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Dissipation of *S*-metolachlor and butachlor in agricultural soils and responses of bacterial communities: Insights from compound-specific isotope and biomolecular analyses

Running title: Chloroacetanilide dissipation and bacterial communities in laboratory soil microcosms

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Abstract

The soil dissipation of the widely used herbicides *S*-metolachlor (SM) and butachlor (BUT) was evaluated in laboratory microcosms at two environmentally relevant doses (15 and 150 µg/g) and for two agricultural soils (crop and paddy). Over 80% of SM and BUT were dissipated within 60 and 30 days, respectively, except in experiments with crop soil at 150 µg/g. Based on compound-specific isotope analysis (CSIA) and observed dissipation, biodegradation was the main process responsible for the observed decrease of SM and BUT in the paddy soil. For SM, biodegradation dominated over other dissipation processes, with changes of carbon isotope ratios ($\Delta\delta^{13}\text{C}$) of up to 6.5‰ after 60 days, and concomitant production of ethane sulfonic acid (ESA) and oxanilic acid (OXA) transformation products. In crop soil experiments, biodegradation of SM occurred to a lesser extent than in paddy soil, and sorption was the main driver of apparent BUT dissipation. Sequencing of the 16S rRNA gene showed that soil type and duration of herbicide exposure were the main determinants of bacterial community variation. In contrast, herbicide identity and spiking dose had no significant effect. In paddy soil experiments, a high (4:1, V/V) ESA to OXA ratio for SM was observed, and phylotypes assigned to anaerobic Clostridiales and sulfur reducers such as Desulfuromonadales and Syntrophobacterales were dominant for both herbicides. Crop soil

microcosms, in contrast, were associated with a reverse, low (1:3, V/V) ratio of ESA to OXA for SM, and Alphaproteobacteria, Actinobacteria, and Bacillales dominated regardless of the herbicide. Our results emphasize the variability in the extent and modes of SM and BUT dissipation in agricultural soils, and in associated changes in bacterial communities.

Keywords: Chloroacetanilide herbicides; Carbon isotope fractionation; Pesticide degradation; Bacterial diversity

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Introduction

Chloroacetanilides represent one of the top ten pesticide classes used worldwide (FAOSTAT, 2013). They are widely used as selective, pre-emergent suppressors to control a variety of broadleaf weeds and annual grasses in crops, and represent 4.2% of global pesticide use (Pereira et al., 2009; Fenner et al., 2013). *S*-metolachlor (SM), in particular, is one of the most intensively used chloroacetanilides in the EU and the US. Butachlor (BUT), in contrast, is mostly used in South America and Asia (Kim et al., 2013). Both SM and BUT are persistent in soil. Together with their transformation products, mainly ethane sulfonic acid (ESA) and oxanilic acid (OXA) derivatives (Baran and Gourcy, 2013), they represent a risk for downstream non-target ecosystems. The extent of their transport to aquatic ecosystems mainly depends on dissipation processes in soil. However, knowledge on the fate of SM and BUT in soil is still limited, especially in relation to non-target soil organisms such as bacteria, some of which may also contribute to dissipation by degrading these compounds.

Several exploratory investigations of the response of soil bacterial communities to chloroacetanilide herbicides and their transformation products have been reported (Chen et al., 2003, 2009; Min et al., 2001; Saha et al., 2012; Zhou et al., 2012, Elsayed et al., 2015). The few bacterial isolates capable of degrading SM and BUT that have been reported (e.g., Liu et al., 1999; Kim et al., 2013) have not been characterized in terms of the enzymes and corresponding genes associated with chloroacetanilide degradation. However, the advent of high-throughput DNA sequencing allows exploration of the relationship between pesticide exposure of soil ecosystems and the soil microbial compartment in renewed detail (Imfeld and Vuilleumier, 2012; Aravindraja et al., 2013). This approach may provide a better understanding of microbial diversity in contaminated soils, including the capacity of microorganisms for pesticide degradation (Cycoń et al., 2017), and the influence of pesticide

exposure. Nevertheless, a reference microbial ecotoxicological evaluation of soils contaminated with chloroacetanilide herbicides is still lacking.

In this context, compound-specific isotope analysis (CSIA) of pesticides in soil may contribute to teasing apart degradative dissipation processes from non-degradative phenomena including sorption, dilution and offsite transport (Elsner and Imfeld, 2016; Alvarez-Zaldívar et al., 2018). Chloroacetanilide pesticides feature both light and heavy stable isotopes of their constitutive elements (e.g., C, H, and N). Degradative processes result in enrichment of heavier isotopes in the residual, non-degraded fraction of the molecules of interest (Elsner et al., 2012). In contrast, non-degradative dissipation processes generally do not result in significant isotope fractionation (Schmidt et al., 2004; Alvarez-Zaldívar et al., 2018). In soil environments, CSIA has recently been applied to characterize the degradation of several pyrethroid herbicides (Xu et al., 2018) and of the fungicide metalaxyl (Masbou et al., 2018b). CSIA has been used to characterize dissipation of chloroacetanilide herbicides in wetlands (Elsayed et al., 2014) and at the catchment scale (Alvarez-Zaldívar et al., 2018), but so far not specifically for soil.

Here, we designed a laboratory microcosm study on two agricultural soils to study SM and BUT dissipation and associated bacterial communities. The soils originate from two regions with distinct agriculture systems and agricultural practices, and have contrasting characteristics, including water content. The objective of this study was to evaluate and compare, using CSIA and 16S rRNA amplicon sequence analysis under controlled laboratory conditions, (1) the contribution of processes responsible for SM and BUT dissipation in soil, and (2) soil bacterial communities in relation to herbicide physico-chemical properties, levels of SM and BUT application, and exposure time.

1 Materials and methods

1.1 Chemicals

Chloroacetanilide standards of SM and BUT (analytical grade, purity: 99.6%), metolachlor ESA and OXA and butachlor ESA (purities: 98%, 98% and 96.9%, respectively), metolachlor-*d*₁₁, alachlor-*d*₁₃ (analytical grade purity: > 97%), dichloromethane (DCM), acetonitrile (ACN), ethyl acetate (EtOAc) (HPLC grade, purity: > 99.9%) and anhydrous magnesium sulfate (reagent grade: > 97%) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). SM and BUT standards were used for both quantification and isotopic analysis. Butachlor OXA (analytical grade purity: 99.4%) was obtained from Cluzeau Info Labo (CIL, Sainte-Foy-La-Grande, France). Physico-chemical properties of SM and BUT are

provided as Appendix A Table S1. Stock solutions of SM and BUT for soil spiking and standards for chromatographic analysis were prepared in DCM and ACN, respectively. S-metolachlor ESA and OXA were dissolved in ACN, and butachlor ESA and OXA were prepared in DCM, as 1 g/L stock solutions. Aliquots were stored at -20°C. Primary-secondary amine (PSA) was supplied by Supelco (Bellefonte, PA, USA).

