non-metric multidimensional scaling analysis (NMDS), according to Bray-Curtis distance matrix in order to test the dissimilarity between treatment groups (Figure 3). Bacterial community was ordered according to the disturbance levels promoted by the serial dilution ("dilution-to-extinction approach"): NS >> D1 >> D3 >> D6 >> SS in the GS and CS. PERMANOVA ratified the bacterial community segregation in 5 different clusters in each soil (p value MC <0.001) (Table S2).

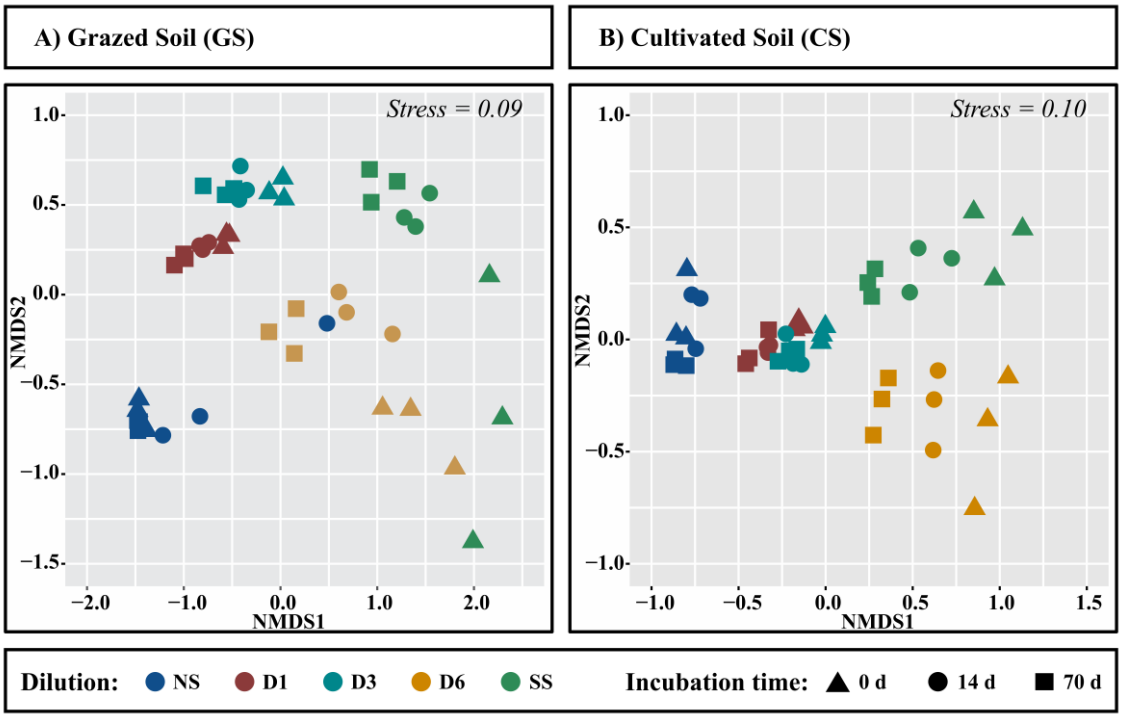

Figure 3. Non-metric multidimensional scaling of the bacterial community at ASV table in the grazed soil (GS, without long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application) at 0, 14, and 70 d of incubation (n = 3). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

Atrazine dissipation

The long-term of herbicide application had a strong effect on the ability of the soil microbial community to biodegrade atrazine, as well as the bacterial diversity disturbance levels (Figure 4). In CS, mineralization was the main dissipation route of the atrazine. After 70 d, the CS-NS treatment mineralized about 12-fold more atrazine compared to GS-NS. In GS, the depletion diversity impact was lower, with a reduction in the mineralization rate 2.3 to 3-fold in the relation to NS versus all disturbance scenarios (D1, D3, D6 and SS) (Figure 4 and Table S2). For soil GS, the bacterial diversity depletion had a greater impact on the atrazine mineralization rate. Minor effect of diversity depletion was observed in the D1 and D3 when compared with NS, which mineralized 1.1 and 1.2-fold more atrazine than the D1 and D3 bacterial communities. While at more drastic levels of community disturbance (10^{-6} dilution and sterile soils) the ability to mineralize atrazine was reduced by 31.2 and 34.3-fold compared to NS treatment (Table S3).

The GS presented a low capacity to biodegrade atrazine (mineralization), and only <5% of the applied radioactivity (AR) was released as $^{14}C\text{-}CO_2$ (Figure 4). Whereas the CS reached emission rates of $^{14}C\text{-}CO_2$ ~ 52% of the AR after 70 d. In general, the CS-NS treatment showed a faster rate of dissipation compared to the other treatments. DT_{50} value was only 4 d in the CS-NS treatment versus 22 d in GS-NS. The CS-D1 and CS-D3 treatments presented DT_{50} values lower than GS-NS (7.8 and 11 versus 22 d). At GS, DT_{50} values of atrazine doubled in the comparison of SS versus NS. While D6 versus NS, DT_{50} values was 1.6-fold higher (37 versus 22 d) (Figure 4 and Table 1).

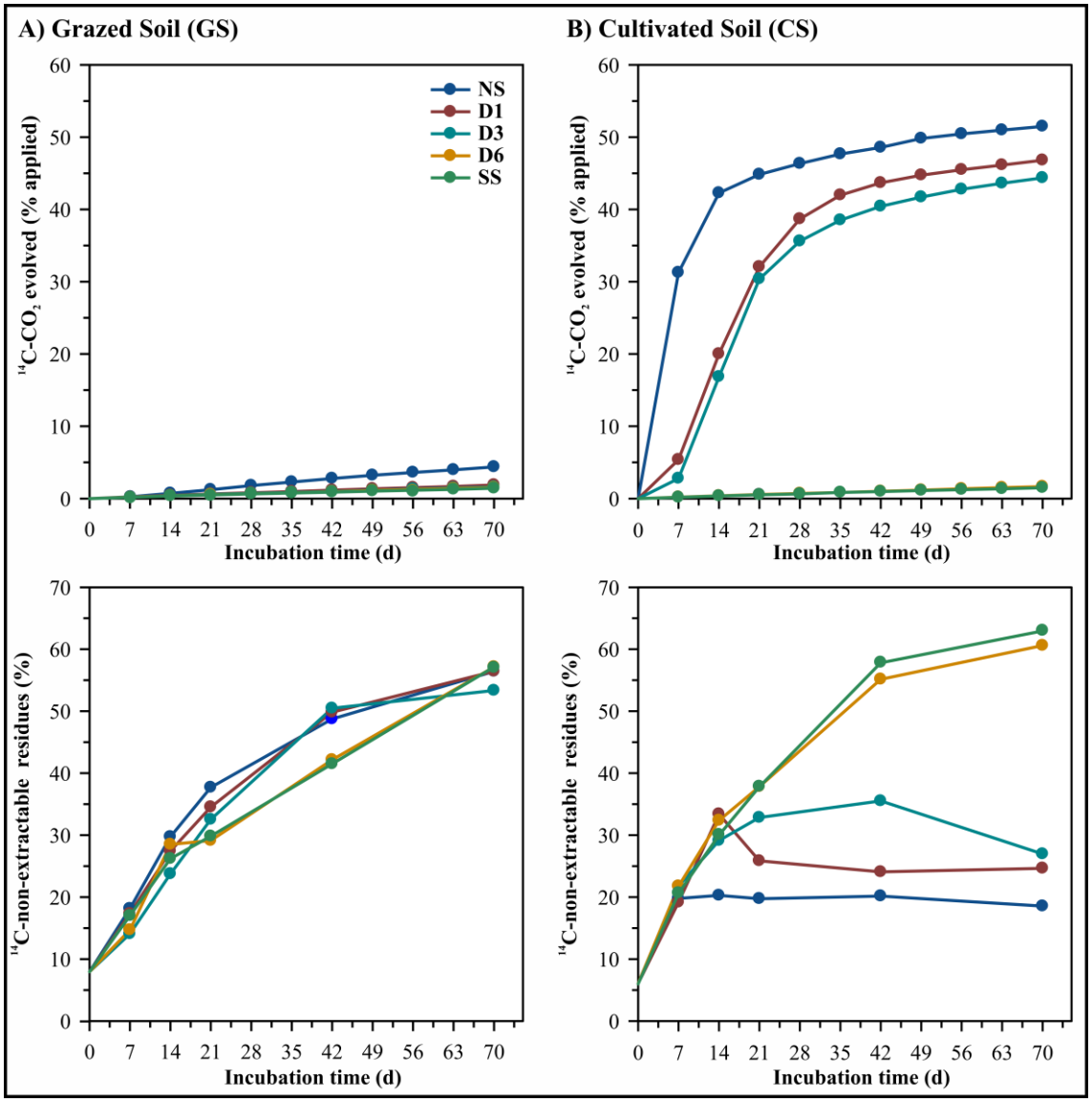

Figure 4. ^{14}C-CO_2 evolved (atrazine mineralization rate) and non-extractable residues (NER) in the grazed soil (GS, without long-term of atrazine application) (A) and Cultivated Soil (CS, with a long-term of atrazine application) (B) (n=2) after 70 d of incubation. NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

The extracts analysis revealed that atrazine is degraded into three distinct and unidentified metabolites (Figure 5). Atrazine dissipation profile was shown to be similar in all treatments of GS soil and in CS-D6 and CS-SS. Met1 metabolite (Rf = 0.4) was detected only in the GS-NS, GS-D1 communities at concentrations < 3% of AR. Higher Met1 concentrations were found in CS-D1, CS-D3 and CS-D6 (~3-18% of AR). The Met2 metabolite (Rf = 0.6) was detected in concentrations ~10-12% of the AR in all treatments of GS and in CS-D6 and CS-SS. Meanwhile, the Met3 metabolite was detected in concentrations ~10% of the RA in the GS inoculated with the NS, D1 and SS communities only 7 d. In the CS, Met3 was detected in more relevant concentrations (> 10% of AR) in the disturbed communities (D1, D3 and D6) and in the sterile soil (SS) also at 7 d after application of the herbicide. The remaining radioactivity in the extracts (ATZ - Rf = 0.9) showed similar behavior in all GS communities, as well as in the worst scenarios of the CS bacterial diversity loss (D6 and SS). ^{14}C-ATZ residues recovered in the extracts was about 20 – 30% of AR. In the CS-NS, CS-D1 and CS-D3 bacterial communities, ^{14}C-ATZ residues in the extracts was found at 3.9, 3.8 and 4.6% of the AR after 70 d, respectively (Figure 5).

