Biodegradation and photooxidation of phenolic compounds in soil - a compound-specific stable isotope approach

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Abstract

Phenolic compounds occur in a variety of plants and can be used as model compounds for investigating the fate of organic wastewater, lignin, or soil organic matter in the environment. The aim of this study was to better understand and differentiate mechanisms associated with photo- and biodegradation of tyrosol, vanillin, vanillic acid, and coumaric acid in soil. In a 29 d incubation experiment, soil spiked with these phenolic compounds was either subjected to UV irradiation under sterile conditions or to the native soil microbial community in the dark. Changes in the isotopic composition ($\delta^{13}C$) of phenolic compounds were determined by gas chromatography–isotope ratio mass spectrometry and complemented by concentration measurements. Phospholipid-derived fatty acid and ergosterol biomarkers together with soil water repellency measurements provided information on soil microbial and physical properties. Biodegradation followed pseudo-first-order dissipation kinetics, enriched remaining phenolic compounds in ^{13}C, and was associated with increased fungal rather than bacterial biomarkers. Growing mycelia rendered the soil slightly water repellent. High sample variation limited the reliable estimation of apparent kinetic isotope effects (AKIEs) to tyrosol. The AKIE of tyrosol biodegradation was 1.007 ± 0.002. Photooxidation kinetics were of pseudo-zero- or first-order with an AKIE of 1.02 ± 0.01 for tyrosol, suggesting a hydroxyl-radical mediated degradation process. Further research needs to address $\delta^{13}C$ variation among sample replicates potentially originating from heterogeneous reaction spaces in soil. Here, nuclear magnetic resonance or nanoscopic imaging could help to better understand the distribution of organic compounds and their transformation in the soil matrix.

Highlights

- Biodegradation of phenolic compounds was associated with increased fungal biomarkers
- Photooxidation of phenolic compounds was potentially catalyzed by hydroxyl radicals
- Dissipation kinetics could be described by a pseudo-zero order rate law
- High variation in $\delta^{13}C$ between soil samples calls for further method development

Keywords

Polyphenols, allelochemicals, olive mill wastewater, soil fungi, stable isotopes, metabolic pathways

1. Introduction

Natural phenolic compounds occur in a variety of plants such as olives, grapes, or tea, and thus function as precursors for plant residues, lignin, soil organic matter (SOM), or organic wastewater (Justino et al., 2010; Siqueira et al., 1991). This makes phenolic compounds, specifically phenolic alcohols and hydroxycinnamic acid derivatives, an important group of model compounds for facilitating a better understanding of the environmental fate and turnover processes of plant residues in soil (Glaser, 2005). Such fate studies become particularly relevant when plant pomaces or organic wastewater is applied to soil.

Olive mill wastewater (OMW), for instance, is particularly rich in phenolic compounds and serves as a natural fertilizer in the Mediterranean due to its high content in potassium, manganese, and calcium (Barbera et al., 2013). While low concentrations of phenolic compounds may repel pests or stimulate beneficial organisms, phenolic compounds can adversely affect soil fauna when released extensively (Kurtz et al., 2015; Peikert et al., 2015). In semi-arid and Mediterranean climates, the fate of phenolic compounds originating from OMW largely depends on the application season and the time passed since the last application, ranging from not any to complete dissipation after 6–18 months (Steinmetz et al., 2015). Buchmann et al. (2015) suggested that the transformation of phenolic OMW constituents may involve three subprocesses, including (1) mineralization of easily accessible phenolic compounds, (2) transformation of more recalcitrant phenolic substances, and (3) immobilization or incorporation of phenolic compounds into SOM. However, current understanding remains contrasting in terms of the extent bacteria and fungi may be involved in biodegradation of phenolic OMW constituents under various environmental conditions (Laor et al., 2011; Peikert et al., 2017). In addition, OMW spread on topsoil and exposed to sunlight has been assumed to induce photooxidation and polymerization of phenolic compounds, potentially rendering soil water repellent (Tamimi et al., 2016).

The objective of our study was to better understand natural biodegradation and photooxidative processes of phenolic compounds in soil to identify unique characteristics of both transformation mechanisms in

the field, for instance, after an OMW application. Based on the assumptions by Tamimi et al. (2016) and Peikert et al. (2017), we hypothesized that (1) natural biodegradation of phenolic compounds is more facilitated by fungi rather than bacteria and that (2) differences in dissipation kinetics of biological and photooxidative processes lead to distinct isotope fractionation of phenolic compounds during degradation.

In order to investigate this, we spiked an olive orchard soil with four phenolic compounds typically found in OMW, namely tyrosol, vanillin, vanillic acid, and coumaric acid (Buchmann et al., 2015; Justino et al., 2010), and let them be degraded for 29 d either by the native soil microbial community in the dark or by ultraviolet (UV) irradiation under sterile conditions. The duration of the experiment was chosen based on previous studies that found more than 80% of OMW-derived phenolic compounds degraded within the first 30 days after application (Buchmann et al., 2015; Peikert et al., 2017). To track the degradation of phenolic compounds, we analyzed changes in their isotopic carbon composition ($\delta^{13}C$) using gas chromatography–isotope ratio mass spectrometry (GC–IRMS; Elsner, 2010). Compound-specific carbon stable isotope analysis has been proven useful to detect abiotic or biotic transformation reactions of organic compounds based on their specific isotope fractionation during degradation (Elsner et al., 2005). However, making phenolic compounds amenable for routine GC–IRMS measurements requires prior derivatization to decrease their polarity. Therefore, we first developed and validated a derivatization procedure suitable for subsequent GC–IRMS measurements. Our analyses were complemented with soil respiration measurements to assess the overall soil metabolic activity. Ergosterol and phospholipid-derived fatty acid (PLFA) biomarkers were used as indicators for fungal biomass and soil microbial community composition, respectively. Water drop penetration time (WDPT) measurements served as a proxy for soil water repellency.

2. Materials and methods

2.1. Experimental setup

Incubation conditions were aimed to reflect average field conditions of a typical OMW application scenario, in an olive orchard in the Northern Negev, Israel, during spring (March to May). Incubation temperature and relative humidity were set to 22 ± 1 °C and 32 ± 1 %, respectively. A sieved Israeli olive

orchard topsoil (0–10 cm, , <2 mm mesh) as previously used elsewhere (Buchmann et al., 2015; Steinmetz et al., 2015) served as reference soil. Briefly, the fine soil contained 56 % sand, 13 % silt, and 31 % clay (loessial sandy clay loam) with a bulk density of 1.17 g cm^{-3} and an effective cation exchange capacity of 33 $mmol_c$ kg^{-1} at a soil pH_{H2O} of 7.9. Organic and inorganic carbon were 5 ± 1 g kg^{-1} and 27 ± 6 g kg^{-1}, respectively, with a bulk $\delta^{13}C$ of –8.5 ‰.