1.2 Experimental soils

Soil samples were collected on August 6-7, 2015 and December 31, 2014, respectively, from two crop and paddy soils with different agricultural practices and histories of SM and BUT application. Soil sampling and physico-chemical characterization of soils are detailed in Appendix A Section S2. Samples of a silty-clay arable crop soil were obtained from a well-investigated field site at Alteckendorf, Alsace, France (see Lefrancq et al., 2017 for site characteristics). This site has been cultivated with sugar beet, corn and wheat, and SM has been applied for several years, while BUT has never been used.

Samples of a clayey paddy soil were collected from the rice farming area near the city of Rasht, Guilan Province, Iran (longitude: 49°31'56.29"E; latitude: 37°13'37.02"N; altitude: 13 m) on the southern coastline of the Caspian Sea (Appendix A Section S2). Along with BUT (application rate of 4 L/ha; Armanpour and Bing, 2015), the organophosphate insecticide diazinon (30 kg/ha) and the fungicide edifenphos (1 L/ha) are the most currently applied pesticides in the area (Alighardashi et al., 2017), whereas application of SM has not been reported.

1.3 Experimental set-up and operations

Three series of soil experiments were set up in (1) non-sterile (biotic) and (2) sterile (abiotic) soil spiked with the herbicides at two concentration levels of 15 and 150 µg/g (spiked soil experiments) to evaluate herbicide dissipation, and (3) in non-sterile soil adjusted with sterile-filtered deionized water (non-spiked soil experiments), as controls to evaluate the effect of experimental conditions and incubation on bacterial communities. The experimental design is summarized in Appendix A Section S3. For sterile spiked experiments, soil was autoclaved three times at 24-hr intervals.

Soil microcosms consisted of about 20 g (crop) or 10 g (paddy) air-dried soil in 20 mL crimped glass vials with silicone/natural PTFE caps (Interchim®, France). A 0.2 µm syringe filter (Rotilabo®, Carl Roth®, France) mounted on a syringe tip was stuck through

the vial cap to allow soil-atmosphere exchanges in the vials while limiting water loss and avoiding contamination (see Appendix A Fig. S2).

Each experiment was spiked with aqueous stock solutions of SM or BUT. For this, herbicide stock solutions in DCM were added to distilled water and stirred for 6 hr until complete DCM evaporation. Resulting herbicide aqueous solutions were filter-sterilized through 0.2 μm syringe filters for soil spiking. The final herbicide concentrations of 15 or 150 $\mu\text{g/g}$ were equivalent to environmental or severe chronic concentrations, respectively (Armanpour and Bing, 2015, Lefrancq et al., 2017). Spiking of aqueous solutions was adjusted to reach a typical field water content of 20% (W/W) or saturated conditions of 100% (W/W) in crop and paddy soil experiments, respectively (See Appendix A Table S3). To homogenize soil microcosms, spiked aqueous solutions were added between soil layers (i.e., 4 layers of soil and 5 layers of spiked aqueous solution), and microcosms were vortexed for one minute and shaken for 1 hr.

All experiments were incubated for 85 days in the dark at 30°C. Sampling was carried out by a sacrificial approach, on days 0, 15, 30, 60, and 85 for non-sterile spiked soil experiments, on days 0, 30, and 85 for sterile spiked soil experiments, and on days 0 and 85 for non-spiked soil experiments (see also Appendix A Table S4). Non-sterile spiked soil experiments were carried out in triplicate for each soil, herbicide dose, and sampling day. Sterile spiked and non-spiked soil experiments were performed in duplicate. Thus, 176 experiments including 120 microcosms with non-sterile spiked soil, 48 experiments with sterile spiked soil, and 8 experiments with non-sterile non-spiked soil were performed in total.

1.4 Extraction and quantification of herbicides and transformation products

Herbicides were extracted from soil with ethyl acetate, and clean-up was performed using PSA, as described previously (Masbou et al., 2018b). Concentrations of SM and BUT, and their transformation products (TPs) OXA and ESA, were measured using GC-MS and LC-MS, respectively, accounting for extraction recoveries. Extraction and quantification procedures of SM, BUT, and their TPs are described in Appendix A Section S4.

1.5 CSIA analysis of SM and BUT

The carbon isotope composition ($\delta^{13}\text{C}$) of SM and BUT was analyzed using a GC-C-IRMS system consisting of a gas chromatograph (TRACE™ Ultra, ThermoFisher Scientific, Germany) coupled via a GC IsoLink/Conflow IV interface to an isotope ratio mass

spectrometer (DeltaV Plus, ThermoFisher Scientific, Germany). The oxidation furnace of the interface was set to a temperature of 1000°C. A TG-5MS column (60 m × 0.25 mm ID, 0.25 µm film thickness) was used for chromatographic separation, with helium as the carrier gas at a flow rate of 1.5 mL/min. The column was held at 50°C for 1 min, then heated at a rate of 15°C/min to 150°C, followed by ramping at 2°C/min to 250°C and then at 20°C/min to 300°C, and held for 3 min. Samples (3 µL volume) were injected into a split/splitless injector operated in splitless mode, and held at 280°C.

$\delta^{13}\text{C}$ values were calibrated using a three-point calibration against the Vienna Pee Dee Belemnite (VPDB) standard using international reference material AIEA600, USGS40, and USGS41 ($\sigma < 0.05\text{‰}$). An in-house pesticide mixture standard and a BTEX standard of known isotopic composition were measured every ten samples to verify the quality of measurements. Carbon isotope ratios were reported in δ notation, in parts per thousand (‰) relative to the international carbon isotope standard VPDB. Reference carbon isotope signatures of standards were obtained using an elemental analyzer-isotopic ratio mass spectrometer (EA-IRMS, Flash EA IsoLink™ CN, Thermo Fisher Scientific, Bremen, Germany). Details of IRMS methods are described in Appendix A Section S5.

The linearity range, i.e., the range of the injected amount of compound for which $\delta^{13}\text{C}$ values stay within a $\pm 0.5\text{‰}$ interval, was established at about 25 to 200 ng of carbon (Appendix A Table S10). The method used for extraction from soil did not cause significant fractionation of SM ($\Delta\delta^{13}\text{C} < 1\text{‰}$) (Appendix A Table S9). In contrast, the extraction method significantly altered $\delta^{13}\text{C}$ values for BUT, likely mainly because of low extraction recovery of BUT. Isotopic data obtained from BUT experiments were not further analyzed for this reason (see Appendix A Section S6 for more details).

1.6 Bacterial community analysis

1.6.1 DNA extraction

Total DNA was extracted from two independent soil replicates for each soil condition using the PowerSoil® DNA isolation kit (MO BIO, Carlsbad, CA, USA) following the manufacturer's instructions. DNA concentrations were determined fluorometrically with the Qubit® dsDNA HS assay kit (Invitrogen, Carlsbad, CA, USA). Each replicate sample was subsequently analyzed independently by PCR and sequence analysis.

