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RESEARCH ARTICLE

Metagenomic analyses reveal no differences in genes involved in cellulose degradation under different tillage treatments

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One sentence summary: Metagenomic analysis of reduced and conventionally tilled soils reveals the stability of cellulose-degrading communities in soil.

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ABSTRACT

Incorporation of plant litter is a frequent agricultural practice to increase nutrient availability in soil, and relies heavily on the activity of cellulose-degrading microorganisms. Here we address the question of how different tillage treatments affect soil microbial communities and their cellulose-degrading potential in a long-term agricultural experiment. To identify potential differences in microbial taxonomy and functionality, we generated six soil metagenomes of conventional (CT) and reduced (RT) tillage-treated topsoil samples, which differed in their potential extracellular cellulolytic activity as well as their microbial biomass. Taxonomic analysis of metagenomic data revealed few differences between RT and CT, and a dominance of *Proteobacteria* and *Actinobacteria*, whereas eukaryotic phyla were not prevalent. Prediction of cellulolytic enzymes revealed glycoside hydrolase families 1, 3 and 94, auxiliary activity family 8 and carbohydrate-binding module 2 as the most abundant in soil. These were annotated mainly to the phyla of *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. These results suggest that the observed higher cellulolytic activity in RT soils can be explained by a higher microbial biomass or changed expression levels but not by shifts in the soil microbiome. Overall, this study reveals the stability of soil microbial communities and cellulolytic gene composition under the investigated tillage treatments.

Keywords: Metagenomics; Cellulose degradation; Agriculture; Microbial Communities; Cellulases; Reduced Tillage

INTRODUCTION

The most abundant organic polymer on earth is cellulose. As a key component of plant cell walls, it is highly abundant in all plants, mostly in combination with hemicellulose and xylan (Varner and Louis 1989). The degradation of cellulose is an essential ecosystem service and it is of key value to gain more quantitative and qualitative understanding of the role of cellulose breakdown within the carbon cycle and in a changing environment. Cellulases are in general hydrolytic and divided into three major types: β-1,4-endoglucanases (EC 3.2.1.4), β-1,4-exoglucanases and β-glucosidases (EC 3.2.1.21) (Lynd *et al.* 2002). Exoglucanases are further divided into cellobiohydrolases or

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cellobiosidases (EC 3.2.1.91 and EC 3.2.1.176) and cellodextrinases (EC 3.2.1.74). These cellulases differ in their binding and cutting sites on the cellulose fibres or oligomers. Besides hydrolysis, other catalytic modes of action to cut cellulose chains have been identified, namely oxidoreduction, mediated by the enzyme cellobiose dehydrogenase (CBH; EC 1.1.99.18, Langston *et al.* 2011) or induced by a quinone- or glycopeptide-mediated Fenton reaction (Baldrian and Valášková 2008), and phosphorolytic degradation (EC 2.4.1.20 and EC 2.4.1.49, Reichenbecher, Lottspeich and Bronnenmeier 1997). Cellulases generally consist of a catalytic module, classified into glycoside hydrolase (GH) families, and often harbour a carbohydrate-binding module (CBM), whereas cellulases with oxidoreductive catalytic modules are classified as the auxiliary activity (AA) family of proteins. The classification into modules is based on amino acid sequence similarity (Henrissat 1991), and a comprehensive description of these module families can be found in the CAZy database (Carbohydrate-active enzymes database; www.cazy.org, Lombard *et al.* 2014).

Different agricultural practices such as tillage and fertilization strategies influence world-wide carbon cycles in soil (Schimel 1995; Schlesinger and Andrews 2000; Lal 2004). Tillage, a key component of modern agriculture, disperses added nutrients throughout the soil and aerates the soil, but also affects soil aggregates (Abdollahi, Munkholm and Garbout 2014). There is a strong ongoing debate regarding how different tillage treatments impact not only yields, but also soil nutrients and the diversity and activity of soil microbial communities. To clarify this question, several long-term agricultural experiments have been established to assess the adaptation of soils to different tillage treatments. In this context, it was shown that reduced tillage (RT) leads to a higher carbon content and higher microbial biomass in the topsoil compared with conventional tillage (CT) (Alvarez *et al.* 1995; Kandeler, Tscherko and Spiegel 1999; van Groenigen *et al.* 2011; Küstermann, Munch and Hülsbergen 2013).

However, it is currently unclear how different tillage treatments influence the phylogenetic structure of the soil microbial community, in particular those which drive the degradation of polymers, like cellulose, which are major constituents of plant residues used to improve soil quality. Only a few studies have compared conventional tillage with no-tillage systems, and these studies have detected several differences in taxonomy and functionality of the associated microbial communities (Souza *et al.* 2013; Carbonetto *et al.* 2014). For example in a long-term (26 years) experiment, deep tillage practice led to a lower microbial diversity in the top 10 cm of the soil (Silva *et al.* 2013). However, no in-depth analysis focusing on microbes catalysing carbohydrate degradation has been performed so far in agricultural systems.

Thus, in this study, we assess the impact of different tillage treatments on microbial communities which drive cellulose degradation in soil. Samples were taken from the topsoil (0–10 cm) of a long-term field experiment, where a split plot-based study has been established with conventional (CT; 25 cm working depth) and reduced tillage (RT; 8 cm working depth) treatments (Meyer-Aurich *et al.* 2009; Küstermann *et al.* 2013). One month before sampling, corn had been harvested and the remaining plant litter had been incorporated into the soil with the different tillage treatments. Due to the different tillage depths the amount of plant residues and consequently the amount of straw in the topsoil differed between the two treatments (Tebrügge *et al.* 1999). To assess the influence of different tillage treatments on the enzymatic activity of key cellulolytic enzymes in the soil, the potential enzymatic activities of β-glucosidase and cellobiohydolase were measured using methylumbeliferone-labelled substrates.