The incubation experiment was set up in triplicates by transferring each 10 g of air-dried soil into screw cap glass jars equipped with a 0.1 µm air filter (99.8 % bacterial filtration efficiency; EN 14683, 2014) and a septum port for sterile injections. After a 14-d preincubation period in the dark, one subset of incubation jars was spiked with the phenolic compounds to initiate biodegradation by the native soil microbial community. The spiking solution contained each 10 mg of tyrosol, vanillin, vanillic acid, and *m*-coumaric acid dissolved in 500 µL of a 1:1 mixture of methanol and ultrapure water (see supplementary material S1 for purities and suppliers of all chemicals used in this study). With that, a nominal content of 1 g kg^{-1} of each phenolic compound in soil was obtained, which reflects typical contents of phenolic compounds in topsoil directly after OMW application (Buchmann et al., 2015; Sierra et al., 2001). Another subset of preincubated soil was sterilized by autoclaving at 121 °C for 20 min prior to spiking with phenolic compounds. After two days, the soil was autoclaved a second time to prevent potential recolonization from spores. During incubation, soils were kept sterile and irradiated with UV light (7 W m^{-2} UVA and 0.02 W m^{-2} UVB, CLEO Performance N, Philips, Amsterdam, Netherlands) for 16 h per day to trigger photooxidative degradation. In addition to these two treatments, sterilized soil spiked with phenolic compounds and incubated in the dark (without UV) was used to quantify sorption or complexation processes. Incubation jars filled with plate-count agar (5 g L^{-1} peptone, 2.5 g L^{-1} yeast extract, 1 g L^{-1} glucose, 15 g L^{-1} agar, pH 7.0) were used to verify sterility by visual absence of colony-forming units. Sterilized and non-sterilized soils treated with a clean methanol–water mixture free of phenolic compounds served as controls.

During the experiment, the soil matrix potential was kept at −63 kPa (pF = 2.8) by tempering weight losses of incubation jars with the addition of ultrapure water. The soil was sampled by sacrificing incubation jars (n = 6) at days 0, 1, 2, 4, 8, 15 and 29. All samples were freeze-dried at a constant weight and stored at −20 °C for further analyses.

2.2. Extraction and quantification of phenolic compounds

Phenolic compounds were extracted from 2 g of soil with 10 mL of methanol for 24 h at 400 rpm in an orbital shaker. Preliminary experiments showed that this extraction procedure yielded highest and most reproducible recoveries compared to accelerated solvent extraction, microwave-induced extraction, ultrasonic extraction, and sequential extraction with hexane and methanol (see supplementary material S2). After sedimentation, supernatants were filtered through 0.45 µm syringe filters.

Phenolic compounds were separated by high-performance liquid chromatography (HPLC, Series 1200, Agilent, Santa Clara, USA) on a C_{18} reversed-phase column (Nucleodur C18 Pyramid, 5 µm, Macherey-Nagel, Düren, Germany). The mobile phase consisted of acidified ultrapure water (0.2 % formic acid) and a gradually increasing percentage of acetonitrile (5 % for 5 min, 5 to 40 % for 13 min; 40 to 90 % for 6 min with a final 1-min hold; 0.8 mL min^{-1} total flow). Phenolic compounds were quantified with a UV detector at 280 nm against external standards. All chromatograms were screened manually for unidentified peaks to be investigated by gas chromatography–mass spectrometry (GC–MS) as described in the following section.

2.3. Compound-specific stable isotope analysis of phenolic compounds

Prior to GC–IRMS analysis, phenolic compounds needed to be derivatized into their respective ethers and esters (Elsner et al., 2012; Reinnicke et al., 2010). For that, standard test mixtures of the four phenolic compounds (10–500 mg L^{-1}) with and without soil matrix were prepared and their susceptibility to various derivatization agents was evaluated. These were trimethylsulfonium hydroxide (TMSH) according to a method by Reinnicke et al. (2010), trimethylanilinium hydroxide (TMAH) based on Rompa et al. (2004), BF_3 (Maier et al., 2014; Meier-Augenstein, 1997), and a Purdie methylation with CH_3I modified by Lehmann et al. (2017). Derivatization agents were added in varying molar excess (10–400 fold) and phenolic standard solutions were left for either 30 or 60 min at 30, 40, 60, and 80 °C, respectively, in hermetically crimped vials (ND11 with aluminum seal and butyl/PTFE septum, VWR, Darmstadt, Germany). Phenolic derivatives were identified and their reaction yield was checked by GC–MS (GC Trace Ultra + DSQII-MSD, Thermo Fisher Scientific, Bremen, Germany). Specifically, 0.5–2.5 µL sample volume were injected into various inlet liners either splitless at constant temperature (150,

200, or 250 °C) or by purging excess solvent for 0.1 min (80 mL min^{-1} split flow) prior to rapid injector heating with a 14 °C s^{-1} ramp from 100 to 250 °C. Tested liner types included straight, tapered, and baffled glass liners, stainless steel as well as glass liners filled with glass wool or a carbofrit. Optionally, carbofrit liners were packed with 250 µm or 400–600 µm silanized glass beads (see supplementary material S1 for materials). The analytes were separated on a 60 m × 0.25 mm capillary column (5 % phenyl-arylene, 95 % dimethylpolysiloxane, 0.25 µm film thickness, ZB-5MS, Phenomenex, Aschaffenburg, Germany) equipped with 2-m deactivated fused silica guard column (Phenomenex, Aschaffenburg, Germany) in order to avoid film decomposition by derivatization agents. The oven program was adjusted by optimizing chromatographic resolution R_s as defined in Equation 1. Therein, t_{R1} and t_{R2} [min] are the retention times of adjacent peaks, and w_{b1} and w_{b2} [min] are corresponding peak widths taken from integration thresholds (Meyer et al., 2015).

$$R_s = 2 \cdot \frac{t_{R2} - t_{R1}}{w_{b2} + w_{b1}} \quad (1)$$

If applicable, methods producing sufficiently high signal intensities were examined for signal stability and linearity by repeatedly measuring standard series of phenolic model compounds both via GC–MS and GC–IRMS (Trace GC Ultra with GC IsoLink coupled to a ConFlo IV and Delta V Advantage IRMS, Thermo Fisher Scientific, Bremen, Germany). In order to increase reproducibility, test mixtures were spiked with 2-naphthol as internal standard (IS) for tyrosol and vanillin derivatives. Vanillic acid and coumaric acid derivatives were IS-corrected with biphenyl-2-carboxylic acid. The ISs were selected for their structural similarity to the analytes while ensuring their absence in natural soil and sufficient chromatographic separation.