1.6.2 Illumina amplicon sequencing

Bacterial diversity was evaluated for each sample by targeting the V3-V4 hypervariable region of the 16S rRNA gene, amplified using an optimized and standardized 16S amplicon library protocol (Metabio®), GenoScreen, Lille, France), and primers modified from Klindworth et al. (2012). Briefly, PCR was carried out using 5 ng total DNA with bar coded primers at final concentrations of 0.2 µmol/L using the following thermal profile: 95°C for 5 min, then 30 cycles of 94°C for 30 sec, 50°C for 40 sec, 72°C for 1 min, followed by one cycle of 72°C for 10 min, and 4°C hold. Products from the first stage amplification were added to a second PCR. Primers for the second PCR were designed based on Illumina Nextera PCR primers. The second stage amplification was run in the same conditions as in the first stage, except for 10 cycles only. Obtained amplicon libraries were cleaned up with an in-house magnetic bead-based protocol, quantified, and multiplexed at equal concentrations. Sequencing was performed using a 250-bp paired-end sequencing protocol on the Illumina MiSeq platform (Illumina, San Diego, USA) at GenoScreen. Raw paired-end reads were subjected to: (1) quality filtering with the PRINSEQ-lite PERL script (Schmieder and Edwards, 2011) by truncation of bases from the 3' end with quality Phred score <30; (2) paired-end reads assembly using FLASH (Magoč and Salzberg, 2011) with a minimum length overlap of 30 bases and 97% overlap identity; and (3) search and removal of both forward and reverse primer sequences using CutAdapt, with no mismatches allowed in primers sequences. Data were deposited to the ENA archive, BioProject accession number PRJNA594264.

1.7 Data analysis

1.7.1 Dissipation kinetics

Pesticide data in each experiment were fitted to an exponential decay single first-order (SFO) model according to NAFTA guidelines (US-EPA). Dissipation rates (k) were estimated when the model provided a significant fit ($R^2 > 0.6$ and $p < 0.05$) and $n \geq 5$ sampling points were available.

1.7.2 Carbon stable isotope notation and calculation

The average isotope value of the residual, non-degraded fraction of herbicide varies according to the Rayleigh equation (Eq. (1)):

$$\frac{(^{13}\text{C}/^{12}\text{C})}{(^{13}\text{C}/^{12}\text{C})_0} = \frac{\delta^{13}\text{C}+1}{\delta^{13}\text{C}_0+1} = f^\varepsilon \quad (1)$$

where f is the residual substrate fraction, $\delta^{13}C$ is the corresponding isotope value and $\delta^{13}C_0$ is the initial isotope value of SM at the beginning of the experiment ($f = 1$).

The isotopic fractionation factor ε relates changes of isotope ratios to the extent of transformation. The ε factor was determined for SM based on data obtained from the entire incubation period (days 0 - 85) using a linear regression according to Eq. (2):

$$\ln \frac{\delta^{13}C_{t+1}}{\delta^{13}C_0+1} = \varepsilon \ln f \quad (2)$$

Values of carbon isotopic fractionation for SM were determined if (1) isotope fractionation during the incubation period was significant ($\Delta\delta^{13}C > 2\text{‰}$), (2) the linear regression of the Rayleigh plot was significant ($R^2 > 0.6$, $P < 0.05$), and (3) $n \geq 5$ sampling points were available.

1.7.3 Quantification of SM and BUT degradation in soil experiments

The relative contribution of abiotic and biotic dissipation of SM and BUT in spiked soil experiments was estimated by comparing dissipation in non-sterile (biotic) soil and in sterile (abiotic) soil, and from CSIA data in the case of SM. The dissipated fraction ($1 - f$) at time t (D_t , %) of SM and BUT in sterile (abiotic) soil ($D_{\text{abiotic},t}$, %) defines the contribution of aging and abiotic degradation, i.e., sorption-desorption processes, while the observed dissipation in non-sterile (biotic) experiments ($D_{\text{biotic},t}$, %) additionally features biotic degradation. Thus, the extent of biodegradation (B_t , %) of SM or BUT at time t (days) was estimated as:

$$B_t = (D_{\text{biotic},t} - D_{\text{abiotic},t}) \quad (3)$$

In Eq. (3), it is assumed that the extent of sorption and abiotic degradative processes is equal in sterile and non-sterile soil experiments. Standard deviations associated with $B_{\text{SM},t}$ values were obtained via the error propagation method.

The extent of biodegradation of SM at time t in non-sterile soil experiments was also estimated from obtained $\delta^{13}C$ values ($B_{\text{SM},t(\text{CSIA})}$, %) using the inverse form of the Rayleigh equation:

$$B_{\text{SM},t(\text{CSIA})} = 1 - f = \left[\frac{(\delta^{13}C/^{12}C)_t}{(\delta^{13}C/^{12}C)_0} \right]^{1/\varepsilon} = \left[\frac{\delta^{13}C_{t+1}}{\delta^{13}C_0+1} \right]^{1/\varepsilon} \quad (4)$$

An isotopic fractionation factor ε (‰) relating changes of isotope ratios to the extent of transformation (Elsner et al., 2005) could not be reliably determined from our experiments due to poor fitting with the Rayleigh model ($R^2 < 0.6$, $P > 0.05$; $n < 5$) (data not shown). The previously reported literature value of -1.5 ± 0.5 ‰ for SM (Alvarez-Zaldívar et al., 2018) was used to estimate $B_{\text{SM},t(\text{CSIA})}$. The uncertainty of the enrichment factors (between -1‰ to -

2‰) was used to calculate a range of minimum and maximum $B_{SM,t}$ (CSIA) values. No significant isotope fractionation under abiotic conditions was assumed, as supported by $\Delta\delta^{13}\text{C} < 2\text{‰}$ in sterile soil experiments (data not shown).

1.7.4 Illumina MiSeq data processing

Obtained 16S rRNA gene sequences were analyzed by GenoScreen (Lille, France) using an in-house automated pipeline based on QIIME v1.9.1 involving chimera detection and elimination, sequence clustering into operational taxonomic units (OTUs) at 97% identity threshold, cleaning, and taxonomic affiliation of the OTUs using the Greengenes database (<http://greengenes.secondgenome.com>), followed by computation of the relative abundance of each identified phylotype. Between 1000 and 4000 OTUs were found in soil samples. Filtering for dominant taxa (minimum relative abundance 0.03%) was then carried out on all samples. Filtering include removal of artefactual OTUs detected by analysis of a mock synthetic community included in the sequencing. For analysis and comparison of bacterial diversity in the different samples, sequence datasets were subsampled by random selection to ensure equally sized data sets in all samples. Shannon (H'), inverse Simpson (S), and Chao1 richness (S_{Chao1}) indices were calculated as previously described (Babcsányi et al., 2017). Statistical analysis was performed with *R* (version 3.3.2). Nonmetric multidimensional scaling (NMDS), based on Bray-Curtis dissimilarities of Hellinger-transformed data was used to visualize dissimilarities between bacterial communities in different soil microcosms. Analysis of similarities (ANOSIM, $n = 55$ samples) based on Bray–Curtis dissimilarities of samples was used to infer statistical differences between groups of community profiles.

