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Microbial responses to chitin and chitosan in oxic and anoxic agricultural soil slurries

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Abstract

Chitin is the second most abundant biopolymer in terrestrial ecosystems and is subject to microbial degradation. Chitin can be deacetylated to chitosan or can be hydrolyzed to *N*,*N*′-diacetylchitobiose and oligomers of *N*-acetylglucosamine by aerobic and anaerobic microorganisms. Which pathway of chitin hydrolysis is preferred by soil microbial communities has previously been unknown. Supplementation of chitin stimulated microbial activity under oxic and anoxic conditions in agricultural soil slurries, whereas chitosan had no effect. Thus, the soil microbial community likely was more adapted to chitin as a substrate. In addition, this finding suggested that direct hydrolysis of chitin was preferred to the pathway that starts with deacetylation. Chitin was apparently degraded by aerobic respiration, ammonification, and nitrification to carbon dioxide and nitrate under oxic conditions. When oxygen was absent, fermentation products (acetate, butyrate, propionate, hydrogen, carbon dioxide) and ammonia were detected, suggesting that butyric and propionic acid fermentation were along with ammonification likely responsible for apparent anaerobic chitin degradation. In total, 42 different *chiA* genotypes were detected of which twenty were novel at an amino acid sequence dissimilarity of > 50 %. Various *chiA* genotypes responded to chitin supplementation and affiliated with a novel deep-branching bacterial *chiA* genotype (anoxic conditions), genotypes of *Beta*- and *Gammaproteobacteria* (oxic and anoxic conditions), and *Planctomycetes* (oxic conditions). Thus, this study provides evidence that detected chitinolytic bacteria were catabolically diverse and occupied different ecological niches with regard to oxygen availability enabling chitin degradation under various redox conditions at the level of the community.

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1 Introduction

Chitin is a globally abundant biopolymer and is subject to rapid turnover in terrestrial ecosystems (Gooday, 1990a). Chitin consists of alternating β-1-4-linked *N*-acetylglucosamine (GlcNAc) residues and is a structural component of fungi, protists, and arthropods (Gooday 1990a, b; Martínez et al., 2009). In aerated soils (i.e., including many agricultural soils), fungi and arthropods are the main sources of chitin. Soluble hydrolysis products are sources of energy, carbon, and/or nitrogen for chitinolytic and saccharolytic soil microorganisms (Gooday, 1990b; Keyhani and Roseman, 1999; Geisseler et al., 2010; Kellner and Vandenbol, 2010).

Chitin can be initially hydrolyzed by exo- and endochitinases (EC 3.2.1.14) to *N*,*N*′-diacetylchitobiose ($[GlcNAc]_2$) and longer oligomers of GlcNAc. $[GlcNAc]_2$ is subsequently cleaved (i.e., by β-*N*-acetylglucosaminidases; EC 3.2.1.30) into *N*-acetylglucosamine (GlcNAc). An alternative hydrolysis pathway starts with the deacetylation of chitin to chitosan that is then hydrolyzed to glucosamine (GlcN) by chitosanases (EC 3.2.1.132) and glucosaminidases (EC 3.2.1.30) (Gooday, 1990b; Beier and Bertilsson, 2013). Two previous studies suggested that the hydrolysis via initial deacetylation might be predominant in estuarine sediments (Hillman et al., 1989a, b). Nonetheless, for aquatic and terrestrial ecosystems it has not been evaluated which chitin hydrolysis pathway prevails in microbial communities (Beier and Bertilsson, 2013). Deacetylation of chitin would produce chitosan, which is less abundant in nature than chitin (Gooday, 1990a; Raafat et al., 2008) and known to be toxic for microorganisms in pure culture (Raafat et al., 2008; Šimůnek et al., 2012). Therefore, the degradation pathway that starts with deacetylation is likely not a dominant mechanism of chitin hydrolysis at the community level.

Cultured chitinolytic bacteria are members of *Acidobacteria*, *Actinobacteria*, *Bacteriodetes* (*Cytophaga)*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Firmicutes* (Gooday, 1990b; Yang et al., 2005; Someya et al., 2011; Foesel et al., 2013). Detection of genes encoding selected chitinases has been employed in soils and other envi-

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ronments to assess chitinolytic bacteria at the community level (Cottrell et al., 1999; Ramaiah et al., 2000; Williamson et al., 2000; Metcalfe et al., 2002; LeCleir et al., 2004; Hobel et al., 2005; Xiao et al., 2005; LeCleir et al., 2007; Hjort et al., 2010; Beier et al., 2011; Peter et al., 2011; Cretoiu et al., 2012; Köllner et al., 2012; Beier and Bertilsson 2013; Kielak et al., 2013). A single bacterium can harbor multiple copies and also various different chitinase genes, e.g. *Streptomyces coelicolor* A3 possesses nine genes of glycoside hydrolases family (GH) 18 chitinases and two of GH19 (Saito et al., 2003). In total, there are over 100 enzyme families of glycoside hydrolases which can be distinguished. Nonetheless, most of the known hydrolases that can attack chitin (i.e., so called chitinases) belong to GH 18 or 19, whereby the latter is mainly restricted to plants. GH18 is dominated by chitinase genes of chitinolytic *Bacteria* (Cohen-Kupiec and Chet, 1998; Karlsson and Stenlid, 2009). Few chitinase-like proteins are affiliated with GH 23 and 48 (Fujita et al., 2006; Arimori et al., 2013). GH18 is divided into subfamilies A, B, and C (Henrissat and Bairoch, 1993; Suzuki et al., 1999; Cantarel et al., 2009; Karlsson and Stenlid, 2009). Established primers that have been used for environmental detection of microbial chitin degraders were designed to target chitinase genes belonging to subfamily A of GH18 (i.e., the so called *chiA* gene) (Williamson et al., 2000; Metcalfe et al., 2002; LeCleir et al., 2004; Hobel et al., 2005; Xiao et al., 2005). Beyond their role in the degradation of chitin through soil microorganisms, chitinases are involved in formation and changes of cell walls of fungi and the exoskeleton of arthropods, or can act in plants as a defense system against pathogens (Gooday 1990b; Patil et al., 2000; Kasprzewska, 2003; Seidl, 2008). The large diversity of known *chiA* genotypes that has been discovered in each previous environmental study reflects the broad functional and large organismal diversity. However, many genotypes might not have been discovered to date (Beier and Bertilsson, 2013; Kielak et al., 2013).

The majority of known *chiA* genotypes that have been detected in soil affiliate with *Actinobacteria* and to a minor extent with *Firmicutes* (Metcalfe et al., 2002; Ikeda et al., 2007; Hjort et al., 2010). In contrast to this general finding, supplementation of shrimp shell residues to agricultural soil can stimulate a rapid response of *Beta- and*

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Gammaproteobacterial chiA-like genotypes, suggesting that not only classic microbial chitin degraders can be relevant for chitin degradation in soil (Kielak et al., 2013).

