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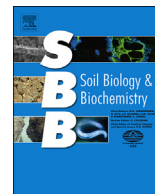
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# Microbial decomposition of $^{13}\text{C}$ -labeled phytosiderophores in the rhizosphere of wheat: Mineralization dynamics and key microbial groups involved

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## ABSTRACT

Being low molecular weight carbon (LMW-C) compounds, phytosiderophores (PS) released by strategy II plants are highly susceptible to microbial decomposition. However, to date very little is known about the fate of PS in soil. Using in-house synthesized  $^{13}\text{C}_4$ -2'-deoxymugineic acid (DMA), the main PS released by wheat, we investigated DMA mineralization dynamics, including microbial incorporation into phospholipid fatty acids (PLFA), in the wheat rhizosphere and bulk soil of two alkaline and one acidic soil. Half-lives of the intact DMA molecule (3–8 h) as well as of DMA-derived C-compounds (8–38 days) were in the same order of magnitude as those published for other LMW-C compounds like sugars, amino acids and organic acids. Combining mineralization with PLFA data showed that between 40 and 65% of the added DMA was either respired or incorporated into soil microbial biomass after 24 h, with the largest part of total incorporated DMA- $^{13}\text{C}$  being recovered in gram negative bacteria. Considering root growth dynamics and that PS are mainly exuded from root tips, the significantly slower mineralization of DMA in bulk soil is of high ecological importance to enhance the Fe scavenging efficiency of PS released into the soil.

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## 1. Introduction

The release of phytosiderophores (PS) by graminoid plants (referred to as strategy II) is considered a highly efficient strategy for acquiring Fe, particularly when growing in alkaline soils. Forming strong chelates with Fe, but also with other trace metals, phytosiderophores have been found to efficiently solubilize Fe from

*Abbreviations:* Phytosiderophores (PS), 2'-deoxymugineic acid (DMA); water content (WC), diethylene triamine pentaacetic acid (DTPA) phospholipid fatty acid (PLFA); stable isotope probing (SIP), low molecular weight carbon (LMW-C).

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insoluble Fe oxides and soils (Reichard et al., 2005; Schenkeveld et al., 2014a). With the organic ligand preventing Fe from re-precipitating, strategy II plants then take up the entire Fe(III)-PS complex.

As low molecular weight organic C compounds (LMW-C), PS are susceptible to microbial degradation once released from roots into the soil. von Wiren et al. (1993) showed that the recovered amount of Fe-chelating compounds released by maize was significantly reduced when plants were grown on a limestone substrate under non-axenic conditions compared to axenic conditions. Moreover in nutrient solution cultures the uptake of PS-Fe was significantly decreased when the culture medium was inoculated with different bacterial mixtures (Barnes et al., 1992;

Crowley et al., 1992). Furthermore, von Wiren et al. (1995) demonstrated that the depletion of the apoplastic Fe pool of sorghum (low PS exudation) was significantly reduced in non-axenic culture, while little difference in apoplastic Fe depletion was found between axenically and non-axenically grown barley (high PS exudation) indicating a strong effect of both release rate (quantity) and microbial activity on the Fe scavenging efficiency of PS. Also, Watanabe and Wada (1989) isolated six strains of mugineic acid decomposing bacteria from barley roots but found none in the culture solution. Overall, only a handful of studies investigated different aspects of microbial degradation of PS, with the majority of studies being carried out in solution culture or in artificial substrates. To the best of our knowledge, only (Shi et al., 1988) reported a decrease in ammonium carbonate extractable mugineic acid by 72% from a barley rhizosphere soil incubated for 12 h at room temperature, pointing to efficient microbial utilization of PS. Microbial utilization of PS has strong implications for soil PS concentrations and Fe solubilization, the latter of which was shown to increase with the amount of PS added (Reichard et al., 2005; Schenkeveld et al., 2014a).

All these results suggest that microbial decomposition plays a significant role in the functional efficiency of PS in soil. So far, PS exudation rates and PS concentrations in the rhizosphere were mostly derived from studies of plants grown in zero-Fe nutrient solution culture (e.g. Römheld, 1991). In a recent study however, we showed that zero-Fe hydroponic conditions lead to a significant overestimation of PS exudation rates compared to soil grown plants, resulting in much lower rhizosphere concentrations under natural soil growth conditions than originally anticipated (lower  $\mu\text{M}$  instead of mM range) (Oburger et al., 2014). In another recent study, Schenkeveld et al. (2014b) proposed the conceptual 'window for Fe acquisition' model, which describes the influence of soil processes on the efficiency of Fe mobilization by PS, both in terms of extent and duration. They identified a number of processes and parameters that either increased Fe mobilization by PS from soil (e.g. increased PS release rate from the root, increased Fe release rate from the soil, and increased soil-Fe solubility) or decreased it (e.g. increased adsorption of PS ligands and metal-PS complexes, increased complexation of competing metals, and increased (bio) degradation of the PS ligand) While a few studies exist describing PS-metal complex formation and sorption behavior (Reichard et al., 2005; Schenkeveld et al., 2014a, 2014b) and the influence of soil microbes thereon (Takagi et al., 1988; Schenkeveld et al., 2014b), the mineralization dynamics of PS in soil have not yet been investigated in detail.

In the past decades, experimental procedures using stable or radioisotopes have been found to be a powerful tool to trace the fate of C-compounds in soil. Recently Namba et al. (2007) published a chemical synthesis method of the phytosiderophore 2'-deoxy-mugineic acid (DMA) which has been applied by our collaborators and adapted to produce several grams of synthetic DMA as well as mg quantities of  $^{13}\text{C}_4$ -DMA (for details on DMA synthesis and molecule structure see Walter et al., 2014). The synthesized  $^{13}\text{C}$ -DMA enabled us for the first time to monitor DMA mineralization dynamics in soil. The objectives of this study therefore were to investigate  $^{13}\text{C}$ -DMA derived  $^{13}\text{CO}_2$  release in the rhizosphere (wheat, *Triticum aestivum* cv. Tamaro) and bulk soil of two calcareous and one acidic soil and to determine the main microbial groups responsible for DMA mineralization using PLFA-SIP (stable isotope probing) analysis. DMA mineralization gradients in the rhizosphere of wheat with increasing distance to the root surface were also investigated. The obtained data were then used to elucidate the dynamics of DMA partitioning between soil matrix (liquid & solid), and microbial uptake, the latter further divided into respiratory use and incorporation into biomass (PLFA) in different soils.

## 2. Materials and methods

### 2.1. Experimental soil and plant growth conditions

Experimental soils were collected from sites located in Austria (Lasee (Lass), Siebenlinden (SL)), and Spain (Santomera (Sant)). These soils are already described in detail in previous Fe deficiency studies (Schenkeveld et al., 2008, 2010; Oburger et al., 2014). While the soils from Lasee and Santomera are highly calcareous and were chosen according to their low DTPA-extractable Fe concentration (determined after Loeppert and Inskeep, 1996), the soil from Siebenlinden is acidic (pH 4.7) and has comparatively higher DTPA-extractable Fe concentrations (Table 1). Soils were fertilized with ( $\text{mg kg}^{-1}$ ):  $\text{NH}_4\text{NO}_3$  (530),  $\text{K}_2\text{HPO}_4$  (720),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (410),  $\text{H}_3\text{BO}_4$  (4),  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$  (0.8) and allowed to equilibrate for a week at 60% of the maximum water holding capacity prior to planting. All plant experiments were conducted in the greenhouse with an average day/night temperature of 27/20 °C and a 16-h photoperiod at 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR).

### 2.2. $^{13}\text{C}_4$ -DMA decomposition dynamics in three different soils

For comparing DMA decomposition and distribution of DMA derived  $^{13}\text{C}$  within PLFA biomarkers of the microbial community, wheat (*Triticum aestivum* cv Tamaro) was grown in rhizoboxes that were separated into two compartments (C1: 3 cm  $\times$  12 cm; and C2: 4  $\times$  12 cm respectively) by two layers of a 30  $\mu\text{m}$  nylon mesh holding a 2 mm layer of soil between the nylon membranes. This rhizobox-setup has been described in detail in Fitz et al. (2003b). Four rhizoboxes per soil (C1: 300 g dry weight (dwt.); C2: 500 g dwt., sieved < 2 mm) were prepared and four 2-day old wheat seedlings were planted into the smaller compartment C1, while C2 was maintained as root free bulk soil compartment. Wheat plants were grown for 37 days in the greenhouse as described above. Water content was kept constant by 4 glass fiber wicks (TRIPP Kristallo Rundschnur, 4 mm, IDT, Germany, covered by polyethylene tubes (diameter 4 mm) to prevent evaporation) inserted into the rhizoboxes and connected to a water reservoir. The day before harvest, soil samples were taken to determine the water content in each rhizobox to ensure accurate correction for it. The average water content was  $24 \pm 4\%$  (of dwt, mean  $\pm$  SD) across all rhizoboxes and compartments. At harvest, bulk soil and rhizosphere soil were collected from the unplanted and planted compartments respectively, for each rhizobox separately. By the time of harvest, the planted compartments were densely rooted and therefore the entire soil was considered as rhizosphere soil. To separate rhizosphere soil from roots, plant roots were gently shaken, the loose soil was collected and root debris was removed. The soil from the 2-mm separation layer was also considered rhizosphere soil as the 30  $\mu\text{m}$  nylon mesh restricts root growth but not the penetration of root hairs. Subsequently subsamples (2 g dwt.) from each rhizobox were weighed into 50 ml PE-tubes and either 120  $\mu\text{L}$  of the in-house synthesized  $^{13}\text{C}_4$ -DMA solution (30  $\mu\text{M}$ ) (Walter et al., 2014) or deionized water as control was immediately added to the soils and the vials closed using air-tight suba seal rubber septa (Sigma Aldrich). An additional set of samples was prepared from the Santomera rhizosphere soil where 120  $\mu\text{L}$  of 300 and 3000  $\mu\text{M}$   $^{13}\text{C}_4$ -DMA solution were added. Samples were incubated at 20 °C in the dark for a period of seven days. With an average initial soil water content (WC) of  $24 \pm 4\%$ , the addition of 1.8 nmol DMA per g soil (2 g soil plus 120  $\mu\text{L}$  30  $\mu\text{M}$  DMA) resulted in an initial DMA concentration in the soil solution of  $6.0 \pm 0.8 \mu\text{M}$ , as well as  $60 \pm 8 \mu\text{M}$ , and  $596 \pm 79 \mu\text{M}$  (all mean  $\pm$  SD), in the Santomera rhizosphere soil (2 g soil plus 120  $\mu\text{L}$  of 300  $\mu\text{M}$  and 3000  $\mu\text{M}$  DMA respectively) if immediate adsorption