We explored the influence of long-term conventional and reduced tillage on the taxonomic and functional diversity of soil microbial communities, which are involved in cellulose degradation. As it is well accepted that cellulases occur ubiquitously in all kingdoms (http://www.cazy.org, Lombard *et al.* 2014) and show a high genetic diversity, we employed a shotgun sequencing approach to identify the different cellulases present and the microbes harbouring these genes in a qualitative (diversity) and semi-quantitative manner. The analysed top soils differed in their amount of total organic carbon (Küstermann *et al.* 2013). Therefore, we hypothesized that a higher relative abundance and diversity of genes coding for cellulases would be found in soil samples from RT compared with CT.

MATERIALS AND METHODS

Site description and soil sampling

Soil was sampled from an agricultural field experiment, established in 1992 at a research farm in Scheyern, 40 km north of Munich (Germany) (Meyer-Aurich *et al.* 2009; Küstermann *et al.* 2013). From this field experiment, two tillage treatments were analysed: CT with a mouldboard plough (25 cm working depth) and RT with a rotary harrow (8 cm working depth). The experiment has been set up with three independent replicates (split plot design). Ten year average precipitation and temperature are 792.3 mm and 8.3°C respectively (2000–2010). The soil is a Luvisol with a pH of 6.3, consisting of 2.2% coarse sand, 17.0% fine sand, 55.4% silt and 25.4% clay.

The soil was sampled in November 2012, 1 month after corn harvest and incorporation of the plant residues into the soil. For each plot, a composite sample of five soil cores to a depth of 10 cm was taken with a soil auger of 5 cm diameter, and sieved with a 3 mm sieve. Afterwards, a subset of the soil was mixed and immediately stored on dry ice and subsequently at –80°C before DNA extraction. The remaining soil was stored at 4°C.

Enzymatic activity assays, biomass and chemical measurements

Potential enzymatic activity was measured 1 day after sampling of soil stored at 4°C. The potential cellulose degradation activity of the microbial community was assessed by adding methylumbeliferone (MU)–β-D-glucoside and MU–β-cellobioside to soil solutions (Pritsch *et al.* 2005). The optimum substrate concentration (C_{opt}) and incubation time (T_{opt}) for degradation of both substrates were determined during a pilot experiment; $C_{opt} = 800$ μM MU–ß-cellobioside and 500 μM MU–ß-D-glucoside, $T_{opt} = 120$ min for both. Fluorescence measurement after incubation of the substrate with soil solution was done with a spectrophotometer at an excitation wavelength of λ = 365 nm and an emission wavelength of λ = 450 nm. Fluorescence values were corrected for soil autofluorescence or fluorescence inhibition by soil particles (quenching) and background noise.

For measurement of microbial carbon, water-extractable organic carbon (WEOC) was determined before and after chloroform fumigation (Joergensen 1996). For determination of WEOC, soils were mixed with 0.01 M $CaCl_2$ in a 1:5 ratio, incubated in an overhead shaker for 40 min at room temperature, filtered,

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and stored at –20°C until measurement with the Dimatoc 100 (DimatecAnalysentechnik GmbH, Germany).

For measurement of total carbon (TC) and nitrogen (TN), soil was dried at 40°C for ~5 days and ground to a powder by shaking in a tissue lyser at 30 Hz for 3–9 min. A 20–25 mg aliquot of ground soil powder was weighed in duplicate, wrapped in aluminium and subjected to elemental analysis using an Elementar Vario EL III instrument in combustion mode.

DNA isolation and pyrosequencing

Soil DNA was extracted from 300 mg of frozen soil samples using the DNA isolation kit 'Genomic DNA from soil' NucleoSpin Soil Kit (Macherey-Nagel, Germany) according to the protocol of the manufacturer. DNA was stored at –20°C until further processing. Library preparation was performed according to the Roche protocol 'Rapid Library Preparation Method Manual' using Roche Molecular Identifier (MID) adaptors as barcodes. Amplification of DNA via emulsion PCR was done according to the Roche protocol 'emPCR Method Manual-Lib-L Large Volume (LV)'. Pyrosequencing was carried out on the Genome Sequencing (GS) FLX+ instrument, using a GS FLX Titanium sequencing kit XL+. Image and signal processing was performed by the Roche software provided. The sequences are deposited in SRA under the BioProject ID: PRJNA235154.

Real-time PCR assays

Quantitative real-time PCR (qPCR) was performed using primers for amplification of the bacterial 16S rRNA gene (Bach *et al.* 2002) and the fungal internal transcribed spacer (ITS) regions (White *et al.* 1990). The reaction mixtures (total volume 25 μl) for quantification of the 16S rRNA gene consisted of 12.5 μl of Power SYBR Green master mix (Life Technologies), 5 pmol of each primer, 0.5 μl of 3% bovine serum albumin (BSA) and 2 μl of soil DNA template. The reaction mixtures (total volume 25 μl) for quantification of the ITS gene consisted of 12.5 μl of Power SYBR Green master mix (Life Technologies), 10 pmol of each primer and 2 μl soil DNA template. For quantification, standard curves were calculated using serial dilutions (10^2–10^6 gene copies μl^{-1}) of a plasmid containing *Fusarium oxysporum* DNA (for fungal qPCR) or *Pseudomonas putida* (for bacterial qPCR). The PCR detection limit was assessed to 10 gene copies μl^{-1}. In order to prevent PCR inhibition, the optimal dilution for each amplification assay was determined by dilution series of DNA extracts (data not shown). The qPCR assays were performed in 96-well plates. All PCR runs started with a hot start at 95°C for 10 min, then either 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s for ITS samples or 40 cycles of 95°C for 45 s, 58°C for 45 s and 72°C for 45 s for 16S rRNA qPCR. Finally, melting curve analyses were carried out for all samples by a final cycle of 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. The amplification efficiency was calculated from the formula $Eff = [10^{(-1/slope)} - 1] \times 100$, and resulted in the following efficiencies: 16S rRNA, 102%; and ITS, 87%.