2 Results

2.1 Dissipation of SM and BUT in soil

Except for the crop soil at 150 $\mu\text{g/g}$, over 80% of SM and BUT was dissipated within 60 and 30 days, respectively, in all experiments with non-sterile soil spiked with SM or BUT. Dissipation rates were generally independent of spiking dose or soil type (**Tables 1 and 2**). Dissipation rates of SM (0.03 ± 0.01 to $0.05 \pm 0.02 \text{ day}^{-1}$) were slightly slower than those of BUT (0.05 ± 0.01 to $0.12 \pm 0.02 \text{ day}^{-1}$). SM dissipation was lower in sterile soil experiments than in non-sterile soil experiments (**Tables 1 and 2**; Appendix A Section S7). Changes in $\delta^{13}\text{C}$ values were not significant ($\Delta\delta^{13}\text{C} < 2\text{‰}$ -data not shown) in sterile spiked soils, suggesting that degradative processes did not contribute significantly to overall SM dissipation.

Substantial changes in isotope composition of SM became noticeable in both crop and paddy soil after 60 or 85 days (**Table 1**). Changes in $\delta^{13}\text{C}$ values for SM after 85 days were generally lower in non-sterile crop soil experiments ($\Delta\delta^{13}\text{C}$ up to $(4.2 \pm 0.7)\text{‰}$) than in paddy soil experiments. In non-sterile paddy soil experiments, changes in $\delta^{13}\text{C}$ values at day 60 were slightly larger at 150 $\mu\text{g/g}$ ($\Delta\delta^{13}\text{C} = 6.5\text{‰} \pm 0.2\text{‰}$) than at 15 $\mu\text{g/g}$ ($\Delta\delta^{13}\text{C} = 4.8\text{‰} \pm 0.6\text{‰}$).

Biodegradation was estimated based on observed differences between the dissipated fraction of the herbicides in sterile and non-sterile soil experiments (Eq. (3)). Biodegradation accounted for dissipation of up to $(60 \pm 10)\%$ and $(36 \pm 6)\%$ of SM and $(57 \pm 5)\%$ and $(37 \pm 11)\%$ of BUT in non-sterile paddy and crop soil, respectively (**Tables 1 and 2**). The extent of SM biodegradation estimated based on isotopic data (Eq. (4)), however, suggested considerably larger values ranging from 79% to 100% SM biodegradation (**Table 1**). For both SM and BUT, biodegradation was more pronounced in experiments with paddy soil than with crop soil (**Tables 1 and 2**).

2.2 Transformation products of SM and BUT

Patterns of SM and BUT transformation products differed widely as a function of soil type (Appendix A Section S7). In the case of SM, high concentrations of ESA and OXA were detected in non-sterile spiked experiments (Appendix A Fig. S3b). Average concentrations of transformation products (ESA + OXA) were similar in non-sterile spiked experiments with crop or paddy soil. However, a 4:1 (V/V) ESA:OXA ratio was observed in biotic experiments in paddy soil in 6 out of 8 points, contrasting with a ratio of 1:3 (V/V) in crop soil in 5 out of 8 points (Appendix A Fig. S3b). In experiments with non-sterile paddy soil, average concentrations of SM OXA, normalized to initial concentrations of SM, were 3 times higher in soil spiked at 150 $\mu\text{g/g}$ than at 15 $\mu\text{g/g}$. ESA and OXA in sterile experiments remained one order of magnitude lower than in non-sterile experiments (Appendix A Fig. S3b).

In the case of BUT, in contrast, low concentrations of BUT ESA and OXA were generally detected in experiments with crop soil. Maximal concentrations were observed at day 15 and in non-sterile paddy soil at the higher spiking dose of 150 $\mu\text{g/g}$ for BUT ESA (3 times higher than OXA). Higher concentrations of ESA were noted at both spiking doses at day 30 as well (Appendix A Fig. S3a). Notably, ESA was also detected in significant amounts in sterile soil experiments with paddy soil at day 85.

2.3 Changes in bacterial communities following herbicide exposure

2.3.1 Diversity indices

In total, 3,767,137 high-quality sequences were obtained from the 28 non-sterile soil samples. The mean number of sequences per sample was 57,077. The sequencing depth was sufficient to survey the bacterial diversity in both soils (Appendix A Figs. S4a and S4b). Rarefaction curves of diversity indices reached asymptotes, confirming that the sequencing depth was sufficient to capture the diversity extent of soil bacterial communities (Appendix A Fig. S4c-f).

Overall, herbicide identity and application dose did not significantly affect the bacterial diversity in experiments with either crop or paddy soil ($p > 0.05$) (Appendix A Table S11), as assessed by analysis of dominant OTUs defined at the 97% identity threshold. Herbicide exposure in crop soil did not affect bacterial diversity throughout the experiment ($p > 0.05$). In paddy soil, in contrast, exposure to either SM or BUT (at both 15 or 150 $\mu\text{g/g}$ spiking doses) was associated with decreases in Shannon, inverse Simpson, and S_{Chao1} indices by 17%, 66%, and 28% respectively after 15 days, while no significant changes were observed in non-spiked experiments ($p < 0.05$). Subsequently, bacterial diversity in paddy soil did not change further until the end of the experiment (day 85, $p > 0.05$).

2.3.2 Bacterial community composition

Soil type was the main driver of community composition (**Fig. 1a**, clusters P and C; $p = 0.001$). Bacterial composition differed significantly in experiments with investigated crop and paddy soils, and did not vary much over the time course of experiments in the absence of herbicide exposure (i.e., in non-spiked, **Fig. 1a**). Krona charts provide details of bacterial community composition in non-spiked crop and paddy soils (Appendix A Figs. S5 and S6, respectively). In non-spiked crop soil, bacterial composition was dominated by Proteobacteria in all experiments, mainly Alpha- and Gammaproteobacteria, and by Actinobacteria, in particular Actinomycetales, Solirubrobacterales, and Gaiellales (**Fig. 1b**; Appendix A Fig. S5). In non-spiked paddy soil, bacteria were also predominantly affiliated to Proteobacteria, mainly strictly anaerobic and sulfate reducers such as Deltaproteobacterial Syntrophobacteraceae, and a minority of phylotypes assigned to Gamma- and Betaproteobacteria as well as Firmicutes (**Fig. 1c**; Appendix A Fig. S6).

Duration of herbicide exposure was the main second-order driver of community change for both investigated soils. In experiments with paddy soil, exposure to herbicides rapidly and significantly altered bacterial communities, in contrast to what was observed in non-spiked experiments (**Fig. 1a**, cluster P_A; $p = 0.001$). Three distinct clusters were apparent

for the bacterial community in experiments with paddy soil (**Fig. 1a**, clusters P_B, P_C, and P_D; $p= 0.001$). For crop soil, in contrast, changes in bacterial composition following herbicide exposure occurred more slowly, with profiles at days 60 and 85 differing from those at day 15 and from non-spiked experiments (**Fig. 1a**, clusters C_A and C_B; $p= 0.001$). Overall, however, neither type of herbicide or spiking dose had significant effects on soil bacterial communities with both soils ($p > 0.05$).