The non-extractable residues (NER) formation consisted of the primary atrazine dissipation route in the GS treatments (NER ~ 55% of AR) and the worst scenarios of bacterial diversity depletion in the CS (D6 and SS), with NER ~ 60% of RA (Figure 4).

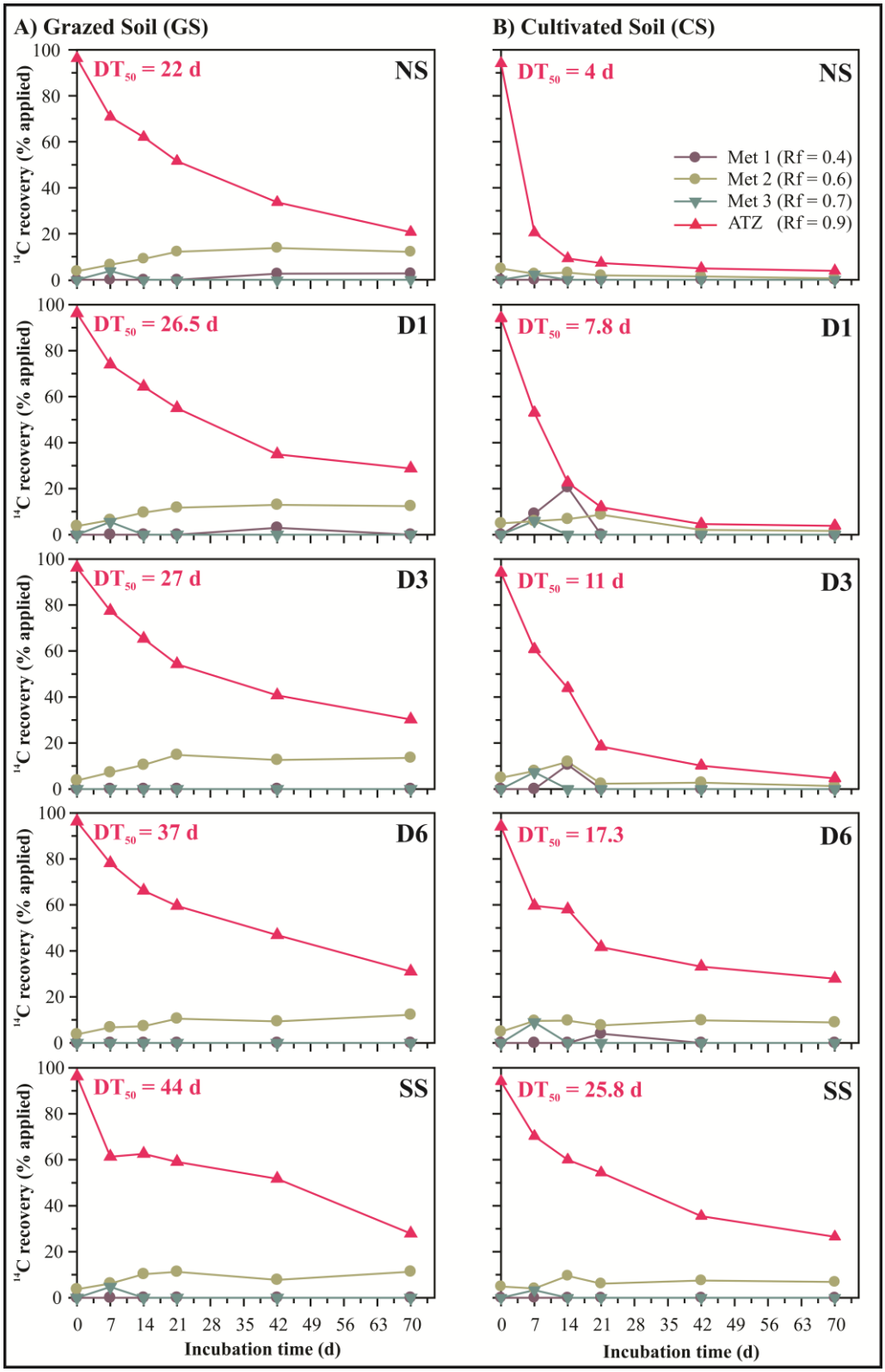

Figure 5. ^{14}C-atrazine, DT_{50} values and its metabolites (Met1, Met2, and Met3) in the grazed soil (GS, without long-term of atrazine application) (A) and Cultivated Soil (CS, with a long-term of atrazine application) (B) after 70 d of incubation (n = 2). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil. DT_{50} = degradation rate of 50 percent of the target compound (half-life), expressed in days.

Co-occurrence network analysis, Species Classification and FAPROTAX profile

Network co-occurrence analysis revealed the complexity of the connections between members of the bacteria community in the GS and CS with different levels of bacterial diversity depletion. The GS bacterial communities showed a lower degree of complexity and interactions than the CS bacterial communities. The complexity and interactions of the bacterial communities was greater in the GS-NS (nodes = 156 and Edges = 1947) and CS-NS (nodes = 172 and edges = 2725) treatments (Figure 6).

The GS bacterial community's complexity has been greatly reduced with the bacterial diversity depletion. This resulted in more simplified interaction networks, having as key groups (Betweenness centrality) members of Proteobacteria, Actinobacteria, Acidobacteria and Firmicutes. Whereas, CS communities showed a higher degree of complexity, as well as a greater number of key groups (Figure S4).

Multinominal species classification test (CLAM) showed the proportion of specialist, generalist, and rare groups in the NS, D1, D3, D6 and SS bacterial communities of the GS and CS (Figure 7). For GS, the proportions of specialist groups in NS versus D1, D3, D6 and SS were 51.1% versus 18.8%, 65.1% versus 13.7%, 72.4% versus 8.6%, and 54.5% versus 14.0%, respectively (Figure A). A similar trend was also observed in the NS versus D6 and NS versus SS contrasts in CS. Meanwhile, the NS versus D1 and NS versus D3 showed more balanced proportions of specialist groups between the contrasts (Figure 7B).

The sequences annotation in the FAPROTAX database revealed a total of 22 and 28 different functions in the GS and CS, respectively (Figure 8). NS treatments showed an increase in the function abundances (Z-score ranging from -1.8 to 1.8). Most abundant functions in the bacterial communities were those associated with energy obtaining (aerobic chemoheterotrophy, anoxygenic photoautotrophy, anoxygenic photoautotrophy sulfur oxidizing, photoautotrophy, photoheterotrophy, and phototrophy), nitrogen cycle (aerobic ammonia oxidation, aerobic chemoheterotrophy, aerobic nitrite oxidation, nitrate denitrification, nitrate denitrification, nitrate respiration, and nitrification) (Figure 8).

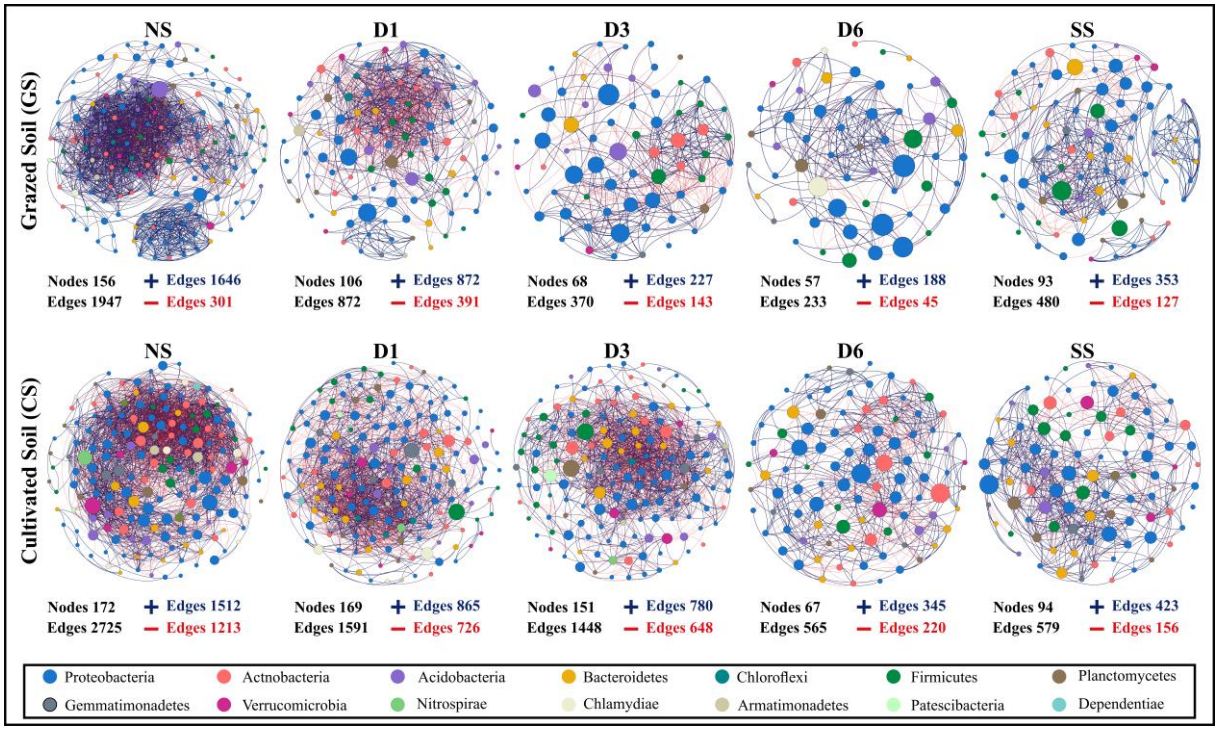

Figure 6. Network co-occurrence analysis of bacterial communities in the grazed soil (GS, without long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application) after 70 d of microcosm incubation. A connection stands for Spearman correlation with magnitude > 0.7 (positive correlation – blue edges) or < −0.7 (negative correlation – red edges) and statistically significant ($p < 0.05$). Each node represents taxa affiliated at phylum level, and the size of the node is proportional to the Betweenness centrality. Network properties are presented in table 3. NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