Soil type, water content, temperature, substrate availability, and most significantly soil pH are environmental factors known to influence soil chitinolytic communities (Manucharova et al., 2006, 2011; Terahara et al., 2009; Yaroslavtsev et al., 2009; Kielak et al., 2013). Oxygen availability is another important factor that affects soil microbial communities. Oxygen distribution is heterogeneous and dynamic and depends on moisture, aggregate size, and properties of biogeochemical interfaces in aerated soils (Or et al., 2007). Aerated agricultural soils are largely oxic. Nonetheless, microbial anaerobiosis occurs in microzones of such soils (Küsel and Drake, 1995; Wagner et al.,1996; Picek et al., 2000; Pett-Ridge and Firestone, 2005), and presence or absence of oxygen can differentially impact on the stimulation of microbial processes involved in the degradation of the biopolymer cellulose in aerated agricultural soil, consequently leading to different active bacterial taxa (Schellenberger et al., 2010, 2011, 2012).Thus, contrasting energy-conserving microbial metabolisms occurring at close proximity to each other can simultaneously contribute to the overall degradation of biopolymers in soil.

Aforementioned considerations suggest (i) that chitin in soil is not primarily hydrolyzed via deacetylation to chitosan, (ii) that previously unknown *chiA* genotypes occur, and (iii) that different chitinolytic taxa are active under oxic and anoxic conditions in aerated agricultural soil. These three hypotheses were tested by investigation of oxic and anoxic soil slurries of a temperate agricultural soil that were either supplemented with chitin or chitosan. Microbial processes associated with chitin and chitosan hydrolysis and degradation were determined, and metabolic responses and identities of chitinolytic microorganisms were assessed by analyzing the gene marker *chiA*.

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2 Materials and methods

2.1 Sampling site and soil properties

The upper 20 cm layer of an aerated agricultural soil was sampled in April 2011 and April 2012, and stored under dark and moist conditions at 2 °C, and processed within a week. The sampling site is located on the research farm "Klostergut Scheyern" near Munich, Germany (48°30.0′ N, 11°20.7′ E). The mean annual precipitation was 803 mm with a mean temperature of 7.4 °C over a 30 yr period (Sommer et al., 2003). The soil type was a Dystric Cambisol (FAO classification system) (Fuka et al., 2008).

2.2 Chitin, chitosan, *N*,*N*'-diacetylchitobiose, *N*-acetylglucosamine, and glucosamine supplemented soil slurries

Soil slurries were prepared by mixing soil with sterile oxic and anoxic water (ratio 1 : 2.5) in a total volume of one litre in sterile rubber-stoppered 2 L flasks. Soil from April 2011 was used in experiments with GlcNAc and GlcN, and soil sampled in April 2012 was used in experiments with chitin, chitosan, and $[GlcNAc]_2$. Slurries were placed on ice and flushed with sterile argon (100 %, Riessner-Gase-GmbH, Lichtenfels, Germany) or sterile air for one hour. Soil slurries were homogenized on an end-over-end shaker for 1.5 h at 5 °C and were then divided in 80 mL-aliquots in rubber-stoppered 0.5 L flasks with sterile argon or air as atmosphere. Treatments were conducted in triplicates. For chitin- and chitosan-supplemented slurries, 0.2 g of grounded chitin or chitosan (acetylation degree ≥ 95 % and 15–25 % respectively) (Sigma-Aldrich® GmbH, Germany) was added at the onset of incubation. The applied amount of biopolymers was similar to the added amount of cellulose to soil slurries of the same site in a previous study (Schellenberger et al., 2010). Chitin- and -chitosan-treatments were incubated for 41 days as these substrates are large, sterile, and insoluble crystals, which need to be colonized and hydrolyzed before they can be utilized as a carbon, nitrogen, and energy source, i.e., a process that is much slower than the microbial degra-

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dation of soluble hydrolysis products of these biopolymers. Such soluble *N*-sugars were supplemented with a final concentration of 250 µM (GlcNAc, GlcN; AppliChem GmbH, Darmstadt, Germany) and 125 µM ($[GlcNAc]_2$; Megazyme, Bray, Ireland) and incubated for two days to test the ability of the soil microbial community to metabolize such typical chitin and chitosan hydrolysis products. Therefore, the amount of supplemented carbon was higher in chitin and chitosan treatments and corresponded to 400 µmol, and 360 µmol g_{carbon} $soil_{DW}^{-1}$, respectively, whereas in the $[GlcNac]_2$, GlcNAc, and GlcN supplemented treatments only 8.7 µmol and 6.5 µmol g_{carbon} $soil_{DW}^{-1}$ were added, respectively. The flasks were incubated in the dark on an end-over-end shaker (60 rounds min^{-1}) at 20 °C. Organic acids, sugars, inorganic anions, and gases were measured as described below. No products were detected in the chitosan treatment within 41 days. Therefore, GC measurements were conducted after 156 days to check if chitosan was not degraded or if degradation was hampered.

2.3 Chemical analytics

Soil moisture content was determined by weighing sieved (mesh size 2 mm) soil before and after drying at 105 °C for 48 h. Total ammonium, iron, manganese, nitrate, and sulfate concentrations were determined by ion chromatography (Center of Chemical Analytics of Bayreuth Center of Ecological and Environmental Research at the University of Bayreuth, Germany). pH was measured with a pH meter (U457-S7/110 combination pH electrode; Ingold, Germany). Liquid samples (containing soil) and gas samples were taken with sterile syringes, i.e., sampling time points can be retrieved from Figs. 1, 2, and S1. Liquid samples were centrifuged at 13 000 g (Himac CT15E, Hitachi Koki Co., Ltd., Tokyo, Japan) for 15 min and the supernatant was filtrated (HPLC nylon filter, pore volume 0.2 µm, Infochroma, Zug, Switzerland). Organic acids and sugars were determined by high-performance liquid chromatography with an ion exclusion column using 4 mM phosphoric acid as eluent (1090 series II with UV detector; Hewlett Packard, Palo Alto, CA) (Wüst et al., 2009). Carbon dioxide, hydrogen gas, and

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methane were measured with a gas chromatograph (Multigas analyser SRI 8610C, SRI Instruments, Torrance, CA) equipped with a thermal conductivity detector (TCD) and a helium ionization detector (HID). The injected gas sample was simultaneously separated on two columns. Carbon dioxide and methane were separated on a HayeSep-D column (2 m by 1/8 in; SRI Instruments, Torrance, CA) coupled to the TCD. Hydrogen gas and methane were separated with a molecular sieve column 13X (2 m by 1/8 in; Restek, Bellefonte, PA, USA) and detected with the HID. The carrier gas was helium at a flow rate of 40 (TCD) and 20 mL (HID) min^{-1}, injector and column temperatures were 30 °C and 60 °C, respectively. Chromatograms were integrated and analyzed with PeakSimple (SRI Instruments, Torrance, CA, USA).