After chromatographic separation, phenolic derivatives were combusted to CO_2 at 1000 °C in a freshly oxidized CuO−NiO reactor tube (Thermo Fisher Scientific, Bremen, Germany). Reactor tube reoxidation was performed after each measurement by flushing oxygen over the CuO–NiO catalyst for 20 s followed by a 120 s He (grade 5.0) vent. The isotopic composition of CO_2 was converted by software (Isodat 3.0, Thermo Fisher Scientific, Bremen, Germany) into compound-specific $\delta^{13}C$ relative to Vienna Pee Dee Belemnite and in-house references (Equation 2).

$$\delta\ ^{13}C = \frac{^{13}C/\ ^{12}C_{Sample} - \ ^{13}C/\ ^{12}C_{Reference}}{^{13}C/\ ^{12}C_{Reference}} \quad (2)$$

Derivatization introduces foreign carbon atoms into the phenolic molecule, changing the $\delta^{13}C$ of the analyte. Therefore, phenolic derivatives were corrected with bracketing standards of known isotopic composition as verified by elemental analyzer–isotope ratio mass spectrometry (EA–IRMS, Flash HT with SmartEA coupled to the same IRMS system, Thermo Fisher Scientific, Bremen, Germany) using Equation 3 (Maier et al., 2014); *n* is the number of carbon atoms in the respective molecule.

$$\delta\ {}^{13}C_{Sample} = n_{Derivative,\ Standard} \cdot \left(\frac{\delta\ {}^{13}C_{Derivative,\ Sample} - \delta\ {}^{13}C_{Derivative,\ Standard}}{n_{Standard}}\right) + \delta\ {}^{13}C_{Standard} \qquad (3)$$

In addition, $\delta^{13}C$ values of phenolic derivatives were corrected with the $\delta^{13}C$ of their respective IS to reduce potential isotope shifts from chromatographic effects or incomplete combustion in the reactor tube. IS correction was performed by subtracting the $\delta^{13}C$ of the IS determined by EA–IRMS from those obtained from GC–IRMS measurements. This difference was then applied to the $\delta^{13}C$ values of the sample.

For method validation, we purchased a second set of phenolic model compounds from different suppliers as a reference (see supplementary material S1) and determined their $\delta^{13}C$ composition both by EA–IRMS and GC–IRMS after IS-correction. At last, the GC–IRMS method was applied to the soil samples of our incubation experiment and the variation in $\delta^{13}C$ within sample replicates was evaluated.

2.4. Soil respiration, biomarkers, and water repellency

Soil respiration was recorded at days 0 and 29 of the incubation study using the MicroResp method without substrate addition (Campbell et al., 2003). To this end, four aliquots of sample were transferred into wells of a deep well microtiter plate. The soil was equilibrated at 20 °C for 16 h to reduce artifacts from manual soil perturbation. Subsequently, the well plate was covered air-tight with a detection microtiter plate filled with CO_2-sensitive indicator agar (12.5 mg L^{-1} cresol red, 150 mM KCl, 2.5 mM $NaHCO_3$) and incubated for 25 h. After incubation, the color change of the indicator was quantified with a plate reader (Infinite M200, TECAN, Männedorf, Switzerland) at 572 nm.

Fungal biomass at days 0 and 29 was estimated from free ergosterol contents extracted by physical disruption (Gong et al., 2001). Accordingly, 5 mL of methanol were added to 1 g of freeze-dried soil together with 1 g of acid-cleaned glass beads (equivalent amount of beads with 150–250 µm and 700–

1000 µm diameter, Carl Roth, Karlsruhe, Germany). The suspension was agitated for 1 h at 400 rpm in an orbital shaker. Suspended particles were allowed to sediment and the supernatants were filtered through 0.45 µm syringe filters. After extraction, ergosterol was eluted isocratically (1 mL min^{-1} methanol) at 33 °C via HPLC equipped with a C_{18} reversed-phase column (LiChrospher 100 RP 18-5µ, CS Chromatographie-Service, Langerwehe, Germany) and quantified with a UV detector at 282 nm.

In order to assess changes in the composition of the soil microbial community, freeze-dried soils sampled at the first and last day of the incubation experiment (day 0 and 29) were analyzed for PLFA biomarkers according to Bligh and Dyer (1959) and Butte (1983). In brief, phospholipids were extracted from 2 g soil using a mixture of 2 mL chloroform, 4 mL methanol, and 1.6 mL phosphate buffer. Extracts were agitated for 1 h in an overhead shaker (16 rpm) and purified via solid phase extraction (SPE) with a modified polar polystyrene-divinylbenzene copolymer (Chromabond, Macherey-Nagel, Düren, Germany). Phospholipids were transesterified with TMSH (0.25 M in methanol). The resulting fatty acid methyl esters (FAMEs) were quantified by gas chromatography with flame ionization detector (CP-3800, Varian, Darmstadt, Germany). External standards for FAME quantification were *i*15:0 and *i*17:0 for Gram+ bacteria, 16:1ω7c, 18:1ω9c and 18:1ω7c for Gram− bacteria, 16:1ω5c for mycorrhiza, and 18:2ω6c for fungi (Zelles, 1999). Deuterated 18:0 and deuterated 14:0 were used as IS.

Soil water repellency potentially induced by polymerization of phenolic compounds was assessed by taking WDPTs on fresh soil at day 0 and 29. To this end, droplets of deionized water (100 µL) were placed onto the soil surface and the time to complete penetration of each droplet was determined. WDPTs were classified according to Bisdom et al. (1993).

2.5. Data evaluation

Data processing and statistical analyses were performed using R (version 3.5.1). All results are presented as mean ± 95 % confidence interval (CI). Limits of detection (LODs) and limits of quantification (LOQs) were calculated from blank measurements in accordance with German standard DIN 32645 (2008) as implemented in the R package “envalysis” (version 0.3.3, code publicly available from https://doi.org/cn74). Changes in phenolic compounds, soil biomarkers, respiration, and water repellency

during incubation were statistically evaluated by 95 % CI testing (Wheeler et al., 2006) as implemented in the R package "drc".