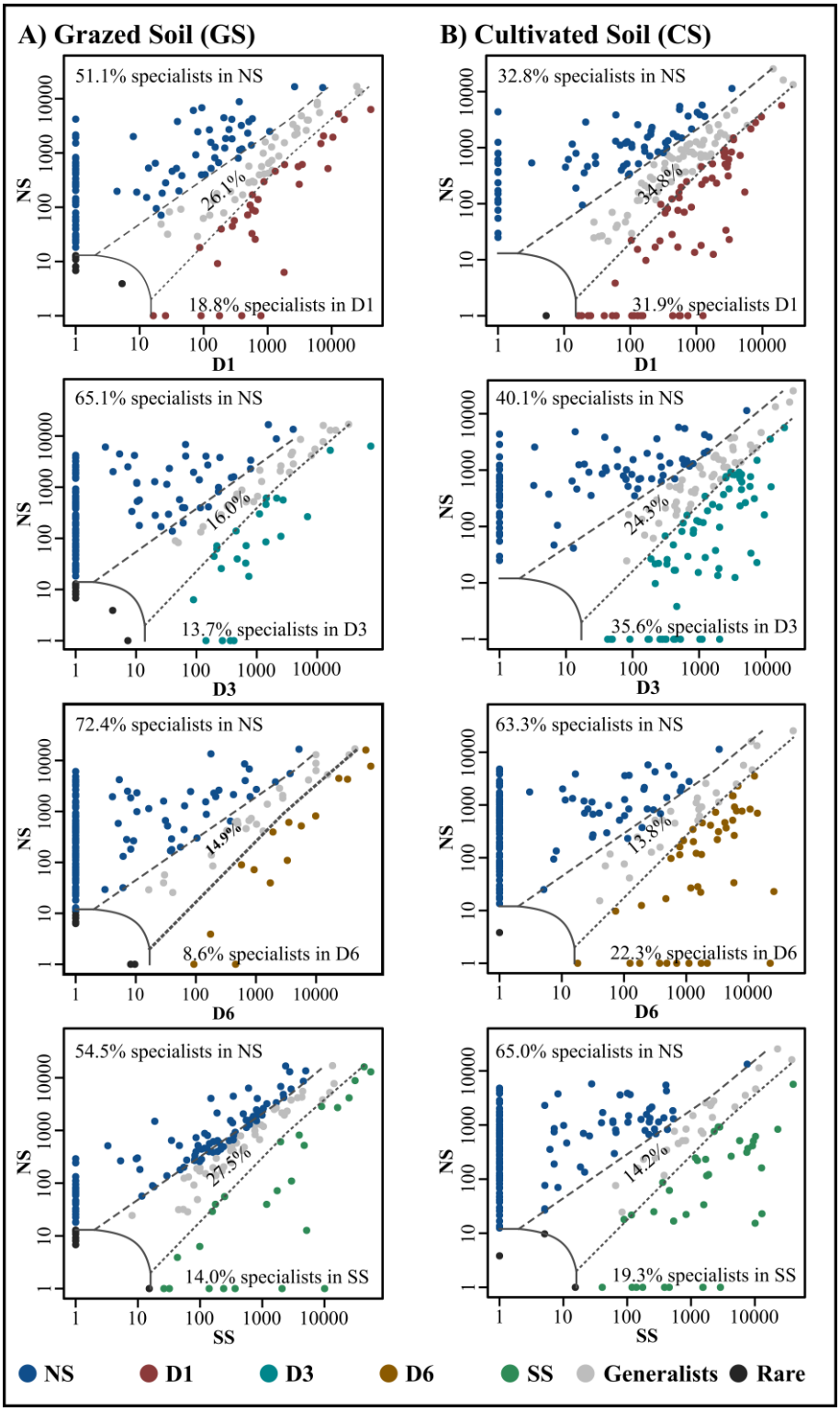

Figure 7. Multinominal species classification test (CLAM) for niche occupancy in the grazed soil (GS, without long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application). The pairwise treatment test were evaluated as following: NS bacterial versus D1, D3, D6 and SS bacterial communities. NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

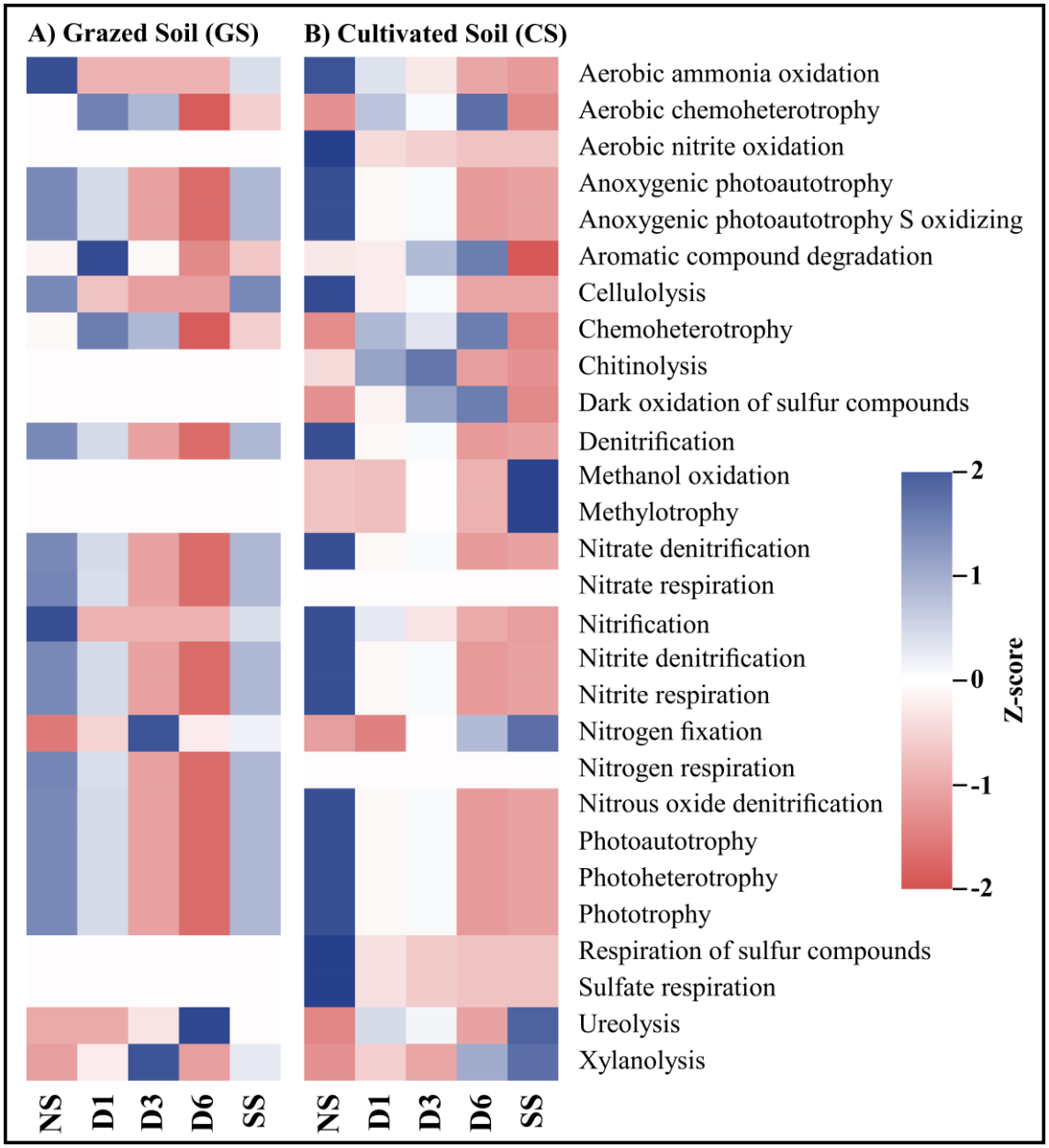

Figure 8. Heatmap showing the differential abundance of predicted functional categories in the grazed soil (GS, without long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application) along five bacterial diversity gradients (NS, D1, D3, D6, and SS) based on FAPROTAX database. The color key relates the heatmap colors to the standard score (z-score), which represents the deviation from row mean in units of standard deviation above or below the mean. NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

Relation between bacterial community and atrazine biodegradation

Long-term atrazine application has played a crucial role in the bacterial community's ability to breakdown the herbicide molecule in different bacterial diversity levels (Figure 9). GS bacterial community presented a smaller number of groups correlated with the atrazine mineralization at genus level (Figure 9A), as well as at family level (Figure S5). Top genera correlated with atrazine degradation in the GS were: *Anaeromyxobacter*, *Candidatus_Solibacter*, *Chitinophaga*, and *Inquilinus* ($-0.7 \leq R_{SPEARMAN}$ $^{14}C\text{-}CO_2 \geq 0.7$ and P value < 0.05) (Figure 9A). While the CS presented a bacterial community more effective in the degradation of atrazine. In total, 36 genera (of the 149 identified) and 32 families (of the 91 assigned to the sequences) correlated with the atrazine biodegradation rate ($-0.7 \leq R_{SPEARMAN}$ $^{14}C\text{-}CO_2 \geq 0.7$ and P value < 0.05), presenting a greater number of correlations between the abundance of these taxa and the emission of $^{14}C\text{-}CO_2$, which resulted in strong negative correlations with ER, NER, Met1, Met2, ATZ, and DT_{50} (Figure 9B). Twelve bacterial genera correlated more strongly ($-0.8 \leq R_{SPEARMAN}$ $^{14}C\text{-}CO_2 \geq 0.8$ and P value < 0.05) with ATZ mineralization, for example, *Ralstonia*, *Nitrospira*, *Hirschia*, *MND1*, *Candidatus_Koribacter*, *OM27_clade*, *Arenimonas*, *Occallatibacter*, *Bryobacter*, *UTBCD1*, *Ellin6067*, and *Crenobacter* (Figure 9B).