2.4 Extraction of nucleic acids

Nucleic acids were extracted from 0.4 g soil slurry using cetyltrimethylammonium bromide (CTAB) and phenol-chloroform-isoamyl alcohol (25 : 24 : 1) (pH 8.0) based on a published protocol (Griffiths et al., 2000). Liquid samples were taken at t_0 and t_{END} from each replicate with sterile syringes and centrifuged at 13 000 g (1–15 K Sartorius microcentrifuge, Sigma, Osterode am Harz, Germany) for 15 min. The pelleted soil was used for extraction. Lysis was achieved by bead beating two times at 5.5. m s^{-1} in a Bead Beater (FastPrep FP 120, Thermo Savant, USA) for 30 s using zirconium beads (0.5 g Ø 0.1 mm, 0.5 g Ø 0.5 mm; CarlRoth, Karlsruhe, Germany).The following steps were conducted according to the published protocol (Griffiths et al., 2000) and combined RNA and DNA extracts were dissolved in RNAse and DNAse-free water. RNA was removed by treatment with RNase (Fermentas GmbH, Germany). DNA was quantified using the Quant-iT™ PicoGreen® dsDNA kit (Invitrogen, Germany) and stored at −20 °C.

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2.5 TRLFP analysis

Primer ChiA_F2 was labeled with the infrared dye "Dyomics 681" for terminal restriction fragment length polymorphism (TRFLP) analysis (Microsynth AG, Balgach, Switzerland). The endonucleases *Msp*I, *Hha*I, *Hae*III and *Alu*I were tested in silico with *chiA* dataset retrieved from samples of the conducted slurry incubations (2.6). *Alu*I (data not shown) revealed highest genotype resolution in silico with MEGA version 5 and the REPK Web Tool (Collins and Rocap, 2007; Tamura et al., 2011) and was used for further analyses. Notably, the TRF resolution by *Msp*I was low and yielded many short TRFs below 40bp which are not reliable detectable by the used sequencer (NEN 4300, LICOR, USA). Therefore a parallel digestion with *Msp*I according to a previous study was omitted (Hijort et al., 2009). Restriction digestion of PCR products was conducted at 37° C with *Alu*I endonuclease (New England Biolabs GmbH, Frankfurt am Main, Germany) for four hours. Single stranded DNA was removed by digestion with Mung Bean Nuclease (New England Biolabs GmbH, Frankfurt am Main, Germany). Remaining double stranded DNA was quantified with PicoGreen (Quant-iT™ PicoGreen® dsDNA Kit, Invitrogen, Germany), and TRFLP analyses were performed using a NEN4300 LiCOR DNA sequencer as previously described (Hamberger et al., 2008). Gels were analysed using GELQUEST (version 3.1.7, SequentiX GmbH, Klein Raden, Germany). The fluorescence values of TRFs were determined by normalization of the fluorescence value of a detected TRF against the fluorescence value of the respective TRF in the TRFLP profile with the lowest total fluorescence. TRFs with relative frequencies below 3 % were excluded from further analysis as low abundant genotypes are more prone to PCR biases (such as preferential amplification). Taxa that were represented by TRFs were identified in silico by manual assignment to clone insert sequences by searching for the first restriction site in a *chiA*-like gene dataset (MEGA, version 5) (Tamura et al., 2011).

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2.6 Chitinase (*chiA*) gene libraries

A fragment of *chiA* genes was amplified with primers ChiA_F2 (5'-CGT GGA CAT CGA CTG GGA RTW YCC-3') and ChiA_R2 (5'-CCC AGG CGC CGT AGA RRT CRT ARS WCA-5') (Hobel et al., 2005). Five *chiA* libraries were prepared from pooled DNA extracts of t_0 and t_{End} samples from each substrate supplemented treatment. PCR-premix containing *Taq*-DNA polymerase and all components except for primers and DNA template (MasterMix, 5 PRIME GmbH, Hamburg, Germany) were used for PCR according to previously published protocols (Hobel et al., 2004). End concentrations of primers were 1.0 µM. In total 35 cycles were run consisting of a denaturation (45 s, 95 °C), an annealing (45 s, 42 °C), and an elongation step (90 s, 72 °C). Non-purified PCR products were either (a) commercially cloned and vector inserts were sequenced (LGC Genomics, Berlin, Germany) or (b) cloned into *Escherichia coli* JM 109 (Promega, Madison, USA) competent cells using the CloneJET PCR Cloning Kit (Thermo Scientific, Erlangen, Germany). Inserted chitinase gene sequences were reamplified using vector specific primers pJET1.2 forward 5'-CGACTCACTATAGGGAGAGCGGC-3' and pJET1.2 reverse 5'-AAGAACATCGATTTTCCATGGCAG-3' and commercially sequenced (Macrogen, Europe). All quality-checked (i.e., manual removal of chimera, checked identity as being a potential *chiA* sequences by BLAST Search in nucleotide database of Genbank) *chiA* sequences (per library between 16 and 67 sequences) were combined in one dataset, which was used to assign detected TRFs to OTUs and to evaluate the richness of genotypes. Identification of microbial taxa solely by *chiA* on genus or species level is limited due to obvious incongruencies between *chiA* and organismal phylogenies at these low taxonomic ranks (Karlsson and Stenlid, 2009). Therefore, a threshold value of 50 % amino acid dissimilarity was used to group translated ChiA sequences into operational taxonomic units (OTUs) using the software DOTUR (Schloss and Handelsman, 2005). This similarity cut-off threshold refers to the taxonomic rank "class".

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2.7 Phylogenetic analysis of *chiA* sequences

The retrieved dataset of partial *chiA* gene sequences (206 sequences) was edited, translated into amino acid sequences and aligned using CLUSTALW and MUSCLE algorithms in MEGA version 5 (Tamura et al., 2011). The alignments were refined manually. A similarity-based distance matrix was calculated using an alignment of amino acid sequences. Phylogenetic trees were constructed from all sequences, their closest related genotypes (BLAST analysis using the latest version of the GenBank nucleotide database) (Altschul et al., 1990), and distantly related genotypes using MEGA. The *chiA* tree was calculated using translated amino acid sequences by applying the Neighbor Joining algorithm implemented in MEGA 5 (Tamura et al., 2011). Partial deletion with a site coverage cut off value of 80 % was chosen for gaps and missing data treatment. The topology of the Neighbor Joining tree was confirmed with MEGA-implemented Maximum Likelihood and Maximum Parsimony algorithms, using the same dataset. Tree branches in which reference sequences grouped together with sequences of a single OTU were condensed (Fig. 5).