**Table 1**  
General soil parameters of the experimental soils: pH, calcium carbonate content (CaCO<sub>3</sub>) soil organic carbon (SOC), diethylenetriamine pentaacetic acid (DTPA) – extractable Fe concentrations, and acid ammonium oxalate extraction (amorphous) Fe and Al oxides concentrations (Loeppert and Inskeep, 1996).

	pH CaCl <sub>2</sub>	CaCO <sub>3</sub> g kg <sup>-1</sup>	SOC g kg <sup>-1</sup>	Clay mg kg <sup>-1</sup>	Fe (DTPA) mg kg <sup>-1</sup>	Fe (AAO) mg kg <sup>-1</sup>	Al (AAO) mg kg <sup>-1</sup>
Santomera	7.8	499	7.3	306	4.9	0.5	0.7
Lassee	7.7	138	16	268	4.8	1.1	1.7
Siebenlinden	4.7	9	13	103	47	2.8	1.9

processes are not taken into account.

The overall experimental layout was as follows: (i) <sup>13</sup>C DMA – 6 μM (final rhizosphere soil solution concentration): 3 soils × 4 replicates × 2 soil treatments (rhizosphere/bulk soil) × 7 time points = 168 samples, control: 3 soils × 4 replicates × 2 soil treatments × 3 time points (0.5, 24, 168 h = 72 samples). (ii) <sup>13</sup>C DMA – 60 and 600 μM: 1 soil (Santomera only) × 4 replicates × 1 soil treatment (rhizosphere only) × 2 concentrations × 7 time points = 56 samples, resulting in a total number of 296 samples.

### 2.2.1. DMA derived <sup>13</sup>CO<sub>2</sub> release

Gas samples were taken at 0.5, 2, 8, 24, 48, 96, 168 h after <sup>13</sup>C-DMA addition using a 30 ml syringe. The collected gas was transferred to evacuated glass vials (12 ml exetainers) and CO<sub>2</sub> concentration as well as the <sup>13</sup>C/<sup>12</sup>C ratio analyzed by headspace sampler (GasBench II, Thermo Fisher) coupled to a gas-isotope ratio mass spectrometer (Delta V Advantage, Thermo Fisher). <sup>13</sup>CO<sub>2</sub> production in the labeled samples during the given incubation time was calculated by (1) converting the obtained δ<sup>13</sup>C values into atom percent of the heavy isotope (AT%<sup>H</sup>) with δ<sup>13</sup>C<sub>sample</sub> being the measured δ<sup>13</sup>C notations and R<sub>pDB</sub> being the isotopic ratio of the fossil carbon standard PeeDee Belemnite (Eq (1))

$$AT\%^H = \frac{\delta^{13}C_{sample} + 1000}{\delta^{13}C_{sample} + 1000 + \frac{1000}{R_{pDB}}} \quad (1)$$

(2) by calculating the atom percent enrichment (APE in %) in the labeled sample compared to the unlabeled control (Eq (2))

$$APE = AT\%_{labeled\ sample}^H - AT\%_{control}^H \quad (2)$$

and (3) by multiplying APE with the measured CO<sub>2</sub> concentration in the sample (Eq (3)).

$$^{13}CO_2 [nmol\ g^{-1}\ t^{-1}] = APE * CO_2\ tot [nmol\ g^{-1}\ t^{-1}] \quad (3)$$

with t representing the individual incubation time. The amount of <sup>13</sup>CO<sub>2</sub> released was expressed as percentage (%) of DMA-<sup>13</sup>C mineralized to <sup>13</sup>CO<sub>2</sub>.

### 2.2.2. Accounting for DMA-derived <sup>13</sup>CO<sub>2</sub> trapped in the alkaline soils

Using radiolabeled <sup>14</sup>C-organic acids Ström et al. (2001) demonstrated that a significant proportion of organic acid derived <sup>14</sup>CO<sub>2</sub> can be trapped as Ca(H<sup>14</sup>CO<sub>3</sub>)<sub>2</sub> in alkaline soils, leading to an underestimation of C substrate mineralization. The same authors also presented a method to determine the amount of <sup>14</sup>CO<sub>2</sub> trapped in an alkaline soil which is based on dissolving the soil's carbonate content through the addition of HCl and capturing the released CO<sub>2</sub> and <sup>14</sup>CO<sub>2</sub> in a series of NaOH traps. However this method cannot be applied using stable isotopes, as the sensitivity of the headspace-IRMS is unlikely to be sufficient at the extremely high background <sup>12</sup>CO<sub>2</sub> concentrations (i.e. CO<sub>2</sub> released from carbonate soil after HCl addition). Therefore we used <sup>14</sup>C-citrate as a proxy for DMA and adapted the method introduced by Ström et al. (2001) to our experimental conditions to determine the amount on

citrate derived <sup>14</sup>CO<sub>2</sub> trapped in both alkaline soils over time. Citrate has also been found to efficiently solubilize Fe (e.g. Oburger et al., 2011) and despite the lower metal complex affinity compared to DMA, sorption and complexation behavior can be expected to be similar. Our setup differed in the sampling procedure of the gas headspace. Unlike Ström et al. (2001) we did not use NaOH traps placed directly into the incubated sample to capture the released <sup>14</sup>CO<sub>2</sub>, as this would result in a different (and uncomparably low) CO<sub>2</sub> partial pressure as in our stable isotope setup. Hence we added 120 μL of 60 μM <sup>14</sup>C-citrate (concentration corresponds to the same in terms of total C as added in the DMA experiment at 30 μM) to 2 g of the Lassee and Santomera soils (same water content as for the stable isotope experiment) as well as 6000 μM <sup>14</sup>C-citrate to the Santomera soil only and incubated the samples for 0.5, 2, 24 and 168 h. Gas samples (20 ml) were taken via a syringe as described above (stable isotope experiment), but the samples were transferred to rubber-sealed 50 ml vials containing 1 ml 1 M NaOH and the gas trap was left to react for 24 h. Immediately after taking the gas sample, 10 ml of 4 M HCl were added to the soil and the evolved <sup>14</sup>/<sup>12</sup>CO<sub>2</sub> was captured by passing air over the sample and subsequently through 5 consecutive tubes containing each 5 ml and a 6th tube containing 10 ml of 2 M NaOH for one hour using a similar setup as described by Ström et al. (2001). The <sup>14</sup>C activity in all NaOH traps was then determined by liquid scintillation counting using UltimaGold™ XR scintillation fluid (PerkinElmer Corp., Shelton, USA) and a TriCarb 2910 TR liquid scintillation counter (PerkinElmer, Inc. Waltham, MA, USA). We also tested the acidic Siebenlinden, but like already demonstrated by Ström et al. (2001), no <sup>14</sup>CO<sub>2</sub> release upon HCl addition was found (data not shown). Using the obtained data, a CO<sub>2</sub> correction factor to account for the respired CO<sub>2</sub> trapped in the carbonate system of the soil was calculated as follows:

$$CO_2\ correction\ factor = \frac{^{14}C\ activity\ (gas\ headspace)}{^{14}C\ activity\ (gas\ headspace + soil)} \quad (4)$$

Assuming no isotopic fractionation, total CO<sub>2</sub> (Fig. 2) as well as <sup>13</sup>CO<sub>2</sub> release (Fig. 3) from Santomera and Lassee soils were corrected accordingly.