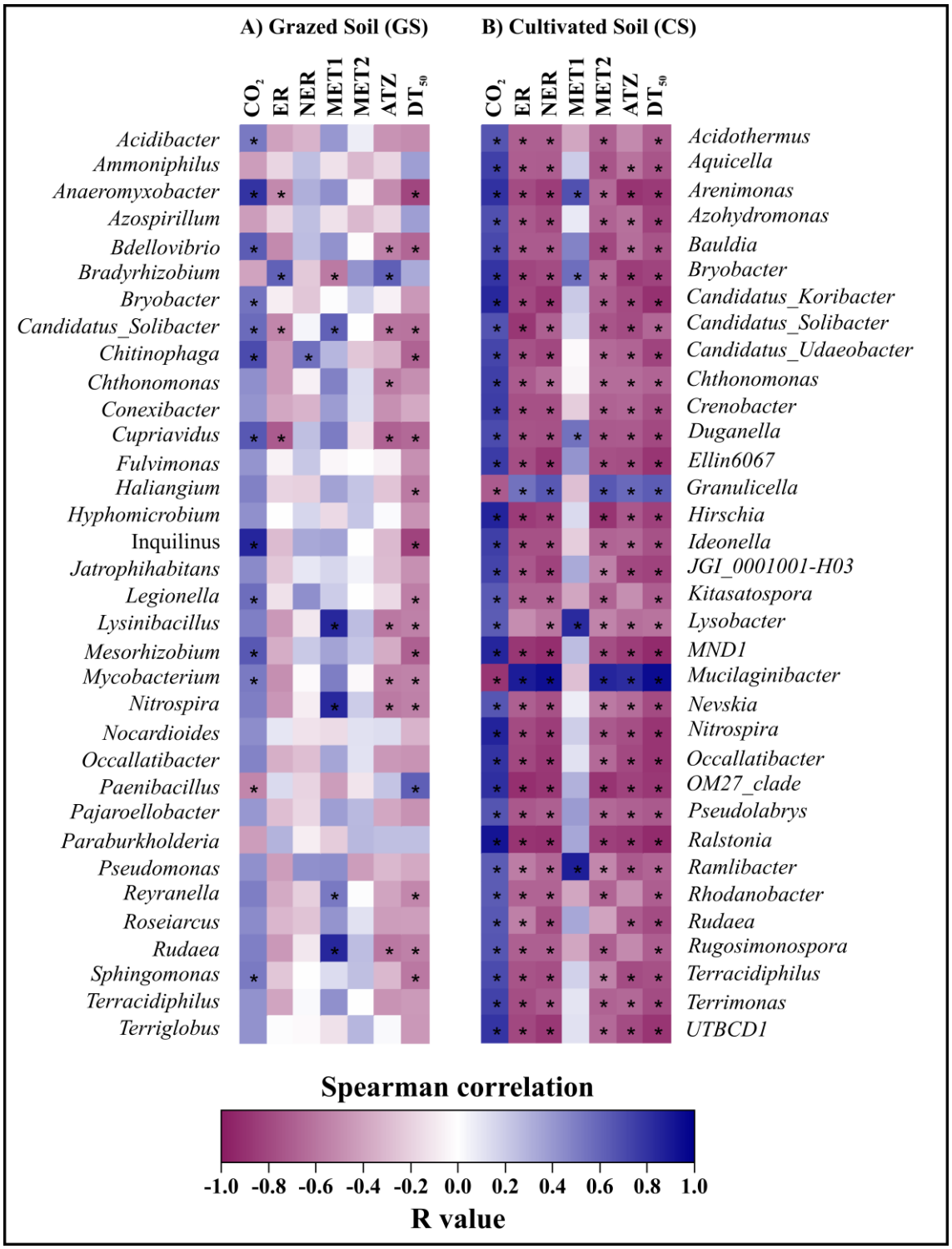

Figure 9. Spearman correlation matrix of soil bacterial community at Genus level and atrazine dissipation parameters in the grazed soil (GS, without long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application). *Significant correlation at P value < 0.05.

Discussion

Brazilian Cerrado is naturally composed by low fertility soils (Embrapa, 2018; Ngolo et al., 2019). Advances in conservation and cultivation practices, such as no-till and soil fertility correction via the input of lime and mineral fertilizers, made agriculture viable in this biome (Lemes et al., 2020; Spera et al., 2016). Compared to grazing (GS), cultivation (CS) and no-till system improved the physical-chemical attributes of the soil, as well as nutrient and organic matter accumulation (Mello and Van Raij, 2006; Nunes et al., 2018). It contributed to slightly higher ATZ sorption to the CS due to its higher OC, even though sorption was low in both soils ($K_{d\text{-}app}$ < 3 L kg^{-1}). Several studies have already reported ATZ low sorption even in tropical soils, having high metal (oxy)hydroxide contents (Inoue et al., 2006; Leal et al., 2019), in which it may reach surface and subsurface water bodies (Barizon et al., 2020; Singh et al., 2018). Land use and adopted agricultural management practices, such as pesticide, fertilizer, and corrective amendments to soils under no-till, also impacted soil bacterial community composition, structure, and bacterial diversity. Alpha-diversity of the bacterial community [Shannon-Weiner (H) and Simpson (1-D) indexes] showed a distribution pattern according to the disturbance degree promoted by the dilution-to-extinction approach (de Souza et al., 2021; Wertz et al., 2006). The diversity depletion effect was more accentuated in the GS, without occurrence of bacterial diversity recovery. Meanwhile, the CS showed a slight recovery of bacterial diversity (H), mainly in the D1 and D3 disturbance degrees in relation to NS. In general, the 1-D index showed greater variation among GS communities, suggesting a dominance of bacterial groups (Figure 2).

Pesticide repeated applications are an important modulator of microbial communities in the soil (Daam et al., 2019; Singh et al., 2018), modifying both diversity, composition, and structure, as well as the dominance of groups within soil communities with a long-term of pesticide use (Fan et al., 2020; Fang et al., 2015; Liu et al., 2020). Qu et al. (2018) reported a significant reduction in the bacterial alpha-diversity (H and 1-D) of sediment communities on acidic lakes that received the atrazine application. While Fang et al. (2015) observed depletion of bacterial diversity in the first atrazine application, regardless of the dose applied (1, 2 and 5 times the field dose). The authors also observed that the exposure time to the herbicide impacted the bacterial diversity, since 50 d after the 2nd and 3rd application, the H and 1-D indices showed increases in the treated communities when compared to the control, indicating that exposure repeated to the ATZ can promote an increase in the abundance of dominant bacterial

groups in the community. This fact corroborates our results since the CS bacterial diversity showed a faster recovery and was higher than GS (Figure 1).

The bacterial community composition (beta-diversity) was affected by land use (GS or CS and diversity depletion (Figure 2). The soil is a highly favorable environment for microbial life (Trivedi et al., 2019), since it has a high diversity and richness of species (Cameron et al., 2018; Maron et al., 2018), which are often distributed within the phyla Proteobacteria, Actinobacteria Acidobacteria, Planctomycetes, Chloroflexi, Verrucomicrobia, Bacteroidetes and Firmicutes (Delgado-Baquerizo et al., 2018). In the present study, the most abundant phyla were Proteobacteria > Bacteroidetes > Firmicutes > Actinobacteria > Acidobacteria > Verrucomicrobia > Planctomycetes > Gemmatimonadetes in both soils (GS and CS). The diversity disturbance levels also favored the dominance of Proteobacteria, Bacteroidetes and Firmicutes groups, with abundance increases in relation to NS and SS of ~ 51.8 – 72.8%, ~ 8.5 – 15.2% and 13.1 – 22.5%, respectively (Figure 2A). In the worst scenarios of bacterial diversity disturbance (D6 and SS), the phyla Armatimonadetes, Dependentiae, Chlamydiae, Nitrospirae were completely suppressed (Figure 2A). In fact, recent studies have shown a strong impact of the pesticide use on soil microbial communities (Daam et al., 2019; Feld et al., 2015; Singh et al., 2018).

Pesticides has a direct effect on the diversity, taxonomic and functional composition of microbial populations (Poursat et al., 2019; Singh et al., 2018), since most of these substances often do not occur in the environment and can cause deleterious effects to some members of the community or favor other microbial groups by eliminating competitors (Jacobsen and Hjelmsø, 2014). For example, Fernandes et al. (2020) reported that the bacterial community of the soil treated with atrazine had a dominance of Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi and Planctomycetes, without difference in their abundance in comparison with control (non-atrazine application). While Bhardwaj et al. (2020) reported a greater abundance of Proteobacteria (84–96% of total abundance) and Firmicutes (1–13% of total abundance) in contaminated soil mesocosms, observing a slight reduction in the abundance of these groups compared to the control treatment. Proteobacteria, Actinobacteria and Firmicutes were also the dominant groups in soils exposed to increasing doses of atrazine (3, 6 and 15 mg kg^{-1}) followed by repeated applications (Fang et al., 2015). The phyla Proteobacteria and Actinobacteria are bacterial groups widely distributed in the environment (Delgado-Baquerizo et al., 2018) and their members are known for their high biotechnological potential, acting in promoting plant growth, nutrient cycling, biological control, and pollutant degradation (Dube et al., 2019; Fierer et al., 2007).