2.8 Statistical analyses

Canonical Correspondence Analysis (CCA) was conducted to group TRF patterns and to correlate single TRFs with TRF patterns by PAST software package (Hammer et al., 2001). CCA allows to statistically analyze different treatments with not normally distributed data (Schütte et al., 2008). Relative abundances of TRFs were used as variables and correlated with TRF patterns of each treatment replicate (Figs. 4 and S5) using the same data as presented in Fig. 3. Presentation option "Scaling 2" was chosen to emphasize the relationships between single TRFs and TRF patterns. For treatments with eigenvalues (λ) ≥ 0.3 for both axes and positive substrate degradation, triplot presentation was used to evaluate the relative influence of single TRFs on TRF patterns. Selected TRFs were subsequently tested for significant increase from t_0 to t_{END} with Mann-Whitney U test (Table S1).

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2.9 Nucleotide sequence accession numbers

Partial *chiA* gene sequences of the current study were deposited at EMBL nucleotide database with accession numbers HG315747 to HG315952.

3 Results

3.1 Soil properties

Several soil parameters were determined in freshly collected samples. The C/N ratio was 6.9 ± 0.1. Soil pH (measured in water) was 6.6 ± 0.1, and the gravimetric water content was 17.7 % (±0.8 %) and 21.9 % (±1.0 %) for samplings in 2011 and 2012, respectively. Ammonium, nitrate, and sulfate concentrations were 0.06 ± 0.15, 3.54 ± 0.14, and 0.03 ± 0.14 µmol g^{-1}_{soilDW} in soil samples of 2011, and 0.42 ± 0.17, 10.50 ± 2.5, and 0.17 ± 0.17 µmol g^{-1}_{soilDW} in soil samples of 2012, respectively. Ferrous iron was not detectable, and total amounts of iron and manganese were 40.0 ± 15.8 and 5.3 ± 3.1 µmol g^{-1}_{soilDW}, respectively.

3.2 Effect of oxygen on microbial activities in chitin and chitosan supplemented agricultural soil slurries

The effect of supplementation of substrates on the concentration of formed potential products was substantial and exceeded that of controls by at least twofold. Apparent degradation products of chitin were detected under oxic and anoxic conditions within 41 days (Fig. 1a), whereas no effect on microbial product formation was apparent within same period in chitosan supplemented slurries (Fig. 1b). Nonetheless, after more than five months carbon dioxide was formed in oxic and anoxic chitosan supplemented slurries (Fig. S1) suggesting that chitosan degradation was substantially delayed and slower compared with chitin degradation, and that the soil microbial community had only a limited capability to degrade chitosan.

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Stimulating effects of chitin supplementation on the microbial product formation were detected after one week under oxic and after three weeks under anoxic conditions suggesting slower chitin degradation when oxygen was absent (Fig. 1a). Under oxic conditions, chitin was apparently dissimilated to carbon dioxide (Fig. 1a). 17 % of the presumably degraded nitrogen of chitin was recovered as ammonium and nitrate (data not shown). Oxygen concentration was spot-checked in the course of the experiment and decreased from 21 % to 10 % after 41 days. Under anoxic conditions, measured products were acetate (8.9 %), propionate (0.2 %), butyrate (0.6 %), molecular hydrogen (0.4 %), and carbon dioxide (1.7 %), suggesting that various different fermentation metabolisms were active. Ethanol, a typical microbial fermentation product, was not detected. Potential products of chitin hydrolysis ($[GlcNAc]_2$ and GlcNAc) were below the detection limit, and thus did not exceed concentrations of 30 µM. Methane production was not detected, and pH was stable in oxic treatments at values around 5.2 (chitin) and 6.3 (chitosan), and slightly increased in the anoxic treatments from 5.4 to 6.4 (chitin) and 6.1 to 6.8 (chitosan). End point measurements after 41 days in the anoxic treatments (chitin and chitosan) revealed that high amounts of ferrous iron (9.7 ± 0.5 mM) were formed.

3.3 Effect of oxygen on microbial activities in $[GlcNAc]_2$, GlcNAc, and GlcN supplemented agricultural soil slurries

The capability of the soil microbial community to metabolize typical chitin and chitosan hydrolysis products was tested by supplementation of $[GlcNAc]_2$, GlcNAc, and GlcN to soil slurries. The decrease of $[GlcNAc]_2$ and GlcNAc concentrations was slower in anoxic incubations suggesting a slower uptake and metabolization rates under anoxic conditions (Fig. 2). GlcNAc transiently accumulated in $[GlcNAc]_2$-supplemented slurries indicating that most of the $[GlcNAc]_2$ was extracellularly hydrolyzed, since GlcNAc was detectable in the liquid phase of the slurries (Fig. 2a). Carbon dioxide was the major product in $[GlcNAc]_2$- and GlcNAc-supplemented slurries under both oxic and anoxic conditions. Formation of acetate occurred in anoxic GlcNAc- and GlcN-

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supplemented slurries (Fig. 2b and c). Similar to incubations with chitin, methane production was not detected. pH was stable in the oxic treatments at values around 5.1 ($[GlcNAc]_2$) and 6.1 (GlcNAc, GlcN) and slightly increased in the anoxic treatments from 5.1 to 5.5 ($[GlcNAc]_2$) and from 6.1 to 6.4 (GlcNAc, GlcN). Substantial release of ammonium was not observed whereas detectable nitrate concentrations stayed constant or even decreased over time in both oxic and anoxic treatments. These observations suggested that within the short incubation period no substantial net production of inorganic nitrogen compounds (as observed in the long term incubation with chitin [Fig. 1a]) occurred. GlcN stimulated microbial activity under both oxic and anoxic conditions. However, product formation was delayed compared to slurries that were supplemented with $[GlcNAc]_2$ and GlcNAc (Fig. 2).

3.4 *chiA* TRFs responding to chitin supplementation

The response of *chiA* genotypes to the supplementation of chitin, chitosan, $[GlcNAc]_2$, GlcNAc, and GlcN was evaluated by TRFLP analysis (Figs. 3 and S4). DNA extracts from each replicate of a treatment were analyzed to assess the variability of genotype diversity in the soil slurries and further analyzed by CCA and Mann-Whitney-U test to identify changed TRFs (Figs. 4, S5 and Table S1).

An effect of substrate supplementation and/or oxygen availability on *chiA* TRF patterns was not evident for $[GlcNA]_2$, GlcNAc, and GlcN supplemented slurries. Likely, the short time period (2 days) did not allow for substantial growth, and thus there were no detectable changes in TRF patterns (Figs. S4 and S5). In the chitosan supplemented slurries T_{End} patterns under oxic conditions were separated according to CCA plots from t_0 TRF patterns and the T_{End} controls (Figs. 3b and S5a). A change due to chitosan degradation was not likely, since potential degradation products were not detected at that time point (Fig. 1b), whereas after 156 days a net increase of carbon dioxide was observed in both oxic and anoxic treatments (Fig. S1) suggesting a strongly delayed degradation of chitosan.