Dissipation kinetics were assessed by fitting zero-order (Equation 4) and first-order rate equations (Equation 5) to phenolic soil contents c_t [mg kg^{-1}] changing with incubation time t [d]; c_0 is the initial phenolic content [mg kg^{-1}] at time $t = 0$ [d]. k_{zero} [mg kg^{-1} d^{-1}] and k_{first} [d^{-1}] are the dissipation rates.

$$c_t = c_0 - k_{\text{zero}} \cdot t \quad (4)$$

$$c_t = c_0 \cdot \mathrm{e}^{-k_{\text{first}} \cdot t} \quad (5)$$

The resulting regression models were evaluated for their goodness of fit using corrected Akaike information criteria (AICs, from R package "MuMIn") and adjusted R^2. 95 % CI testing of prediction intervals was used to check for significant differences between the expected rate equation and its potentially better fitting alternative (Wheeler et al., 2006).

In addition, ln-transformed linear models were used to estimate isotope enrichment ε [‰] occurring during degradation of phenolic compounds as defined by the Rayleigh equation (Equation 6). The Rayleigh equation relates isotope ratios at incubation time t ($\delta^{13}C_t$ [‰]) with respect to initial $\delta^{13}C_0$ [‰] with the fraction c_t/c_0 of remaining parent compound (Elsner, 2010).

$$\ln\left(\frac{\delta\ ^{13}C_t + 1}{\delta\ ^{13}C_0 + 1}\right) = \varepsilon \cdot \ln\left(\frac{c_t}{c_0}\right) \quad (6)$$

On this basis, Equation 7 was used to estimate a compound-averaged apparent kinetic isotope effect (AKIE) by relating ε to the number of carbon atoms n in each phenolic compound (Elsner et al., 2005).

$$\text{AKIE} \approx (1 + n \cdot \varepsilon)^{-1} \quad (7)$$

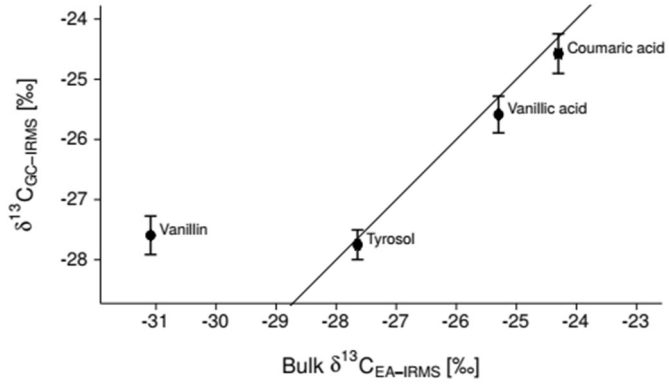
The AKIE comprises isotope effects of all steps of a reaction chain. A rate-determining but hardly fractionating step such as formation of an enzyme–substrate complex could mask the intrinsic kinetic isotope effect of the bond-changing reaction typically used to identify a certain reaction mechanism. In such cases, the AKIE is smaller than expected which needs to be taken into account when interpreting results (Elsner et al., 2005).

3. Results and discussion

3.1. Development and validation of a derivatization method to analyze phenolic model compounds by GC–IRMS

Off-line derivatization with TMSH at more than 100-fold excess for 30 min at 40 °C followed by splitless injection of 1.5 µL into a 250 °C glass bead liner produced best results in terms of $\delta^{13}C$ signal sensitivity and reproducibility. Stable $\delta^{13}C$ values were obtained for peak amplitudes greater than 500 mV. Multiple injections of phenolic standard mixtures (n = 32) into the GC–IRMS resulted in a precision of <0.19 ‰ measurement variation (95 % CI, see supplementary material S3 for figures). Baseline separated peaks with a chromatographic resolution $R_s \geq 2$ (Meyer et al., 2015) were achieved for tyrosol, vanillic acid, and coumaric acid derivatives when applying the following GC oven program: 80 °C (1 min hold), 10 °C min^{-1} ramp to 140 °C (5 min hold), 2 °C min^{-1} ramp to 180 °C, and a final 25 °C min^{-1} ramp to 300 °C (1 min hold). The vanillin derivative interfered with a derivatization by-product (R_s = 1) and peaks remained chromatographically inseparable from one another when extending the oven program.

EA–IRMS measurements showed that bulk $\delta^{13}C$ values of the second set of phenolic reference compounds used for method validation were about 1 ‰ lighter than $\delta^{13}C$ values of the standard mixture used for standard bracketing (coumaric acid was by 0.16 ‰ heavier; see supplementary material S3 for comparisons). Compound-specific $\delta^{13}C$ values obtained by GC–IRMS measurements after correction with bracketing standards and IS accurately reproduced EA–IRMS measurements of tyrosol, vanillic acid, and coumaric acid reference compounds (Figure 1). GC–IRMS measurement uncertainties were typically below 0.3 ‰. Only vanillin deviated from the identity line by 3 ‰ which was most likely due to the insufficient chromatographic separation. For this reason, vanillin was excluded from further isotope analyses.



281 *Figure 1: Validation of GC–IRMS measurements against EA–IRMS measurements of a phenolic reference*
282 *mixture, the solid line is the 1:1 line; error bars indicate 95 % CIs (the variability of bulk* $\delta^{13}C_{EA-IRMS}$
283 *values was < 0.05 ‰)*

284

285 Our preferred GC–IRMS procedure is in line with findings by Reinnicke et al. (2010), who established a
286 GC–IRMS method for the compound-specific stable isotope analysis of (4-chloro-2-
287 methylphenoxy)acetic acid and bentazone after derivatization with TMSH. The authors stressed the
288 importance of using excess TMSH in order to avoid irreproducible derivatization-induced isotope shifts
289 potentially originating from spatial isolation of analytes and derivatization agent in the porous space of
290 the glass bead liner (Reinnicke et al., 2010). This effect may also explain the analyte carry-over we
291 observed unless we added a cleaning run after every fifth sample injection. Unpacked liners generally
292 performed worse in terms of reproducibility. Liners packed with glass wool showed even higher carry-
293 over effects than glass bead liners.

294 In comparison to TMSH, derivatization with TMAH and BF_3 resulted in lower measurement sensitivities
295 due to a substantial background noise from phenyl and fluoric mass fragments. The low turnover of the
296 modified Purdie reaction rendered the detection of phenolic compounds at concentrations < 200 mg L^{-1}
297 impossible. Lehmann et al. (2017) initially developed and applied the method for $\delta^{18}O$ of carbohydrates
298 in the g L^{-1} range. Since we expected analyte concentrations in our experiment to be 2–3 orders of
299 magnitude lower than the concentrations used by Lehmann et al. (2017), derivatization of phenolic
300 compounds with TMSH and hot injection into a glass bead liner was the superior method for our
301 experiment, and thus used for all further GC–IRMS analyses. After correction with bracketing standards

and IS, multiple injections of the same sample were both precise and accurate with deviations smaller than 0.3 ‰ (see above).