Analysis of sequencing data and prediction of cellulolytic enzymes

Sff-files were separated by MID adaptors, using 454/Roche SFF Tools. Trimming of reads was carried out using a modified DynamicTrim (Cox, Peterson and Biggs 2010) as supplied by MG-RAST (Meyer *et al.* 2008) using the following settings: minimum Phred score = 15, maximum number of bases below minimum Phred score = 5, minimum read length = 50. Remnant adaptor sequences and duplicated reads were removed using Biopieces (www.biopieces.org) and cd-hit (Fu *et al.* 2012), respectively. For taxonomic and functional annotation, filtered reads were blasted against both the National Center for Biotechnology Information (NCBI) non-redundant protein database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (June 2011) using Diamond (Buchfink, Xie and Huson 2014) as well as against the Silva SSU database using Blastn (expected (e)-value threshold = 10^{-4}). Mapping of the top 25 blast results (i.e. hits with the lowest e-value) to taxonomic and functional annotations was carried out with the MetaGenome ANalyzer (MEGAN, Version 5.2.3) software (http://ab.inf.uni-tuebingen.de/software/megan5/). During the MEGAN analysis, the following settings were used: Min support = 1, Min score = 50, Top% = 10, Min-Complexity Filter = 0. Visualization of data was performed in R (R Core Team 2013). To obtain a relative abundance of reads, the number of reads was divided by the total number of filtered reads per sample and multiplied by 100.

For prediction of catalytic modules (GH and AA) and CBMs in the reads, protein open reading frames were predicted using FragGeneScan (Rho, Tang and Ye 2010) and translated to protein sequences. Different families of catalytic modules and CBMs harboured by the key enzymes in cellulose degradation, β-1,4-endoglucanases, β-1,4-exoglucanases, β-glucosidases, cellodextrin phosphorylases and cellobiose dehydrogenases, were selected from the CAZy database (www.cazy.org, Lombard *et al.* 2014). Protein Hidden Markov Model (HMM) logos can be used to scan protein sequences using hmmscan (Eddy 2011). For the selected families, several HMM logos are available in the Protein family (Pfam) A 26.0 database (Punta *et al.* 2012) and in the DataBase for automated Carbohydrate-active enzyme Annotation (dbCAN, Yin *et al.* 2012), but can also be built personally using hmmbuild (which is contained within the HMMER package version 3.0 (March 2010), packaged together with hmmscan; http://hmmer.org/) and an alignment file with sequences containing the corresponding module domain as input (alignment files were generated using sequences from CAZy for each corresponding family).

Because the selected module families also contain a varying amount of enzymes other than cellulases, we decided to validate the specificity of these HMM logos for cellulases by scanning them against a list of positive or negative cellulase sequences. These lists have been assembled by downloading CAZy entries for all glycoside hydrolase, carbohydrate-binding and auxiliary activity families that contain cellulolytic proteins. A list of 1283 entries was then classified as positive, having a matching EC number (as mentioned in the Introduction) and a list of 1390 entries as negative where the EC number did not match. Of the positive sequences, 662 were eukaryotic. For the families AA8 and AA9, entries that mentioned activity on cellulose in the name were included in the positive list. Taking all GenBank IDs for these positive and negative entries, the corresponding protein sequences were downloaded from NCBI. Finally, these lists were scanned with the three sets of HMM logos (from Pfam A, dbCAN or personally built), and the HMM logos that annotated most of the positive sequences (see Supplementary Table S1 in bold) were used to scan the translated protein sequences from the metagenomes (e-value threshold = 10^{-4}).

To limit the number of false-positive predictions, we refined the search for cellulases by performing an additional quality control step, i.e. blasting all HMM-annotated protein sequences against the above-mentioned positive sequences database using blastp (e-value threshold = 10^{-5}). To obtain a relative abundance

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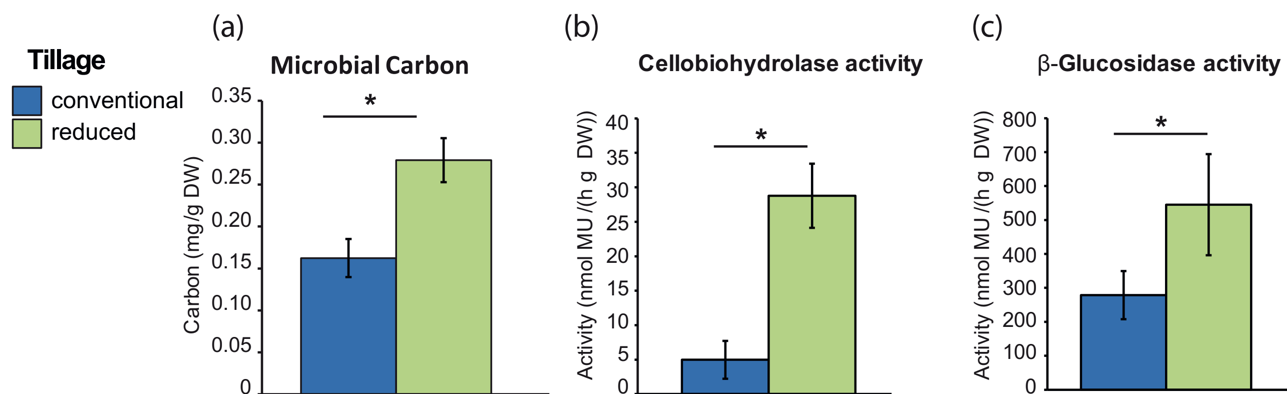

Figure 1. Reduced tillage increases microbial carbon and potential cellobiohydrolase and β-glucosidase activities in the topsoil. **(a)** The amount of microbial carbon detected in soils with conventional and reduced tillage treatments, in mg per g dry weight of soil. **(b, c)** The amount of methylumbelliferone (MU) produced in nmol per h and g dry weight of soil, that has been released by the enzymatic degradation of MU–β-cellobioside (b) or MU–β-D-glucoside (c). Significant differences between conventional and reduced tillage were determined by unpaired *t*-test statistics (*$P < 0.05$, $n = 3$).

of reads, the number of reads was divided by the total number of filtered reads per sample and multiplied by 100 000.