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Several TRFs responded positive towards chitin supplementation and led to a shift in the TRF patterns (Figs. 3a and 4). T_{End} TRF patterns of chitin supplemented slurries were different from t_0 patterns and the respective controls at t_{End} under both oxic and anoxic conditions (Fig. 4a). Thereby, the TRF patterns under anoxic condition exhibited a larger variability. Under oxic conditions, TRF 114bp and 54bp positively responded and correlated with the shift of TRF patterns (Fig. 4a), whereby TRF 54bp had the bigger influence (Fig. 4b). Under anoxic conditions TRFs 137bp and 188bp responded positively and were responsible for the shift. TRF 264bp was detected under oxic and anoxic conditions (Figs. 3a and 4) and correlated with the shift of the patterns under oxic and anoxic conditions. TRFs 114bp, 188bp, and 264bp significantly increased their relative abundance (Table S1). The response of TRF 114bp was less significant ($p \leq 0.20$) and was found in only two of three replicates (relative abundance 10.7[±10.2]%). TRF 137bp influenced the pattern for one replicate (k, Fig. 4) under anoxic conditions due to its high relative abundance (14.6 %) but the increase of its relative abundance was not significant ($p \leq 0.51$, Fig. 4 and Table S1).

3.5 Diversity of *chiA*-like genotypes

In total, 206 *chiA*-like genotypes were detected and grouped into 42 OTUs (Figs. 5, S2, and S3). Based on the cut off value (similarity ≥ 50 %), rarefaction analysis revealed sufficient sampling depth, although a plateau indicative of a complete coverage of genotype diversity was not fully reached (Fig. S2). The high number of detected OTUs suggested a large *chiA* genotype richness in the microbial community of the investigated agricultural soil. Twenty two OTUs affiliated with *chiA*-genotypes of cultivated species (similarity ≥ 50 %), and were assigned to *Beta-* and *Gammaproteobacteria* (OTU 2, 12, 19, 42), *Actinobacteria* (OTU 7 and 20), *Acidobacteria* (OTU 4 and 26), *Bacteriodetes* (OTU 9, 14, and 28), *Firmicutes* (OTU 10 and 15), *Planctomycetes* (OTU 1), *Chloroflexi* (OTU 11), and microeukaryotes (OTU 17, 21, 24, 25, 41, 27, and 37) (Fig. 5). Whereas, OTUs 3, 5, 6, 8, 13, 16, 18, 22, 23, 29–36, and 38–42 repre-

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sented novel *chiA* genotypes based on their high dissimilarity to known *chiA* genotypes (dissimilarity > 50 %) and their separate branching (Figs. 5 and S3).

Eukaryote-like *chiA* sequences accounted for a minor fraction of detected *chiA* OTUs and were closest related to those of either fungi (*Basidiomycota*; OTU 17, 25 and 41), *Amoebozoa* (OTU 24), or diatoms (*Heterokontophyta*, OTUs 21 and 27) (Figs. 5 and S3). *Basidomycota* are known to comprise chitinolytic species (Gooday, 1990a, b; Tracey, 1955). *Bacteria* might have outcompeted chitinolytic *Basidiomycota* in soil slurries based on data of the current study (Figs. 3a, 4 and 5). Nonetheless, it should be noted that used primers were developed for targeting bacterial *chiA*-sequences, and thus conclusions with regard to eukaryotic genotypes are limited.

4 Discussion

4.1 Microbial response to chitin and chitosan supplementation in agricultural soil slurries

Degradation of chitin and chitosan flakes was slow in a previous field experiment and detectable degradation and a substantial loss of flakes mass was observed after 50 and 180 days, respectively (Sato et al., 2010). In the current study, stimulation of microbial activity in chitosan supplemented slurries was substantially delayed compared to the rapid stimulation by chitin under both oxic and anoxic conditions (Figs. 1 and S1). The fast response towards chitin as observed in a study by Sato and coauthors (Sato el al., 2010) can be explained by a higher physical accessibility of the used biopolymers in the current study (since grounded chitin was used) and by an unknown effect of the divergent incubation conditions employed in both studies. The response of *chiA* genotypes towards and the rapid stimulation of microbial activity in supplemented agricultural soil slurries suggested that the soil microbial community was better adapted to chitin as substrate than to chitosan. That is likely, as chitin is more abundant than chitosan in nature. For example, with few exceptions chitin occurs ubiquitously in fungi

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whereas chitosan is only found in *Zygomycetes* (Gooday, 1990a; Raafat et al., 2008). In agreement with this, in GlcN (i.e., a potential product of chitosan hydrolysis) supplemented slurries, product formation was delayed compared with slurries that were supplemented with potential products of chitin hydrolysis, i.e., $[GlcNAc]_2$ and GlcNAc (Fig. 2). The relevance of deacetylation and subsequent chitosan hydrolysis for soil communities has not experimentally been addressed in previous studies. The experimental data suggest that chitin was largely not deacetylated, and that it is likely that hydrolysis without prior deacetylation was the preferential pathway of chitin breakdown of the investigated soil microbial community.

4.2 Effect of oxygen on community metabolism in chitin supplemented treatments

Chitin stimulated microbial activity without apparent delay under oxic conditions with carbon dioxide being the sole detected carbonaceous product. Stimulation of the microbial activity under anaerobic conditions was evident after three weeks (Fig. 1b) suggesting a slower degradation compared to aerobic degradation. Potential products of chitin hydrolysis ($[GlcNAc]_2$ and GlcNAc) could not be detected suggesting an efficient consumption of hydrolysis products leading to low steady state concentrations, i.e. $< 30\,\mu M$. The generally lower degradtion rate of *N*-sugars under anoxic conditions likely explains the lower rate of apparent chitin degradation under anoxic conditions.

Detected anaerobic products were indicative for mixed acid, and propionic and butyric acid fermentation (Buckel, 2005; White, 2007). Propionate and butyrate were not detected in the short term experiments with $[GlcNAc]_2$, GlcNAc, and GlcN suggesting that these products were intermediates in the sequence of products of anaerobic microbial degradation of chitin, which became detectable when the sequential reactions became decoupled due high substrate input combined with long term incubation. Acetate production might have been additionally associated with syntrophic fermentation and acetogenesis (Wagner et al., 1996; Drake et al., 2009). The same degradation products were detected under anoxic conditions when cellulose was supplemented

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to soil slurries in the same soil (Schellenberger et al., 2010). Ferrous iron formation suggested that ferric iron reducers consumed a part of fermentation products, which was also found with anaerobic cellulose degradation in the same soil (Schellenberger et al., 2010). Nitrate formation was likely caused by nitrification of released ammonium (Schulten and Schnitzer, 1998; Kowalchuk and Stephen, 2001; Erguder et al., 2009) and nitrate consumption in the anoxic treatments due to dissimilatory nitrate reduction (Kraft et al., 2011).The C/N ratio as well as the high initial nitrate concentrations (Figs. 1 and 2) indicate that nitrogen was not limiting for microbial metabolism in the investigated agricultural soil. This likely explains why nitrogen from chitin was not fully utilized, and 17 % were recovered as ammonium and nitrate. At the community level, anaerobic metabolism of chitin and *N*-sugars in the soil slurries was largely similar to that of anaerobic cellulose degradation in soil slurries of the same soil, i.e., the only divergent observation was net release of ammonium from chitin through ammonification.