In general, the most abundant families were Burkholderiaceae, Solirubrobacteraceae, Rhodanobacteraceae, Xanthobacteraceae, Rickettsiaceae, Chitinophagaceae, Micropepsaceae, Caulobacteraceae, Planococcaceae, Paenibacillaceae, and Acidobacteriaceae (Figure 2), with differential occurrence of Burkholderiaceae, Solirubrobacteraceae, Rhodanobacteraceae, Xanthobacteraceae, which presented significant abundance increasing (Figure S3 and S4). Soil microbiome is widely variable and responsive to spatial and temporal oscillations (Liu et al., 2018), in addition to management practices (Trivedi et al., 2019). In general, the systems of monoculture and continuous use of agrochemicals have contributed to the simplification of soil microbial communities (Landis, 2016), which results in the loss of taxonomic and functional diversity, as consequence the production capacity and soil quality are reduced (Cameron et al., 2018; Philippot et al., 2010). The dilution-to-extinction studies show that the effect of the disturbance is more drastic in groups of less abundant microorganisms (de Souza et al., 2017), whereas groups with high frequency have an advantage within more diluted communities (Wertz et al., 2006), soon present themselves as dominant groups, such as Burkholderiaceae, Solirubrobacteraceae, Rhodanobacteraceae, Xanthobacteraceae that occurred differently in D3, D6 and SS. Similar results were reported by Fernandes et al. (2020) that found significant increases at abundance of Enterobacteriaceae, Burkholderiaceae, Conexibacteraceae, Solirubrobacteraceae, and Gaiellaceae in samples treated with atrazine in relation to control samples.

Similar to our previous study, using the dilution-to-extinction approach and the antibiotic sulfadiazine as the target compound (de Souza et al., 2021), the atrazine application appeared to have a secondary effect on the bacterial community composition, especially in CS treatments. This result can be ratified by the bacterial community structuring in the NMDS analysis and clusters validation by PERMANOVA, which shows the segregation of treatments according to the bacterial diversity disturbance levels (NS > D1 > D3 > D6 > SS). In addition, the segregation pattern in CS communities was more uniform than in GS (Figure 3 and Table 2). This is possibly due to the CS community has bacterial groups already adapted to the herbicide. Therefore, the impact of the herbicide on the microbial population would be more attenuated when compared to the GS (Poursat et al., 2019), which will probably also reflect on the higher capacity of the microbial population to biodegrade the pesticide (Leal et al., 2019; Singh et al., 2018).

ATZ is characterized as a very mobile and bioavailable molecule in the soil (Hsieh et al., 2020; Jablonowski et al., 2010; Martinazzo et al., 2010), which was crucial feature to evaluate the role of bacterial diversity depletion in its biodegradation. Our previous studies with chlorothalonil – fungicide (de Souza et al., 2017) and sulfadiazine – antibiotic (de Souza et al.,

2021) showed low mineralization rates (8 - 12% of the applied dose) and the fast non-extractable residues (NER) formation was the main dissipation route for both molecules. On the other hand, atrazine presented a high mineralization rate and with a greater impact of the bacterial diversity depletion in the CS (Figure 4). These results confirmed our first hypothesis that the effect of bacterial diversity depletion would be minor in the cultivated soil and with long-term atrazine application.

ATZ mineralization rate was very lower in the GS, regardless of bacterial diversity disturbance degree, the $^{14}C\text{-}CO_2$ emitted was less than < 5.0% of the AR. While in the CS, the $^{14}C\text{-}CO_2$ emission rate was greater than 52.0% of RA in NS, presenting atrazine biodegradation recovery at D1 and D3 bacterial diversity depletions, which mineralized 46 and 44% of atrazine after 70 d (Figure 4). Soils with a long-term of atrazine application revealed enormous potential for biodegradation of this herbicide (Jablonowski et al., 2010; Khatoon and Rai, 2020; Krutz et al., 2009; Martinazzo et al., 2010; Sene et al., 2010).

Under conditions of temperate climate and soils with more than 30 y of annual atrazine applications, the mineralized amount of atrazine reached 83% of the AR, whereas in the soil without a history of herbicide use, the $^{14}C\text{-}CO_2$ emission rate was only 10.3% of the AR applied after 92 d of incubation (Jablonowski et al., 2010). A similar trend in atrazine mineralization has also been reported in soils in Brazil. After 85 d of incubation, the $^{14}C\text{-}CO_2$ emission rate was 74% and 82% of the atrazine applied to Rhodic Hapludox and Xanthic Haplustox soils with a long history of use versus less than 5.1% in controls with no history of application (Martinazzo et al., 2010). In a study recent, Leal et al. (2019) also reported a high atrazine biodegradation rate (~ 85% of the AR) in soil adapted to atrazine, which registered a mineralization rate about 8.5-fold higher than in non-adapted soil. These authors also observed a reduction from 85 to 31% in the emission of $^{14}C\text{-}CO_2$ when the straw was kept in the soil. However, these studies have not evaluated the soil microbiota or how the bacterial diversity impacts the dissipation of atrazine.

Considering the long-term of atrazine atrazine (CS) and the bacterial diversity disturbance degrees, we found significant differences in the DT_{50} values (table 1). In the CS-NS soil bacterial community, the DT_{50} was only 4 d against 22 d in GS-NS. Even in the intermediate scenarios (CS-D1 and CS-D3), the DT_{50} was still higher than in the best scenario of the GS bacterial community (NS). Pesticides repeated applications favor the microbial community adaptation, which xenobiotic results in the accelerated degradation (Poursat et al., 2019; Solomon et al., 2013), preventing its accumulation in the environment and mitigating its impact on non-target organisms (Leal et al., 2019; Martinazzo et al., 2010). Leal et al. (2019) reported

the atrazine half-life values based on the mineralization rate in the magnitude of 10 – 43 d for soils with a long-time of atrazine exposure. However, native soil (with no history of atrazine use) showed an extremely low degradation rate (< 1.0% of the applied dose), which made it impossible to estimate the half-life of atrazine. Fang et al. (2015) found a significant reduction in the atrazine half-life from repeated applications, in the first application (dose, two and five times the recommended dose) the magnitude of the half-life was 17.4, 19.1 and 20 d, with a reduction to 12.2, 11.7 and 9.4 from the third application.

The atrazine degradation profile in the extracts revealed the presence of three unidentified metabolites (degradation products) (Met1 – Rf = 0.4, Met2 – Rf = 0.6 and Met3 – Rf = 0.7), with concentrations in the 3 to 20% of the AR. Metabolite formation profile was quite similar in the NS, D1, D3, D6 and SS treatments of the GS and in the D6 and SS treatments of the CS. These results confirm our second hypothesis that the ability to degrade atrazine is extremely sensitive to the bacterial diversity depletion, especially in soils without long-term of herbicide use, but it also depends on the presence of microbial groups what presented ability to cleave the herbicide molecule. In the soil, the most frequently detected metabolites from the atrazine degradation are hydroxyatrazine (HA), deethylatrazine (DEA) and deisopropylatrazine (DIA) (Chowdhury et al., 2021; Solomon et al., 2013). HA was the predominant metabolite both in soils with 10 - 20 y of history atrazine application and in soils with no history, being detected in concentrations close to 30 – 40% of the AR (Jablonowski et al., 2010; Leal et al., 2019). Similarly, Peixoto et al. (2000) also detected hydroxyatrazine as the main degradation product of atrazine, which must correspond to the Met2 in our study.

The low atrazine mineralization rates in GS treatments and in CS-D6 and CS-SS resulted in the quickly non-extractable residue formation (NER), which represented the main route of herbicide dissipation (Figure S5). The long-term of herbicide use is a key factor in the soil microbiota ability to degrade herbicide molecule (Hsieh et al., 2020; Jablonowski et al., 2010). In general, repeated applications exert a selection pressure on the soil's microbial population, favoring groups capable of tolerating the pollutant presence and consequently encoding enzymes capable of partially or totally cleaving the pesticide molecule (Singh et al., 2018; Yang et al., 2010; Zhang et al., 2019). In the adapted microbial groups absence and with ability to degrade the target-compound, other dissipation routes assume a crucial role in pesticide disappearance (Poursat et al., 2019), as reported for GS and the extreme scenarios bacterial diversity loss in CS.

The network analysis revealed a strong simplification of bacterial communities, with a reduction in the number of nodes and edges according to the level of depletion of bacterial

diversity in both soils (GS and CS) (Figure 6). Similar effects were observed in the species classification by niche occupation, with a huge imbalance in the occupation of niches (specialists, generalists and too rare) in all treatments of GS and in the worst CS disturbance scenarios (D6 and SS) (Figure 7). Meanwhile, a total of 28 potential functions were reported from FAPROTAX, with emphasis on functions associated with obtaining energy and nitrogen transformations (Figure 8). Simulations of diversity depletion by dilution-to-extinction mimic the effects of natural events (i.e. drought, inhumations, fires) and anthropogenic disturbances (i.e. pesticides, manure and fertilizer mineral applications, change of land use) on the microbial communities (de Souza et al., 2021; Landis, 2016; Reed and Martiny, 2013).

Through this approach, we observed a strong impact on the complexity of the bacterial community, as well as on the niche occupation and potential functions in both soils. This translated into lower communities' ability to dissipate atrazine, in the GS, CS-D6 and CS-SS. The atrazine is a halogenated aromatic compound what has key step the chlorine atom removal from its molecule (Singh et al., 2018), which requires the presence of specialized microbial groups capable of producing chlorohydrolase enzyme (de Souza et al., 1996; Solomon et al., 2013), which trigger the initial steps its biodegradation. In this case, bacterial population adaptation is a crucial factor, as observed in our results (Figures 4). In addition, the more balanced distribution of specialist groups among the CS-NS, CS-D1 and CS-D3 communities may have favored the molecule dissipation, ratifying that the atrazine biodegradation requires a greater amount of bacterial specialized groups (Feld et al., 2015; Henn et al., 2020).