### 2.2.3. Determination of DMA and DMA-C half lives

The corrected <sup>13</sup>CO<sub>2</sub> mineralization data were used to estimate the half-life of the intact DMA molecule as well as the half-life of DMA-derived C including already assimilated and metabolized DMA-C in the soil. In most soils, LMW C-substrate mineralization showed a biphasic pattern with a rapid phase of substrate-derived CO<sub>2</sub> production followed by a slower second phase of CO<sub>2</sub> evolution (e.g. Nguyen and Guckert, 2001; Oburger et al., 2009). A double first order exponential decay model was therefore fitted to the experimental data for all sites and treatments

$$S_t = [a_1, t=0 e^{(-k_1 t)}] + [a_2, t=0 e^{(-k_2 t)}] \quad (5)$$

where S<sub>t</sub> is the <sup>13</sup>C-label remaining in the soil at any given time, a<sub>1</sub>

and the rate constant  $k_1$  describe the fraction of the added substrate that is taken up and rapidly respired, and  $a_2$  and the rate constant  $k_2$  describe the decomposition of the fraction that is temporarily immobilized either through uptake by the microbial biomass (e.g., formation of new biomass or secondary metabolites) and/or adsorption by the soil solid phase. The duration of the first rapid phase of  $^{13}\text{CO}_2$  release ( $a_1, k_1$ ) approximates the depletion of the intact molecule from the soil while the slower second phase of  $^{14}\text{CO}_2$  production ( $a_2, k_2$ ) can be related to incorporation of the C into the microbial biomass, secondary metabolite formation and microbial turnover. The half-life of pool  $a_1$  (i.e. intact DMA molecule) can be calculated using the equation

$$t_{1/2} = \ln(2)/k_1 \quad (6)$$

The half-life of DMA-derived C were computed numerically by applying the Newton method to Eq (5) and replacing  $S$  by  $(a_1 + a_2)/2$ , as Eq (5) cannot be solved explicitly (Oburger and Jones, 2009). The modeling of DMA mineralization was undertaken with the software package Sigmaplot v12.5 (SPSS Inc., Chicago, IL).

#### 2.2.4. DMA incorporation by the microbial community – $^{13}\text{C}$ PLFA analysis

At each gas sampling, the incubated soil was also collected (destructive approach) and shock frozen by plunging the sample into liquid  $\text{N}_2$ , thereby inhibiting any further microbial activity. Soil samples were subsequently stored at  $-20^\circ\text{C}$  to await PLFA analysis. To compare differences in microbial community structure and  $^{13}\text{C}$  incorporation in microbial biomass, PLFA extraction and analysis was carried out for control and labeled samples incubated for 24 h of all 3 experimental soils and DMA concentrations. Additionally  $^{13}\text{C}$  PLFA concentrations were also determined in labeled samples retrieved after 0.5 h and 168 h incubation from the Lasse soil, only to investigate changes in  $^{13}\text{C}$  incorporation within the microbial community over time.

PLFA extraction was carried out according to Frostegard et al. (1991), but adapted to our laboratory facilities. The used extraction procedure and analysis was described in detail by Watzinger et al. (2014) (supplementary information), except that the GC-IRMS (gas chromatography – isotope ratio mass spectrometry) was an Agilent GC 7890A connected to Delta V Advantage IRMS (Thermo Fisher) with a CuO/NiO/Pt combustion oven and a CTC PAL autosampler and Gerstel PTV injector. It was operated in splitless mode and with a constant He flow of 1.1. ml/min. The GC column temperature was held at  $70^\circ\text{C}$  for 0.9 min and subsequently ramped from  $70$  to  $160^\circ\text{C}$  at  $15^\circ\text{C}$   $\text{minute}^{-1}$ , then to  $245^\circ\text{C}$  at  $2.5^\circ\text{C}$   $\text{minute}^{-1}$  and at  $30^\circ\text{C}$   $\text{min}^{-1}$  to  $290^\circ\text{C}$ , which was hold for 5 min. Due to the low PLFA concentrations in the Santomera soil, the duplicate samples from the 24 h incubation were pooled and 4 g instead of 2 g were extracted with the double amount of chloroform/methanol/citrate buffer solution. After the collection and drying of crude lipid extracts the remaining steps were carried out identically for all samples. The  $\delta^{13}\text{C}$  values of the PLFAs were obtained by correcting the  $\delta^{13}\text{C}$  values of the FAMES

(fatty acid methyl ester) for the methyl-C that was added during methylation (Eq (7))

$$\delta^{13}\text{C}_{\text{PLFA}} = \frac{C_{\text{FAME}} \times \delta^{13}\text{C}_{\text{FAME}} - C_{\text{MeOH}} \times \delta^{13}\text{C}_{\text{MeOH}}}{C_{\text{PLFA}}} \quad (7)$$

where  $C_{\text{FAME}}$ ,  $C_{\text{MeOH}}$ , and  $C_{\text{PLFA}}$  denote the number of carbon atoms in the FAME, methanol, and PLFA, respectively, and  $\delta^{13}\text{C}_{\text{FAME}}$  and  $\delta^{13}\text{C}_{\text{MeOH}}$  are the measured isotope ratios of the FAME and methanol, respectively. The isotopic signature of methanol was  $\delta^{13}\text{C} = -30.1$ . The amount of  $^{13}\text{C}$  incorporated in the microbial fatty acids was calculated by converting the  $\delta^{13}\text{C}$  values into at% and then APE in the labeled samples was calculated (see above:  $^{13}\text{CO}_2$  release) and multiplied by the measured PLFA concentrations (nmol PLFA  $\text{C g}^{-1}$ ). PLFA markers were used as bioindicators for different microbial groups (Table 2) and summarized for further data analysis. The distribution of DMA- $^{13}\text{C}$  within the microbial community was calculated as the percentage share of  $^{13}\text{C}$  incorporated in the PLFA markers of each microbial group of the total  $^{13}\text{C}$  incorporated in all analyzed PLFAs (e.g.  $\sum \text{gram-pos PLFA-}^{13}\text{C} / \sum \text{total PLFA-}^{13}\text{C} \times 100$ ). The soil specific  $^{13}\text{C}$  incorporation into total PLFAs was calculated by dividing the sum of  $^{13}\text{C}$  incorporated across all individual PLFAs (pmol  $^{13}\text{C g}^{-1}$  soil) by the total PLFA C (nmol  $^{12}\text{C g}^{-1}$  soil). To estimate the amount of  $^{13}\text{C}$ -DMA incorporated into the microbial biomass (percentage of total  $^{13}\text{C}$  DMA added) the conversion factor ( $F_{\text{PLFA}}$ ) of 5.8 was used to convert the sum of detected PLFA markers (nmol PLFA- $^{13}\text{C g}^{-1}$ ) to  $\mu\text{g}$  total microbial  $^{13}\text{C}$  per g dry soil (Joergensen and Emmerling, 2006), since PLFAs only constitute a small fraction of total microbial C.

#### 2.3. $^{13}\text{C}$ - DMA mineralization dynamics at a high spatial resolution – rhizosphere gradients

To sample rhizosphere soil at millimeter resolution, the same wheat cultivar was grown in a different rhizobox setup that allows the development of a soil-free root mat that is separated from a soil compartment by a  $30 \mu\text{m}$  nylon mesh. The mesh allows nutrient and solute exchange with the soil but restricts root growth. The experimental setup and growth conditions were described in detail in Oburger et al. (2014). Only the highly calcareous Santomera soil was used in this study and wheat plants were grown for 7 weeks under the conditions as described above. In Oburger et al. (2014) this rhizobox experiment was used to repeatedly and non-destructively measure root DMA release from soil grown plants. In the current study, we harvested the fresh soil from three rhizosphere soil compartments using a custom-made Plexiglas slicing device that enabled us to sample fresh soil adjacent to the root mat in mm-resolution. Slicing device and procedure were described in detail in Fitz et al. (2003a). Soil layers in 0–1, 1–2, 2–3, 3–4 mm distance from the root mat and a bulk soil sample ( $>1.5$  cm distance from the root mat) were collected. Immediately after the collection of the rhizosphere soil layers, six 1.2 g aliquots of fresh soil from each rhizobox and soil layer were weighed into 50 ml PE-tubes and either  $60 \mu\text{L}$  of an in-house synthesized  $^{13}\text{C}_4$ -DMA solution ( $20 \mu\text{M}$ )

**Table 2**  
Assignments of phospholipid fatty acid (PLFA) bioindicators used in this study.

PLFA bioindicators	Specific PLFA markers
Actinomycetes (Act)	10Me 16:0; 10Me 17:0; 10Me 18:0; 12Me 18:0
AM fungi (AM)	16:1 $\omega$ 5
Fungi	18:2 $\omega$ 6.9
Gram-negative bacteria	16:1 $\omega$ 7; 16:1 $\omega$ 6; 17:1 $\omega$ 8; 18:1 $\omega$ 7c/9t; 18:1 $\omega$ 8c; cy17:0; cy19:0
Gram-positive bacteria	i14:0; i15:0; a15:0; i16:0; i17:0; a17:0
Unspecific biomarkers	14:0; 15:0; 16:0; 17:0; 18:0; 18:1 $\omega$ 9c; i17:1 $\omega$ 8; 19:1

or deionized water (control) was added to the soils and the vials were closed using air-tight suba seal rubber septa (Sigma Aldrich). Samples were incubated as described above. With an average initial soil water content of  $16 \pm 2\%$  (mean  $\pm$  SD), the addition of 1.2 nmol DMA per g soil (60  $\mu$ L 20  $\mu$ M DMA) resulted in an estimated DMA concentration in soil solution of  $4.9 \pm 0.6 \mu$ M (mean  $\pm$  SD). Gas samples were taken and analyzed as described above, except no sampling was conducted at 2 h after DMA addition and no PLFA extractions were carried out. The overall experimental layout was as follows:  $^{13}\text{C}$  DMA: 1 soil  $\times$  3 replicates  $\times$  5 soil layers  $\times$  5 time points = 75 samples, control: 1 soil  $\times$  3 replicates  $\times$  5 soil layers  $\times$  2 time points (0.5, 48 h) = 30 samples, resulting in a total number of 105 samples.  $^{13}\text{CO}_2$ -release was calculated and corrected as described above.

#### 2.4. Statistical analysis

All statistical analyses were carried out using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Results were compared using either an independent *t*-test or a one-way ANOVA in combination with the Student-Newman-Keuls post hoc test with  $p < 0.05$ . For each presented data set, the information of the statistical procedure applied is included in the figure and table captions respectively.