4.3 Responding *chiA* genotypes and diversity in chitin supplemented slurries

A broad diversity of *chiA* genotypes (Figs. 5 and S3) indicative of organisms that have the potential to hydrolyze chitin was detected. However, few of the detected *chiA* genotypes were stimulated by chitin supplementation under experimental conditions.

chiA TRFs that were stimulated by chitin supplementation were affiliated with *Betaproteobacteria* (OTU 2; TRFs 114bp and 264bp), *Gammaproteobacteria* (OTU 19; TRF 188bp), *Planctomycetes* (OTU 1, 34; TRF 54bp), and a novel *chiA* genotype (OTU 3; TRF 188bp). TRFs 114bp and 54bp that were detected in oxic treatments likely represented aerobic microorganisms, whereas TRF188bp represented anaerobic and facultative aerobic microorganisms. TRF 264bp that was detected under both oxygen conditions was indicative of facultative aerobic chitinolytic microorganisms. OTU 3 represented a *chiA* genotype of yet unidentified microorganisms that were active under anoxic conditions. Most similar sequences (5–44 % amino acid sequence dissimilarity) and already known genotypes were two environmental sequences from Antarctic lake sediments (L5-24, L11-50), which were most closely related with bacterial *chiA*

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genes (Figs. 5 and S3). Thus, OTU 3 likely represented previously unknown anaerobic chitinolytic *Bacteria*.

OTU 1 positively responded under oxic conditions and affiliated with *Singulisphaera acidiphila*. c*hiA* has been detected in the genome of *S. acidiphila* suggesting that *S. acidiphila* might hydrolyze chitin (Kulichevskaya et al., 2008; Guo et al., 2012). However, *Planctomycetes* have not been shown to be chitinolytic to date (Ivanova and Dedysh, 2012). *Planctomycetes* (including *Singulisphaera acidiphila*) are able to utilize GlcNAc as sole carbon and energy source (Schlesner, 1994; Fuerst et al., 1997; Rabus et al., 2002). Therefore, the physiological function of the *chiA* gene in *S. acidiphila* needs to be considered as elusive based on current knowledge of substrate spectra of *Planctomycetes* isolates. Nonetheless, the current study suggests that *Planctomycetes* were somewhat involved in chitin degradation. A possible role of *Planctomycetes* in the degradation of biopolymers such as chitin and cellulose is in agreement with the detection of a ^{13}C labelled ribosomal RNA of *Planctomycetes* in ^{13}C cellulose supplemented oxic soil slurries of the same agricultural soil (Schellenberger et al., 2010).

Beta- and *Gammaproteobacteria*-like genotypes were abundant genotypes in the *chiA* dataset, and members of these groups were stimulated by chitin supplementation under both oxic and anoxic conditions (Figs. 3 and 4). Supplementation of chitin to agricultural soil slurries also stimulated a rapid response of *Beta-* and *Gammaproteobacteria* (Kielak et al., 2013), whereas a previous field study revealed that chitin supplementation positively correlated with increased abundances of *Actinobacteria* and *Oxalobacteracea* (*Betaproteobacteria*) (Cretoiu et al., 2013). Thus, experimental conditions (for example soil slurries vs. field conditions) obviously affect the activity of certain chitinolytic taxa.

Responding *Betaproteoabacteria*-like *chiA* genotypes of the current study had high similarities with *chiA* sequences of two species of *Oxalobacteracea* (*Janthinobacterium lividum* PAMC 25724 [ZP 10443966.1], *Janthinobacterium sp.* HH01 Jab 2c [ZP 21465866.1]) (Fig. S3). *Oxalobacteracea* (*Betaproteobacteria*) therefore likely play a crucial role in the degradation of chitin in agricultural soils.

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Various *chiA* TRFs positively responded to chitosan supplementation under oxic not under anoxic conditions (Fig. S4a). Stimulation of net product formation did not occur (Fig. 1b). Carbon dioxide production was equal in chitosan supplemented treatments and unsupplemented controls (Fig. 1b). Thus, the stimulation of TRFs was likely not caused by microbial utilization of chitosan. Chitosan might have functioned as substrate analog that triggered growth of certain *chiA* harboring organisms, which utilized non-polymeric substrates and outcompeted those that were active in the absence of chitosan. Although further experiments are required to resolve this issue, lacking net formation of carbon dioxide or any other product suggested that the investigated community was not prone to utilize chitosan.

Notably, most of the detected genotypes did not respond, which included beyond *Actinobacteria*, other classic chitinolytic soil bacteria, such as *Clostridia*. The lack of response does not necessarily mean that the associated taxa were not actively involved in chitin degradation. They might have been active, but did not substantially grow.

4.4 Non-responding *chiA* genotypes

A high fraction (36 %) of *chiA* genotypes that did not respond to substrate supplementation were novel, i.e., they were only distantly related to known genotypes and *chiA* genes of cultivated taxa. Thus, it remains speculative which organisms were represented by those genotypes. 32 % of the not-responding *chiA* genotypes affiliated with *Actinobacteria*, *Bacteriodetes*, *Firmicutes*, *Acidobacteria*, *Chloroflexi*, and Eukaryotes.

Bacteriodetes, Firmicutes and Actinobacteria are known to have chitinolytic members, whereby *Actinobacteria* are often regarded as the most important chitin degraders in aerated soils (Gooday, 1990a, b; Williamson et al., 2000; Krsek and Wellington, 2001; Metcalfe et al., 2002). Therefore, the apparent lack of response of *Actinobacteria* was unexpected and might be related to the experimental conditions of this study. In contrast to aforementioned phyla, chitinolytic lifestyle has not well been established in *Acidobacteria*, which are abundant in soils and difficult to cultivate (Janssen, 2006). Recently, the first chitinolytic strain (*Blastocatella fastidiosa*) was isolated (Foesel et al.,

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2013). Further evidence that chitinolysis is a trait of *Acidobacteria* was revealed by comparative genome analyses of three acidobacterial strains (Ward et al., 2009). The *chiA*-like genes that were detected in the current study were possibly functional chitinases of novel chitinolytic *Acidobacteria*. Future studies are warranted to verify the capability of the chitinolytic lifestyle in this bacterial phylum.