Proteobacteria remained similar in relative abundance for microcosms set up with crop soil over the incubation period, and generally dominated in both crop and paddy soils (**Figs. 1b** and **1c**). In paddy soil experiments, Deltaproteobacteria (mostly anaerobic sulfate and sulfur reducers such as Desulfuromonadales and Syntrophobacterales) increased over the incubation period (data not shown). In paddy soil, herbicide exposure also led to a rapid increase in the relative abundance of Bacteroidetes and Firmicutes, up to 40 and 51% (**Fig. 1c**), respectively, compared to crop soil (up to 11% and 12%, respectively) (**Fig. 1b**). In contrast, no major change in these taxa was observed in non-spiked soil (**Fig. 1c**). In paddy soil, Bacteroidetes were generally dominated by Bacteroidales in both spiked and non-spiked soils (**Fig. 2a**). In contrast, Saprospirales were more abundant within Bacteroidetes in spiked crop soil compared to the non-spiked soil (**Fig. 2b**). In paddy soil, Clostridiales clearly increased with pesticide exposure and became dominant within the Firmicutes, whereas Bacillales dominated in non-spiked soil (**Fig. 2c**). In contrast, Bacillales dominated in both spiked and non-spiked crop soils, although the relative abundance of Clostridiales increased over the incubation period (**Fig. 2d**).

3 Discussion

3.1 SM degradation and BUT sorption are major dissipation processes in agricultural soils

Analysis of SM and BUT dissipation kinetics, CSIA, and patterns of transformation products allowed evaluation of the contribution of biotic and abiotic processes of herbicide dissipation in two different crop and paddy agricultural soils. The contribution of biodegradation to overall dissipation could be estimated for SM and BUT. Biodegradation was identified as the major process responsible for SM and BUT dissipation in the experiments with paddy soil. In contrast, sorption contributed more to SM and BUT dissipation than biodegradation in the experiment with crop soil. Abiotic degradation of BUT possibly also occurred. Taken together, our results show that the contribution of different processes to dissipation depends

on various factors and environmental parameters including soil water content, pesticide water solubility, K_{oc} (Appendix A Table S1) and application doses (Gavrilescu, 2005).

For SM, biodegradation dominated over other dissipation processes. SM biodegradation was more pronounced with paddy soil of higher water content than with crop soil of lower moisture (**Table 1**). This indicates that soil water content may positively impact the extent of SM biodegradation. By acting as a solvent for pesticide mobilization and diffusion, soil water affects sorption-desorption processes, increasing bioavailability, bioactivity, and biodegradation (Bonczek and Nkedi-Kizz, 2007). The soil aqueous phase also solubilizes nutrients and salts, supporting bacterial growth and activity (Haider and Schaffer, 2009) and potentially further enhancing pesticide biodegradation. Indeed, biodegradation of chloroacetanilides was frequently observed under flooded conditions, such as in wetlands (Elsayed et al., 2015) and in aquatic field mesocosms (Graham et al., 2000).

Another important finding is that the extent of biodegradation estimated from dissipation analysis ($B_{SM,t}$) was larger than estimates obtained from isotopic data ($B_{SM,t}^{(CSIA)}$) (**Table 1**). Such discrepancies may be explained in part by how underlying soil characteristics are taken into account, thereby affecting biodegradation estimates. First, the physico-chemical and microbial characteristics of soil used to retrieve isotopic fractionation factors (ϵ) in previous experiments (Alvarez-Zaldívar et al., 2018) may differ from those in the present study. Carbon stable isotope fractionation in the latter study differed from that of the present study, as different soil microbial communities involved in SM degradation may lead different degradation pathways to be dominant. In our experiments, carbon stable isotope fractionation of SM was generally lower than in the study of Alvarez-Zaldívar et al. (2018). Thus, the ϵ reference value used resulted in overestimation of biodegradation.

On the other hand, alternative estimates of biodegradation that were based on observed differences between herbicide dissipation in sterile and non-sterile soil experiments rely on the assumption that abiotic degradation occurs to the same extent in sterile and non-sterile soils. However, soil sterilization by autoclaving may also affect pesticide sorption and abiotic degradation reactions (Ruggiero et al., 2002), in particular by altering soil organic matter (Berns et al., 2008). This resulted in a large uncertainty in estimations of SM degradation in soil, as observed on day 85 (**Table 1**). Thus, biodegradation estimates from dissipation analysis ($B_{SM,t}$) were generally lower, and thus more conservative, than estimates obtained from isotopic data ($B_{SM,t}^{(CSIA)}$), especially for day 85. To sum up, the observed absence of significant isotopic shifts of SM ($\Delta\delta^{13}C < 2\text{‰}$) in sterile soil, suggesting negligible abiotic degradation, the relatively high organic matter content ($> 5\%$) in both crop

and paddy soils (see Appendix A Table S2), and the lower K_{oc} values of SM (e.g. as compared to BUT, SI section A), are all in favor of biodegradation prevailing over sorption for SM in the crop and the paddy soil.

For BUT, in contrast, sorption was likely the main process affecting apparent dissipation in our soil microcosm experiments, (**Table 2**) as suggested previously (Gevao et al., 2000). Pesticide physico-chemical characteristics (Appendix A Table S1) contribute to explaining the observed large differences in dissipation modes for SM and BUT. For instance, dissipation rates of BUT were 2-3 times faster than SM in crop soil (**Tables 1 and 2**), likely reflecting the higher K_{oc} and lower water solubility of BUT (Appendix A Table S1). Thus, BUT sorption and ageing are expected to be particularly prevalent in experiments with crop soil of lower water content. As for SM, however, BUT biodegradation was more pronounced in experiments with paddy soil than with crop soil of lower moisture, except for the experiment with 15 $\mu\text{g/g}$ at day 85 (**Table 2**).

Worthy of note, however, the occurrence of some abiotic herbicide degradation in our experiments cannot be principally excluded. Photolysis can be safely discounted in our study, since experiments were conducted in the dark. Hydrolysis can also be excluded, since the reported hydrolysis half-lives for BUT and SM, of 630 to 1155 days at pH 4 to 7 or > 200 days at pH 1 to 9, respectively (EXTOXNET, 2000; Zheng and Ye, 2001, Masbou et al., 2018a) are far longer than the duration of our experiments. In addition, the isotope fractionation of SM in our experiments with sterile soil was not significant ($\Delta\delta^{13}\text{C} < 2\text{‰}$ within the range of analytical uncertainty, data not shown) while it is known that abiotic hydrolysis can certainly result in pronounced isotopic fractionation. For example, alkaline hydrolysis of SM has been observed previously ($\epsilon_C = -2.8\text{‰} \pm 0.6\text{‰}$) (Masbou et al., 2018a), compared to $\epsilon_C = -1.5\text{‰} \pm 0.5\text{‰}$ for SM degradation under biotic conditions (Alvarez-Zaldívar et al., 2018). In sterile experiments, ESA and OXA levels remained one order of magnitude lower than in biotic experiments (Appendix A Fig. S3), except for the flooded paddy soil experiments with BUT (Appendix A Fig. S3a). In the latter case, the observed production of ESA may have occurred from abiotic dechlorination of BUT by reduced sulfur species under sulfate-reducing conditions, a hypothesis that is also supported by the prevalence of sulfate-reducing bacterial communities in these experiments (see Section 4.2), as also previously suggested (Cai et al., 2007; Graham et al., 2000). These considerations confirm that in our experiments, hydrolysis and photolysis did not contribute significantly to SM and BUT dissipation. Only abiotic dechlorination of BUT in the paddy soil may have

occurred, but is usually considered minor compared to biodegradation and sorption (Chesters et al., 1989).