The functions profile identified from FAPROTAX were impacted by land use and long-term of atrazine application, especially in GS, which presented a reduction in the abundance of functions related to obtaining energy. Pertile et al. (Pertile et al., 2021) observed that application of the herbicides imazethapyr and flumyzin promoted strong changes in the soil functional profile, with a significant increase in the chemoheterotrophy, aerobic chemoheterotrophy functions in the bacterial communities of treated soils in comparison to the control (without herbicide). The increasing in chemoheterotrophic microbial groups may indicate that bacterial communities in adapted environments have a greater capacity to degrade xenobiotic compounds (Fang et al., 2015; Li et al., 2020; Pertile et al., 2021). While in our study there was a significant increase in functions associated with photoautotrophy, photoheterotrophy and metabolism of nitrogen compounds. The increase in abundance of photoautotrophic microbial groups in the community of environments contaminated by xenobiotics may be an indication of the predominance of co-metabolism during its biodegradation (Pertile et al., 2021), especially in the initial steps of the degradation route (de Souza et al., 1996; Henn et al., 2020; Solomon et

al., 2013), where active compound has higher harmful effect in microbial groups (Feld et al., 2015; Singh and Singh, 2014).

For atrazine, the microbial groups presence in the soil that have the *atzA* and *trzN* genes is crucial, since these genes encode chlorohydrolase enzymes that cleave the chlorine atom, facilitating the next steps of atrazine biodegradation (Solomon et al., 2013), this initial steps possibly occur by co-metabolism (Singh et al., 2018). As result of the atrazine mineralization are formed CO_2 and NH_3 (Sene et al., 2010; Singh and Singh, 2014). Therefore, atrazine must have a direct effect on the N cycle (Sene et al., 2010; Sharma et al., 2019), which is confirmed by the increase in functions linked to N metabolism, mainly in the CS-NS, where ~ 52% of the atrazine was mineralized.

The correlation of bacterial community composition with atrazine degradation parameters revealed potential atrazine degraders in both soils, with higher number of bacterial genera correlated with atrazine mineralization in CS (Figure 9). In the soil with history of atrazine use, Fang et al. (2015) reported seven potential atrazine-degrading genera, *Nocardioides, Arthrobacter, Bradyrhizobium, Burkholderia, Methylobacterium, Mycobacterium*, and *Clostridium*. While Fan et al. (2020) reported 12 potential atrazine-degrading bacterial genera in mesocosms treated with atrazine, which included genera *Arthrobacter, Bosea, Bradyrhizobium, Burkholderia, Methylobacterium, Mycobacterium, Nocardioides, Pseudomonas, Rhizobium, Rhodobacter, Shinella, and Streptomyces*. In our work, CS also presented the highest number of bacterial genera correlated with ATZ mineralization, and the largest genera observed has not yet been reported as atrazine degraders in the literature.

Conclusions

The bacterial community was impacted by the history of herbicide use, as well as by the bacterial diversity disturbance levels. The dominant groups in the communities of both soils without and with long-term of atrazine application were Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Acidobacteria, Verrucomicrobia, Planctomycetes and Gemmatimonadetes. The bacterial communities of soil without long-term of atrazine application were more impacted by bacterial diversity the depletion. Meanwhile, the communities of soil with a long-term of atrazine use were more resilient to the bacterial diversity disturbance. This resulted in a greater ability to mineralize atrazine from the NS-CS, D1-CS and D3-CS communities and lower DT_{50} values and the fast formation of three unidentified metabolites. The bacterial diversity disturbance gradient reduced the complexity

of the communities, as well as promoted an imbalance in the distribution of species during the occupation of niches and increases the abundance of functions associated with autotrophy and N cycling. Bacterial communities of adapted soils showed a greater number of microbial groups associated with atrazine biodegradation, with a total of 12 potential genera of atrazine-degrading bacteria.

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Supplementary material

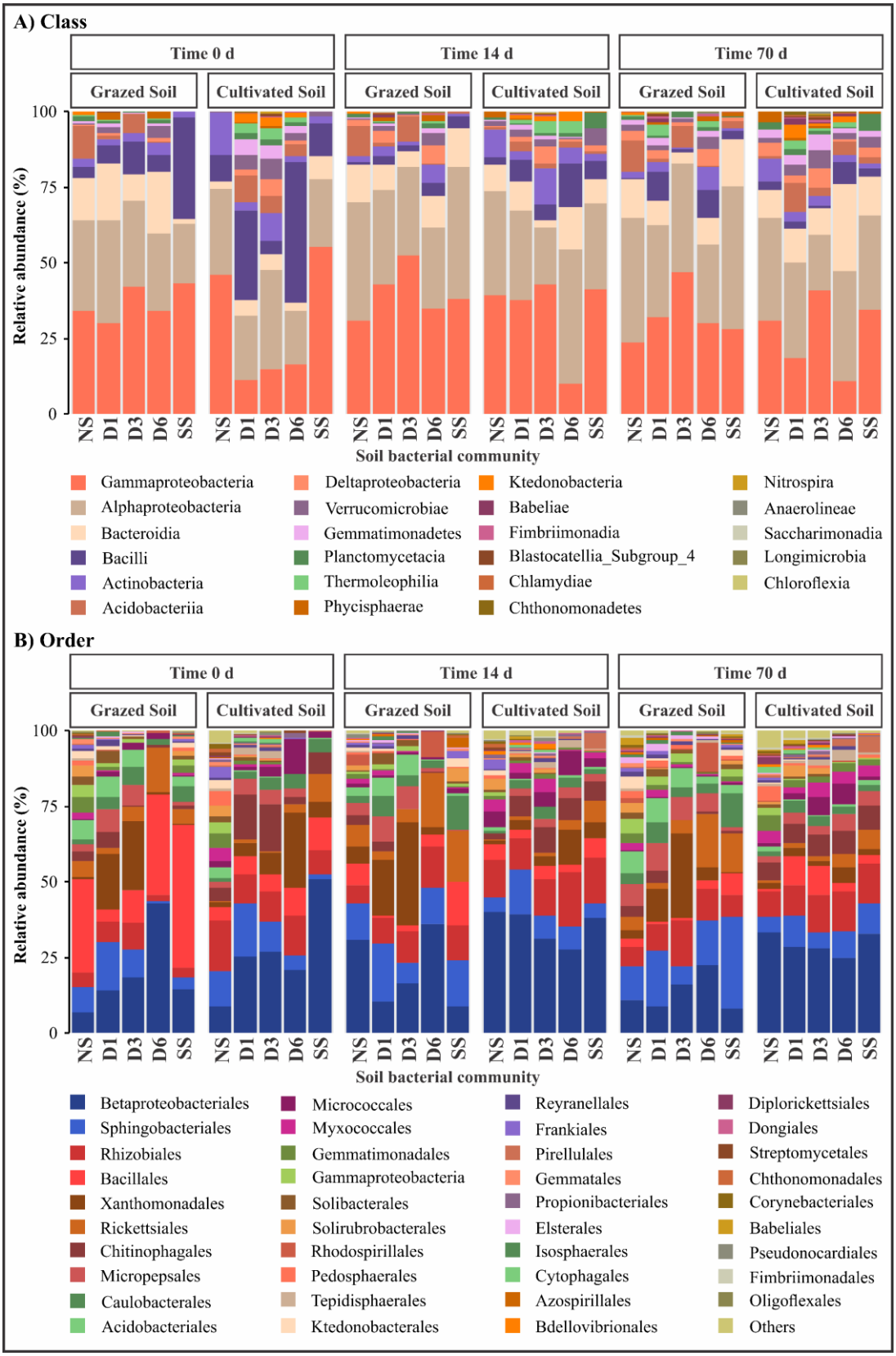

Figure S1. Relative abundance of the bacterial communities at top Class (A) and Order (B) levels in the grazed soil (GS, without long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application) at 0, 14, and 70 d of incubation (n = 3). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

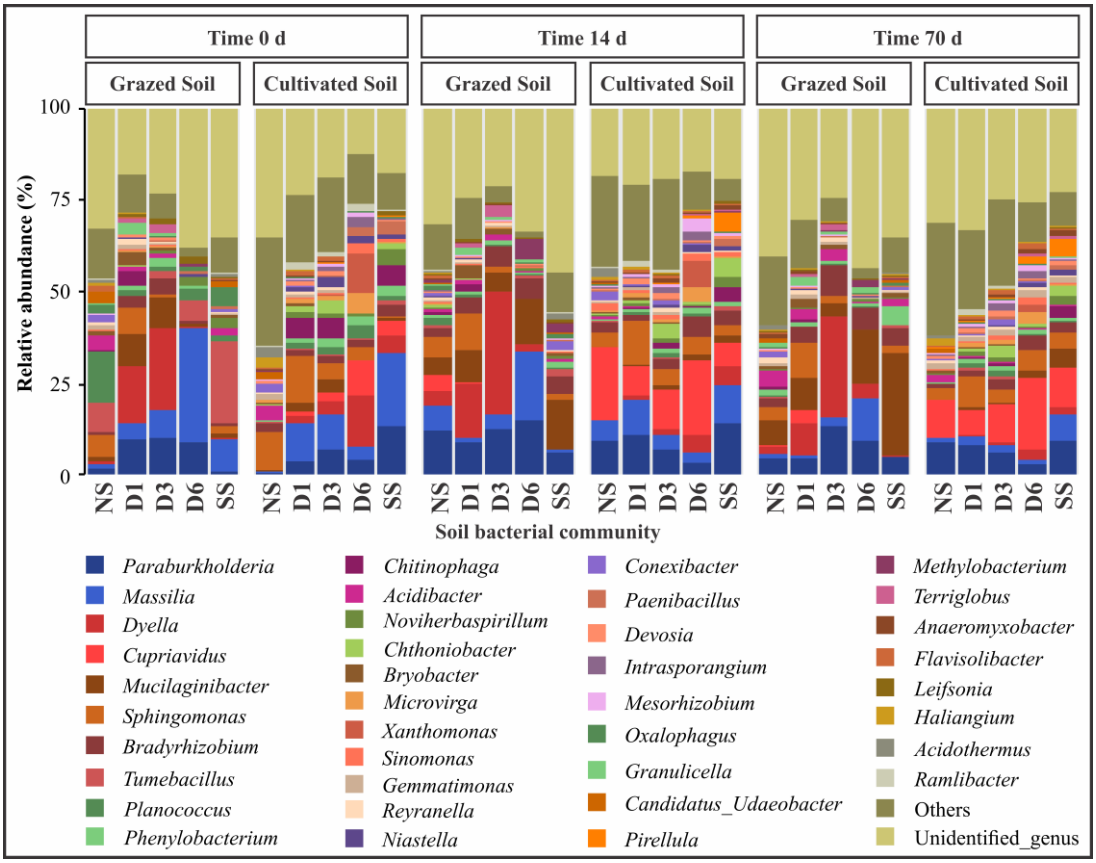

Figure S2. Relative abundance of the bacterial communities at top genus in the grazed soil (GS, without long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application) at 0, 14, and 70 d of incubation (n = 3). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