3.2. Tracking the degradation of phenolic compounds

In non-spiked control soils, phenolic contents were generally below the respective LODs of 2–123 µg kg^{-1} (see supplementary material S3). Soils spiked with phenolic compounds had initial contents of 627–1070 mg kg^{-1} (Figure 2), which generally reflected their recovery rates from soil (see supplementary material S2). In sterilized soil incubated in the dark, these initial levels decreased by about 20 % from day 0 to day 29 of the incubation experiment (see supplementary material S4). This may be explained by sorption of hydroxy, carbonyl (vanillin only), or carboxylic moieties (vanillic acid and coumaric acid) of phenolic compounds to clay minerals or SOM (Buchmann et al., 2015), complexation with metal ions, or manganese-catalyzed polymerization (Gianfreda et al., 2006).

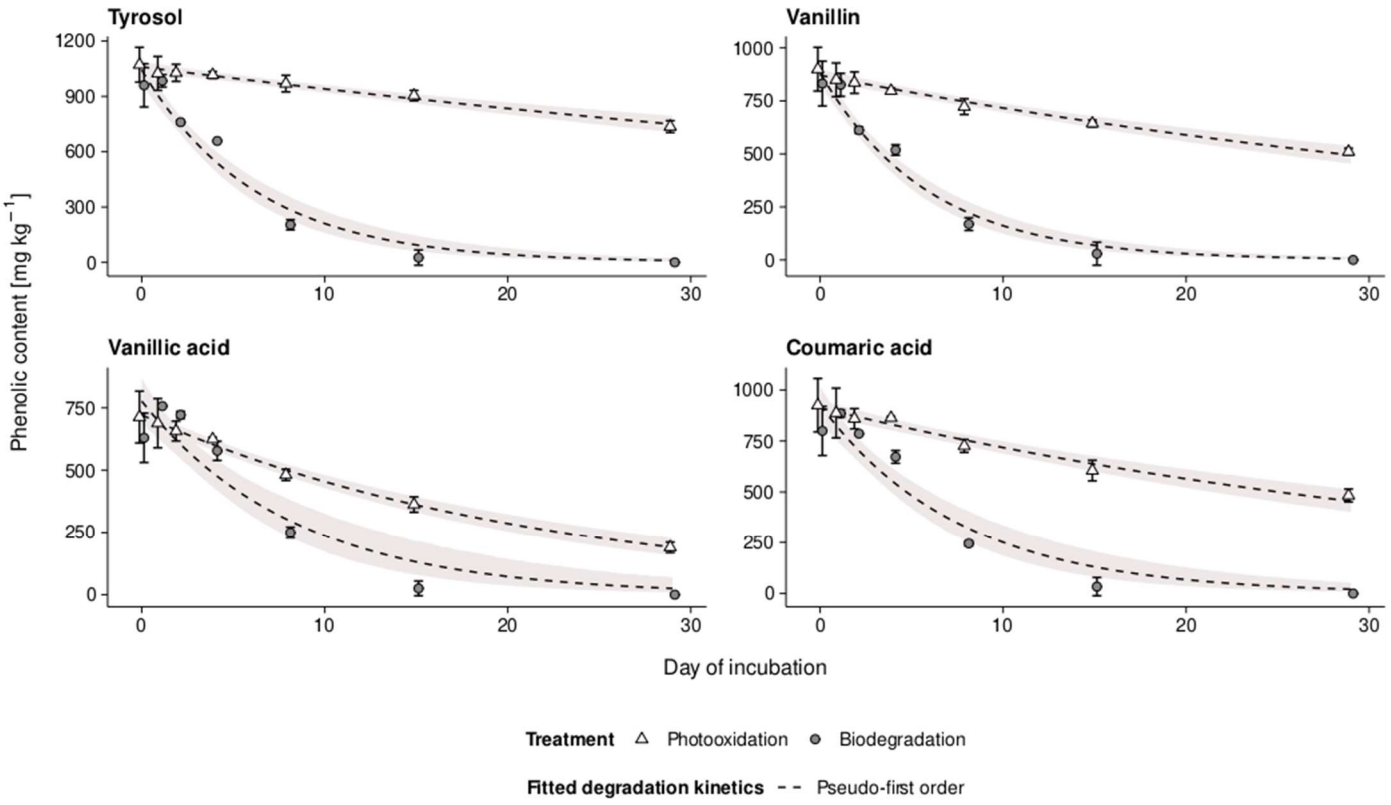


Figure 2: Dissipation kinetics of phenolic model compounds (mean ± 95 % CI) in soil subjected either to photooxidation or biodegradation for 29 d; gray overlays indicate 95 % confidence bands of model fits

When subjected to UV-irradiation, phenolic contents decreased to 26–69 % of their initial content after 29 d of incubation. Photodegradation kinetics were in the best agreement with a first-order rate law (adj.

317 $R^2 \geq 0.849$, Table 1), with vanillic acid being photodegraded the fastest while tyrosol and vanillin were
318 most recalcitrant. Except for vanillic acid, however, the differences to alternative zero-order models were
319 marginal and statistically insignificant (95 % CI testing, see supplementary material S5 for diagnostic
320 plots). Therefore, both models described the dissipation of tyrosol, vanillin, and coumaric acid equally
321 well. However, photodegradation reactions often follow first-order dissipation kinetics since they may
322 not only depend on the number of photons activating a compound for reaction but also on the compound
323 concentration. If rate-limiting, the presence of other reactants such as photocatalysts may even result in
324 dissipation kinetics of higher order (Schwarzenbach et al., 2016).