For taxonomic annotation, all reads with a predicted cellulolytic module, i.e. after scanning against the HMM logos and the positive sequences database, were blasted against the NCBI non-redundant protein database using Diamond (minscore = 50) and mapped with MEGAN5 (parameters as previously announced).

Statistical analysis of sequencing data and diversity analysis

To detect global differences between the metagenomes, principal component analysis (PCA) was performed on the relative order count data of metagenomic reads annotated to the NCBI non-redundant protein database and analysed with MEGAN5. Significant differences of soil measurements and annotated read counts between CT and RT soils were determined by unpaired *t*-test statistics, and adjusted using the Bonferroni correction for metagenomic data, using R (R Core Team 2013). Before testing for differences between soil parameter measurements, QQ plots and Shapiro–Wilk tests for normal distribution were performed. For the PCA plot, the data were square-root transformed, as the QQ plot showed no normal distribution. Differences were regarded as significant when the adjusted *P*-value was smaller than 5% ($P < 0.05$). In addition, only taxa or pathways with a relative abundance of at least 0.05% in one of the replicates were included. Due to the low amount of predicted cellulases, no abundance cut-off was used to detect possible differences between tillage treatments. Shannon indices were calculated using the vegan package in R (Oksanen *et al.* 2013).

RESULTS

Microbial biomass and enzymatic activities

As expected, we found higher total soil carbon and nitrogen contents under conditions of RT ($C = 1.62 \pm 0.13\%$, $N = 0.17 \pm 0.01\%$) compared with CT treatment ($C = 1.08 \pm 0.07\%$, $N = 0.12 \pm 0.01\%$, $P = 0.004$ and $P = 0.004$, respectively). In addition, RT leads to significantly higher microbial biomass compared with soil under CT, as measured by carbon analysis (0.28 ± 0.03 and 0.16 ± 0.02 mg g^{-1} dry weight (DW), respectively, $P = 0.004$, Fig. 1a) and by qPCR of both bacterial ($8.37 \pm 0.49 \times 10^{10}$ and $6.82 \pm 0.19 \times 10^{10}$ copies g^{-1} DW, respectively, $P = 0.007$) and fungal DNA ($5.58 \pm 1.03 \times 10^{8}$ and $3.40 \pm 0.40 \times 10^{8}$ copies g^{-1} DW, respectively, $P = 0.027$). The high ratio of bacterial 16S rRNA genes to fungal ITS genes was not influenced by the treatments. Potential extracellular enzyme activity measurements showed that CT results in a lower activity than RT for both β-glucosidase (278.7 ± 40.9 and 545.1 ± 86.1 nmol MU h^{-1} g^{-1} DW, respectively, $P = 0.049$) and cellobiohydrolase (5.0 ± 1.6 and 28.8 ± 2.7 nmol MU h^{-1} g^{-1} DW, respectively, $P = 0.002$) (Fig. 1b and c).

Taxonomic and functional annotation of metagenomes

Shotgun sequencing of the six DNA libraries (three independent replicates per tillage treatment) resulted in an overall amount of 0.5 Gb of data, which corresponds to a mean of 157 106 filtered reads per replicate with an average length of 410 bp (Supplementary Table S2). To account for the different number of reads between the biological replicates, relative abundances per replicate were calculated. Taxonomic annotation of the metagenomes was performed by blasting all filtered reads against the Silva SSU database and assigning the taxonomic rank with MEGAN5. Due to the low amount of ribosomal sequences in the metagenomes (0.12%), only a classification at kingdom level was performed, and revealed a dominance of Bacteria (86.15%) followed by Eukaryota (13.39%) and Archaea (0.45%) (data not shown). A total of 61% of the reads could be taxonomically annotated to at least super-kingdom level with the NCBI non-redundant protein database. Therefore, all further taxonomic annotations refer to the NCBI database. At super-kingdom level, the majority of reads was mapped to Bacteria (98.03%) followed by Eukaryota (1.08%), Archaea (0.73%) and Viruses (0.05%). Exploratory rarefaction analysis at each taxonomic level revealed an acceptable coverage of diversity on the order level for all six samples (Fig. 2). Taxonomic analysis showed that all soil metagenomes are dominated by *Proteobacteria* and *Actinobacteria* that together make up 26.3% of all reads (Supplementary Fig. S1a). Further prevalent bacterial phyla were *Bacteroidetes* (3.3%), *Acidobacteria* (3.3%), *Verrucomicrobia* (1.7%), *Gemmatimonadetes* (1.4%), *Planctomycetes* (1.3%) and *Chloroflexi* (1.1%). At the order level, *Actinomycetales*, *Rhizobiales*, *Myxococcales*, *Burkholderiales* and *Planctomycetales* accounted for 13.5% of all annotated reads (Supplementary Fig. S1b).

The PCA performed on the relative order count data from the metagenome reads (Supplementary Fig. S2) revealed no clear clustering of replicates from CT and RT soils. In accordance, no significant change in alpha-diversity (Shannon indices: CT,:

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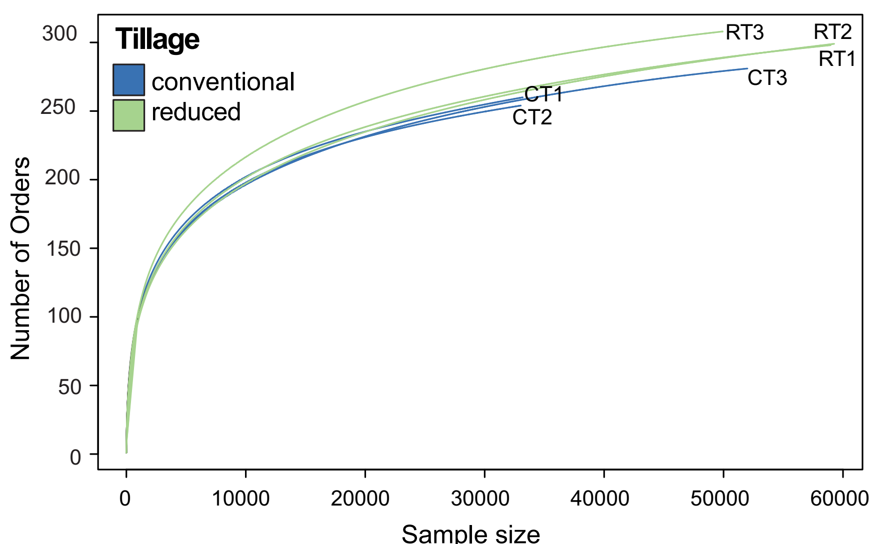