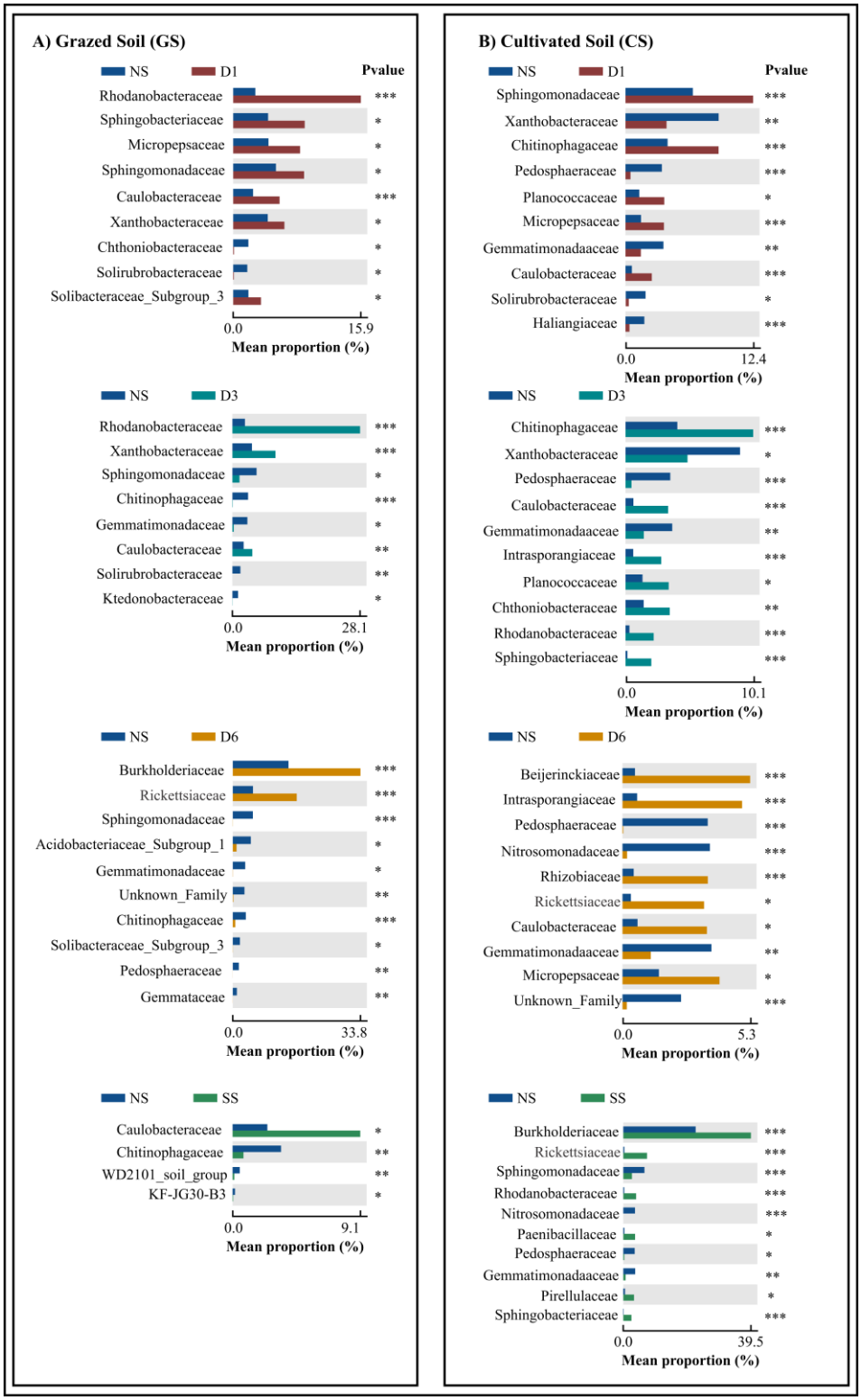

Figure S3. Differential abundance of bacterial community in the grazed soil (GS, without long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil. Two groups comparison by Welch's test at $\alpha < 5\%$. Significant P values rank: *** = 0.001; ** = 0.01; and * = 0.05.

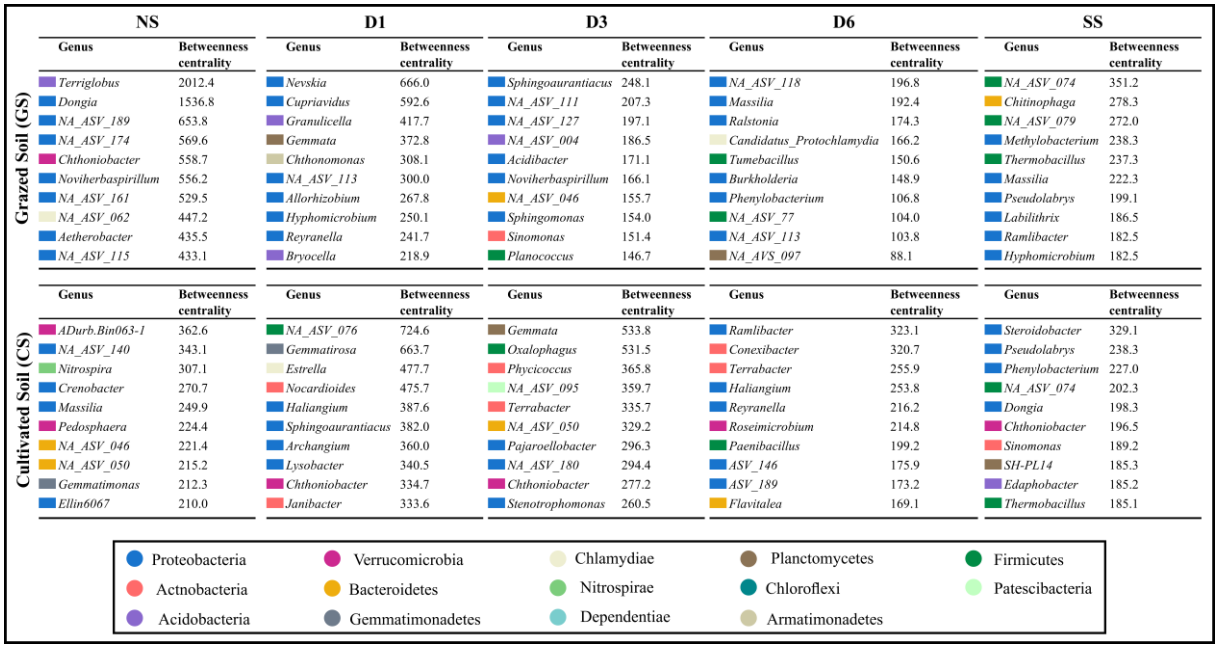

Figure S4. Top 10 betweenness centrality of bacterial community in the grazed soil (GS, without long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application). NS = natural soil; D1, D3, and D6 = dilutions 10^{-1}; 10^{-3}, and 10^{-6}; and SS = sterile soil.

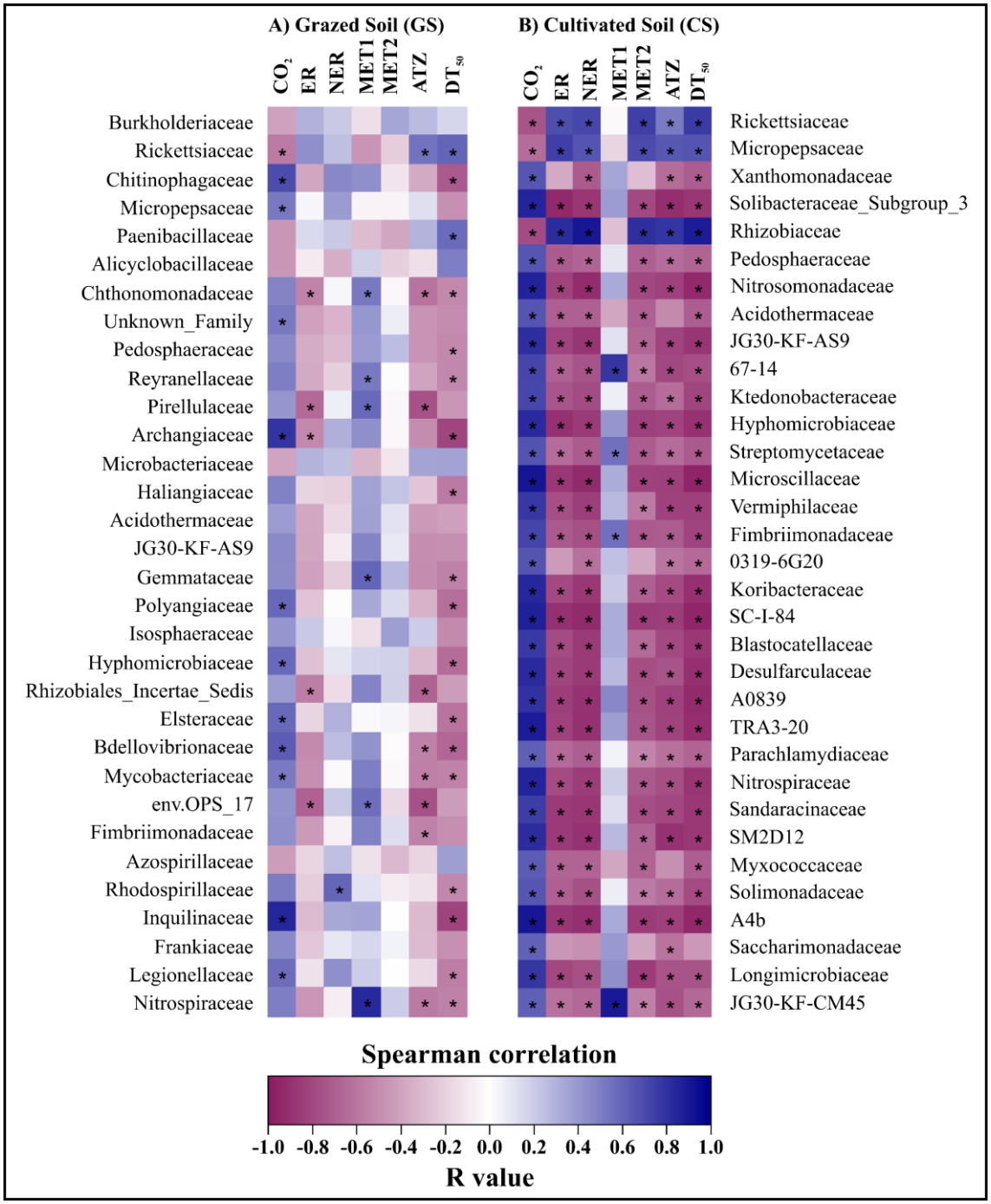

Figure S5. Spearman correlation matrix of soil bacterial community at Family level and atrazine dissipation parameters in the grazed soil (GS, without long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application).