325

326 *Table 1: Dissipation rates with their respective standard error, adjusted R^2 and AIC of the best-fitting*
327 *degradation models depicted in Figure 1*

Compound	**Photooxidation**			**Biodegradation**		
	k_{first} [d^{-1}]	adj. R^2	AIC	k_{first} [d^{-1}]	adj. R^2	AIC
Tyrosol	0.01 ± 0.01	0.849	226.1	0.16 ± 0.02	0.956	240.9
Vanillin	0.02 ± 0.01	0.895	224.9	0.17 ± 0.02	0.965	229.6
Vanillic acid	0.05 ± 0.01	0.945	225.8	0.12 ± 0.02	0.891	248.4
Coumaric acid	0.02 ± 0.01	0.869	238.2	0.13 ± 0.02	0.923	248.2

328 *see supplementary material S5 for alternative zero-order models*

329

330 In non-sterilized soil incubated in the dark, phenolic contents remained constant in the first two days of
331 incubation, followed by complete dissipation (<LODs) towards the end of the incubation period.
332 Biodegradation kinetics were described best by a first-order rate law (adj. $R^2 \geq 0.891$) with vanillic acid
333 degrading most slowly while vanillin was degraded the fastest. Alternative zero-order rate equations had
334 a lower goodness of fit that differed significantly from the expected first-order rate law (95 % CI testing,
335 see supplementary material S5). Deviations from the first-order rate law were found for vanillic acid and
336 coumaric acid, whose contents increased at day 2 before decreasing like all other phenolic compounds.
337 Buchmann et al. (2015) observed similar lag times for coumaric acid and caffeic acid. The authors argued
338 that various phenolic compounds may be biologically transformed into one another. This particularly
339 applies to vanillin which is known to be readily oxidized to vanillic acid by various soil microbes (Neilson

and Allard, 2008). Additionally, vanillic and coumaric acid sorbed to SOM may have been remobilized by soil microbes prior to biodegradation. However, apart from vanillic acid, no further metabolites were detected via HPLC to be identified by GC–MS. This may be attributed to the rapid metabolization of phenolic acids into small, diverse aliphatic compounds no longer amenable to the UV detector of the HPLC or scanning GC–MS (Blum and Shafer, 1988).

3.3. Changes in stable isotope ratios of phenolic compounds during degradation

Initial $\delta^{13}C$ values of tyrosol, vanillic acid, and coumaric acid at day 0 of the incubation experiment were –26.4 ± 0.3 ‰, –23.5 ± 0.4 ‰, and –24.0 ± 0.4 ‰, respectively (Figure 3). During biodegradation, tyrosol became enriched in ^{13}C by about 3.0 ‰. However, sample variation both between and within sample replicates increased to ± 3.5 ‰ towards the end of the incubation experiment at day 15 (see supplementary material S3 comparisons in sample variation). This variation led to a moderate goodness of fit (adj. $R^2 = 0.732$, $p < 0.001$) for the estimation of $\varepsilon = –0.9 \pm 0.3$ ‰. The corresponding AKIE was 1.007 ± 0.002. In contrast, vanillic acid was depleted by about 4.0 ‰ with 3.2 ‰ sample variation at day 15. This resulted in an inverse isotope effect with a derived ε of 1.7 ± 0.7 ‰ and an associated AKIE of 0.987 ± 0.005 (adj. $R^2 = 0.599$, $p < 0.001$). For coumaric acid, changes in $\delta^{13}C$ were small and indistinct with respect to sample variation ($\varepsilon = 0.3 \pm 0.5$ ‰, AKIE = 0.997 ± 0.005, adj. $R^2 = 0.042$, $p = 0.212$).

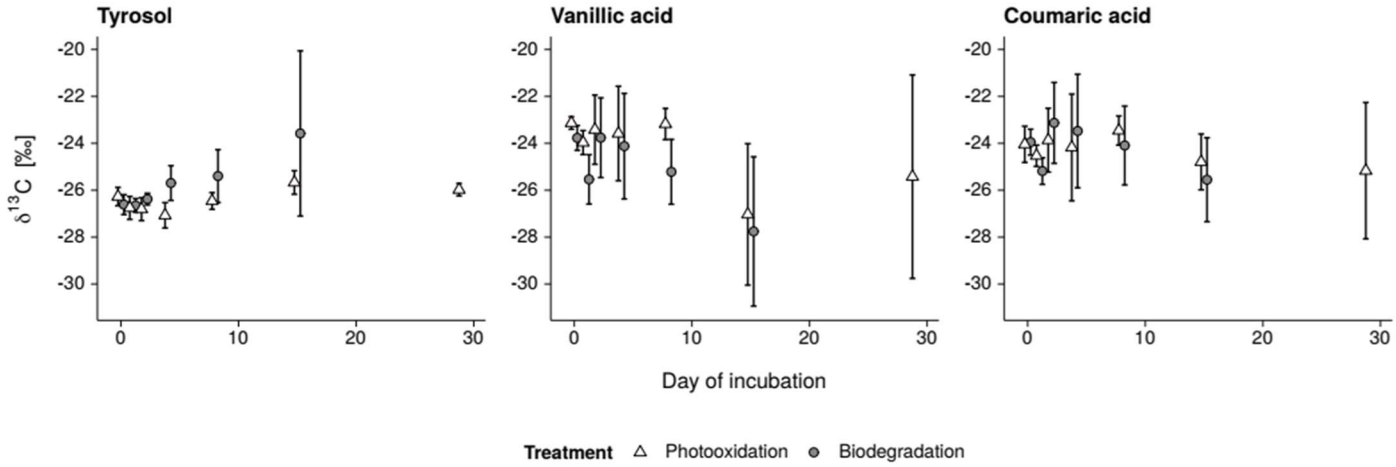


Figure 3: $\delta^{13}C$ of phenolic model compounds (mean ± 95 % CI) during degradation; vanillin was omitted due to unsuccessful method validation

During photooxidation, $\delta^{13}C$ values of dissipating tyrosol varied only by 0.3–0.5 ‰ at each sampling day but remained at a relatively constant level of –25.7 to –27.1 ‰ during incubation. The estimated AKIE was 1.02 ± 0.01 (ε = –3 ± 2 ‰), however, with a considerably worse goodness of fit (adj. $R^2 = 0.264$, $p = 0.010$) compared to biodegradation. For both vanillic acid and coumaric acid, no clear isotope effects were found considering sample variations of up to 4.3 ‰. This resulted in adj. $R^2 < 0.105$ and $p > 0.084$, thereby inflating errors for the estimation of ε and AKIEs.

Although precision and accuracy of replicate standard measurements were within acceptable limits (<0.3 ‰, see above) and comparable to other compound-specific stable isotope analyses (Maier et al., 2014; Reinnicke et al., 2010), the $\delta^{13}C$ variation of soil sample replicates was about 10 times higher. Such high variations have been similarly observed by Dungait et al. (2008) who studied the isotopic carbon composition of phenolic lignin moieties in soil. Due to the heterogeneous nature of soil, substrates are often spatially separated from decomposers or other reaction agents. Moreover, hydroxy or carboxylic moieties of phenolic compounds easily sorb to SOM or clay minerals (Sierra et al., 2007) as indicated by a slight decrease of phenolic compounds incubated in the dark after sterilization. This suggests that distribution processes and transformation reactions in soil are likely to proceed nonuniformly in dependence of the sorption potential of an organic compound. With that, heterogeneous reaction spaces may form and lead to irregular isotope fractionation during compound degradation. Moreover, organic soil constituents can easily cause interferences in $\delta^{13}C$ measurements, for instance, due to incomplete combustion of phenolic compounds in the combustion interface of the GC–IRMS. Follow-up studies should aim at disentangling the potential effects of soil matrix on changes in $\delta^{13}C$, for example, by analysis compound degradation in the presence and absence of different soils.