Finally, and perhaps counterintuitively, higher application doses mostly did not affect the dissipation rates and biodegradation extent of both SM and BUT, or isotopic fractionation of SM (**Table 1**). Dissipation of SM in paddy soil experiments represented an exception, with larger biodegradation extent, and OXA concentrations one order of magnitude higher at 150 $\mu\text{g/g}$ than at 15 $\mu\text{g/g}$ relative to initial concentrations (**Table 1**; Appendix A Fig. S3b). In this case, a higher dose may have increased SM bioavailability, e.g., through saturation of sorption sites, and thus enhanced bacterial degradation. More extensive biodegradation in soil at higher concentrations was also reported previously for some chloroacetanilides (Saha et al., 2012), mesotrione (Crouzet et al., 2010), isoproturon, tebuconazole, and chlorpyrifos (Papadopoulou et al., 2016).

3.2 Response of soil bacterial communities exposed to SM or BUT

Considering how strongly chloroacetanilide bioavailability was likely to be affected by various factors, it was of interest to evaluate if and how the observed trends correlated with bacterial composition and the relative abundance of specific bacterial types. The two soils used in our experiments represent pedologically distinct areas with different agricultural records and physico-chemical properties (Appendix A Section S2). Hence, the observed significant differences in bacterial community composition arising from differences in soil texture and pH, treatment history, and associated vegetation were expected, and in agreement with previous studies (Brockett et al., 2012; Buckley and Schmidt, 2003; Girvan et al., 2004; Nielsen et al., 2010; Smith and Harris, 2007; Tsiknia et al., 2014). Nevertheless, and despite such large initial differences in bacterial community composition in the two soils, exposure to SM and BUT led to detectable changes in soil bacterial community composition in both investigated soils.

A first general finding of our study is that the observed changes in bacterial communities in our experiments were not specific to SM or BUT (**Fig. 1a**), in line with the limited structural differences between SM and BUT, and as previously suggested (Lo, 2010). The occurrence of similar shifts in soil bacterial communities in response to exposure to pesticides of the same chemical family has already been documented, e.g., for carbamates (Morel-Chevillet et al., 1996), isothiocyanates (Warton et al., 2003), and organophosphates (Singh et al., 2005). Our findings suggest that this may also apply to chloroacetanilides. Another finding of interest in our study is the lack of dose-dependent responses of soil

bacterial community composition upon pesticide exposure (**Fig. 1a**). This is in agreement with the observation of similar dissipation rates, degradation, and isotopic fractionation for field-relevant levels of herbicide exposure ranging over 2 orders of magnitude (i.e., from 15 to 150 $\mu\text{g/g}$), in line with previous reports (e.g. Xu et al., 2014).

At a finer level of detail, herbicide exposure in paddy soil experiments, e.g. under flooded conditions, gradually altered the soil bacterial composition towards phylotypes assigned to obligate anaerobic sulfate and sulfur reducers, such as Syntrophobacterales, Desulfuromonadales, and Clostridiales (**Fig. 1c**). We had already observed a higher relative abundance of such taxa in soil exposed to the chloroacetanilide herbicides *rac*-metolachlor, acetochlor, and alachlor under similar anoxic conditions (Elsayed et al., 2015). Dramatic and rapid shifts under conditions of lower oxygen levels in water-saturated soil have also been reported by others (Drenovsky et al., 2004). Prevalence of sulfate-reducing bacteria in paddy soil exposed to BUT has also been reported (Chen et al., 2003; Min et al., 2001; Zhou et al., 2012), and Clostridia have also been found to be dominant in anaerobic soil exposed to alachlor (Lauga et al., 2013). Sulfate-reducing bacterial communities may contribute to explaining why ESA was found here at higher levels than OXA (Cai et al., 2007) (Appendix A Fig. S3), e.g. through dechlorination involving sulfur-containing nucleophiles such as sulfide and thiosulfate (Stamper and Tuovinen, 1998).

In our experiments with crop soil, in contrast, oxic conditions possibly favored the development of aerobic and facultative anaerobic degraders such as Actinomycetales and Sphingomonadales (**Fig. 1b**). Increased abundance of Actinobacteria in soil exposed to the chloroacetanilides alachlor and metolachlor was reported previously (Lauga et al., 2013; Sette et al., 2005), and pesticide-degrading strains affiliated to such phylotypes have also been described (Fuentes et al., 2010; Hussain et al., 2011). It is also worth mentioning, regarding *Bacillus* phylotypes which also became more abundant upon prolonged incubation (**Fig. 1b**), that *Bacillus* strains capable of degrading pesticides including chloroacetanilides have also been reported (Karpouzias and Singh, 2006; Lopez et al., 2005; Wang et al., 2008). Whether strains from such taxa are also key players associated with the degradation of SM and BUT detected in our experiments will require both the isolation of degrading strains from our microcosms, and more generally, improved molecular knowledge of degradation pathways for such pesticides, which currently remain elusive.

4 Conclusions

The modes and extent of dissipation of the two chloroacetanilides SM and BUT in two typical crop and paddy soils were investigated and compared in laboratory microcosm experiments under controlled conditions. The combined analysis of dissipation kinetics and carbon stable isotope fractionation indicates that both degradative and non-degradative processes contributed to the apparent dissipation of SM and BUT. The relative contributions of different processes varied depending on different factors and physico-chemical parameters, and were strongly associated with herbicide K_{oc} and soil water content. Under the water-saturated conditions of paddy soil, biodegradation of SM and BUT was the dominant process. In crop soil of lower moisture, in contrast, ageing emerged as the main driver of BUT dissipation, and SM biodegradation was slower than dissipation in paddy soil. In addition, biodegradation of BUT in crop soil was more limited than that of SM, most likely due to the higher K_{oc} of BUT.

DNA sequence analysis of the 16S rRNA gene showed that bacterial communities in soil were mainly affected by soil characteristics, including water content, and only to a minor extent by herbicide exposure. Herbicide identity or application dose had no significant effect. Phylotypes assigned to obligate anaerobic sulfate reducers were dominant under the water-saturated conditions of paddy soil. This, alongside the observed higher ratio of ESA/OXA produced from SM and BUT, suggested that SM and BUT were also degraded under anoxic conditions. In crop soil, however, mostly bacterial taxa with an aerobic lifestyle were observed. This, together with the observed lower ratio of ESA/OXA in crop soil, confirmed the prevalence of oxic conditions, and most likely of corresponding reactions for SM and BUT degradation,

In conclusion, this exploratory study proposes a robust framework for examination of chloroacetanilide dissipation in relation to soil bacterial communities, and for using bacteria as biomarkers of pesticide contamination and degradation in agricultural soil in future studies.