Figure 2. Rarefaction curves for the metagenomes of conventional and reduced tillage-treated soil. Depicted is the number of detected orders (Blastx (Diamond) of all filtered metagenome reads against the NCBI non-redundant protein database, e-value cut-off = 10^{-4}, assignment of taxonomic rank with MEGAN5) as a function of the sequencing depth, i.e. a randomly chosen amount of reads from the metagenomes for each replicate.

3.58 ± 0.02; RT, 3.55 ± 0.04) between the metagenomes was detected. Significantly different abundances of taxonomically annotated metagenome reads between CT and RT soils are shown in Supplementary Table S3. Genes annotated to originate from the order *Ktedonobacteraceae* were more abundant in CT-treated soil compared with RT-treated soil ($P = 0.003$).

For functional annotation, the metagenomes were mapped to the KEGG database, to which 35.21% of the reads could be annotated, and visualized with MEGAN5. Two-component system, Purine metabolism, ABC transporters, Nitrogen metabolism and Pyrimidine metabolism were among the most abundant pathways and account for 6.14% of all reads (Supplementary Fig. S3). Soils under CT contained significantly more genes involved in Carbohydrate metabolism ($P = 0.008$), Xenobiotics biodegradation and metabolism ($P = 0.0006$), Arachidonic acid metabolism ($P = 0.006$), Tyrosine metabolism ($P = 0.010$) and Drug metabolism ($P = 0.0006$, Table S3).

Prediction of cellulolytic enzymes from metagenomes of conventional and reduced tillage-treated soils

When focusing on the enzymes involved in cellulose degradation within the starch and sucrose metabolism pathway of KEGG, β-glucosidase (EC 3.2.1.21) and endoglucanase (EC 3.2.1.4) were most abundant, with 270 reads (mean relative abundance 0.071 ± 0.008%) for CT and 410 reads (mean relative abundance 0.073 ± 0.003%) for RT annotated to β-glucosidase. A total of 123 reads (mean relative abundance 0.032 ± 0.004%) for CT and 186 reads (mean relative abundance 0.033 ± 0.001%) for RT were annotated to endoglucanase. These numbers revealed no significant difference between CT and RT. Far fewer reads were annotated to exoglucanase (EC 3.2.1.91: CT, 0.001 ± 0.0006%; and RT 0.002 ± 0.0018%) and cellobiose phosphorylase (EC 2.4.1.20: CT 0.002 ± 0.0014%; and RT 0.001 ± 0.0005%) and none to cellobiose dehydrogenase (EC 1.1.99.18). In the KEGG database there are no reference KEGG Orthology groups for cellobiosidase (EC 3.2.1.176) or cellodextrinase (EC 3.2.1.74).

More extensive screening of the metagenomes for cellulases was performed using hmmscan. To identify the most sensitive HMMs for cellulolytic proteins, we benchmarked publicly available as well as personally built HMMs against a set of positive and negative CAZy proteins (see the Materials and Methods). This revealed that most HMMs also detected many negative CAZy proteins. Using the most sensitive HMMs (Supplementary Table S1 in bold), 5906 reads could be annotated to a catalytic module or a CBM. To reduce the number of false-positive predictions using hmmscan, an additional filtering step was performed by scanning the predicted cellulases against the same set of positive CAZy proteins (Supplementary Table S4). After this quality control step, 2021 reads remained with a predicted catalytic or carbohydrate-binding function, which corresponds to 0.214% of the total amount of metagenome reads. The percentage of total annotated cellulases was not different between treatments. A large variety of catalytic modules and CBMs that are involved in cellulose degradation were found in the metagenomes of this agricultural soil; 18 GHs, four AAs and 14 CBMs (Fig. 3). After quality control, most hits were found for GH1, GH3, GH94, AA8 and CBM2. Statistical analysis revealed significantly more reads annotated to CBM11 in CT-treated soil than in RT-treated soil ($P = 0.020$). Conversely, RT-treated soil contained more reads annotated to GH48 than CT soil ($P = 0.032$).

The taxonomic affiliation of the pooled cellulase-annotated sequences (from Fig. 3) is shown in Fig. 4 on phylum and order level, and reflects the overall abundance of phyla in the metagenomes (Supplementary Fig. S1a). A total of 18.56% of all cellulase reads mapped to the *Proteobacteria*, 11.23% to *Actinobacteria* and fewer to *Bacteroidetes* (8.61%), *Cyanobacteria* (2.23%), *Gemmatimonadetes* (2.18%), *Verrucomicrobia* (2.13%), *Acidobacteria* (1.73%), *Firmicutes* (1.53%) and *Chloroflexi* (1.53%). *Actinobacteria*, *Bacteroidetes, Cyanobacteria* and *Firmicutes* show a high abundance here, whereas they are not so abundant in the total metagenome (7.1, 3.3, 0.7 and 0.8% of the total reads, respectively; Supplementary Fig. S1a). Results on order level support the importance of the role which *Actinobacteria* (*Actinomycetales*), *Proteobacteria* (*Rhizobiales* (α), *Burkholderiales* (β), *Xanthomonadales* (γ), *Myxococcales* (δ)), *Bacteroidetes* (*Cytophagales*, *Sphingobacteriales*) and *Verrucomicrobia* (*Verrucomicrobiales*) play in cellulose degradation. Of the reads that were predicted to contain