3.4. Soil water repellency and biological activity

The 29-d incubation experiment considerably influenced soil water repellency, soil respiration as well as ergosterol and PLFA biomarkers (Figure 4). For reference, control soil without phenolic compounds was generally classified as wettable (< 5 s WDPT) according to Bisdom et al. (1993). Photooxidation of phenolic compounds did not change the wetting behavior of the topsoil. Interestingly though, WDPTs increased to 60 ± 80 s after 29 d of biodegradation of phenolic compounds, indicating slight to strong water repellency.

Soil respiration in biodegradation controls, namely non-sterile soils without phenolic compounds, was 16–38 mg kg^{-1} $C{-}CO_2$. Sterile, UV-irradiated controls ranged from 20 to 33 mg kg^{-1} $C{-}CO_2$ (supplementary material S6). Given prior sterilization, the photooxidation treatment generally respired 14–23 mg kg^{-1} $C{-}CO_2$ less than the biodegradation treatment (Figure 4a). However, soil respiration of each treatment did not change significantly within the course of the experiment as indicated by 95 % CI testing.

The ergosterol content in biodegradation controls remained at 2.1–2.2 mg kg^{-1} during incubation (supplementary material S6). Sterile, UV-irradiated controls decreased from initial 1.04 ± 0.06 mg kg^{-1} to 0.34 ± 0.03 mg kg^{-1} ergosterol towards the end of the experiment, which was mostly likely attributed to UV-induced ergosterol degradation. By contrast, biodegradation of phenolic compounds significantly increased the ergosterol content in soil by 3 ± 1 mg kg^{-1} (95 % CI testing, Figure 4b).

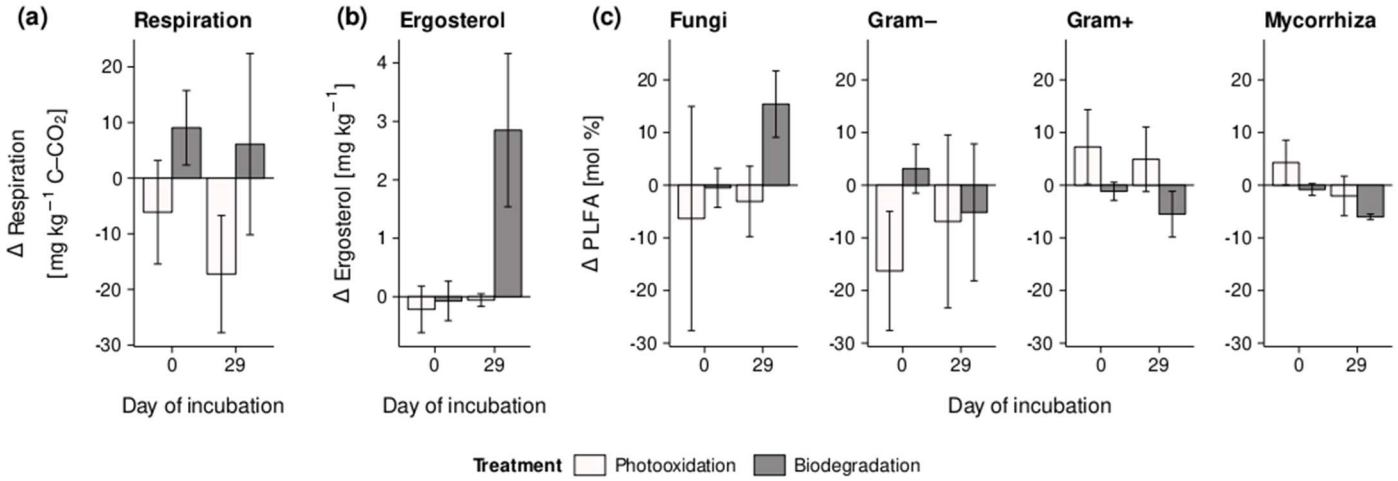


Figure 4: Differences in (a) soil respiration, (b) free ergosterol, and (c) PLFA biomarkers of microbial groups in spiked soils with respect to non-spiked controls (zero line) at the first day (0) and last day (29) of the incubation experiment; error bars indicate 95 % CIs; absolute numbers are given in the supplementary material S6

With respect to PLFA controls (see supplementary material S6 for detailed numbers), Gram–, Gram+, and mycorrhiza biomarkers decreased by about 5 mol % during biodegradation of phenolic compounds (Figure 4c). Consistent with ergosterol, fungal PLFA biomarkers strongly increased by 15 ± 6 mol %. In

408 the photooxidation treatment, changes in microbial biomarkers were mostly indistinct with respect to
409 sample variation.

410 Accompanied by the increase in fungal PLFA and ergosterol biomarkers at day 29 of biodegradation, we
411 observed extensive growth of fungal mycelia or fruiting bodies (sporocarp) in the incubation jars (see
412 supplementary material S4 for images). This increase coincided with an increase in soil water repellency,
413 which suggests that hydrophobic surface of fungal mycelia may have induced soil water repellency rather
414 than photooxidation and polymerization of phenolic compounds as assumed by Tamimi et al. (2016).
415 Owing to the fact that biomarker measurements were restricted to soil samples taken in the beginning
416 and at the end of the experiment, it remains unresolved how the soil microbial community developed
417 over time. Laor et al. (2011) reported a temporary increase in bacteria and fungi during the first 9 d after
418 OMW application. In the following three months, bacterial counts decreased and fungi remained
419 constant. The authors assumed bacteria to preferentially degrade easily available low-molecular-weight
420 compounds while fungi took over for the degradation of more complex substances. However, in our
421 study, soils were spiked only with low-molecular-weight phenolic compounds assumed to be easily
422 available to microorganisms. It is therefore more likely that the initial treatment with phenolic compounds
423 triggered rapid bacterial growth but that more slowly growing fungi eventually survived for their better
424 adaptability to incubation conditions (Peikert et al., 2017). Additionally, phenolic compounds or
425 temporarily present metabolites may have induced toxic effects towards bacteria rather than fungi
426 (Capasso et al., 1995; Mekki et al., 2006). This could further explain why overall soil respiration
427 remained constant at day 29 of biodegradation but was increased compared to controls and the
428 photooxidation treatment as similarly observed by Peikert et al. (2017).