5 Final conclusions

The investigated soil microbial community likely degraded chitin via "direct" hydrolysis, and not by initial deacetylation to chitosan. *Betaproteobacteria* and a novel *Planctomycetes*-like *chiA* genotypes were stimulated by supplemental chitin under oxic and *Beta*-, *Gammaproteobacteria,* and a novel (OTU 3) *chiA* genotype under anoxic conditions. Thus, detected chitinolytic bacteria occupied different ecological niches with regard to oxygen availability. In the same soil also cellulolytic bacteria differentially responded to oxygen availability (Schellenberger et al., 2010, 2011). Thus, aforementioned findings suggest that the catabolic diversity of biopolymer degrading microbes enables continued biopolymer degradation despite fluctuations of oxygen concentration.

Supplementary material related to this article is available online at http://www.biogeosciences-discuss.net/11/2155/2014/bgd-11-2155-2014-supplement.pdf.

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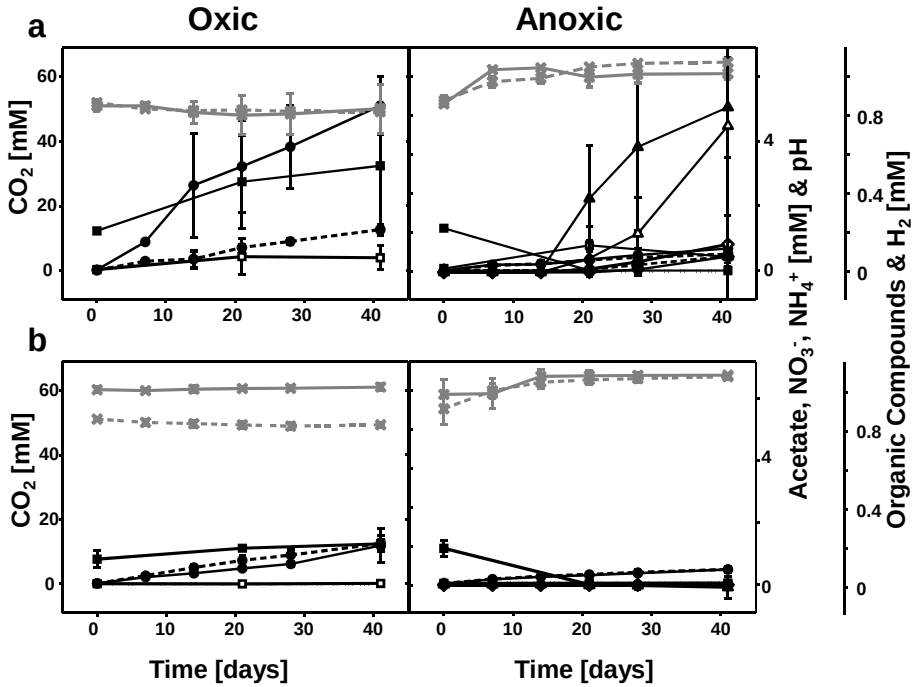

Fig. 1. Product formation of soil slurries supplemented with chitin **(a)** and chitosan **(b)**. Left panels, slurries incubated under an oxic atmosphere. Right panels, slurries incubated under an oxygen-free atmosphere. Closed circle, carbon dioxide. Closed rectangle, nitrate. Open rectangle, ammonium. Closed diamond, propionate. Open diamond, butyrate. Closed triangle, acetate. Open triangle, molecular hydrogen. Grey cross, pH. Error bars, standard deviation of three replicated soil slurries (In some cases these errors were such small that they are masked by the symbol). Dashed lines, values of the controls. Nitrate and ammonium concentrations are gross values of supplemented treatment.

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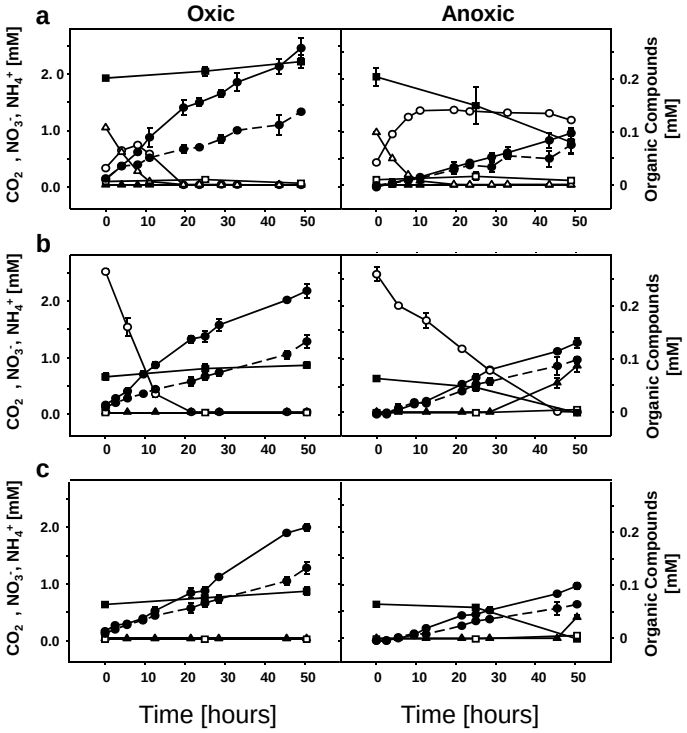

Fig. 2. Product formation of soil slurries supplemented with $[GlcNAc]_2$ **(a)**, GlcNAc **(b)**, and GlnN **(c)**. Left panels, slurries incubated under oxic atmosphere. Right panels, slurries incubated under oxygen-free atmosphere. Closed circle, carbon dioxide. Open circle, GlcNAc. Open triangle, $[GlcNAc]_2$. Closed triangle, acetate. Closed rectangle, nitrate. Open rectangle, ammonium. Error bars, standard deviation of three replicated soil slurries (In some cases these errors were such small that they are masked by the symbol). GlcN was not detectable with the used analytical methods. Dashed lines, values of the controls. Nitrate and ammonium concentrations are gross values of supplemented treatment.