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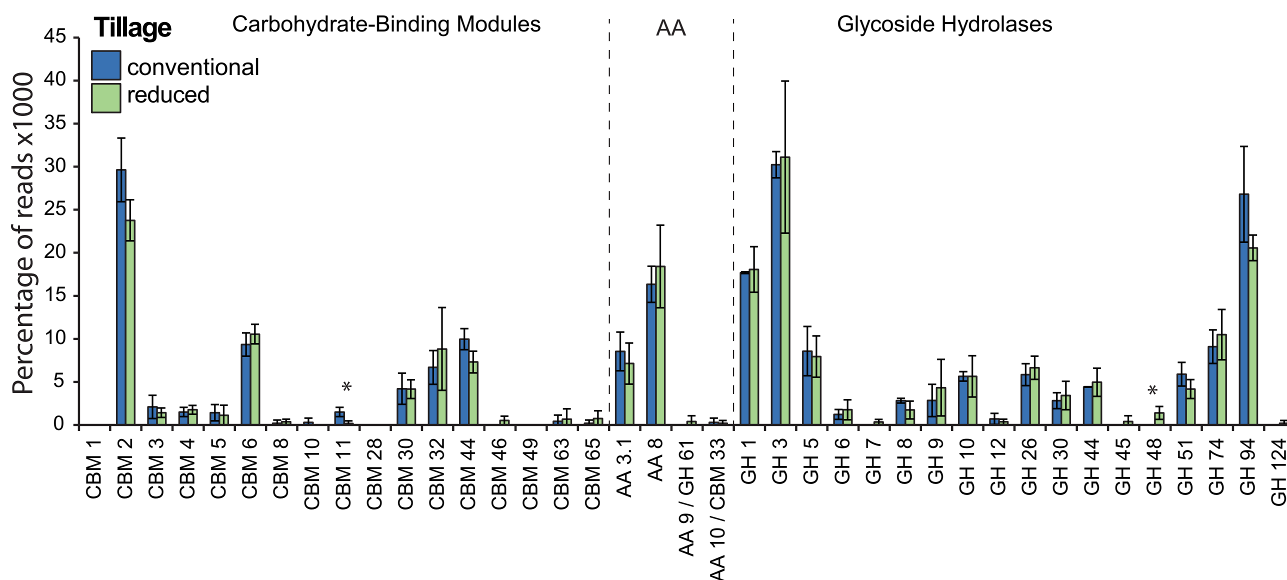

Figure 3. Prediction of cellulases and carbohydrate-binding modules from metagenomes of conventional and reduced tillage-treated soil. Depicted is the percentage of reads multiplied by 1000 annotated to catalytic and carbohydrate-binding modules found in the metagenomes of conventional and reduced tillage-treated soil using HMM modules and after an additional quality control using a curated database. Significant differences between conventional and reduced tillage were determined by unpaired *t*-test statistics (*$P < 0.05$, $n = 3$).

cellulolytic modules, 0.71% mapped to fungi (*Ascomycota* and *Basidiomycota*).

Finally, the taxonomic affiliation for the individual most abundant cellulolytic families (GH1, GH3, GH5, GH94, AA8 and CBM2) and the difference between CT and RT are shown separately in Supplementay Fig. S4. The low number of reads leads to a high variation in the amount of annotated reads among replicates and treatments.

DISCUSSION

Relative abundance and diversity of genes coding for cellulolytic enzymes

We predicted a multitude of cellulolytic enzymes in our study, which reflects the complex nature of cellulose degradation in soil. The functional annotation of cellulase genes agrees with other studies searching for catalytic modules and CBMs in aerobic environments, where genes of GH family 3, 5 and 9 have also been identified (DeAngelis *et al.* 2010; Duan and Feng 2010; Anderson *et al.* 2012; Nyyssönen *et al.* 2013). This is also true for their taxonomic annotation, as cellulolytic microorganisms have been found in the phyla of *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Anderson *et al.* 2012; Berlemont and Martiny 2013; Nyyssönen *et al.* 2013; Woo *et al.* 2013). Also the order of *Actinomycetales* contains well known cellulose degraders (Solans and Vobis 2003; Větrovský, Steffen and Baldrian 2014), as does the order of *Myxococcales* (Reichenbach *et al.* 2006).

To our knowledge, a similar refining analysis on HMM-annotated reads as presented here (Fig. 3) has not previously been done. We find a highly variable specificity of each HMM motif for cellulolytic function, which ranges from 0.0 to 100.0% of specificity (Supplementary Table S4). Our results show that such an analysis is necessary to get a more exact prediction of gene function, as results can depend heavily on the nature of the HMM. However, sequence similarity scoring may not be the optimal method to annotate diverging protein-coding sequences. To define the actual activity of the predicted cellulases, other approaches such as cloning and subsequent functional characterization, transcriptomics and proteomics, are necessary in addition.

To assess further the question of what percentage of predicted cellulases can be more reliably annotated, we compared the sequences predicted to harbour cellulolytic domains (GH and AA) with the sequences that mapped to KEGG modules. This revealed that 19.5% of all sequences predicted by the HMMs before filtering and 28.8% after filtering with the curated database were annotated by the KEGG database as well. This indicates that at least a part of the identified sequences are potentially functional in the soil.