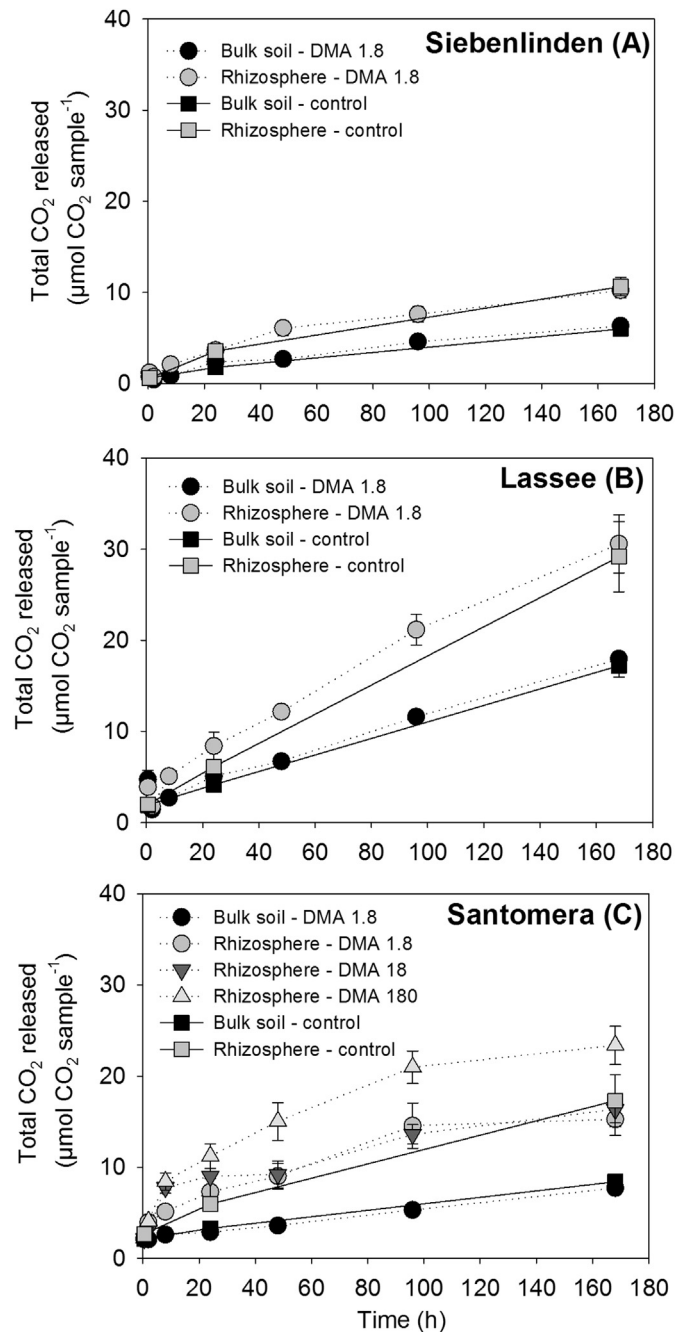
### 3. Results

#### 3.1. $^{13}\text{C}$ -DMA mineralization dynamics in three different soils – $^{13}\text{CO}_2$ release

Despite differences in soil carbonate content, a similar  $\text{CO}_2$  correction factor was determined for both soils (for detailed results see supplementary information, Fig. S1). Since the experimentally obtained correction factors did not significantly differ between the different time points and citrate concentrations applied, the soil specific average  $\text{CO}_2$  correction factor across all investigated time points (Lassee:  $0.20 \pm 0.01$ ; Santomera  $0.19 \pm 0.03$ , mean  $\pm$  SD) was used to correct for the total  $\text{CO}_2$  and DMA-derived  $^{13}\text{CO}_2$  release in the alkaline soils. Except for the first sampling time point (0.5 h), total  $\text{CO}_2$  release was significantly higher in the rhizosphere than in the bulk soil for all soils and treatments (Fig. 1, statistical results see SI-Table S1). The addition of the low and medium concentrations of  $^{13}\text{C}$ -DMA (1.8 & 18 nmol  $\text{g}^{-1}$ ) did not increase total  $\text{CO}_2$  release in the rhizosphere and bulk soil of Santomera compared to the corresponding controls; only the addition of the highest DMA concentration respectively (180 nmol  $\text{g}^{-1}$ , Santomera rhizosphere only) resulted in a significantly enhanced  $\text{CO}_2$  release (Fig. 1C). Generally, background soil respiration (control treatment as an indicator for microbial activity) increased in the order: Siebenlinden (acidic soil) < Santomera < Lassee.

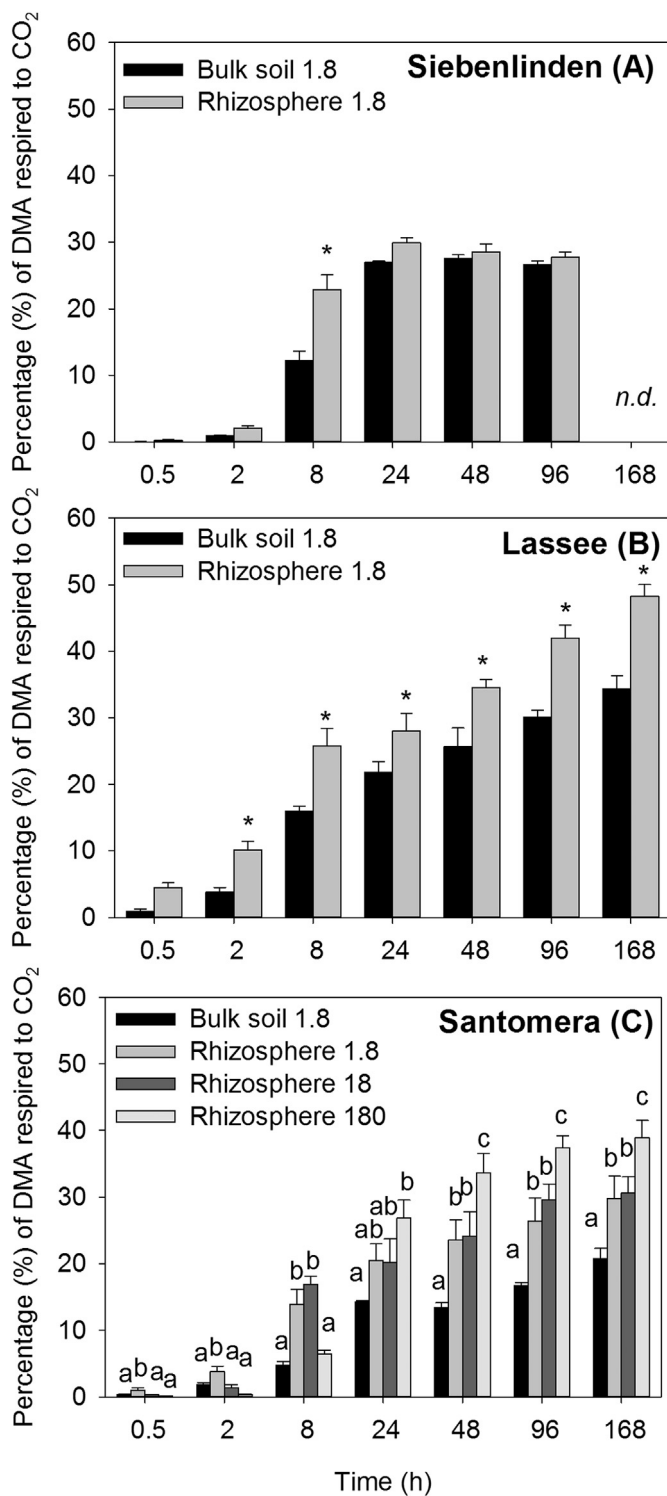
Plotting the percentage of added  $^{13}\text{C}$ -DMA respired to  $\text{CO}_2$  over time (Fig. 2) revealed a significantly faster and overall stronger mineralization of DMA in the rhizosphere than in the bulk soil, particularly in both alkaline soils. Interestingly, a lag phase in DMA-derived  $\text{CO}_2$  release could be observed in the acidic soil (Siebenlinden) at the first two sampling time points for both rhizosphere and bulk soil (0.5 and 2 h), as well as in rhizosphere compared to the bulk soil after 8 h. Mineralization of DMA was also delayed when higher DMA concentrations were added to the Santomera rhizosphere soil (18 nmol  $\text{g}^{-1}$ : 0.5, 2 h; 180 nmol  $\text{g}^{-1}$ : 0.5, 2, 8 h), with the longest delay being observed for the highest DMA concentration.

The double first order kinetic model (Eq (5)) fitted reasonably well to the obtained mineralization data ( $r^2 > 0.86$ , SI-Fig. S2). Calculated half-lives for the intact DMA molecule for the low and medium DMA additions (1.8 & 18 nmol  $\text{g}^{-1}$ ) ranged between 3.1



**Fig. 1.** Total  $\text{CO}_2$  release for rhizosphere and bulk soil with or without  $^{13}\text{C}$ -deoxymugenic acid (DMA) addition (1.8, 18 or 180 nmol  $\text{g}^{-1}$ ) for the acidic soil Siebenlinden (A) and the two alkaline soils Lassee (B) and Santomera (C). Values represent means  $\pm$  SE ( $n = 4$ ). Results from the two alkaline soils were corrected with the experimentally determined factor for respiratory  $\text{CO}_2$  retention by the soil carbonate system (Eq (4)).

and 8.1 h, and showed a tendency (though not statistically significant) of being longer in the bulk soil than in the rhizosphere (Table 3). A 100-fold increase in DMA concentration (Santomera 180 nmol  $\text{g}^{-1}$ ) resulted in an almost tripling of DMA half-life in the Santomera rhizosphere. The half-lives of DMA-derived  $^{13}\text{C}$  ranged between 8 and 38 days in the alkaline soils. Due to the asymptotic behavior of the mineralization data in Siebenlinden and Santomera (highest DMA concentration only) soils, half-lives of DMA-derived C could not be calculated.