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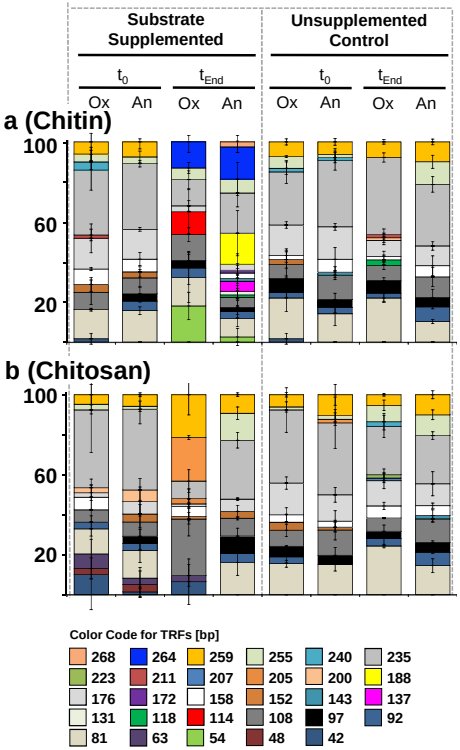

Fig. 3. *chiA* TRFLP patterns of chitin **(a)**, chitosan **(b)** supplemented soil slurries. The corresponding process data are presented in Figs. 1 and 2. In each panel the first four bars represent samples from slurries with substrate and the next four samples from a control experiment without substrate supplementation. Within, the order is as follows: t_0 oxic, t_0 anoxic, t_{END} oxic, and t_{END} anoxic. t_{END} was at 41 days. Per time point three experimental replicates were analysed, i.e., each value of a TRF is based on three DNA extracts. Errors bars, standard deviation (In some cases these errors were such small that they are masked by the symbol).

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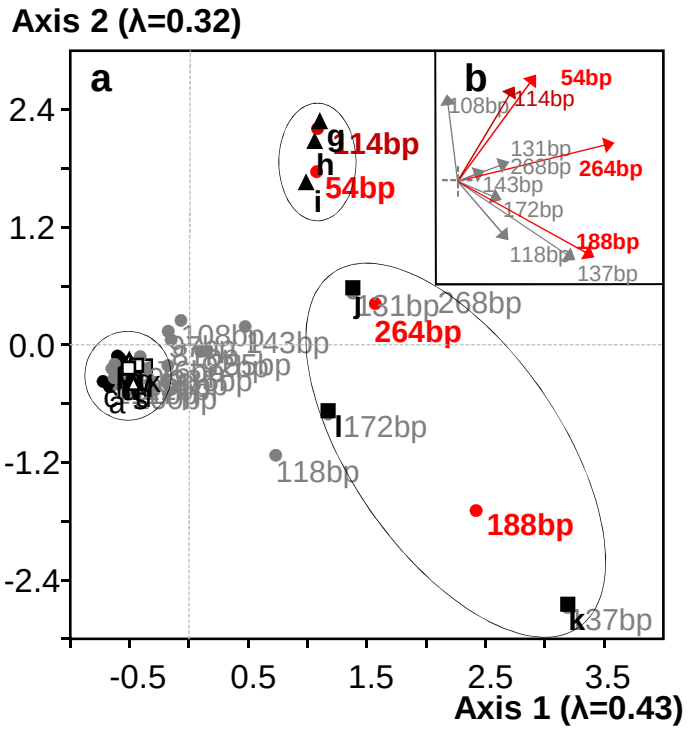

Fig. 4. Effect of chitin supplementation on *chiA* TRF patterns. **(a)** CCA of TRF patterns of each replicate of the chitin treatment (Fig. 3a). Numbers, TRFs. Letters, TRF patterns. TRF patterns of chitin supplemented treatments: t_0 oxic (a, b, c); t_0 anoxic (d, e, f); t_{End} oxic (g, h, i); t_{End} anoxic (j, k, l). TRF patterns of unsupplemented controls: t_0 oxic (m, n,o); t_0 anoxic (p, q, r); t_{End} oxic (s, t, u); t_{End} anoxic (v, w, x). **(b)** Part of the triplot of the respective CCA indicating the relative influence of a given TRF towards the shift. Colored TRFs, significant (Red $p \leq 0.06$, dark red $p \leq 0.2$) correlation of TRF with associated TRF patterns according to measures by Mann-Whitney *U* test (Table S1).

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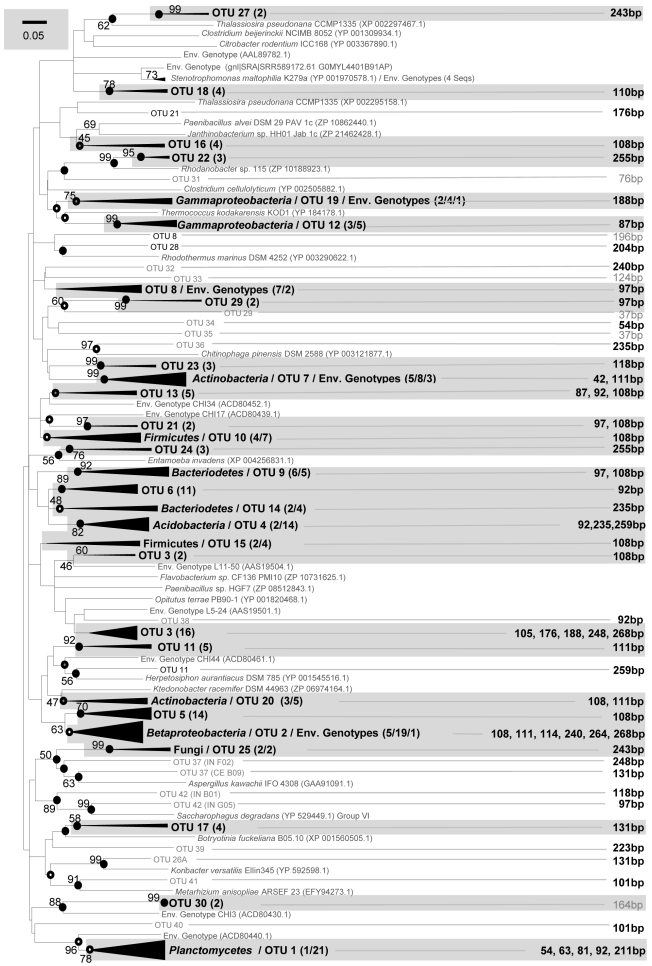

Fig. 5. Caption on next page.

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Fig. 5. Phylogenetic tree of *chiA* OTUs (206 sequences) and references (78 sequences). The expanded tree is presented in Fig. S3. *chiA* gene libraries were prepared from pooled DNA extracts of each substrate treatment and data were combined for the figure. Grey numbers in parentheses, accession numbers of reference sequences. For condensed branches OTUs and taxonomic affiliation of reference sequences are indicated in bold letters and the numbers of organismal reference sequences, OTU sequences and environmental sequences and is given in parentheses. Accession numbers for reference sequences of the condensed branches can be found in Fig. S3. Numbers on the right side, TRFs corresponding to genotypes identified by in silico digestion with *Alu*I. The tree was calculated using translated amino acid sequences with Neighbour Joining algorithm (MEGA 5; Tamura et al., 2007). Percentage values at nodes, bootstrap values of 1000 replicates. Open circles and grey filled circles at nodes, these nodes were confirmed by Maximum Likelihood and Maximum Parsimony algorithms, respectively, using the same dataset. Black circles, confirmation by both algorithms. Scale bar, 5 % sequence divergence.

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