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Table 1 S-metolachlor (SM) dissipation and carbon isotopic fractionation in non-sterile (biotic) soil experiments.

Soil	Dose ($\mu\text{g/g}$)	Time (day)	$D_{\text{biotic},t}$ ^a (%) \pm SD	$\Delta\delta^{13}\text{C}^{\text{b}}$ (‰) \pm SD	$B_{\text{SM},t}$ ^c (%) \pm SD	$B_{\text{SM},t(\text{CSIA})}^{\text{d}}$ (%)	K (day^{-1}) \pm SE
Paddy	15	0	0 ± 13	ns	0 ± 26	ns	
		15	52 ± 9	ns	-	ns	$0.04 \pm$
		30	58^*	ns	40 ± 37	ns	0.02
		60	$92 \pm 4^{**}$	4.8 ± 0.6	-	92 - 99	($n = 5$)
		85	$96 \pm 0^{**}$	4.4 ± 0.0	8 ± 6	87 - 99	
	150	0	0 ± 3	ns	0 ± 3	ns	
		15	43 ± 1	ns	-	ns	$0.03 \pm$
		30	56 ± 2	ns	56 ± 2	ns	0.00
		60	92 ± 1	6.5 ± 0.2	-	96 - 100	($n = 5$)
		85	91 ± 2	5.3 ± 0.1	60 ± 10	93 - 100	
Crop	15	0	0 ± 3	ns	0 ± 34	ns	
		15	56 ± 14	ns	-	ns	$0.05 \pm$
		30	na	ns	na	ns	0.03
		60	92 ± 1	3.0 ± 0.3	-	79 - 96	($n = 4$)
		85	$94 \pm 6^{**}$	4.2 ± 0.7	36 ± 6	89 - 99	
	150	0	0 ± 3	ns	0 ± 3	ns	
		15	41 ± 2	ns	-	ns	$0.03 \pm$
		30	46 ± 9	ns	-3 ± 9	ns	0.00
		60	76 ± 3	3.0 ± 0.3	-	79 - 94	($n = 5$)
		85	83 ± 3	3.5 ± 0.4	20 ± 7	84 - 97	

* Data based on one replicate (no standard deviation).

** Concentrations were below the GC-C-IRMS linear range.

- no measurement

^aDissipated fraction of SM in non-sterile (biotic) soil samples. Means and standard deviations of $n = 3$ replicates.

^bErrors given for $\Delta\delta^{13}\text{C}$ values were calculated *via* error propagation based on ± 1 standard deviation of mean $\delta^{13}\text{C}$ values from ≥ 3 measurements for each sample.

^cThe extent of SM biodegradation ($B_{\text{SM},t}$) (%) was estimated according to Eq. (3). Standard deviations were obtained via the error propagation method.

^dSM biodegradation range ($B_{\text{SM},t(\text{CSIA})}$) (%) was calculated based on the obtained $\Delta\delta^{13}\text{C}$ values and a range of enrichment factors between -1 to -2‰ derived from Alvarez-Zaldívar et al. (2018) (Eq. (4)).

k : dissipation rates (day^{-1}), ns: non-significant biodegradation estimates or isotopic shifts ($\Delta\delta^{13}\text{C} < 2\%$), na: SM concentration could not be measured due to analytical bias in GC-MS measurements, SE: standard error obtained from regression analysis of the first-order kinetic model with $n \geq 4$ sampling dates, SD: standard deviation.

Table 2 Butachlor (BUT) dissipation in non-sterile (biotic) soil experiments.

Soil	Dose ($\mu\text{g/g}$)	Time (days)	$D_{\text{biotic},t}$ ^a (%) \pm SD	$B_{\text{BUT},t}$ ^b (%) \pm SD	$k(\text{day}^{-1}) \pm \text{SE}$
Paddy	15	0	0 ± 28	0 ± 28	0.12 ± 0.02 ($n = 5$)
		15	81 ± 12	-	
		30	92 ± 2	50 ± 2	
		60	97 ± 1	-	
		85	97 ± 0	9 ± 8	
	150	0	0 ± 7	0 ± 45	0.05 ± 0.02 ($n = 5$)
		15	75 ± 0	-	
		30	88 ± 5	57 ± 5	
		60	89 ± 3	-	
		85	92 ± 3	24 ± 3	
Crop	15	0	0 ± 33	0 ± 33	0.11 ± 0.01 ($n = 5$)
		15	82 ± 1	-	
		30	85 ± 4	20 ± 4	
		60	97 ± 1	-	
		85	99 ± 0	23 ± 4	
	150	0	0 ± 29	0 ± 30	0.06 ± 0.01 ($n = 5$)
		15	67 ± 6	-	
		30	72 ± 8	37 ± 11	
		60	96 ± 0	-	
		85	99 ± 0	27 ± 9	

- no measurements

^a Dissipated fraction of BUT in non-sterile (biotic) soil samples. Means and standard deviations of $n = 3$ replicates.

^b The extent of BUT biodegradation ($B_{\text{BUT},t}$) (%) was estimated according to Eq. (3). Standard deviations were obtained *via* error propagation calculation.

k : dissipation rates (day^{-1}), SE: standard error obtained from regression analysis of the first-order kinetic model with $n \geq 4$ sampling dates, SD: standard deviation.