**Fig. 2.** Percentage of  $^{13}\text{C}$ -deoxymugenic acid (DMA) added (1.8, 18 or 180  $\text{nmol g}^{-1}$ ) respired to  $\text{CO}_2$  in rhizosphere and bulk soil of the acidic soil Siebenlinden (A) and the two alkaline soils Lasse (B) and Santomera (C). Values represent means  $\pm$  SE ( $n = 4$ ); n.d. not determined. Results from the two alkaline soils were corrected with the experimentally determined factor for respiratory  $\text{CO}_2$  retention by the soil carbonate system (Eq (4)). Significant differences between rhizosphere and bulk soil at a single time point are marked with an asterisk (\*,  $p < 0.05$ ). Letters a, b, c indicate significant differences between the different concentrations applied to the Santomera soil ( $p < 0.05$ ).

**Table 3**

Half-lives of the intact deoxymugenic acid (DMA) molecule (soil matrix depletion) as well as DMA derived-C (microbial incorporation and turnover) in the different soils. Letters represent significant differences between the different soils (ANOVA,  $p < 0.05$ ). Values represent means  $\pm$  SE ( $n = 4$ ). n.d. – not determined due to asymptotic behavior of the fitted curve.

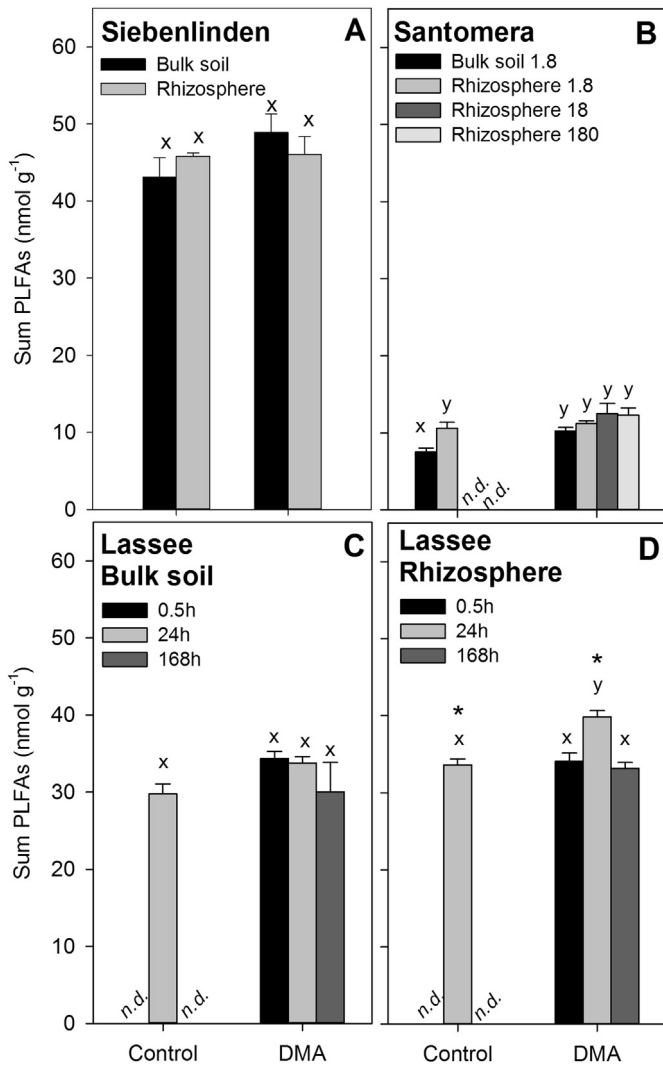
Soil - treatment - $\text{nmol g}^{-1}$ DMA added		DMA half-life Mean $\pm$ se hours	DMA-C half-life Mean $\pm$ se days
Siebenlinden	Bulk 1.8	7.3 <sup>bc</sup> $\pm$ 0.6	n.d.
	Rhizo 1.8	3.9 <sup>ab</sup> $\pm$ 0.5	n.d.
Lasse	Bulk 1.8	3.5 <sup>ab</sup> $\pm$ 0.6	11 <sup>x</sup> $\pm$ 1
	Rhizo 1.8	3.1 <sup>ab</sup> $\pm$ 0.7	8 <sup>x</sup> $\pm$ 1
Santomera	Bulk 1.8	8.1 <sup>c</sup> $\pm$ 0.9	38 <sup>z</sup> $\pm$ 9
	Rhizo 1.8	5.6 <sup>abc</sup> $\pm$ 1.4	28 <sup>yz</sup> $\pm$ 8
	rhizo 18	4.5 <sup>ab</sup> $\pm$ 0.8	22 <sup>xy</sup> $\pm$ 2
	rhizo 180	15.2 <sup>d</sup> $\pm$ 1.6	n.d.

### 3.2. $^{13}\text{C}$ -DMA mineralization dynamics in three different soils – $^{13}\text{C}$ -DMA incorporation by the microbial community

In the rhizosphere of the control treatments of both alkaline soils a significantly higher total PLFA concentration was found compared to the bulk soil, while no difference was observed in the acidic Siebenlinden soil (Fig. 3). The effect of  $^{13}\text{C}$ -DMA addition varied between the soils. While no change was observed in the acidic Siebenlinden soil (Fig. 3A), total PLFA concentrations in the Santomera bulk soil significantly increased at low additions of  $^{13}\text{C}$ -DMA (1.8  $\text{nmol g}^{-1}$ ) to level concentrations observed in the rhizosphere (Fig. 3B). Interestingly none of the  $^{13}\text{C}$ -DMA additions (1.8, 18, 180  $\text{nmol g}^{-1}$ ) resulted in a significant increase in total PLFA concentration in the rhizosphere of this soil. Investigating changes in total PLFA concentrations over time in the Lasse soil revealed no significant effect of low  $^{13}\text{C}$ -DMA additions (1.8  $\text{nmol g}^{-1}$ ) in the bulk soil while a significant increase in total PLFAs after 24 h could be observed in the rhizosphere followed by a drop to the initial PLFA concentrations after 168 h (Fig. 3C & D). Across the soils PLFA concentrations increased in the order Santomera < Lasse < Siebenlinden.

The assignment of single PLFAs to specific microbial groups is marked by ongoing disagreements within the scientific community and even though the chosen PLFA assignments (Table 2) reflect the most common ones found throughout the literature, presented results need to be considered accordingly. Detailed information on  $^{13}\text{C}$  incorporation by individual fatty acids is presented in the supplementary information (Fig. S3). The distribution of community specific and unspecific PLFA biomarkers (presented as percentage of total PLFAs) differed between the soils but was similar between rhizosphere and bulk soil and only rather small changes (however some of them significant) upon DMA addition could be observed (SI Fig. S4, statistical results see SI Table S2). Gram negative bacteria dominated in both alkaline soils (Lasse: 37%, Santomera: 42%), while unspecific PLFA biomarker comprised the largest PLFA fraction in the acidic soil (45%).

Calculating the relative distribution of the total  $^{13}\text{C}$  label incorporated between the different microbial groups revealed that in both alkaline soils between 60% and 73% of DMA-derived  $^{13}\text{C}$  was held in PLFA markers specific for gram negative bacteria, followed by 17–24% incorporated in unspecific bacterial PLFAs and 10% incorporated in PLFAs specific for gram positive bacteria (Fig. 4A, B, C, D). In the acidic soil however about 45% of the total DMA-derived  $^{13}\text{C}$  were recovered in both gram negative and unspecific bacterial



**Fig. 3.** Sum of phospholipid fatty acids (PLFA) in the rhizosphere and bulk soil of wheat growing on the acidic soil Siebenlinden (A) and the two alkaline soils Santomera (B) and Lasse (C,D) after the addition of 1.8 nmol g<sup>-1</sup> <sup>13</sup>C-deoxymugineic acid (DMA) and an incubation time of 24 h (as well as 0.5 and 168 h respectively for the Lasse soil only). Values represent means  $\pm$  SE ( $n = 4$ ). Siebenlinden & Santomera: Letters indicate significant differences across soil compartment and treatments (ANOVA,  $p < 0.05$ ). Lasse soil: Letters indicate significant differences over time across different treatments within each soil compartment (ANOVA,  $p < 0.05$ ). Significant differences between rhizosphere and bulk soil at a single time point are indicated by an asterisk (\*, independent  $t$ -test,  $p < 0.05$ ).