Table S1. 16S rRNA sequencing parameters and reads quality of the bacterial community in the grazed soil (GS) and Cultivated Soil (CS) after 70 d of incubation.

Soil	Dilutions	Input	Filtered	DenoisedF	DenoisedR	Merged	Non chimera	Q score
0 d after atrazine application								
GS	NS	37,664	37,495	35,690	35,676	32,945	32,327	37.0
	D1	32,344	32,239	31,583	31,562	30,585	30,471	37.1
	D3	31,415	31,314	30,870	30,905	30,236	30,098	37.2
	D6	52,370	52,183	51,640	51,779	50,909	49,097	37.0
	SS	40,101	39,924	38,841	38,926	37,261	36,494	37.0
CS	NS	31,750	31,608	27,896	27,930	23,011	22,944	37.0
	D1	30,124	30,022	28,369	28,512	26,375	26,375	37.2
	D3	47,977	47,808	46,582	46,652	44,456	44,212	37.1
	D6	46,556	46,377	45,941	45,959	45,376	44,802	37.1
	SS	44,600	44,445	43,933	43,982	43,258	42,644	37.0
14 d after atrazine application								
GS	NS	31,495	31,403	30,475	30,523	29,061	28,937	37.1
	D1	40,886	40,738	40,006	39,995	38,782	38,512	37.2
	D3	34,318	34,179	33,754	33,734	33,031	32,641	37.1
	D6	37,147	37,030	36,672	36,623	36,112	35,575	37.1
	SS	40,492	40,354	39,243	39,241	37,551	37,302	37.1
CS	NS	35,777	35,627	32,355	32,398	28,637	28,492	36.9
	D1	36,551	36,408	34,697	34,807	32,452	32,360	37.0
	D3	36,772	36,632	35,430	35,429	33,429	33,330	37.0
	D6	28,900	28,805	28,430	28,416	28,028	27,745	36.9
	SS	27,221	27,128	26,726	26,776	26,213	26,058	36.9
70 d after atrazine application								
GS	NS	37,682	37,533	35,656	35,710	32,601	32,407	37.0
	D1	38,540	38,386	37,513	37,497	35,857	35,696	37.1
	D3	31,016	30,904	30,406	30,469	29,674	29,352	37.1
	D6	50,552	50,390	49,806	49,826	49,171	48,922	37.1
	SS	45,666	45,507	44,363	44,435	42,538	42,147	37.2
CS	NS	35,776	35,615	32,229	32,317	28,074	28,021	37.0
	D1	33,550	33,432	31,650	31,834	29,240	29,201	37.0
	D3	42,535	42,336	40,859	40,988	38,348	38,230	36.6
	D6	35,118	34,964	34,392	34,438	33,772	33,664	37.0
	SS	36,241	36,119	35,470	35,573	34,815	34,383	37.0
Total		1,131,139	1,126,906	1,091,477	1,092,912	1,041,797	1,032,439	
Median		36,662	36,520	35,450	35,501	33,230	32,985	37.0
Mean		37,705	37,564	36,383	36,430	34,727	34,415	37.0

GS = Grazed Soil; CS = Cultivated Soil; NS = Natural soil; D1, D3, and D6 = Dilutions 10^{-1}, 10^{-3} and 10^{-6}; SS = Sterile soil.

Table S2. Permutational Multivariate Analysis of Variance (PERMANOVA) using a Monte Carlo test arrangement with 999 permutations and p-value MC < 0.001.

Factors	F test	[(1)] p-value MC
Bacterial community in Grazed Soil (GS)		
Bacterial diversity depletion (BD)	12.75	0.001
Incubation time (IT)	4.23	0.001
BD vs IT	1.74	0.001
NS vs D1	16.67	0.001
NS vs D3	21.74	0.001
NS vs D6	14.43	0.007
NS vs SS	6.84	0.001
D1 vs D3	24.89	0.001
D1 vs D6	25.20	0.001
D1 vs SS	12.69	0.001
D3 vs D6	17.93	0.001
D3 vs SS	11.51	0.001
D6 vs SS	3.78	0.008
Bacterial community in Cultivated Soil (CS)		
Bacterial diversity depletion (BD)	11.11	0.001
Incubation time (IC)	3.76	0.001
BD vs IT	1.66	0.006
NS vs D1	32.13	0.001
NS vs D3	28.79	0.001
NS vs D6	11.11	0.001
NS vs SS	19.96	0.001
D1 vs D3	9.81	0.001
D1 vs D6	8.13	0.001
D1 vs SS	13.70	0.001
D3 vs D6	5.38	0.003
D3 vs SS	9.89	0.001
D6 vs SS	5.12	0.001

[(1)] p-value MC = p value of Monte Carlo test. NS = Natural Soil; D1, D3, and D6 = dilutions 10^{-1}, 10^{-3} and 10^{-6}; SS = sterile soil.

Table S3. Impact of bacterial diversity depletion on the atrazine mineralization in the grazed soil (GS, without long-term of atrazine application) and Cultivated Soil (CS, with a long-term of atrazine application).

Bacterial diversity disturbance	**Mineralization capacity decrease[(a)]**
Grazed Soil (GS)	
D1 vs NS	2.3
D3 vs NS	2.7
D6 vs NS	2.9
SS vs NS	3.0
Mean	2.5
Cultivated Soil (CS)	
D1 vs NS	1.1
D3 vs NS	1.2
D6 vs NS	31.2
SS vs NS	34.3
Mean	16.9
GS vs CS	
NS vs NS	11.8
D1 vs D1	25.0
D3 vs D3	27.7
D6 vs D6	1.1
SS vs SS	1.0
Mean	13.3

[(a)] represents the number of times that atrazine mineralization was reduced after 70 days of incubation. NS = natural soil; D1, D3 and D6 = soils diluted 10^{-1}, 10^{-3} and 10^{-6}; SS = sterile soil.

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4. FINAL REMARKS

For sulfadiazine assay:

- Swine manure application changed completely soil bacterial community structure, but only slightly enhanced its diversity.
- The most abundant phyla in both control soils (NS) were Proteobacteria, Chloroflexi, Actinobacteria, Bacteroidetes, Planctomycetes, Acidobacteria, and Firmicutes, whereas Proteobacteria, Actinobacteria, and Chloroflexi predominated in the perturbed soils.
- Despite these abrupt changes, SDZ dissipation was quite similar in the manured and non-manured soils and NER was the primary route of SDZ dissipation. As a matter of fact, SDZ dissipation was somewhat slower in the manured soil at longer run (DT_{90} = 20 versus 2.1 d) that was supposed to have more adapted soil microbes, thus contradicting our hypothesis.
- The non-amended soil had a greater number of bacterial families that correlated with NER formation and SDZ mineralization, such as Isosphaeraceae, Ktedonobacteraceae, Acidobacteriaceae_(Subgroup_1), Micromonosporaceae, and Sphingobacteriaceae. However, soil bacterial diversity depletion decreased SDZ mineralization (~2-3 times) and initial formation of NER (~1.5-2.5 times).
- SDZ mineralization is a low redundancy soil function, but it is not the case for NER.
- Long term manure application decreased SDZ dissipation.
- If SDZ chemical degradation does happen instantaneously, its biodegradation immediately followed it.
- SDZ biodegradation is favored by a pool of soil bacteria and the newly formed metabolites should favors NER formation since SDZ presented low sorption.
- The depletion in soil bacterial diversity due to soil perturbation is only a transient effect, and most SDZ dissipation recovers up to 42 d. However, this period may be enough to spread resistance genes into the environment.

For atrazine assay:

- The bacterial community was impacted by the history of herbicide use, as well as by the bacterial diversity disturbance levels.
- The dominant groups in the communities of both soils without (GS) and with (CS) long-term of atrazine application were Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Acidobacteria, Verrucomicrobia, Planctomycetes and Gemmatimonadetes.

- The bacterial communities of soil without long-term of atrazine application were more impacted by bacterial diversity the depletion.
- Meanwhile, the communities of soil with a long-term of atrazine use were more resilient to the bacterial diversity disturbance. This resulted in a greater ability to mineralize atrazine from the NS-CS, D1-CS and D3-CS communities and lower DT_{50} values and the fast formation of three unidentified metabolites.
- The bacterial diversity disturbance gradient reduced the complexity of the communities, as well as promoted an imbalance in the distribution of species during the occupation of niches and increases the abundance of functions associated with autotrophy and N cycling.
- Bacterial communities of adapted soils showed a greater number of microbial groups associated with atrazine biodegradation, with a total of 12 potential genera of atrazine-degrading bacteria.