429 ### *3.5. Discussion of degradation processes*

430 Soil biomarkers suggested that the biodegradation of phenolic model compounds was rather driven by
431 fungal than by bacterial degradation. Biodegradation of phenolic compounds was further indicated to
432 follow a pseudo-first-order reaction. Therefore, the reaction rate predominantly depended on the phenolic
433 content. However, the pseudo-first-order reaction may have involved enzymatic catalysis or another
434 reactant available in excess, for example oxygen. These findings are in contrast to those by Piotrowska
435 et al. (2006) and Peikert et al. (2017) who found evidence for the development of suboxic soil conditions

attributed to the initial and rapid aerobic microbial degradation of phenolic compounds or other OMW constituents. But contrary to the experiment conducted by Peikert et al. (2017), our experimental design allowed explicitly for ambient air to be exchanged with the head space of the incubation jars. This suggests that the first rate-limiting step may have involved an oxidation reaction. If rate-limiting, oxidation of aromatic rings typically leads to AKIEs of 1.001–1.006 (Morasch et al., 2002; Vogt et al., 2008), which is comparable with the AKIE of 1.007 ± 0.002 we observed for tyrosol. Moreover, our obtained AKIE was smaller than theoretically derived kinetic isotope effects of 1.01–1.03 for the oxidative cleavage of a C–H bond (Elsner et al., 2005). This further indicates that a non-fractionating, rate-determining step, such as mass transfer or substrate binding, must have preceded the catalytic bond cleavage involved in the oxidative biodegradation of tyrosol which masked the actual kinetic isotope effect (Elsner et al., 2012, 2005).

Although subject to high measurement uncertainty, biodegradation of vanillic acid indicated an AKIE of 0.987 ± 0.005, and hence a depletion of ^{13}C. Such an inverse isotope effect is interesting because natural degradation reactions typically enrich the parent compound in ^{13}C rather than deplete it (Elsner et al., 2005). This deviation may be owed to the experimental design which included both vanillin and vanillic acid as phenolic model compounds. Vanillic acid is a common metabolite of vanillin (Neilson and Allard, 2008). Consequently, the vanillic acid in the soil samples was of two origins: (1) the initial compound added through spiking and (2) the vanillin metabolite. Both vanillic acid species necessarily have a different isotopic composition, which renders the estimation of a reliable isotope enrichment factor impossible. This is a common problem when studying natural systems with a large number of potential compound sources and metabolic pathways. Similar observations were, for instance, made by Sherwood Lollar et al. (1999) and Maier et al. (2014), who studied the degradation of various aromatic compounds by native microbial communities without finding significant isotope effects.

Photooxidation of phenolic compounds was generally slower than biodegradation. Reaction kinetics were described equally well by zero- and first-order rate laws, which suggested the presence of another reactant, potentially a natural photocatalyst. This is consistent with the AKIE of 1.02 ± 0.01 we observed for the photooxidation of tyrosol, and Zhang et al. (2016) found comparable AKIEs of 1.024 and 1.031 for the hydroxyl radical-mediated photooxidation of nitrobenzene and dimethylaniline, respectively. By

contrast, photolytic bond cleavage is less likely to be the rate-limiting step of the degradation reaction, since it only induces AKIEs of 1.002–1.005 (Peng et al., 2013). The hydroxyl radicals required for photooxidation may have been formed by UV-induced photolysis of water with soil TiO2 as photocatalyst ($TiO2(h+) + H2O \rightarrow TiO2 + \bullet OH + H+$) or during photooxidation of SOM with reactive oxygen species (ROS) such as ozone or peroxides ($ROS + SOM \rightarrow SOM–HO\bullet$, Cheng et al., 2016).

Furthermore, Zhang et al. (2016) did not find any indication of compound polymerization during UV irradiation, which has been assumed to induce soil water repellency (Barbera et al., 2013; Tamimi et al., 2016). Here, we identified fungal growth during biodegradation of phenolic compounds as the main driver for soil water repellency.

4. Conclusions

This pilot study investigated the feasibility of detecting changes in $\delta^{13}C$ of selected phenolic compounds, namely tyrosol, vanillin, vanillic acid and coumaric acid, subjected to the sterile photooxidation and biodegradation by a native soil microbial community using compound-specific stable isotope analysis together with soil biomarkers. Our results suggested that biodegradation of phenolic compounds may has been dominated by fungi and most likely followed pseudo-first-order dissipation kinetics. Photooxidation was indicated to follow a mixed pseudo-zero- and first-order reaction probably mediated by hydroxyl radicals.

Although our newly developed GC–IRMS method performed well for laboratory standards, sample variation increased considerably when analyzing vanillin, vanillic acid, and coumaric acid in incubated soil. For tyrosol the method nonetheless allowed for distinguishing biodegradation and photooxidation by AKIEs and to discuss potential degradation mechanisms. In order to exploit the full potential of compound-specific stable isotope analysis, further method development should focus on reducing measurement variation which potentially originated from the heterogeneous structure of reaction spaces in soil. In such heterogeneous matrices, position-specific ^{13}C nuclear magnetic resonance or secondary ion mass spectrometry could help to better understand the nanoscale distribution and transformation of organic compounds. Once resolved, compound-specific stable isotope analysis could offer new opportunities for the identification of biotic and abiotic degradation processes of phenolic compounds in

the field. Such studies may then not be limited to plant residues but could extend to the analysis of allelochemicals exuded by roots or the characterization of lignin or SOM to derive a more comprehensive understanding of plant–soil interactions and SOM quality.

Acknowledgments

This study was part of TRILAT-OLIVEOIL (SCHA 849/13) and supported by the Ministerium für Wissenschaft, Weiterbildung und Kultur Rheinland-Pfalz, Germany, in the framework of the "Research initiative" program, project "AufLand". We thank Kilian Kenngott and Jones Athai for fruitful discussions on the manuscript, Kevin Dolan for English editing as well as Silvia Eichhöfer and Andreas Hirsch for their help in the laboratory.

Conflict of interests

The authors declare no competing conflicts of interest.

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This contribution was published as

Z. Steinmetz, M. P. Kurtz, J. P. Zubrod, A. H. Meyer, M. Elsner, G. E. Schaumann, *Biodegradation and photooxidation of phenolic compounds in soil – A compound-specific stable isotope approach*, Chemosphere 230 (2019) 230, 210-218" DOI: 10.1016/j.chemosphere.2019.05.030