PLFAs, with PLFAs of gram positive bacteria representing only about 5% of the total <sup>13</sup>C label incorporated (Fig. 4A). Saprophytic fungi and AM fungi biomarkers generally held less than 5% of the total <sup>13</sup>C label incorporated in all three experimental soils. However their content in soil and consequently their contribution to DMA decomposition might be underestimated due to the greater  $C_{mic}:PLFA$  ratio of fungi compared to bacteria. Significant differences in <sup>13</sup>C uptake and incorporation were also observed between rhizosphere and bulk soils, particularly in the two alkaline soils. While the investigation of <sup>13</sup>C incorporation dynamics over time in the Lasse soil revealed significantly higher incorporation by actinomycetes and gram positive bacteria, but lower incorporation in gram negative in the bulk compared to the rhizosphere soil after 0.5 h, no differences between rhizosphere and bulk soil were found after 24 h, however after 168 h a significant increase in <sup>13</sup>C

incorporated in gram positive bacteria in the rhizosphere was observed (Fig. 4B,C). Furthermore, increasing <sup>13</sup>C DMA additions caused a significant shift of DMA uptake from gram negative to gram positive bacteria in the rhizosphere of the Santomera soil (Fig. 4D).

Based on PLFA results, we could estimate that overall between 19% and 27% of the total DMA-<sup>13</sup>C added were recovered in microbial biomass after 24 h (Fig. 4 E, F, G, H). Investigating the temporal trend of DMA-<sup>13</sup>C incorporation in the Lasse soil showed a significantly faster and greater incorporation of <sup>13</sup>C in the rhizosphere compared to the bulk soil after 0.5 and 24 h, followed by a decline in the rhizosphere microbial <sup>13</sup>C pool after 168 h. Interestingly, the 10- and 100-fold addition of <sup>13</sup>C-DMA to the Santomera rhizosphere soil had little effect on the relative proportion of the total <sup>13</sup>C label incorporated into microbial biomass. Calculating the extent of incorporation of DMA-derived <sup>13</sup>C after 24 h revealed a significantly higher <sup>13</sup>C incorporation in the microbial biomass of the Santomera soil compared to the other soils (Table 4). Furthermore, significantly higher <sup>13</sup>C incorporation was found in the Santomera bulk soil compared to its rhizosphere soil, while no difference between bulk soil and rhizosphere soil were observed for Siebenlinden and Lasse. A 10- and 100-fold increase in <sup>13</sup>C-DMA addition to the Santomera rhizosphere soil resulted in an almost proportional increase in <sup>13</sup>C incorporation (Table 4).

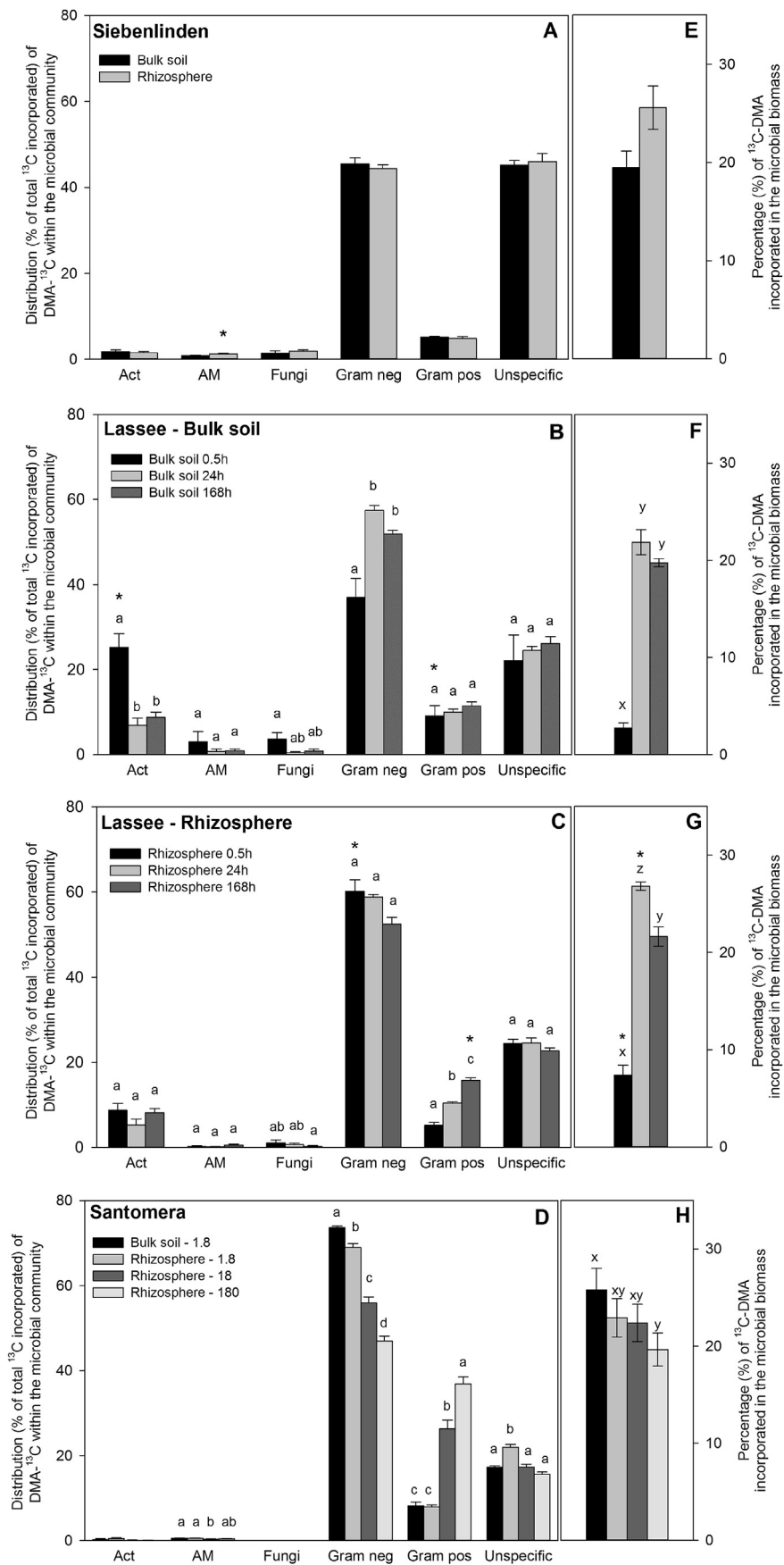
### 3.3. <sup>13</sup>C-DMA mineralization dynamics at high spatial resolution – rhizosphere gradients

The percentage of <sup>13</sup>C-DMA recovered as CO<sub>2</sub> over time in the Santomera bulk and rhizosphere soil sampled with distance from the root surface of wheat grown in rhizoboxes is presented in Fig. 5. Results show that particularly in the first eight hours after <sup>13</sup>C-DMA addition a significantly higher proportion of DMA was mineralized in the soil layer closest to the root surface (0–1 mm distance) compared to the other, more distant rhizosphere soil layers and the bulk soil. Furthermore a clear lag phase of DMA mineralization in the more distant (>1 mm) rhizosphere soil layers and bulk soil for the first 8 h was observed. While after 24 h the bulk soil still showed significantly less DMA mineralization, the difference in DMA decomposition between the different rhizosphere soil layers and the bulk soil was diminished after 48 h.

## 4. Discussion

**General considerations.** The applied <sup>13</sup>C-enriched DMA was not completely and uniformly labeled; during the synthesis procedure only four out of twelve C atoms were labeled with <sup>13</sup>C, with two <sup>13</sup>C atoms being positioned in two carboxylic groups and another two <sup>13</sup>C atoms in the backbone chain of the DMA molecule (for structural details see Walter et al., 2014). Using site specific <sup>13</sup>C labeled amino acids, Apostel et al. (2013) demonstrated that C from the highly oxidized carboxylic groups was mineralized faster, whereas reduced organic C was preferentially incorporated into microbial PLFA biomarkers. Taking this into account, tracing the fate of the applied <sup>13</sup>C<sub>4</sub>-DMA in soil could potentially overestimate DMA-derived CO<sub>2</sub> production over microbial incorporation, because carboxylate-C is over represented as <sup>13</sup>C. From a functional point of view a potential shift between the fraction of DMA being mineralized to CO<sub>2</sub> and incorporated into the microbial biomass is irrelevant as it is the residence time of the intact DMA molecule in the soil that determines its functional efficiency in terms of plant Fe nutrition. Using a conversion factor  $F_{PLFA}$  to convert <sup>13</sup>C PLFA data (pmol PLFA-<sup>13</sup>C g<sup>-1</sup>) to total microbial biomass <sup>13</sup>C (ng <sup>13</sup>C<sub>mic</sub> g<sup>-1</sup> soil) provides an estimate of the total amount of <sup>13</sup>C incorporated





**Fig. 4. A, B, C, D:** Distribution (percentage % of total incorporated) of deoxymugineic acid (DMA)-derived  $^{13}\text{C}$  within the different specific microbial groups: Values represent means  $\pm$  SE ( $n = 4$ ). **E, F, G, H:** Percentage of DMA-derived  $^{13}\text{C}$  incorporated into the microbial biomass in different soils and soil compartments: Values represent means  $\pm$  95% confidence interval ( $n = 4$ ) after applying the phospholipid fatty acid (PLFA)-microbial biomass conversion factor of 5.8. Actinomyces (Act), arbuscular mycorrhiza (AM), gram negative bacteria (Gram neg), gram positive bacteria (Gram pos), PLFAs found in more than one specific microbial group (Unspecific). **A, E:** Acidic soil Siebenlinden after the addition of 1.8 nmol  $^{13}\text{C}$  DMA  $\text{g}^{-1}$  and an incubation of 24 h. Significant differences (independent  $t$ -test,  $p < 0.05$ ) between rhizosphere and bulk soil are indicated by an asterisk (\*). **B, F:** Alkaline soil Lasse –bulk soil and **C, G:** rhizosphere after the addition of 1.8 nmol  $^{13}\text{C}$  DMA  $\text{g}^{-1}$  and an incubation of 0.5, 24 and 168 h respectively. Letters indicate significant differences within each soil compartment across the different time points (ANOVA,  $p < 0.05$ ); Significant differences between rhizosphere and bulk soil at a single time point are indicated by an asterisk (\*, independent  $t$ -test,  $p < 0.05$ ). **D, H:** Alkaline soil Santomera after the addition of 1.8, 18 and 180 nmol  $^{13}\text{C}$  DMA  $\text{g}^{-1}$  respectively and an incubation of 24 h. Letters indicate significant differences across soil compartments and treatments (ANOVA,  $p < 0.05$ ).