In the overall metagenome, 1.08% (NCBI non-redundant protein database) or 13.39% (Silva SSU database) were annotated to eukaryotes. Indeed, the qPCR on the soil DNA showed a very low abundance of fungal ITS sequences, especially compared with bacterial 16S rRNA sequences. These results are comparable with earlier measured ITS sequence abundance in agricultural soil (Ng *et al.* 2012). In accordance with the overall metagenome, 0.93% of the predicted cellulases in the metagenome were annotated to eukaryotes (0.71% were annotated to fungi) when using the NCBI non-redundant protein database. However, 12% of the predicted cellulases were annotated to a CAZy module which contain mostly eukaryotic sequences (GH7, CBM1, CBM49, AA3, AA8 and AA9). Discrepancies between the annotation results of different databases (NCBI versus Silva or NCBI versus CAZy) clearly show the database biases towards sequences from often-studied organisms. In a high-throughput approach, numbers of reads annotated to taxa or functions are only realistic if a sufficient database representation of the corresponding genes exists. Unfortunately, this is not the case, as databases are biased towards culturable bacteria (Wooley, Godzik and Friedberg 2010), and care should be taken when assessing results based on similarity to database contents. In addition, the discrepancy between the annotated abundance of fungi by the Silva SSU database and the qPCR results presented here might be explained by the detection of a different fungal sequence (i.e. 18S rRNA in the Silva SSU database, and ITS by the PCR assay). Besides database

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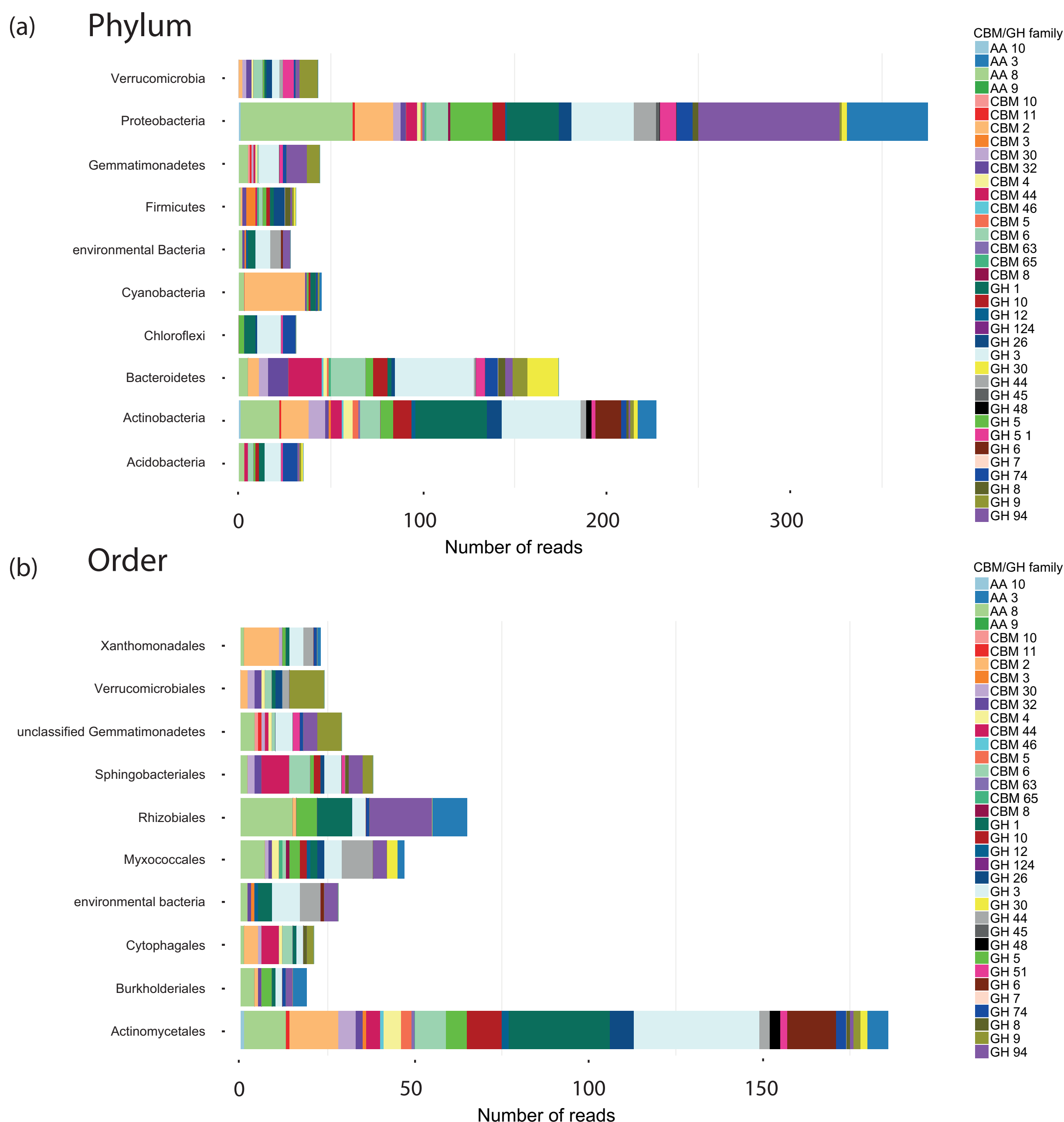

Figure 4. Taxonomic analysis of predicted catalytic and carbohydrate-binding modules. Shown is the number of reads annotated to different phyla **(a)** or orders **(b)** (Blastx (Diamond) against the NCBI non-redundant protein database, assignment of taxonomic ranks using MEGAN5) for each catalytic or carbohydrate-binding module. Presented are the most abundant taxa.

annotation biases, fungal genes contain many intronic sequences, which require long reads for accurate annotation. A transcriptomic approach might resolve the hidden contribution of fungi in the soil metabolic function, as is evidenced by other studies targeting soil transcripts (Kellner, Zak and Vandenbol 2010; Baldrian *et al.* 2012).

The low amount of fungi annotated in the overall metagenome and the cellulase-annotated sequences is striking when one considers the role of fungi in biomass degradation. In forest soils, fungal cellulases are known to be important for cellulose degradation (Bailey, Smith and Bolton 2002; Buée *et al.* 2009). It is, however known that agricultural soils harbour lower amounts of fungi than undisturbed soils, as tillage disrupts soil fungal communities (Miller and Lodge 1997). Furthermore, the yearly application of mineral fertilizers and fungicides negatively influences the amount of fungi in the sampled soil.