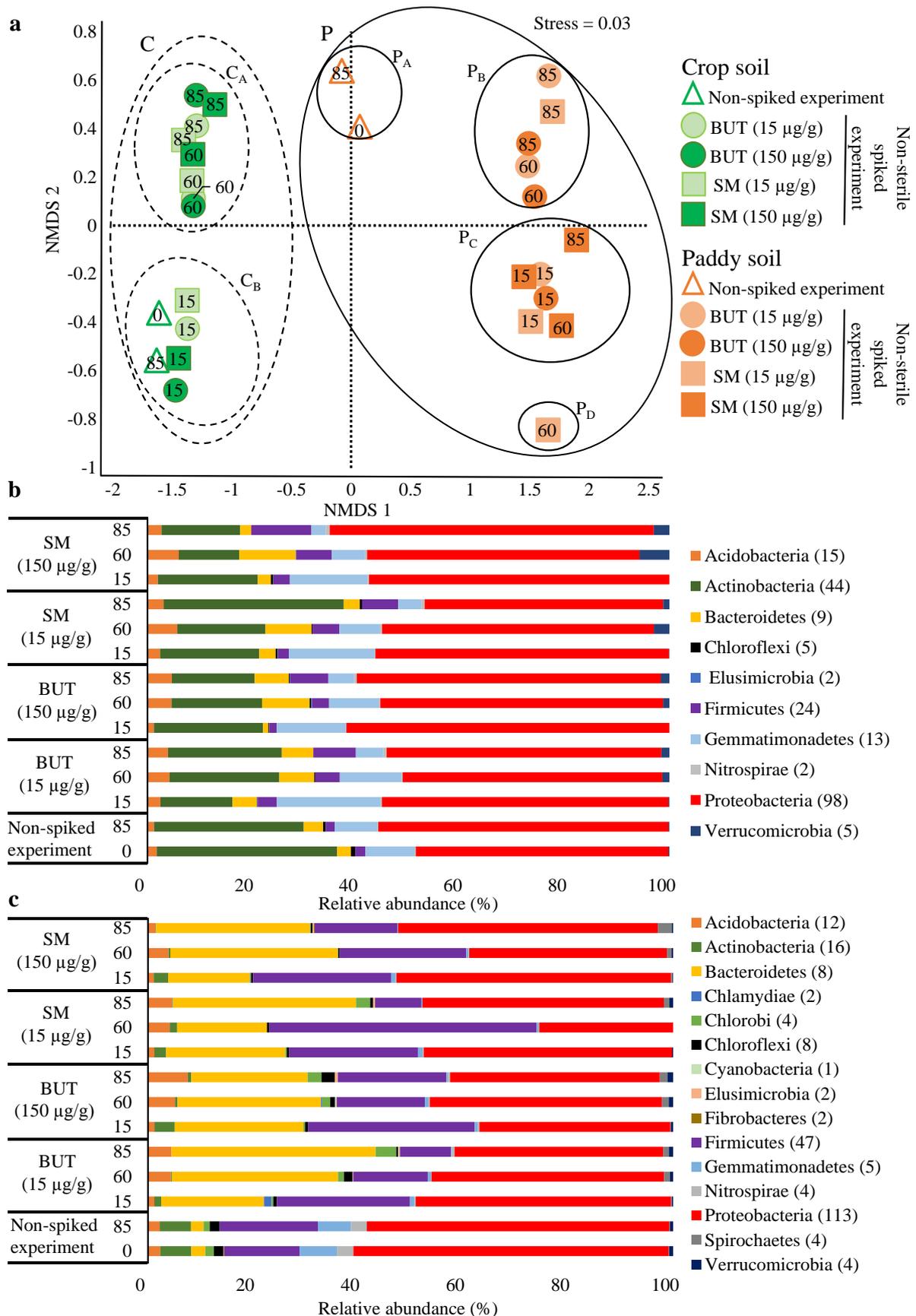


Fig. 1 (a) NMDS ordination of bacterial community profiles. Numbers correspond to sampling time (days). Statistically different clusters (ANOSIM, $p < 0.05$) for paddy and crop

soils are indicated by solid and dotted circles, respectively. A stress value of 0.03 indicates a good fit of original distances/dissimilarities and projected distances in the ordination diagram. Temporal changes (at 15, 60, and 85 days of incubation) in bacterial divisions in (b) crop and (c) paddy soil microcosm experiments for *S*-metolachlor (SM) and butachlor (BUT). Numbers in brackets next to division names indicate the number of recovered OTUs (97% similarity or greater) recovered in that taxon. Incubation time and herbicide amendment are indicated on the *Y* axis.

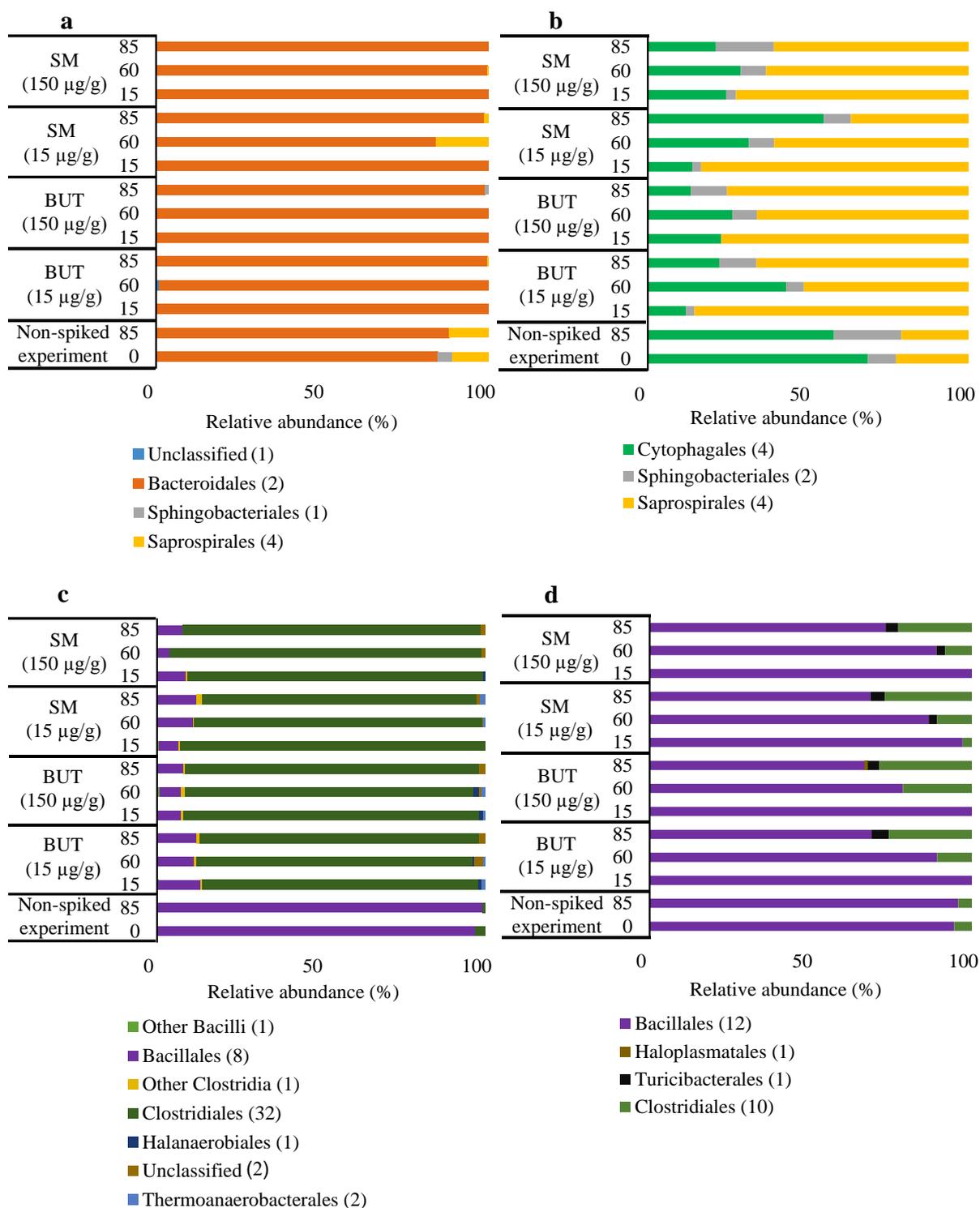


Fig. 2 Order-level changes in Bacteroidetes and Firmicutes. Evolution in relative abundance in Bacteroidetes orders in (a) paddy and (b) crop soil experiments, and Firmicutes orders in (c) paddy and (d) crop soil experiments, respectively. Numbers in brackets in the legend indicate the number of OTUs (97% similarity or greater) recovered in that taxon. Incubation time (15, 60, and 85 days) and herbicide amendment (SM: *S*-metolachlor, BUT: butachlor) are indicated on the Y axis.