**Table 4**

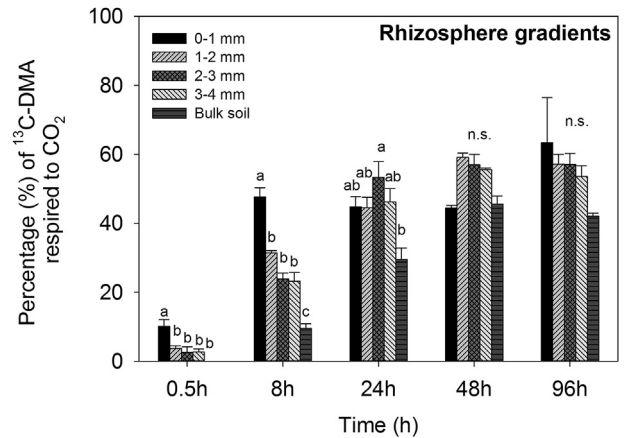
Incorporation of deoxymugenic acid (DMA)-derived  $^{13}\text{C}$  into total PLFA calculated as cumulative  $^{13}\text{C}$  recovered ( $\text{pmol g}^{-1}$  soil) divided by total PLFA-C ( $\text{nmol g}^{-1}$  soil). Letters a,b,c indicate significant differences within either bulk soil or rhizosphere across the different soils after an addition of  $1.8 \text{ nmol } ^{13}\text{C-DMA g}^{-1}$  (ANOVA) and \* indicates a significant difference between rhizosphere and bulk of a single soil (independent *t*-test) with  $p < 0.05$ . Letters x,y,z indicates significant differences across all soils and treatments (ANOVA) with  $p < 0.05$ .

DMA- $^{13}\text{C}$ incorporation after 24 h		
Soil & DMA concentration added	nmol $^{13}\text{C}$ per $\mu\text{mol}$ total microbial C	
	Bulk soil	Rhizosphere
Siebenlinden 1.8–24 h	0.06 <sup>b,x</sup>	0.08 <sup>bc,x</sup>
Lassee 1.8–0.5 h	0.01 <sup>a,x</sup>	0.03 <sup>a,x</sup>
Lassee 1.8–24 h	0.11 <sup>cd,x</sup>	0.12 <sup>dx</sup>
Lassee 1.8–168 h	0.08 <sup>bc,x</sup>	0.08 <sup>bc,x</sup>
Santomera 1.8–24 h	0.41 <sup>f,x</sup>	0.33 <sup>e,x</sup>
Santomera 18–24 h		2.96 <sup>y</sup>
Santomera 180–24 h		26.0 <sup>z</sup>

by the microbial community, since PLFAs are only a small fraction of total microbial C. The applied  $F_{\text{PLFA}}$  is well investigated but based on  $^{12}\text{C}$  data, with  $^{12}\text{C}$  PLFAs being a rather constant pool in soils (Joergensen and Emmerling, 2006). Despite  $\delta^{13}\text{C}$  shifts within the total PLFA pool over time (Fig. 4C,D; see also supplementary information), the  $^{13}\text{C}$  incorporation remained relatively constant between 24 and 168 h in the Lassee soil (Table 4). Additionally, it has been recently shown that PLFA turnover is in the range of 40 days (Malik et al., 2015), much slower than originally anticipated. We therefore assume that the use of the  $F_{\text{PLFA}}$  is valid for the presented  $^{13}\text{C}$  data, particularly for the results obtained after 24 h and 168 h of incubation. However,  $F_{\text{PLFA}}$ -based calculations of  $^{13}\text{C}$  incorporation into the total microbial biomass after 30 min might result in an overestimation of biomass- $^{13}\text{C}$ , since  $^{13}\text{C}$  incorporation into PLFAs has not yet reached a steady state. The presented results from the 0.5 h incubation therefore need to be considered with care (Fig. 4F,G & Table 5C).

**DMA is rapidly decomposed and taken up by the soil microbial community.** Half-life estimates of the intact DMA molecule (depletion from the soil matrix) as well as of DMA-derived C (Table 3) were in the same order of magnitude as observed for other LMW root exudate compounds like sugars, organic acids and amino acids though compound specific half-lives have been found to vary significantly between soils, with particularly soil specific sorption reactions having a strong impact on substrate bioavailability (Boddy et al., 2007; Oburger et al., 2009; Glanville et al., 2012). We found higher respiratory activities (Figs. 1 and 2) and faster LMW-C turnover (Table 3) in the rhizosphere compared to the bulk soil which has been frequently observed in other studies (e.g. Kuzyakov and Cheng, 2001). Nevertheless our PLFA results show that higher heterotrophic respiration in the rhizosphere (Fig. 1) can (e.g. Lassee, Santomera soil) but does not have to (Siebenlinden soil) be related to higher microbial biomass (Fig. 3). Additionally the observed rhizosphere gradient in DMA breakdown (Fig. 5) suggests both an activity gradient (not necessarily resulting from differences in microbial biomass, e.g. Siebenlinden soil) as well as substrate adaptation of the microbial community in the close vicinity of roots further decreasing the DMA residence time in the soil solution. The general lack of correlation between PLFA biomass and respiratory activity in the investigated soils is likely to be related to soil pH with acidity having an impeding effect organic matter mineralization (Curtin et al., 1998).

**Soil organic carbon (SOC) content and the proximity to roots play an important role in the employed microbial C partitioning strategies (allocation to biomass versus respiratory use) of DMA-**



**Fig. 5.** Percentage of  $^{13}\text{C}$ - deoxymugenic acid (DMA) respired to  $\text{CO}_2$  in the rhizosphere of wheat (*Triticum aestivum* cv Tamaro) grown on the calcareous Santomera soil with increasing distance (0–4 mm) from the root surface at a mm-resolution compared to the bulk soil. Letters indicate significant differences between the different soil layers within each time point (ANOVA,  $p < 0.05$ ). Values represent means  $\pm$  SE ( $n = 3$ ).

**C.** Overall only small effects on the total PLFA concentration as an indicator for microbial biomass by the one-time addition of  $^{13}\text{C}$ -DMA to the different soils could be observed (Fig. 3). Although a significant increase in total PLFAs was found in the Santomera bulk soil after the addition of  $1.8 \text{ nmol DMA g}^{-1}$ , even a 100-times higher DMA addition ( $180 \text{ nmol g}^{-1}$  corresponds to  $26 \mu\text{g C g}^{-1}$ ) did not result in an increase of total PLFA concentration after 24 h in the Santomera rhizosphere soil (data not shown). Adding increasing glucose concentrations ( $25\text{--}416 \mu\text{g C g}^{-1}$ ) to a permanent grassland soil ( $17 \text{ g kg}^{-1}$  SOC), Dungait et al. (2011) also didn't find any significant increase in PLFA concentrations after 120 h, except for the highest glucose addition. However when the same glucose amendments were performed with an arable soil ( $11 \text{ g kg}^{-1}$  SOC), concentrations of PLFA biomarkers specific for actinomycetes and gram-positive bacteria significantly increased for all glucose additions, suggesting different ecological strategies of microbes between these soils, with r-strategists directly and rapidly respiring added C (grassland) while K-strategists first incorporate LMW-C into intracellular reserves (arable soil). Similar to Dungait et al. (2011) we found a significantly higher  $^{13}\text{C}$  incorporation in the bulk soil with the lowest SOC content (Santomera, Tables 1 and 4) indicating a higher abundance of K-strategists. This is also reflected in the similar percentage of DMA- $^{13}\text{C}$  recovered in microbial biomass across all soils and treatments (Fig. 4 E, F, G, H), despite the significantly lower PLFA abundance in Santomera soil compared to the other soils (Fig. 3). Furthermore the higher  $^{13}\text{C}$  incorporation in the Santomera bulk soil (Table 4) after 24 h suggests a shift from dominating K-strategists to an increasing number of r-strategists in the rhizosphere due to the more constant C supply (i.e. root exudation) in the vicinity of roots. No such differences between rhizosphere and bulk soils were found in the soils with higher SOC content.

**Gram negative bacteria play a significant role in the biodegradation of DMA in all three soils.** Irrespective of soil compartment (rhizosphere or bulk soil), incubation time or DMA concentration (Fig. 4 A, B, C, D), 45–73% of total  $^{13}\text{C}$  label recovered in PLFAs were associated with gram-negative bacteria. While in both alkaline soils (Santomera and Lassee), biomarkers specific for gram-negative bacteria also constituted the largest PLFA fraction, they made up only about 20% of total PLFAs in the acidic soil (SI-Fig. S4). Using  $^{13}\text{C}$ -enriched glucose, fumaric acid and glycine in an

**Table 5**

Partitioning of deoxymugeneic acid (DMA) -derived  $^{13}\text{C}$  into the relevant pools (gas phase, microbial biomass, soil matrix) (A) in the two alkaline (Santomera, Lasse) and the acidic soil (Siebenlinden) after 24 h (B) in the Santomera soil after different DMA concentrations being added (1.8, 18, 180  $\mu\text{mol g}^{-1}$ ) and (C) in the Lasse soil after 0.5, 24 and 168 h of incubation. \*corrected for  $\text{CO}_2$  trapped in the alkaline soils.