Influence of tillage treatment on soil metagenomes

We hypothesized to find a higher abundance and diversity of cellulase genes in RT soil compared with CT soil, based on the assumption of higher substrate availability under RT. Few significant differences were found between the metagenomes, e.g. genes related to GH48 were exclusively found in RT soil, whereas CT soil harboured more CBM11 genes (Fig. 3). GH48 family proteins are generally harboured by bacteria and

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anaerobic fungi, and are always present in cellulosomes. Furthermore, GH48 cellulases are considered the key component of various cellulolytic systems (Olson *et al.* 2010; Izquierdo, Sizova and Lynd 2010; Devillard *et al.* 2004). CBM11 family proteins are only known to be present in bacterial cellulases. However, so far not much is known about the function of cellulase diversity or specific groups of cellulases in the degradation process in soil. Future research will have to elucidate the specific contribution of each family. Another finding is that more reads were annotated to the class *Ktedonobacteria* in CT soil than in RT soil (Supplementary Table S3). *Ktedonobacteria* are Gram-positive, aerobic organisms that produce branched vegetative mycelia (Yokota *et al.* 2012). However, not much is known about their physiology and growth conditions or their ecological importance. Using the KEGG database for functional annotation of the metagenomes, we detect significantly more reads for carbohydrate metabolism and biodegradation of xenobiotics in CT soil compared with RT soil. This is an interesting finding but difficult to interpret as there are many downstream pathways and none of them differs between tillage treatments.

Most detected differences were, however, based on low read counts, questioning their ecological relevance. For this, a metagenomic assessment with higher resolution or more biological replicates can be helpful to perform more powerful statistics. In general, this in-depth analysis of soil metagenomes from RT and CT soils reveals no global differences in taxonomic and functional content, and none in alpha-diversity.

This is to our knowledge the first metagenomic analysis of the same agricultural soils under different tillage practices. It is therefore plausible that a change in tillage practice has far less impact on a functioning microbial community than a comparison of tillage versus no-tillage, for which several differences in microbiome structure and taxonomic composition were detected (Souza *et al.* 2013; Carbonetto *et al.* 2014). In this respect we can hypothesize that conditions below 10 cm in the RT soil in this study might resemble conditions under no-tillage. Indeed, Angers *et al.* showed that the surface layer and deeper soil layers can be differentially affected by tillage treatment (Angers *et al.* 1997). In the subsoil of RT, no extra carbon is incorporated. Accordingly, no differences in soil organic carbon between CT and RT were observed at deeper soil levels (18–25 cm) at this experimental site (Küstermann *et al.* 2013). To confirm whether the subsoil of RT is affected in a similar way to no-tillage soil, future research is required.

Our results indicate that tillage intensity does not strongly influence microbial community structure in the time frame of 20 years, and imply that these microbial communities in agricultural soils are stable to some extent in response to long-term differences in tillage treatments. The impact of different tillage treatments might prove less selective for soil microbial community members than other environmental factors such as the regional climate. This theory is in agreement with a recent meta-analysis of reduced tillage systems in organic farming (Cooper, personal communication).

In contrast to the similarity between metagenomes, microbial biomass, fungal and bacterial signature gene copies and cellulase enzyme activity, which were determined per gram of soil, were clearly lower in CT than RT topsoil. In agreement with these observations, a higher fungal biomass in reduced compared with conventionally tilled soil has been reported previously (van Groenigen *et al.* 2010; Murugan, Koch and Joergensen 2013). Due to a difference in microbial biomass, a higher bacterial 16S rRNA and fungal ITS abundance and enzymatic activity in RT compared with CT soils might be an indirect result of tillage. Alternatively, the higher enzymatic activity might be due to a higher abundance of cellulase enzymes in RT soil.

Several other explanations for the discrepancy between the enzyme activity measurements and the metagenomic analysis exist. One potential explanation is that the soil microbiome might primarily react to differences in tillage treatments on a transcriptional level. The metagenomic approach does not reveal the active members of a given microbial community and it is likely that these will be different under CT and RT practice. A metatranscriptomic or metaproteomic approach would be required to reveal the effect of tillage on the expression or translation of specific cellulase genes. The advantage of these approaches is that the active organisms are addressed directly, but the disadvantage is that such measurements can fluctuate considerably over time. Furthermore, it is known that sequencing depth influences the detection of rare species (Wooley *et al.* 2010), so it is conceivable that in this study many rare microbial species have been missed that contribute to cellulose degradation. However, if these species have a very low abundance, it is unlikely that their role in degradation is ecologically significant.

Another explanation for the observed discrepancy is the sampling time. The incorporation of plant litter leads to a high input of carbon into the soil. It is conceivable that at the time of sampling after 1 month, the whole microbial community might be profiting from an increased labile carbon pool and there would be little competition between microorganisms. This is in agreement with findings in a field study by Fu *et al.*, which showed that soil respiration was still increasing after 30 days of crop residue incorporation, under both no-tillage and conventional tillage (Fu *et al.* 2000). Sampling at a later time point (e.g. half a year) might reveal stronger differences between degradation potential of microbial communities of CT and RT soils due to the lower amount of carbon left in the topsoil of CT compared with RT.

Outlook

Cellulose degradation replenishes the labile carbon pool in soil and is an important ecosystem service. In this study, we addressed the question of how different tillage treatments affect cellulose-degrading communities in soil in an agricultural setting. As expected, we identified a high diversity of cellulases in soil and several glycoside hydrolase families with high abundances, but did not find evidence that tillage treatment strongly influences cellulose-degrading communities at the level of DNA.

We detected only few differences in the microbial communities of CT- versus RT-treated soils as well as in the abundance of cellulolytic genes. The differences observed in potential enzymatic activities could not be explained by changes in diversity and might be driven by the increased microbial biomass present at RT sites or different expression patterns. However, as the present study is based purely on functional predictions, a confirmation of the results by more targeted methods that confirm the cellulolytic activities is needed, e.g. by using isotopically labelled cellulose as substrate and subsequent stable isotope probing analysis, or a transcriptomic approach further combined with degenerate primer gene capture. Finally, further quantification of genomic or transcribed cellulases in response to different biotic and abiotic factors, as well as studies that improve our understanding on the dynamics of these microbial communities in time and space, will be a crucial step to better understand to what extent the large diversity of cellulolytic enzymes is relevant for efficient cellulose degradation.

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SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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