Pools	(A) Partitioning of 1.8 nmol $^{13}\text{C}$ DMA $\text{g}^{-1}$ after 24 h					
	Santomera		Lasse		Siebenlinden	
	Bulk soil	Rhizosphere	Bulk soil	Rhizosphere	Bulk soil	Rhizosphere
% Of total $^{13}\text{C}$ added						
$^{13}\text{C}$ respired	14*	20*	22*	28*	27	30
$^{13}\text{C}$ in microbial biomass	26	23	22	27	19	26
$^{13}\text{C}$ soil matrix (solid + liquid)	60	57	56	45	54	44
Pools	(B) Partitioning of increasing concentrations of $^{13}\text{C}$ -DMA after 24 h					
	Santomera					
	Bulk soil 1.8	Rhizosphere 1.8	Rhizosphere 18	Rhizosphere 180		
% Of total $^{13}\text{C}$ added						
$^{13}\text{C}$ respired	14*	20*	20*	27*		
$^{13}\text{C}$ in microbial biomass	26	23	22	20		
$^{13}\text{C}$ soil matrix (solid + liquid)	60 (30 + 30 <sup>a</sup> )	57(27 + 30 <sup>a</sup> )	58(38 + 20 <sup>a,b</sup> )	53(43 + 10 <sup>a</sup> )		
Pools	(C) Partitioning of 1.8 nmol $^{13}\text{C}$ DMA $\text{g}^{-1}$ over time					
	Lasse 0.5 h		Lasse 24 h		Lasse 168 h	
	Bulk soil	Rhizosphere	Bulk soil	Rhizosphere	Bulk soil	Rhizosphere
% Of total $^{13}\text{C}$ added						
$^{13}\text{C}$ respired	1*	4*	22*	28*	34*	48*
$^{13}\text{C}$ in microbial biomass	3	7	22	27	20	22
$^{13}\text{C}$ soil matrix (solid + liquid)	96	89	56	45	46	30

<sup>a</sup> Data retrieved from (Schenkeveld et al., 2014b).

<sup>b</sup> Estimate as DMA soil solution concentration in our study lies between 30 and 100  $\mu\text{M}$ .

artificial root system, Paterson et al. (2007) could not only show that gram-negative bacteria and fungi were most strongly affected by the different root exudate compounds but also that glycine was only used by a small part of the microbial community. While 60% of glycine-derived  $^{13}\text{C}$  was recovered in gram-negative bacteria, the utilization of glucose and fumaric acid was more widespread across the microbial community. This is consistent with our findings as phytosiderophores are non-proteinaceous amino acid compounds and the mineralization strategy/dynamics can therefore be expected to be similar to other amino acids.

More rapid assimilation and turnover of  $^{13}\text{C}$ -enriched root exudates by gram-negative bacteria compared to other microbial groups was also observed in other studies investigating  $^{13}\text{C}$  incorporation in PLFAs after  $^{13}\text{CO}_2$  pulse labeling of grassland sites (Treonis et al., 2004; Balasooriya et al., 2014). However in both studies also saprophytic fungal biomarkers showed rapid  $^{13}\text{C}$  enrichment, though compared to our soils the relative abundance of fungal PLFAs was much higher in these studies. Dungait et al. (2011) on the other hand observed a greater  $^{13}\text{C}$  incorporation in gram-positive bacteria, which were significantly more abundant than gram-negative and fungal biomarkers in the investigated soils. This shows that despite gram-negative bacteria and fungi generally being more competitive in decomposing root derived LMW-C compounds, the partitioning of labile root exudate compounds will strongly be influenced by the relative abundance of the different microbial groups.

**The functionality of DMA in soil is strongly affected by (pH dependent) sorption and by microbial decomposition.** As demonstrated by Reichard et al. (2005), the negatively charged DMA ligand strongly interacts with positively charged mineral surfaces, with sorption increasing with decreasing pH, affecting its bioavailability to plants and microbes. In addition to the lower soil pH, the acidic soil (Siebenlinden) contains the highest ammonium oxalate extractable Fe and Al contents (Table 1), which provide a

measure for the amorphous Fe and Al-oxide phases in the soil. These amorphous minerals have a relatively large specific surface area compared to more crystalline phases and generally represent the dominant soil reactive phase for anion adsorption. This suggests stronger retention of DMA by the acidic soil matrix slowing down its microbial degradation as reflected by the asymptotic behavior of  $^{13}\text{CO}_2$  release from Siebenlinden soils (Fig. 2A). On the other hand, in addition to the higher microbial biomass/activity, the higher SOM content of the alkaline Lasse soil might have further prevented strong adsorption of DMA, leading to a higher bioavailability and consequently to faster microbial breakdown in this soil compared to the other alkaline soil (Santomera, Tables 1 and 3). Calculating the partitioning of  $^{13}\text{C}$ -DMA between the relevant pools and processes i.e. respiration, microbial biomass, and soil matrix (solid & liquid) revealed that between 40 and 56% of DMA added has either been mineralized or metabolized after 24 h in all 3 experimental soils irrespective of initial DMA concentration added (Table 5ab). Schenkeveld et al. (2014b) showed in a soil interaction experiment with Santomera soil, applying a soil to solution ratio of 1, that across a wide range of DMA concentrations (3–100  $\mu\text{M}$ ) about 60% of DMA was rapidly (<0.25 h) adsorbed by the soil matrix of the Santomera soil, under sterile and non-sterile conditions. Furthermore it was found that the remaining DMA concentration (i.e. about 40% of total added) in solution was relatively constant in the sterile treatment over time. However, in the non-sterile treatment, only between 30% (3 & 30  $\mu\text{M}$ ) and 10% (100  $\mu\text{M}$ ) of the DMA added were recovered in soil solution after 24 h. Additionally the same authors generally observed a more rapid depletion of the free DMA ligand than of certain metal-DMA complexes (Cu-DMA and Ni-DMA) before total DMA (free ligand and metal complex) was completely removed from the soil solution after 48–96 h, depending on the concentration added. Combining the data from Schenkeveld et al. (2014b) with our results indicates that both sorption and metal complexation of DMA are initially the dominant

processes affecting DMA speciation, but that the continuous removal of DMA by the microbial community may rapidly trigger DMA desorption (solution buffering) as well as a shift towards less decomposable metal DMA complexes (Cu-DMA and Ni-DMA in the case of Santomera soil). Whether or not microbes can directly take up the entire metal-DMA complex remains to be investigated. The faster depletion of the free DMA ligand than the DMA-metal complexes however suggests dissociation of the metal complex prior to microbial uptake or at least preferential uptake of the free DMA ligand. Schenkeveld et al. (2014b) also observed a clear lag phase of 4–8 h before the remaining DMA concentration in soil solution started to deviate between the sterile and non-sterile treatments. They attributed this finding to the emergence of the microbial community from stationary phase, adaptation to the new environmental conditions, and use of new substrates. The more rapid mineralization of DMA in the rhizosphere (Fig. 2) as well as declines in DMA decomposition with increasing distance from the root surface (Fig. 5) supports the hypothesis of lower microbial activity and/or greater time needed for substrate adaptation of the bulk soil microbial community. However investigating the time dependent DMA mineralization dynamics in the Lasse soil showed that between 1% (bulk soil) and 4% (rhizosphere) of DMA was already respired to CO<sub>2</sub> after 0.5 h (Fig. 4F & G); when including the estimates from <sup>13</sup>C DMA biomass incorporation between 4 and 11% of DMA were recovered in the microbial pool (i.e. respiration + biomass). Assuming a similar lag phase for the depletion of DMA from soil solution in other soils than Santomera, these results suggest a rapid replenishment of DMA removed by microbial uptake from the DMA fraction adsorbed to the soil matrix (i.e. desorption), mitigating a change in soil solution concentration. This is supported by a recently published study (Walter et al., 2016) that showed that a fraction (57–69%, depending on the DMA concentration added and the initial interaction time) of DMA/DMA-metal complexes adsorbed to Santomera soil (same as used in this study) was readily desorbable within 1 h in the presence of a sterilizing agent (NaN<sub>3</sub>). Our results consequently indicate that microbial uptake of DMA occurs faster and to a greater extent than can be observed from monitoring changes in soil solution concentrations only as desorption of initially adsorbed DMA will buffer the soil solution concentrations.

## 5. Conclusion

Results of this study confirm that microbial decomposition strongly reduces the time window of Fe acquisition by PS in soil. DMA mineralization in soil was found to be rapid and comparable to other LMW root exudate compounds like sugars, amino and organic acids with half-lives of the intact DMA molecule ranging between 3 and 8 h. Like amino acids, the largest fraction of DMA-C was recovered in gram-negative bacteria. The slower DMA mineralization in bulk soil compared to rhizosphere soil is of particular ecological importance, considering that the majority of DMA is released at the root tips that slowly grow into bulk soil parts with lower microbial activity. Although sorption and metal complexation initially appear to govern PS speciation, our data shows that microbial degradation occurs faster than can be observed from monitoring the decline in DMA soil solution concentration only, due to desorption processes buffering the soil solution.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2016.04.014>.

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